R&D Note

Study of stationary phase metabolism via isotopomer analysis of amino acids from an isolated protein**

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ABSTRACT

Microbial production of many commercially important secondary metabolites occurs during stationary phase, and methods to measure metabolic flux during this growth phase would be valuable. Metabolic flux analysis is often based on isotopomer information from proteinogenic amino acids. As such, flux analysis primarily reflects the metabolism pertinent to the growth phase during which most protein is synthesized. In order to investigate central metabolism and amino acids synthesis activity during stationary phase, addition of fully-^{13}C-labeled glucose followed by induction of green fluorescent protein (GFP) expression during stationary phase was used. Our results indicate that *Escherichia coli* was able to produce new proteins (i.e., GFP) in the stationary phase, and the amino acids in GFP were mostly from degraded proteins synthesized during the exponential growth phase. Among amino acid biosynthetic pathways, only those for serine, alanine, glutamate/glutamine, and aspartate/asparagine had significant activity during the stationary phase.

KEYWORDS

secondary metabolites, metabolic flux, ^{13}C, green fluorescent protein, proteinogenic amino acids

INTRODUCTION

Cell-wide studies have come to play an important role in many metabolic engineering endeavors that seek to use microbial systems for the production of commercial chemicals [1-3]. For the rational design of metabolic pathways in the host cells, isotopomer-based flux analysis can be used to assess the flux of carbon through central metabolic pathways [4, 5], and often utilizes proteinogenic amino acids to obtain the labeling information about their precursors in the central metabolism. Isotopomer-based flux analysis is only pertinent to the exponential phase, the growth phase during which majority of the cellular protein is generated [6]. However, optimizing microbial host strains requires metabolism information relevant for the stationary phase in which many products of interest may be produced [7, 8]. To perform such flux analysis, one cannot simply use the proteinogenic amino acids from cells harvested during the stationary phase because their labeling patterns mainly reflect the metabolism when the amino acid was synthesized, i.e., the exponential phase.

Recently, liquid chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry have been developed to obtain flux data relevant to stationary growth phase based on the ^{13}C labeling information in rapidly turned over metabolites (e.g., oxaloacetate)[9, 10]. These techniques are methodologically challenging. After quenching of cellular metabolism, metabolites must be extracted taking extreme care that these molecules do not undergo any leakage or degradation[11-15]. On the other hand, in our previous study using the model organism *E. coli* expressing a plasmid-borne gene encoding an S-tagged green fluorescent protein (GFP), we found that amino acids from the purified GFP could serve as a proxy for data from total protein[16]. Here we utilize this strategy to examine important aspects of metabolism when cells enter stationary phase by inducing GFP at different growth stages.

MATERIALS AND METHODS

E. coli growth and metabolite assays. The culture conditions have been described in our previous study[16]. In brief, all *E. coli* BLR(DE3) pET30-GFP cultures were grown in M9 minimal medium supplemented with 2% glucose (2 grams of glucose in 100 mL of medium) and a trace metal solution[17]. Kanamycin (50 μg/mL) was used for strains harboring pET30-GFP. All cultures were inoculated with 0.1% volume and grown at 37°C with shaking at 200 rpm. Two sets of cultures (4×100 mL each) were used in this study. The first set of cultures was grown in minimal medium containing 2% singly labeled 1-^{13}C-glucose (99% pure, Cambridge Isotope Laboratories, MA). The second set of cultures was grown in minimal medium with 2% unlabeled glucose. When the OD_{600} of the cultures reached 5.8 (stationary phase), 0.1 g of fully-labeled ^{13}C-glucose was added to each of the four 100-mL cultures growing in unlabeled glucose medium (16 hours after inoculation). Then isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich, MO) was added to a final concentration of 1 mM to both sets of cultures to induce production of recombinant S-tagged GFP. Cell growth was monitored by measuring OD_{600}. Extracellular metabolites (including acetate, lactate, ethanol and formic acid) were measured by HPLC equipped with a UV detector (1200 Series HPLC, Agilent Technologies, CA) and Bio-Rad HPLC Organic Acid Analysis Column (Aminex HPX-87H Ion Exclusion Column, 300mm x 7.8mm, Cat# 125-0140, CA). Extracellular metabolite data were useful for metabolic flux analysis (Supplementary materials).

Protein purification. After 24 hours of GFP induction, cells from 400 ml cultures were harvested by centrifugation. The GFP protein was purified using a modified version of the S-tag rEK Purification Kit protocol (Novagen Inc., Madison, WI). Each cell pellet from the 100 mL culture

was treated as follows. After resuspension in 20 mL of binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100), cells were lysed via sonication. The soluble GFP was separated by centrifugation (small amount of GFP remaining in the insoluble fraction could be extracted using binding buffer with 8M urea), and the concentration of urea in the soluble GFP solution was adjusted to 2M. Using end-over-end rotation, 6 mL of S-protein agarose slurry was mixed with this total protein sample for 3 hours. The S-protein agarose was washed three times with 20 mL of binding buffer containing 2M urea. The resin was then resuspended in 6 mL of the same buffer, and 60 units of rEK (recombinant enterokinase) were added to cleave the target protein from the washed resin. After a 21-hour digestion, the rEK was removed from the mixture by binding with 3 mL of EKapture agarose for 20 minutes using end-over-end rotation. After centrifugation (500 × g for 5 minutes), GFP was obtained in the supernatant and could be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Isotopomer analysis and flux analysis. The GC-MS protocol for isotopomer measurement has been reported previously[18]. In brief, protein pellets (from 50 mL culture) were hydrolyzed and the resulting amino acid mixture was derivatized in tetrahydrofuran (THF) and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich, St. Louis, MO). The isotopomer labeling was analyzed using a gas chromatograph (Model 6890, Agilent, Wilmington, DE) equipped with a DB5 column and a mass spectrometer (Model 5973 Network, Agilent, Wilmington, Delaware). Two types of positively charged species were obtained: unfragmented amino acids, $[M-57]^+$, and fragmented amino acids that have lost their α carboxyl group, $[M-159]^+$. The natural abundance of isotopes was corrected using a published algorithm

before using the data for calculating the label distribution[19]. The enrichment of isotopic labeling fraction (F) in amino acids can be defined as the equation below:

$$F = \frac{\sum_{i=0}^{i=I} i \cdot M_i}{I} \qquad (1)$$

where I is the number of carbons in the amino acid; i (from 0~I) represents unlabeled, singly labeled, doubly labeled isotopomers; and Mi is the fraction of the isotopomer with i labeled carbons. Isotopic labeling fraction (F) can be used to investigate the amino acid synthesis during stationary phase.

The isotopomer model for metabolic flux analysis has been developed as described before[20]. The biochemical pathways for *E. coli* central carbon metabolism include the tricarboxylic acid (TCA) cycle, the glyoxylate shunt, C1 metabolism, glycolysis, and the pentose phosphate (PP) pathway. The detailed flux analysis algorithms and flux results for this study are in the supplementary materials.

RESULTS AND DISCUSSION

Isotopomer information from proteinogenic amino acids is commonly used to assess cellular metabolic fluxes [5, 9, 21-23] in exponential growth phase by assuming that cell growth is in a pseudo steady state[6]. To study stationary phase metabolism, *E. coli* BLR(DE3) pET30-GFP was cultured in glucose minimal medium. When the cell optical density stopped increasing, the cells were considered to be in stationary phase. Samples of the biomass and protein were collected from the exponential phase, early stationary phase, and late stationary phases (Fig. 1). This set-up allowed a comparison of the isotopomer distribution in amino acids from several different samples; 1) total protein from exponential phase, 2) total protein in early stationary phase, 3)

total protein in late stationary phase, and 4) purified GFP induced during stationary phase. The data for 14 key amino acids (that represent the central metabolic pathways) across all the samples listed above clearly indicate that the isotopomer distributions of amino acids from total protein extracted from stationary-phase cells are similar to those extracted from exponentially-growing cells (difference is less than 3%, Table 1). This is not surprising because it can be assumed that the majority of the protein present in stationary-phase cells was synthesized during the exponential growth phase. Thus, even if the stationary growth is expected to result in very different flux through metabolic pathways, the isotopomer information from proteinogenic amino acids may not reflect such changes. Hence, proteinogenic amino acids from these samples cannot be used to determine the metabolic fluxes specific to the stationary phase.

We have previously demonstrated that amino acids derived from a single protein yield data similar to that from total protein in a sample[16]. To investigate the stationary phase metabolism, we used such a single purified protein, a P_{lac}-driven, S-tagged GFP, induced specifically during the stationary phase. The S-tag enabled simple and efficient purification of the GFP (Supplementary Fig. S1). By derivatizing and analyzing the isotopomer distribution in the amino acids of this protein, one may observe the metabolism specific to stationary-phase cells. However, our results indicate that the isotopomer distribution in amino acids derived from purified GFP produced during the stationary phase is very similar to the data from total protein harvested at different growth stages (Table 1). Metabolic fluxes calculated based on such amino acids labeling (Supplementary Fig. S2 and S3) would therefore suggest that central metabolic pathways actively degrade glucose for energy and biomass production during stationary phase (i.e., a typical exponential phase metabolic status).

It is likely that the amino acid biosynthetic pathways are much less active during stationary phase and some portion of amino acids used in the production of the stationary phase-GFP are from proteins degraded during exponential phase. In order to quantify the percentage of each amino acid that is freshly synthesized during stationary phase, the culture was grown in unlabeled glucose and fully labeled ^{13}C-glucose was added when the culture reached stationary phase, followed by induction of the GFP. The addition of labeled glucose only in stationary phase ensured that only amino acids from active biosynthetic pathways would contain the labeled carbons. Analysis of GFP expressed exclusively in stationary phase allowed us to assess the fraction of each given amino acid in this protein that was newly synthesized (contained label) versus predominantly recycled from degraded proteins (unlabeled). Our results show an enrichment in labeled carbon in all amino acids above the background (1.1%), but much lower than the labeled carbon percentage in the stationary-phase medium (8.0%) (Table. 2). The unlabeled amino acids were assumed to be recycled from proteins synthesized during exponential phase, while the labeled amino acids, were assumed to be synthesized during the stationary phase. Table 2 shows the fraction of freshly synthesized amino acids during stationary phase in total GFP amino acids, calculated based on the ^{13}C enrichment (Equation 1). Alanine (45%), serine (53%), glutamate/glutamine (36%), and aspartate/ asparagine (28%) were found to have a ^{13}C enrichment that was significantly high. The rest of the amino acids had <25% ^{13}C enrichment and thus are considered to have been derived almost exclusively from the recycled amino acids from exponential phase protein. Specifically in the case of serine, there was significant labeling difference between the two growth phases (Table 1), suggesting a change in pathways during growth from exponential to stationary phase. *E. coli* is known to have two

serine synthesis pathways (i.e., Glycerate-3-P→serine; glycine→serine); and the relative activity of the two pathways are highly affected by cell growth rates [24].

CONCLUSIONS

The use of concurrent GFP induction and labeled glucose addition allowed us to pinpoint the amino acids with active biosynthesis during stationary phase. This study also confirmed that the isolated GFP, though expressed only in the stationary phase, contained both amino acids synthesized during stationary phase and recycled amino acids from protein synthesized during the exponential phase. As a result, the fluxes during stationary phase cannot be directly assessed using proteinogenic amino acids from such a sample. To perform ^{13}C based flux analysis of the stationary metabolism, free metabolites in central metabolic pathways (such as oxaloacetate) rather than amino acids should be used.

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FINANCIAL INTEREST

Jay D. Keasling has a consulting relationship with and a financial interest in Amyris and a financial interest in LS9, two biofuels companies.

Figures and Figure Captions

Figure 1. Biomass growth and glucose consumption. The symbols are •, OD_{600}; o, glucose concentration. Fully-labeled glucose was added at the end of exponential phase, 1 hour before the addition of IPTG. Note: 1. sample taken during the exponential phase; 2. addition of IPTG; 3. sample taken during late stationary phase.

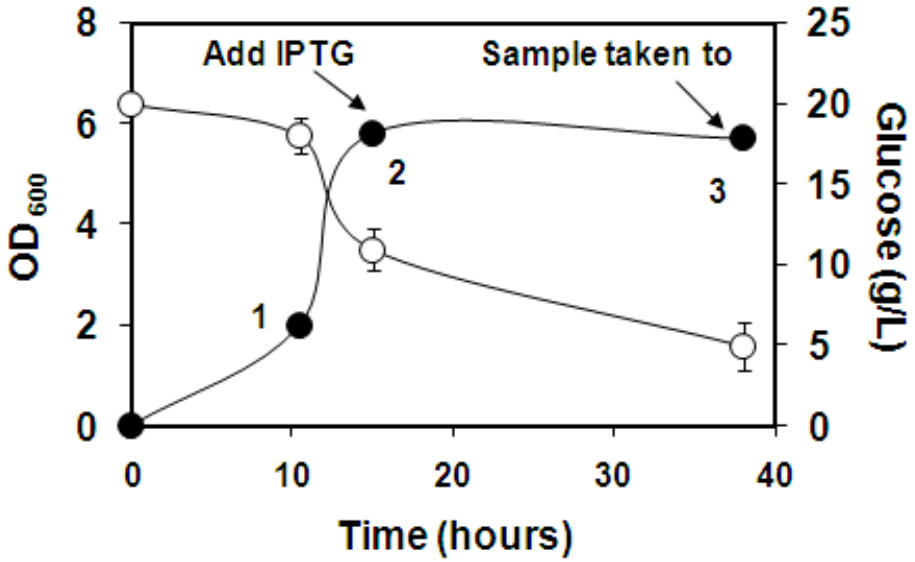

Table 1. Amino acid isotopomer analysis of proteinogenic amino acids (1-^{13}C glucose, n=2).

Amino acids		GFP stat phase		Exponential phase		Early stat phase		Late stat phase	
		57	**159**	**57**	**159**	**57**	**159**	**57**	**159**
Ala	M0	0.56	0.58	0.55	0.56	0.55	0.55	0.55	0.54
	M1	0.42	0.39	0.43	0.40	0.43	0.40	0.43	0.41
	M2	0.01	0.02	0.01	0.04	0.01	0.05	0.01	0.04
Gly	M0	0.92	0.99	1	1	0.99	0.99	0.98	0.99
	M1	0.08	0.01	0	0	0.01	0.01	0.02	0.01
Val	M0	0.37	0.35	0.31	0.29	0.31	0.30	0.31	0.30
	M1	0.49	0.46	0.48	0.43	0.50	0.43	0.51	0.43
	M2	0.15	0.17	0.19	0.17	0.19	0.18	0.17	0.18
Leu	M0	Peak overlap	0.23	Peak overlap	0.20	Peak overlap	0.20	Peak overlap	0.20
	M1		0.42		0.42		0.41		0.42
	M2		0.27		0.29		0.30		0.31
	M3		0.08		0.09		0.08		0.07
Ile	M0	Peak overlap	0.26	Peak overlap	0.23	Peak overlap	0.23	Peak overlap	0.24
	M1		0.46		0.46		0.47		0.46
	M2		0.23		0.25		0.25		0.23
	M3		0.05		0.06		0.04		0.04
Met	M0	0.24	0.28	0.21	0.27	0.21	0.27	0.21	0.27
	M1	0.39	0.39	0.40	0.41	0.38	0.41	0.40	0.41
	M2	0.25	0.25	0.26	0.22	0.29	0.21	0.26	0.23
	M3	0.10	0.06	0.10	0.08	0.08	0.08	0.08	0.07
Ser	M0	**0.46**	**0.69**	0.55	0.59	0.58	0.57	0.52	0.52
	M1	**0.49**	**0.29**	0.41	0.39	0.40	0.41	0.48	0.48
	M2	**0.04**	**0.01**	0.02	0.02	0.02	0.02	0	0
	M3	**0.01**	**0.0**	0.01	0	0.01	0.01	0	0
Phe	M0	0.29	0.26	0.25	0.26	0.26	0.25	0.27	0.26
	M1	0.40	0.45	0.46	0.43	0.47	0.44	0.45	0.43
	M2	0.30	0.24	0.23	0.25	0.23	0.24	0.23	0.22
	M3	0.02	0.04	0.03	0.05	0.03	0.03	0.04	0.04
Asp/Asn	M0	0.39	0.48	0.36	0.45	0.36	0.44	0.38	0.47
	M1	0.46	0.43	0.49	0.44	0.47	0.45	0.45	0.41
	M2	0.14	0.08	0.11	0.10	0.15	0.09	0.15	0.09
	M3	0.01	0	0.05	0	0.01	0.01	0.01	0.02
Glu/Gln	M0	0.27	0.34	0.26	0.30	0.26	0.29	0.25	0.30
	M1	0.45	0.46	0.45	0.45	0.44	0.45	0.44	0.47
	M2	0.24	0.19	0.24	0.21	0.25	0.22	0.27	0.21
	M3	0.04	0.01	0.04	0.03	0.04	0.04	0.04	0.02
His	M0	0.27	0.34	0.29	0.39	0.31	0.41	0.32	0.42
	M1	0.41	0.48	0.40	0.44	0.41	0.46	0.41	0.43
	M2	0.26	0.16	0.23	0.14	0.22	0.10	0.21	0.09
	M3	0.07	0.01	0.06	0.03	0.05	0.03	0.06	0.02
Tyr	M0	0.29	0.27	0.25	0.27	0.26	0.28	0.27	0.27
	M1	0.39	0.45	0.43	0.42	0.44	0.43	0.40	0.44
	M2	0.24	0.20	0.21	0.23	0.19	0.21	0.22	0.20
	M3	0.04	0.06	0.05	0.04	0.05	0.04	0.04	0.05

Note: The standard error for the GC-MS measurement is ~0.01; stat = stationary. M0, M1, M2… are fractions of unlabeled, singly labeled, and doubly labeled amino acids respectively.

Table 2. The use of concurrent GFP induction and labeled glucose addition to pinpoint the amino acids with active biosynthesis during stationary phase (Percentage of labeled carbon in amino acid backbone, i.e., M-57, was based on GFP amino acid labeling from stationary phase).

Amino acids	M0	M1	M2	M3	M4	Isotopic enrichment fraction (F)	% of fresh amino acids in total amino acids**
Ala	0.93	0.03	0.01	0.03	-	4.7%	45 ± 6%
Gly	0.95	0.04	0.01	-	-	3.0%	24 ± 3%
Val	0.92	0.06	0.01	0.01	0	2.2%	14 ± 2%
Leu*	0.93	0.06	0.01	0	0	1.6%	6 ± 1%
Iso*	0.95	0.04	0.01	0	0	1.3%	3 ± 1%
Met	0.91	0.09	0	0	0	1.8%	9 ± 1%
Ser	0.93	0.02	0.01	0.04	-	5.3%	53 ± 8%
Phe	0.90	0.07	0.02	0.01	0	1.6%	6 ± 1%
Asp	0.91	0.06	0.02	0.01	0	3.3%	28 ± 4%
Glu	0.87	0.07	0.05	0.01	0	4.0%	36 ± 5%
His	0.91	0.05	0.03	0.01	0	2.3%	15 ± 2%
Tyr	0.83	0.09	0.03	0.03	0.02	3.6%	31 ± 4%

*: M-57 for Leu and Iso overlapped with other amino acids, so M-159 labeling data were used in the table. **: in the stationary phase, about 8 ± 1 % of total glucose in the medium was fully labeled. Natural ^{13}C abundance is 1.1%. % of fresh amino acids in total amino acids was calculated by: (F-1.1%) / 8%.

REFERENCES

1. Becker, J.; Klopprogge, C.; Wittmann, C., Metabolic responses to pyruvate kinase deletion in lysine producing *Corynebacterium glutamicum*. *Microb Cell Fact* **2008,** 7, 8.
2. Grotkjaer, T.; Christakopoulos, P.; Nielsen, J.; Olsson, L., Comparative metabolic network analysis of two xylose fermenting recombinant *Saccharomyces cerevisiae* strains. *Metab Eng* **2005,** 7, (5-6), 437-44.
3. Luttik, M. A.; Vuralhan, Z.; Suir, E.; Braus, G. H.; Pronk, J. T.; Daran, J. M., Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: quantification of metabolic impact. *Metab Eng* **2008,** 10, (3-4), 141-53.
4. Sauer, U., Metabolic networks in motion: 13C-based flux analysis. *Mol Syst Biol* **2006,** 2, 62.
5. Stephanopoulos, G., Aristidou, AA, Nielsen, J, *Metabolic Engineering Principles and Methodologies*. San Diego: Academic Press, : 1998.
6. Sauer, U.; Lasko, D. R.; Fiaux, J.; Hochuli, M.; Glaser, R.; Szyperski, T.; Wuthrich, K.; Bailey, J. E., Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. *J Bacteriol* **1999,** 181, (21), 6679-88.
7. Ro, D. K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D., Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **2006,** 440, (7086), 940-3.
8. Atsumi, S.; Liao, J. C., Metabolic engineering for advanced biofuels production from Escherichia coli. *Curr Opin Biotechnol* **2008,** 19, (5), 414-9.
9. Iwatani, S.; Van Dien, S.; Shimbo, K.; Kubota, K.; Kageyama, N.; Iwahata, D.; Miyano, H.; Hirayama, K.; Usuda, Y.; Shimizu, K.; Matsui, K., Determination of metabolic flux changes during fed-batch cultivation from measurements of intracellular amino acids by LC-MS/MS. *J Biotechnol* **2007,** 128, (1), 93-111.
10. Toya, Y.; Ishii, N.; Hirasawa, T.; Naba, M.; Hirai, K.; Sugawara, K.; Igarashi, S.; Shimizu, K.; Tomita, M.; Soga, T., Direct measurement of isotopomer of intracellular metabolites using capillary electrophoresis time-of-flight mass spectrometry for efficient metabolic flux analysis. *J Chromatogr A* **2007,** 1159, (1-2), 134-41.
11. Bolten, C. J.; Kiefer, P.; Letisse, F.; Portais, J. C.; Wittmann, C., Sampling for metabolome analysis of microorganisms. *Anal Chem* **2007,** 79, (10), 3843-9.
12. Villas-Boas, S. G.; Hojer-Pedersen, J.; Akesson, M.; Smedsgaard, J.; Nielsen, J., Global metabolite analysis of yeast: evaluation of sample preparation methods. *Yeast* **2005,** 22, (14), 1155-69.
13. Winder, C. L.; Dunn, W. B.; Schuler, S.; Broadhurst, D.; Jarvis, R.; Stephens, G. M.; Goodacre, R., Global metabolic profiling of *Escherichia coli* cultures: an evaluation of methods for quenching and extraction of intracellular metabolites. *Anal Chem* **2008,** 80, (8), 2939-48.
14. Wittmann, C.; Kromer, J. O.; Kiefer, P.; Binz, T.; Heinzle, E., Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria. *Anal Biochem* **2004,** 327, (1), 135-9.
15. Taymaz-Nikerel, H.; de Mey, M.; Ras, C.; ten Pierick, A.; Seifar, R. M.; van Dam, J. C.; Heijnen, J. J.; van Gulik, W. M., Development and application of a differential method for reliable metabolome analysis in Escherichia coli. *Anal Biochem* **2009,** 386, (1), 9-19.

16. Shaikh, A. S.; Tang, Y. J.; Mukhopadhyay, A.; Keasling, J. D., Isotopomer distributions in amino acids from a highly expressed protein as a proxy for those from total protein. *Anal Chem* **2008,** 80, (3), 886-90.
17. Neidhardt, F. C.; Bloch, P. L.; Smith, D. F., Culture medium for enterobacteria. *J Bacteriol* **1974,** 119, (3), 736-47.
18. Tang, Y. J.; Meadows, A. L.; Kirby, J.; Keasling, J. D., Anaerobic central metabolic pathways in *Shewanella oneidensis* MR-1 reinterpreted in the light of isotopic metabolite labeling. *J Bacteriol* **2007,** 189, (3), 894-901.
19. Wahl, S. A.; Dauner, M.; Wiechert, W., New tools for mass isotopomer data evaluation in 13C flux analysis: mass isotope correction, data consistency checking, and precursor relationships. *Biotechnol Bioeng* **2004,** 85, (3), 259-68.
20. Tang, Y. J.; Martin, H. G.; Dehal, P. S.; Deutschbauer, A.; Llora, X.; Meadows, A.; Arkin, A.; Keasling, J. D., Metabolic flux analysis of *Shewanella spp*. reveals evolutionary robustness in central carbon metabolism. *Biotechnol Bioeng* **2009,** 102, (4), 1161-9.
21. Wiechert, W., 13C metabolic flux analysis. *Metab Eng* **2001,** 3, (3), 195-206.
22. Szyperski, T., 13C-NMR, MS and metabolic flux balancing in biotechnology research. *Q Rev Biophys* **1998,** 31, (1), 41-106.
23. Heinzle, E.; Yuan, Y.; Kumar, S.; Wittmann, C.; Gehre, M.; Richnow, H. H.; Wehrung, P.; Adam, P.; Albrecht, P., Analysis of 13C labeling enrichment in microbial culture applying metabolic tracer experiments using gas chromatography-combustion-isotope ratio mass spectrometry. *Anal Biochem* **2008,** 380, (2), 202-10.
24. Perrenoud, A.; Sauer, U., Impact of global transcriptional regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on glucose catabolism in *Escherichia coli*. *J Bacteriol* **2005,** 187, (9), 3171-9.