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Proteomic Response Analysis of *Escherichia coli* in Stress Environments

Yang-Hoon Kim, Kyung Yeon Han, and Jeewon Lee

Department of Chemical and Biological Engineering, Korea University, Anam-Dong 5-1, Sungbuk-Ku, Seoul 136-713, South Korea

Introduction

During the last decades, many research efforts have focused extensively on major cold-shock proteins and their expression mechanisms on the levels of transcription, mRNA stability, and translation efficiency at low temperature (1,2). Recently, understanding the cold-shock response in *Escherichia coli* is an important issue for both the development of industrial strains and the improvement of the solubility and stability of heterologous proteins overexpressed in cells in industrial scale fermentation processes (3-5). The performance of the major *E. coli* cold-shock promoter in directing the synthesis of recombinant proteins at low temperatures has been reported in batch fermentations using plasmid-encoded transcriptional gene fusion between the cspA promoter region and the lacZ gene (5). Although there have been some reports on the comprehensive view of the physiological state and responses of *E. coli* metabolism upon the cold-shock stress using transcriptomic or proteomic tools (6-9), these approaches for building a quantitative dynamic analysis and the metabolic pathway are still at an early stage of development.

In the present study, the overall protein expression pattern of the cold-shocked *E. coli* K-12 strain was studied during fed-batch fermentation through a comparative analysis of its proteomic responses to a temperature downshift from 37°C to 20°C. As for selected interesting proteins, peptide masses analyzed by automated matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometry were matched with the theoretical peptide masses of the *E. coli* ExPASy database, and their functional characteristics in the bacterial cellular metabolisms were then elucidated.

Materials and Methods

Bacterial strains and culture media

The bacterial strain employed for proteomic response analysis on cold-shock was *E. coli* K-12. The composition of semi-synthetic medium used for batch operations per liter was prepared as previously described by Lee et al. (10). All batch and fed-batch bioreactor experiments were conducted in 5-l laboratory

fermentors at pH 6.8 and 37°C, and dissolved oxygen was controlled above 40% of saturation to avoid oxygen limitation. The operating conditions of fed-batch bioreactors and data acquisition and control were the same as previously described by Lee et al.. Cells were grown to mid-exponential phase at 37°C and then were shifted to 20°C.

Sample preparations for two-dimensional electrophoresis.

During the temperature downshift culture, cells were harvested at the different time courses and adjusted to OD_{600} at 0.8 with 10 mM Tris buffer (pH 8.0). Cell pellets were resuspended in lysis buffer (8 M urea, 4% w/v CHAPS, 40 mM Tris, and protease inhibitor cocktail solution. After sonication for 2 min on ice, the cell debris was removed by centrifugation at 25750 × g for 60 min. 700 g proteins were resuspended in rehydration solution (8 M urea, 2% w/v CHAPS, 0.005% w/v bromophenol blue; final volume, 350 l) for two-dimensional electrophoresis (2-DE).

2-DE and image analysis

The first step of 2-DE, IEF was performed on a Pharmacia Biotech IPGphor Electrophoresis System at 20℃. The samples were loaded with intracellular protein concentrations of 700 g for coomassie brilliant blue stained gels with Seeblue plus2 marker (Invitrogen, ca, USA). IEF was performed for 2 h at 500 V, for 0.5 h at 1000 V, for 0.5 h at 2000 V, for 0.5 h at 4000 V, and finally maintained 70000 V·h at 8000 V in the IPGphor for analytical and preparative gels. Equilibrated gel strips were placed on a 12.5% polyacrylamide gel, and the second dimensional separation was carried out using a PROTEAN II Xi cell system (Bio-rad, Ca, USA) in a cold chamber at 4℃. Coomassie brilliant blue stained gels were scanned using UMAX powerlook 1100 scanner. ImageMaster software v 4.01 (Amersham Pharmacia Biotech, NJ, USA) was used for the gel image analysis including quantification of the spot intensities that is done on a volume basis.

MALDI-TOF mass spectrometric analysis and protein identification

MALDI-TOF mass spectrometry analysis was carried out as the previous protocol (11). Protein analyses were performed using MALDI-TOF mass spectrometry system (Voyager DE-STR, PE Biosystem, Framingham, MA). Spectra were calibrated using a matrix and tryptic autodigestion ion peaks as internal standards. Peptide mass fingerprints were analyzed using the MS-Fit (http://prospector.ucsf.edu/). The accessibility of such data has been revolutionized by the use of protocols such as SWISS-2D-PAGE (http://www.expasy.org/).

Results And Discussion

Proteome profiling of *E. coli* fed-batch culture to low temperature.

Prior to 2-DE analysis, *E. coli* K-12 was cultivated in a batch bioreactor (5 l), and was shifted from 37℃ to 20℃ in the middle of the exponential growth phase. After the temperature downshift, the growth of the cell ceased for about 4 h, after which the bacteria reinitiated exponential growth, but at a lower rate. As shown in Fig. 1, the culture broths of each in the 37℃ control culture (N) and the low-temperature culture (C) were harvested after 4, 9, and 15 h of temperature downshift. For each protein sample, 2-D

SDS-PAGE was repeated 4-8 times, and after coomasie blue-staining, an average 2-DE gel image was constructed for comparative image analysis using the ImageMaster software. Approximately, 350 spots were visualized on every average gel image, and the 2-DE gel images at 37℃ and 20℃ were systematically compared at each time point. In the present study, to obtain significant candidates of stress-related proteins, the results focused on the following proteomic behavior of low temperature: (1) the synthetic levels of 26 proteins were significantly induced after the temperature downshift, and the high levels were maintained during the whole period of low-temperature growth; and (2) 31 proteins were significantly repressed for a prolonged time after the temperature downshift from 37℃ to 20℃ occurred. Thus, these proteins would be useful as reliable marker proteins for the assessment of cold-stress response on the low culture temperature. The time-course variation of each protein was relatively estimated using the spot intensity at the N4 phase as 1 (Table 1). The different expression ratios above 1 indicate induction, and those below 1 indicate repression. The MALDI-TOF analysis for each protein spot and the subsequent peptide mass finger-printing using the *E. coli* ExPASy database enabled identification, as listed in Table 1, and those in the list are involved in energy metabolisms, several cellular molecule biosynthetic pathways and catabolism, cell processes, flagellar biosynthesis and motility, and protein translation and folding.

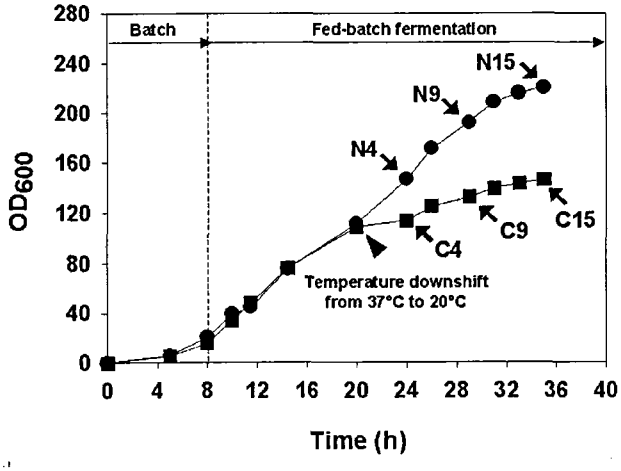

Fig. 1 Time profiles of cell growth during fed-batch cultures.
●, 37℃ control culture; ■, temperature downshift (37℃→20℃) culture. Culture samples were taken from the bioreactor at three different growth phases (4 h, 9h, and 15h) after temperature downshift.

Energy metabolisms

Uncontrolled or deregulated metabolic pathways by cellular stresses lead to metabolic imbalance and unoptimal productivity (6,7), the alternation of metabolic enzyme synthesis could disturb the balanced metabolite flux and might exert a significant burden on optimal cellular growth (12). Although cell growth was arrested by the strong repression of the metabolic activity of the cell, as shown in Fig. 1, the present study showed induction of several proteins of energy metabolism after the temperature downshift compared with proteome profiles in the 37℃ control culture (Fig. 2). Interestingly, the protein level of pyruvate kinase (PykF) catalyzing the conversion of phosphoenolpyruvate into pyruvate was extremely upregulated during the whole period in a cold environment. It was also observed that the synthesis levels of isocitrate dehydrogenase (Icd), malate dehydrogenase (Mdh), and Succinyl-CoA synthetase alpha chain (SucD),

which play important roles as key metabolic enzymes in the tricarboxylic acid (TCA) cycle, was highly promoted in the low-temperature culture. The increased synthesis of glycolytic proteins and TCA-cycle-related enzymes might be caused by the cellular effort to overcome the metabolic imbalance in cellular pathways, discussed above.

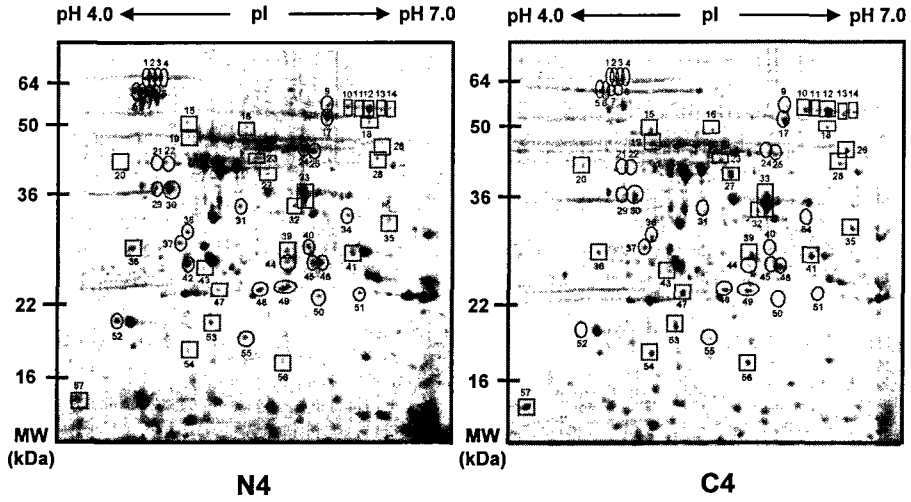

Fig. 2 Coomassie blue-stained 2-D SDS-PAGE gel showing 57 protein spots at 4 h after temperature downshift listed in Table 1. (The rectangular indicated induced proteins upon temperature downshift from 37℃ to 20℃, respectively. The circular indicated repressed proteins upon temperature downshift from 37℃ to 20℃.).

Amino acid biosynthesis and degradation

Through a comparative proteome analysis of *E. coli* fed-batch culture, it was observed that the expression level of 18 metabolic enzymes, which were involved in the amino acid biosynthesis and degradation, significantly changed after a temperature downshift, as shown in Table 1 and Fig. 2. Nine metabolic enzymes out of the 18 proteins were highly induced all the way during the whole period of low-temperature growth. The synthesis level of key enzymes for the biosynthesis of lysine, DapE, DapB and DapF, were strongly increased in low-temperature growth. In contrast, consistently, it was found that three key enzymes for histidine biosynthesis were significantly repressed probably to decrease the conversion of histidine, if any. In this regard, the analyses in this paper strongly imply that the alternation of these enzymes, which were involved in amino acid biosynthesis and degradation, might result from the cellular effort to overcome the amino acid imbalance, and that there must be more favorable conditions to keep up the level of cellular metabolisms by cold-shock stress during low-temperature cultivation.

Protein translation and folding

In the present study, two major molecular chaperones, heat shock protein Hsp70 (DnaK) and 60 kDa chaperonin (GroEL), which are key factors for the structural flexibility of proteins and for cell viability, were significantly downregulated in a cold condition, as shown in Fig. 2 and Table 1 (spot 1 and 2, and spot 5-8, respectively). DnaK is a major, well-studied protein of the heat shock response. Expression of DanK or

molecular chaperones in general, however, has not been identified in the cold-shock response (13,14). Previous studies have shown that the cellular content of GroEL decreases as growth temperature decreases, and that the overproduction of heat shock proteins (DnaK, GroEL, and GroES) generally reduced the cell viability in low temperature, while a trigger factor (TF) increased the enhance viability of *E. coli* at 4℃ (15). The effects of TF on GroEL-dependent protein degradation and on GroEL's binding to unfolded proteins were much greater when cells were grown at 20℃ than at 37℃ (16). On the other hand, it was also observed that the synthesis of 30S ribosomal protein S1 (RpsA), which is needed to translate mRNA, was significantly inhibited after a temperature downshift. Interestingly, DnaK chaperone affect ribosome biogenesis in vivo (17), and the purified proteins, have been shown to facilitate 30S ribosomal subunit assembly (18). Thus, the results of this study seem to presume that the alternated level of DnaK and RpsA by temperature downshift will be strongly reflected in the formation of the 30S ribosomal subunit assembly and the protein translation machinery. On the other hand, since some of the proteins that are induced during cold shock are involved in successful protein folding, the response may prepare cells to reinitiate protein synthesis during exposure to cold temperature (19). This study revealed that the synthesis of peptidyl-prolyl cis-trans isomerase (PpiB) was strongly activated all the way during the whole period of low-temperature growth. It is well known that PpiB is important for helping protein folding or degradation; it could also be important for the adaptation of *E. coli* at low temperatures (3,15).

Flagellar biosynthesis and motility

Many bacteria swim by using flagella, helical propellers driven by a reversible rotary motor in the cell membrane. Rotation is powered by a transmembrane gradient of ions, usually protons (20). It has been known that flagellar proteins are transiently induced following heat shock, salt, and acid stress of limitation of glucose (20,21). It was observed that three major components of the flagellar biosynthesis and motility system were highly induced after a temperature downshift, namely: flagellin (FliC), flagellar assembly protein (FliH), and flagellar motor switch protein (FliN). The ATP synthase alpha chain (AtpA) was also more strongly activated at 20℃ than at 37℃. If the change in AtpA is indicative of ATP synthase activity, this may indicate that ATP generation and proton import are simultaneously induced at a low temperature. According to previous reports, the increase of the proton motive force strongly drives the flagellar rotation (20). On the other hand, it is surprising to note that the synthesis levels of S-ribosylhomocysteinase (LuxS), which was involved in the synthesis of autoinducer-2 (AI-2) from *E. coli*, was significantly activated after the temperature downshift. It was reported that the luxS/AI-2 quorum-sensing system activates the expression of genes involved in the assembly of flagellar and motility through the activation of flhDC transcription (22). Taken together, global upregulation of flagellar proteins, ATP synthase, and LuxS by cold-stress response might play a major role in flagellar biosynthesis, mobility, and transport of *E. coli* K-12.

References

1. Etchegaray JP, Inouye M (1999) J Bacteriol 181: 1827-1830

2. Yamanaka K, Inouye M (2001) Genes Cells 6: 279-290
3. Phadtare S, Inouye M (2004) J Bacteriol 186: 7007-7014
4. Qing G, Ma LC, Khorchid A, Swapna GVT, Mal TK, Takayama MM, Xia B, Phadtare S, Ke H, Acton T, Montelione GT, Ikura M, Inouye M (2004) Nat Biotechnol 22: 877-882
5. Vasina JA, Peterson MS, Baneyx F (1998) Biotechnol Progr 14: 714-721
6. Durrschmid K, Marzban G, Durrschmid E, Striedner G, Clementschitsch F, Cserjan-Puschmann M, Bayer K (2003) Electrophoresis 24: 303-310
7. Kim YH, Park JS, Cho JY, Cho KM, Park YH, Lee J (2004) Biochemical J 381: 823-829
8. Perrot F, Hebraud M, Junter GA, Jouenne T (2000) Electrophoresis 21: 1625-1629
9. Polissi A, Laurentis WD, Zangrossi S, Briani F, Longhi V, Pesole G, Deho G (2003) Res Microbiol 154: 573-580
10. Lee J, Saraswat V, Koh I, Park YH, Rhee SK (2001) FEMS Microbiol Lett 295: 127-132
11. Majoul T, Bancel E, Triboi E, Hamida JB, Branlard G (2003) Proteomics 3: 175-183
12. Kumar JK, Tabor S, Richardson CC (2004) Proc Natl Acad Sci USA 101: 3759-3764
13. Jones PG, Cashel M, Glaser G, Neidhardt FC (1992) J Bacteriol 174: 3903-3914
14. Lelivelt MJ, Kawula TH (1995) J Bacteriol 177: 4900-4907
15. Kandror O, Goldberg AL (1997) Proc Natl Acad Sci USA 94: 4978-4981
16. Kandror O, Sherman M, Rhode M, Goldberg AL (1995) EMBO J 14: 6021-6027
17. Charollais J, Dreyfus M, Iost I (2004) Nucleic Acids Res 32: 2751-2759
18. Maki JA, Schnobrich DJ, Culver GM (2002) Mol Cell 10: 129-138
19. Thieringer HA, Jones PG, Inouye M (1998) BioEssays 20: 49-57
20. Maurer LM, Yohannes E, Bondurant SS, Radmacher M, Slonczewski JL (2005) J Bacteriol 187: 304-319
21. Tang H, Billings S, Wang X, Sharp L, Blair DF (1995) J Bacteriol 177: 3496-3503
22. Sperandio V, Torres AG, Kaper JB (2002) Mol Microbiol 43: 809-821

Table. 1 The 57 proteins involved in 350 proteins showing significantly prolonged difference in intracellular protein level (i.e. spot intensity) during the whole period of low temperature growth

Spot No.	Gene name	Protein name	Accession number[a]	% Sequence Coverage[b]	Protein level ratio (fold change)[c]					
					N4/N4	N9/N4	N15/N4	C4/N4	C9/N4	C15/N4
Energy metabolism										
ATP-proton motive force interconversion										
10	pntA	NAD(P) transhydrogenase subunit alpha	P07001	78.8	1	1.2	0.9	1.2	3.1	3.2
11	atpA	ATP synthase alpha chain	P00822	75.8	1	1.4	1.3	2.2	3.1	8.2
Glycolysis										
12	lpdA	Dihydrolipoyl dehydrogenase	P00391	70.6	1	1.1	1.2	2.3	2.4	2.8
18	pykF	Pyruvate kinase I	P14178	75.4	1	0.05	0.01	3.1	8.1	8.5
45	tpiA	Triosephosphate isomerase	P04790	74.8	1	1	1.3	0.4	0.1	0.1
50	eda	KDPG aldolase	P10177	74.3	1	1	1	0.01	0.05	0
Pentose phosphate pathway										
9	zwf	Glucose-6-phosphate dehydrogenase	P22992	75.4	1	1.2	1.2	0.2	0.3	0.4
TCA										
13	citF	Citrate lyase alpha chain	P75726	75.3	1	0.1	0.05	2.5	3.1	9.2
14	citF	Citrate lyase alpha chain	P75726	76.1	1	1.1	1.2	2.3	6.5	13.1
19	icd	Isocitrate dehydrogenase [NADP]	P08200	73.8	1	7.7	4.9	7.3	9.1	16.6
33	mdh	Malate dehydrogenase	P06994	71.5	1	0.6	0.6	1.5	1.8	1.9
35	sucD	Succinyl-CoA synthetase alpha chain	P07459	57.3	1	1.1	1	6.2	11.2	11.1
Glyconeogenesis										
24	sdaB	L-serine dehydratase 2	P30744	72.5	1	1	1	0.3	0.2	0
Glyoxylate bypass cycle										
34	glxR	2-hydroxy-3-oxopropionate reductase	P77161	74.6	1	1.3	1.3	0.2	0.3	0.2
52	allA	Ureidoglycolate hydrolase	P77731	56.9	1	1.3	1.4	0.05	0.2	0.1
Cell process										
Chaperones										
1	dnaK	Heat shock protein Hsp70	P04475	72.9	1	4.1	5.2	0.05	0.35	0.05
2	dnaK	Heat shock protein Hsp70	P04475	75.2	1	3.5	3.2	0.3	0.41	0.2
5	groEL	60 kDa chaperonin	P06139	70.7	1	1.5	1.5	0.5	0.2	0.05
6	groEL	60 kDa chaperonin	P06139	69.8	1	1.2	1.2	0.7	0.3	0.2
7	groEL	60 kDa chaperonin	P06139	72.3	1	1.2	1.1	0.12	0.05	0.07
8	groEL	60 kDa chaperonin	P06139	67.1	1	1.1	1.2	0.3	0.05	0.01
56	ppiB	Peptidyl-prolyl cis-trans isomerase B	P23869	76.6	1	2.1	1.9	4.2	9.3	11.2
Marcomolecules										
***Peptidoglycan* biosynthesis**										
38	murI	Asp/Glu racemase	P22634	68.7	1	1.6	3.1	0.6	0.05	0.08
Protein translation										
3	rpsA	30S ribosomal protein S1	P02349	75.4	1	2.5	3.3	0.4	0.6	0.3
4	rpsA	30S ribosomal protein S1	P02349	72.2	1	2.5	2.4	0.4	0.2	0.1
Cell motility										
20	fliC	Flagellin	P04949	58.8	1	1	1	2.1	7.2	7.1
36	fliH	Flagellar assembly protein FliH	P31068	75.3	1	1.3	1.5	1.3	5.2	6.1
57	fliN	Flagellar motor switch FliN protein	P15070	75.8	1	0.3	0.9	2.2	4.2	4.5
Unknown protein										
55	yjfY	Hypothetical protein yjfY [Precursor]	P39307	57.1	1	1.5	1.5	0.2	0.2	0.1

Spot No.	Gene name	Protein name	Accession number[a]	% Sequence Coverage[b]	Protein level ratio (fold change)[c]					
					N4/N4	N9/N4	N15/N4	C4/N4	C9/N4	C15/N4
Small molecule biosynthesis and degradation										
Amino acid biosynthesis										
16	glnA	Glutamine synthetase	P06711	75.1	1	0	0	3.3	4.2	4.8
22	hisC	Histidinol-phosphate aminotransferase	P06986	70.1	1	2.1	7.5	0.5	0.9	0.8
23	proA	Gamma-glutamyl phosphate reductase	P07004	57.4	1	0.9	0.8	1.5	3.1	3.5
26	serA	D-3-phosphoglycerate dehydrogenase	P08328	68.8	1	0.4	0.5	10.1	9.5	18.4
27	dapE	Succinyl-diaminopimelate desuccinylase	P24176	76.6	1	1.2	1.4	12.3	17.3	19.5
28	carA	Carbamoyl-phosphate synthase small chain	P00907	75.8	1	0.4	0.2	1.7	2.5	2.7
29	ybiK	Putative L-asparaginase [Precursor]	P37595	79.8	1	1.3	2.1	0.2	0.3	0.2
31	cysM	Cysteine synthase B	P16703	78.8	1	1.5	2.3	0.05	0	0.2
37	trpA	Tryptophan synthase alpha chain	P00928	65.6	1	2.1	4.4	0.8	0.1	0.2
39	dapB	Dihydrodipicolinate reductase	P04036	74.2	1	3.3	2.5	3.2	5.5	7.3
40	cysH	Phosphoadenosine phosphosulfate reductase	P17854	69.5	1	1.2	1.3	0.2	0.2	0.01
41	dapF	Diaminopimelate epimerase	P08885	60.5	1	1	0.5	1.8	2.2	3.8
42	hisA	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	P10371	59.1	1	1.1	1.5	0	0	0
46	proC	Pyrroline-5-carboxylate reductase	P00373	75.5	1	1.2	1.4	0.7	0.2	0.4
47	aroD	3-dehydroquinate dehydratase	P05194	75.8	1	1.2	1.7	9.3	14.2	13.4
48	hisH	Imidazole glycerol phosphate synthase subunit hisH	P10375	76.5	1	1.5	2.4	0.8	0.05	0.3
49	argB	Acetylglutamate kinase	P11445	79.1	1	1	1.1	0.4	0.03	0.2
54	folA	Dihydrofolate reductase	P00379	78.4	1	1.2	1.8	8.1	21.1	20.5
Amino acid degradation										
15	aspA	Aspartate ammonia-lyase	P04422	73.3	1	0.5	0	4.1	8.3	13.6
17	gabD	Succinate-semialdehyde dehydrogenase	P25526	57.3	1	1.1	1.2	0.8	0.4	0.3
21	garK	Glycerate kinase 2	P23524	69.9	1	2.5	8.2	0.6	0.9	0.8
25	aspC	Aminotransferase	P00509	75.4	1	1.2	1.2	0.5	0.05	0.1
30	glyQ	Glycyl-tRNA synthetase alpha chain	P00960	78.4	1	1.1	1.3	0.6	0.5	0.4
32	tdh	L-threonine3-dehydrogenase	P07913	59.2	1	1	1	1.3	1.6	3.6
Fatty acid metabolism										
43	atoD	Acetate CoA-transferase alpha subunit	P76458	57.8	1	1.3	2.1	1	7.3	9.5
51	atoA	Acetate CoA-transferase beta subunit	P76459	70.2	1	2.3	2.8	0	0	0
Regulatory function										
53	luxS	S-ribosylhomocysteinase	P45578	71.1	1	0.5	1.1	4.5	8.9	7.4
Cell survival										
44	surE	Survival protein SurE	P36664	74.4	1	0.9	1.4	0.1	0.05	0.05