COMMUNICATION TO THE EDITOR

Proteome Analysis to Assess Physiological Changes in *Escherichia coli* Grown Under Glucose-Limited Fed-Batch Conditions

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Abstract: Proteome analysis was used to compare global protein expression changes in *Escherichia coli* fermentation between exponential and glucose-limited fed-batch phase. Two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry were used to separate and identify 49 proteins showing >2-fold difference in expression. Proteins upregulated during exponential phase include ribonucleotide biosynthesis enzymes and ribosomal recycling factor. Proteins upregulated during fed-batch phase include those involved in high-affinity glucose uptake, transport and degradation of alternate carbon sources and TCA cycle, suggesting an enhanced role of the cycle under glucose- and energy-limited conditions. We report the upregulation of several putative proteins (*ytfQ*, *ygiS*, *ynaF*, *yggX*, *yfeX*), not identified in any previous study under carbon-limited conditions.
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INTRODUCTION

Escherichia coli fermentations are widely used in the biotechnology industry for the production of various recombinant products (Yee and Blanch, 1992). However, relatively few studies have looked at global *E. coli* responses to carbon limitation under fed-batch (Yoon et al., 2003) and continuous culture (Hua et al., 2004; Wick et al., 2001) conditions. Most previous studies on *E. coli* response to carbon availability were performed in shake flask cultures (Blum et al., 1990; Chuang et al., 1993; Groat and Matin, 1986; Nystrom, 1994; Schultz and Matin, 1991; Weichart et al., 1993) where cells primarily grow under saturating nutrient levels and are transiently nutrient-limited for only a brief period, before complete nutrient deprivation. Thus, for understanding the regulatory differences in gene expression between starvation and "hunger" responses, it is necessary to perform studies under controlled nutrient-limited environments (Ferenci, 1996, 1999).

In this study, we used proteome analysis [two-dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry] to evaluate qualitative and quantitative differences in protein expression between exponential and fed-batch phase in glucose-limited *E. coli* fermentations. Under glucose limitation, we observed the upregulation of many genes that have only been reported at the transcript level in earlier global gene expression studies. We also report the increased expression of several genes which, until now, have not been associated with carbon-limited conditions. These include genes involved in carbon compound metabolism (*fucI*, *cpdB*, *add*, *garR*), oligopeptide transport (*mppA*, *oppF*), cell processes (*pspA*, *arcA*), and many genes with putative/unknown functions (*ytfQ*, *ygiS*, *ynaF*, *yggX*, *yfeX*).

MATERIALS AND METHODS

Details available at http://www.umbc.edu/proteome

Fermentation

Overnight cultures (OD_{600}~1.0) of wild type *E. coli* K12 strain W3110 [F^-, IN(*rrnD*-*rrnE*) *rph*-1, λ^-] in LB media were used to inoculate (0.1% v/v) glucose (5 g/L) minimal media (same as fermentation). At OD_{600}~1.0, this culture was used to inoculate (0.5% v/v) the fermentors. Media was adapted from (DeLisa et al., 1999) and prepared according to (Riesenberg et al., 1991). Fermentations were conducted in 20 L BioFlow IV fermentor (New Brunswick Scientific Co., Inc., Edison, NJ) with 10 L working volume (all media components, except glucose, added for 12 L), at 37°C, 1 bar head pressure, 600 rpm agitation, 1 vvm air supply and controlled pH of 6.8; antifoam was added as needed.

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2-DE

Intracellular protein samples were prepared according to http://www.expasy.org with the following modifications. In lysis buffer, 0.5 mM DTT was used instead of DTE and 1.5 mM PMSF for Pefabloc SC. Nucleic acids were digested with nuclease buffer [Tris-HCl buffer pH 8.0 0.5M, $MgCl_2$ 50 mM, Dnase I 1.0 mg/mL, Rnase A 0.25 mg/mL (Worthington Biochemical Corporation, Lakewood, NJ)]. Protein samples were solubilized overnight in sample buffer (SB) [Tris-HCl buffer pH 8.0 67 mM, Urea 7M, Thiourea 2M, CHAPS 4% w/v, DTT 1% w/v, IPG buffer 3–10 NL 2% v/v, Bromophenol Blue (BPB)] and diluted with 100 μL rehydration buffer [SB with 10% v/v Glycerol instead of Tris-HCl buffer]. Samples were in-gel rehydrated in 18 cm pH 3–10 NL IPG strips [350 μL per strip with 100 μg (analytical) or 400 μg (preparative) protein] in reswelling tray for 16 h and focused in Multiphor II (analytical) or IPGPhor (preparative) apparatus (equipment and reagents from Amersham Biosciences, Piscataway, NJ) at 20°C, for a total of ~30–35 kVh, according to manufacturer's instructions. Focused strips were equilibrated in buffer [Tris-HCl buffer pH 8.8 50 mM, Urea 6M, Glycerol 30% v/v, SDS 2% w/v, BPB], for 20 min with 1% w/v DTT, followed by 20 min with 2.5% w/v Iodoacetamide; loaded on slab gels and overlaid with 0.5% w/v agarose. Large-format (18.5 × 19 cm), 1.5 mm thick, 12% T/2.67% C, continuous Tris-HCl linear gradient gels, with PDA as crosslinker, were cast using Multi-gel casting chamber (Bio-Rad Laboratories, Hercules, CA), according to manufacturer's instructions. SDS–PAGE separation was performed at constant current (12 mA/gel for 45 min, 30 mA/gel until end of run), at 12°C using PROTEAN II XL electrophoresis cell (Bio-Rad laboratories) according to manufacturer's instructions.

Gels were silver stained according to protocol adapted from (Blum et al., 1987) and (Shevchenko et al., 1996). Stained gels were digitized using GS-800 imaging densitometer (Bio-Rad Laboratories) and the images were analyzed using Melanie 3.0 (Genebio S.A., Geneva, Switzerland). For each sample, three gels were pair-wise matched and composite gels containing spots common to all three gels were created. Composite gels were then compared to determine quantitative differences in protein expression between samples. Spot quantity was expressed in vol% (volume of a spot/total volume of all the spots in a gel) to minimize error due to staining variations.

MALDI-TOF

Gel pieces were destained according to (Gharahdaghi et al., 1999). In-gel digestion protocol was adapted from (Shevchenko et al., 1996), with prolonged extraction steps. Peptide samples were desalted using C18 zip-tips (Millipore Corporation, Billerica, MA), according to manufacturer's instructions. Peptides were analyzed in saturated α-cyano-4-hydroxycinnamic acid solution in 70% acetonitrile/0.1% trifluoroacetic acid using Autoflex series MALDI-TOF (Bruker Daltonics, Inc., Billerica, MA) in linear, positive mode. Spectra were calibrated, externally using Leu-5-enkephalin and Insulin B oxidized chain and, internally using trypsin autolytic peak–[MH+] m/z 2212.42. Proteins were identified through peptide mass fingerprinting in NCBInr *E. coli* database using ProFound database search engine (Genomic Solutions, Ann Arbor, MI). Criteria for confident identification were that the protein should (a) be highlighted by ProFound, (b) match within 0.05% peptide mass tolerance, (c) have at least 20% sequence coverage, and (d) match at least four peptides; except few high and low MW proteins which satisfied only three out of four criteria–(a), (b) and either (c) or (d), respectively.

RESULTS AND DISCUSSION

Fermentation

Fed-batch biomass and specific growth rate plots are shown in Figure 1a. Initial glucose (5 g/L) was consumed in ~12 h, after which glucose was fed at ~30 g/h based on specific glucose consumption rate at the end of batch phase (Fig. 1b). Glucose was limiting (<10 mg/L) for ~6 h, until other media components became limiting. Dissolved oxygen was non-limiting (>60%) throughout the fermentation (Fig. 1b). Multiple fermentations were run to ensure biological reproducibility and to estimate variations in protein expression from experimental differences. Fermentations were reproducible with matching biomass growth [$\mu_{Batch} = 0.66 \pm 0.03\ h^{-1}$] and glucose consumption profiles.

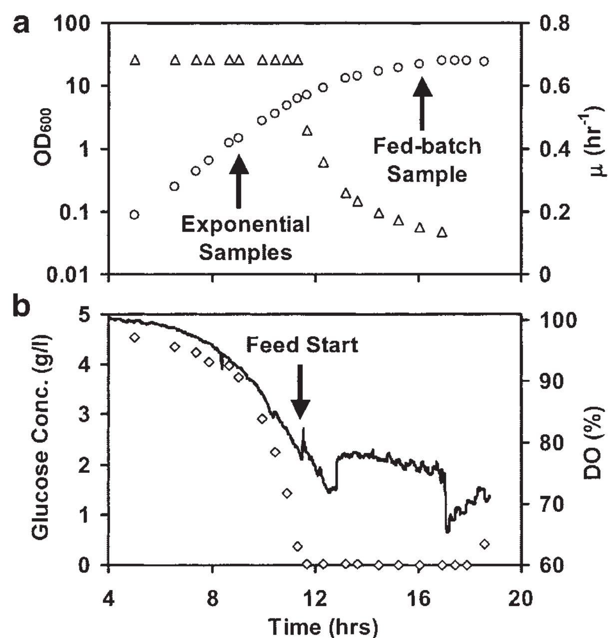

Figure 1. **a**: Optical density at 600 nm (OD_{600}) and specific growth rate (μ, h^{-1}) are plotted against fermentation time (h). Arrows indicate sample harvest times for proteome analysis. **b**: Glucose concentration (g/L) and dissolved oxygen (DO, %) are plotted against fermentation time (h). Arrow indicates glucose feed start time.

Proteome Analysis

Reproducibility of proteome methods was tested as follows (data available at http://www.umbc.edu/proteome). We compared replicate gels run from the same sample to estimate run-to-run variability in protein expression. We found that 93% of spots were matched within a differential expression range of 0.5–2.0. In addition, we compared exponential phase gels from multiple fermentations to estimate batch-to-batch variability in protein expression. In this case, 91% of the spots were matched within a two-fold differential expression ratio. Based on these studies, only spots with >2-fold difference in protein expression were considered as significantly up- or down-regulated.

In this study, 2-D gels of exponential and fed-batch phase protein samples were compared. We found 60 protein spots with significantly higher expression (>2-fold) under glucose limitation, and 30 spots with higher expression in exponential phase. Out of these, 57 spots (fed-batch, 47; exponential, 10) were identified using MALDI-TOF mass spectrometry, and the proteins were classified based on their physiological functions according to GenProtEC (Serres et al., 2004) (Tables I and II). Interestingly, 14% of identified spots were isoforms of other proteins (GlcB, GatZ, TnaA, GatY, OppA). Spots of the same protein had similar molecular weight with differing pI, suggesting isoforms with potential post-translational modifications (Lee and Lee, 2003). Some protein spots (TnaA, MglA) appeared only on fed-batch gels and were not detectable on exponential phase gels (data available at http://www.umbc.edu/proteome).

Although 2-DE is a powerful technique capable of providing a global snapshot of the cellular responses to various external stimuli at the protein level, it is not possible to analyze the entire proteome. In this study, we detected approximately 1,000 protein spots on each 2-D gel, which represents only ~25% of the *E. coli* proteome. This limitation is evident in the case of several upregulated operons (*gatYZABCD*, *fadBA*, *mglBAC*, *oppABCDF*, *glcDEFGBA*) where not all proteins were identifiable on the 2-D gel (Table II). Some of these are membrane proteins (*gatC*, *mglC*, *oppB*, *oppC*) and hence likely not detected using our protocol optimized for cytoplasmic proteins. Also, a two-fold difference in expression is not required to effect changes in biological functions or processes, although it minimizes the occurrence of false positives. Thus, some up- or down-regulated proteins would have been missed in our analysis due to the conservative two-fold differential expression threshold. Therefore, it is very unlikely that all the genes of a pathway or operon/regulon, affected by the imposed condition, will be detected using the 2-DE approach. Hence, in the following discussion, genes upregulated were taken to imply the potential operation of pathways or activation of the operon/regulon.

Genes Upregulated in Exponential Phase

Genes upregulated in exponential phase include (ribo)nucleotide biosynthesis enzymes, a translational factor, and amino acid biosynthetic enzymes, among others (Table I).

Growth rate dependent regulation of many of the upregulated pyrimidine (*carA*, *pyrB*, *pyrI*, *pyrD*) and purine (*guaB*) nucleotide biosynthetic enzymes has been reported at the transcriptome level (Yoon et al., 2003). In this study, there was a four-fold difference in growth rate between exponential [μ_{Batch} 0.66 h^{-1}] and fed-batch [$\mu_{Fed\text{-}batch}$ 0.15 h^{-1}, Fig. 1a] phase. Upregulation of *carA*, coupled with increased expression of *pyrBI* and *pyrD* genes suggests the channeling of the common intermediate, carbamoyl phosphate, towards pyrimidine nucleotides (CTP, UTP) than arginine biosynthesis (Neuhard and Kelln, 1996).

Ribosome recycling factor (*rrf*) catalyzes the disassembly of ribosome–mRNA–tRNA post termination complex and has been shown to stimulate protein synthesis in vitro (Ryoji et al., 1981). Hence, its upregulation during exponential growth is likely for enabling rapid protein synthesis.

Table I. Proteins showing significantly higher expression (>2-fold) in exponential phase as compared to fed-batch phase, identified using MALDI-TOF mass spectrometry.

Sub-functional groups	Gene name/ b number	Protein ID	Peptides matched	Sequence coverage	Log (fed-batch/ exponential)
		Nucleotide biosynthesis			
Pyrimidine	*carAb0032*	Carbamoyl phosphate synthetase	12	49%	−0.34
	pyrDb0945	Dihydro-orotate dehydrogenase	11	35%	−0.31
	pyrBb4245[a]	Aspartate carbamoyltransferase catalytic chain	8	25%	−0.37
	pyrIb4244[a]	Aspartate carbamoyltransferase regulatory chain	7	61%	−0.39
Purine	*guaBb2508*	IMP dehydrogenase	16	46%	−0.38
		Translational apparatus			
	rrf/b0172	Ribosome recycling factor	10	63%	−0.42
		Amino acid biosynthesis			
	trpD/b1263	Anthranilate synthase component II	15	40%	−0.34
	metE/b3829	Tetrahydropteroyltriglutamate methyltransferase	15	16%	−0.36
		Cell processes and global regulation			
	tolB/b0740	TolB protein [precursor]	11	37%	−0.31
	fliC/b1923	Flagellin	14	40%	−0.43

[a]Belong to *p* operon.

Table II. Proteins showing significantly higher expression (>2-fold) in fed-batch phase as compared to exponential phase, identified using MALDI-TOF mass spectrometry.

Sub-functional groups	Gene name/ b number	Protein ID	Peptides matched	Sequence coverage	Log [fed-batch/ exponential]
		Carbon compound utilization			
Carbohydrate degradation	*gatY/b2096*[b,e]	Tagatose bisphosphate aldolase 1	>8	>24%	>0.54
	gatZ/b2095[a,e]	Putative tagatose-6-phosphate kinase	>20	>54%	>0.44
	fucI/b2802	L-fucose isomerase	11	20%	0.52
Fatty acid degradation	*fadA/b3845*[f]	Acetyl-CoA transferase	11	40%	0.32
	fadB/b3846[f]	Fatty oxidation complex alpha subunit	23	37%	0.89
Amino acid degradation	*dadX/b1190*	Alanine racemase, catabolic	7	20%	0.66
	tnaA/b3708[a]	Tryptophanase	>18	>46%	Infinity[d]
		Transport and binding proteins			
Carbohydrate transport-PTS dependent	*gatA/b2094*[e]	PTS system, galactitol-specific IIA component	7	69%	0.54
	gatB/b2093[e]	PTS system, galactitol-specific IIB component	5	63%	0.70
	manX/b1817	PTS system, mannose-specific IIAB component	11	47%	0.47
Carbohydrate transport-binding protein dependent	*rbsB/b3751*	D-Ribose binding protein	15	59%	0.64
	malK/b4035	Maltose/maltodextrin transport ATP-binding protein	7	31%	0.70
	mglA/b2149[g]	Galactoside transport ATP-binding protein	6	15%	Infinity[d]
	mglB/b2150[g]	D-Galactose D-glucose binding protein	10	42%	0.54
Amino acid and oligopeptide transport	*argT/b2310*	Lysine-, arginine-, omithine-binding periplasmic protein	16	68%	0.39
	oppA/b1243[c,h]	Periplasmic oligopeptide binding protein (precursor)	>9	>23%	>0.35
	oppF/b1247[h]	Oligopeptide transport ATP-binding protein	16	50%	0.54
	mppA/b1329	Periplasmic murein peptide-binding protein precursor	15	31%	0.48
Putative transport	*ytfQ/b4227*	Putative D-ribose transport periplasmic binding protein	9	36%	0.89
	ygiS/b3020	Putative oligopeptide transport periplasmic binding protein	10	27%	0.51
		Central intermediary metabolism			
Nucleotide and nucleoside conversions	*cpdB/b4213*	2′,3′-cyclic-nucleotide 2′-phosphodiesterase precursor	6	15%	0.37
	add/b1623	Adenosine deaminase	10	31%	0.33
	udp/b3831	Uridine phosphorylase	7	38%	0.31
Glycolate degradation	*glcB/b2976*[a,i]	Malate synthase G	>19	>33%	>0.43
	glcG/b2977[i]	Protein GlcG (encoded within glycolate utilization operon)	3	22%	0.60
	garR/b3125	2-hydroxy-3-oxopropionate reductase	9	50%	0.48
Gluconeogenesis	*pckA*/b3403	Phosphoenolpyruvate carboxykinase	31	58%	0.57
Acetate catabolism	*acs/b4069*	Acetyl-CoA synthetase	16	28%	0.66
	hmpA/b2552	Dihydropteridine reductase	7	18%	0.33
	dxs/b0420	1-deoxyxylulose-5-phosphate synthase; flavoprotein	8	19%	0.74
		Energy metabolism			
TCA cycle	*gltA/b0720*	Citrate synthase	7	21%	0.57
	sdhA/b0723	Succinate dehydrogenase, flavoprotein subunit	13	32%	0.54
	fumA/b1612	Fumarate hydratase class I (aerobic)	22	47%	0.41
		Cell processes and global regulation			
	arcA/b4401	Aerobic respiration control protein	13	49%	0.32
	pspA/b1304	Phage shock protein A	9	49%	0.36
Putative	*ynaF/b1376*	Nucleotide binding protein (subunit of UspF homodimer)	7	42%	0.57
	yggX/b2962	Protein YggX (possible role in oxidative stress protection)	6	43%	0.46
		Putative, hypothetical, and unknown proteins			
	yqeF/b2844	Probable acetyl-CoA acetyl transferase	7	23%	0.40
	yfeX/b2431	Hypothetical protein YfeX	14	66%	0.38

a–c, protein isoforms appearing as multiple spots on gel.
e–i, multiple genes from the same operon.
[a]Two spots.
[b]Three spots.
[c]Four spots.
[d]Spot was not detectable in exponential phase gels.
[e]*gatYZABCD*.
[f]*fadBA*.
[g]*mglBAC*.
[h]*oppABCDF*.
[i]*glcDEFGBA*.

Increased expression of the methionine biosynthetic enzyme, MetE, is likely because of the amino acid's vital role in synthesis of the methyl group donor, *S*-adenosylmethionine, and in initiation of peptide synthesis (Greene, 1996).

Overall, the genes upregulated in exponential phase equip cells for (a) increased nucleotide biosynthesis, and (b) enhanced protein synthesis. Exponentially dividing *E. coli* cells are known to maintain a higher concentration of ribosomes, necessary for supporting rapid protein synthesis at high growth rates (Bremer and Dennis, 1996), and our results are consistent with this type of regulation.

Genes Upregulated in Fed-Batch Phase

Genes upregulated in fed-batch phase include proteins involved in, carbon compound utilization, transport and binding, central intermediary and energy metabolism, among others (Table II, Fig. 2). Many of the upregulated genes are known to be under catabolite repression (Busby and Kolb, 1996; Kumari et al., 2000; Nobelmann and Lengeler, 1996; Park and Gunsalus, 1995; Park et al., 1994; Ramseier et al., 1995; Stern et al., 1984).

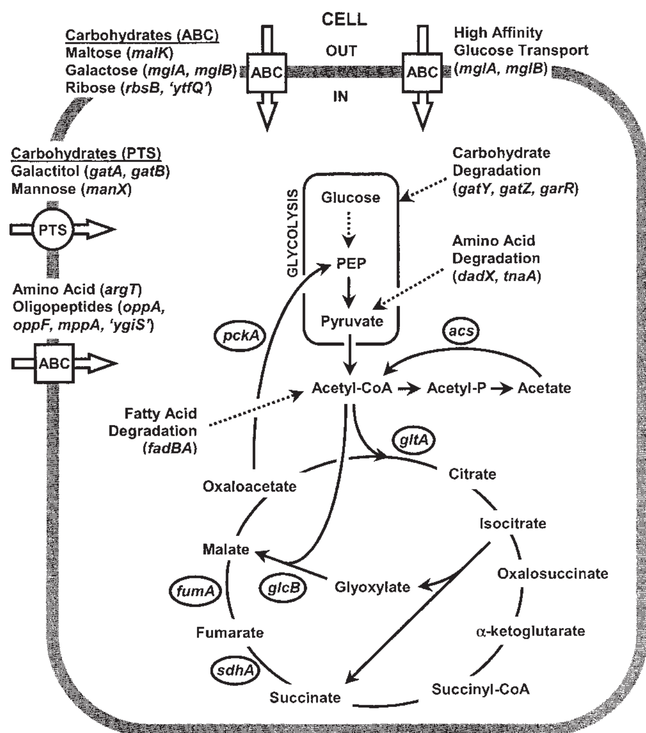

Figure 2. Schematic showing genes upregulated, along with reactions catalyzed by them and processes they are involved in, under glucose-limited conditions in fed-batch phase. Solid line arrows indicate single step metabolic reactions, while dotted line arrows indicate processes with multiple steps. Single step reaction genes upregulated are circled, while those upregulated in general processes are indicated in parentheses under each category. The following genes, upregulated in fed-batch phase and listed in Table II, are not shown in the above schematic: *fucI*, *cpdB*, *udp*, *glcG*, *dxs*, *hmpA*, *arcA*, *pspA*, *ynaF*, *yggX*, *yqeF*, *yfeX*.

Carbon Compound Utilization

Carbon compound utilization proteins upregulated include those involved in degradation of carbohydrates (*gatY*, *gatZ*, *fucI*), fatty acids (*fadA*, *fadB*), and amino acids (*dadX*, *tnaA*) (Table II).

GatY and GatZ are involved in galactitol degradation and catalyze the dissociation of D-tagatose 1,6-biphosphate to glycolytic intermediates (Nobelmann and Lengeler, 1996). Galactitol PTS transport proteins, GatA and GatB, were also upregulated in fed-batch phase. Upregulation of the *gat* operon under glucose-limited conditions has been reported in earlier *E. coli* transcriptome (Hua et al., 2004; Yoon et al., 2003) and proteome (Wick et al., 2001) studies. GatY was also found to be induced in the presence of acetate and under low pH conditions (Kirkpatrick et al., 2001) suggesting the involvement of Gat proteins in other cellular functions. But, if the genes are upregulated solely for utilization of galactitol upon its availability, then one could argue that it is wastage of cellular energy since galactitol is not a by-product of *E. coli* metabolism and hence is not likely to be present during growth on minimal media.

Other upregulated proteins include FucI, involved in degradation of L-fucose and D-arabinose, and *fadBA* encoded subunits of the fatty acid β-oxidation multienzyme complex. Fucose is widely present in bacterial polysaccharides, such as colanic acid (Andrianopoulos et al., 1998) and *E. coli* is capable of growing on fucose as the sole carbon and energy source. Non-differentiating bacteria are known to degrade membrane phospholipids and utilize the derived fatty acids as endogenous nutrient and energy sources during starvation (DiRusso and Nystrom, 1998). Interestingly, only one other study has reported the upregulation of *fadBA* operon under glucose limitation, rather than starvation (Hua et al., 2004).

Two amino acid degradation enzymes (*tnaA*, *dadX*) were also upregulated under glucose limitation. *E. coli* is capable of utilizing amino acids as sole sources of carbon and energy (McFall and Newman, 1996). TnaA catalyzes the degradation of L-tryptophan, L-cysteine, and L-serine, pyruvate being the end product of all reactions (McFall and Newman, 1996). The *tnaAB* operon also encodes the L-tryptophan specific permease–TnaB. Upregulation of *tnaA* and *dadX* transcripts was also reported in glucose-limited chemostat cultures of *E. coli* (Hua et al., 2004). DadX protein plays a vital role in supply of D-alanine for cell wall peptidoglycan biosynthesis (Reitzer, 1996). It catalyzes the conversion of L-alanine to the D-form, which is further degraded to pyruvate by DadA, also encoded in the *dadAX* operon (McFall and Newman, 1996).

When the primary carbon source is in limited supply, upregulation of various catabolic enzymes allows cells to utilize alternate carbon compounds immediately when they become available. In fact, *E. coli* cultivated in glucose-limited chemostats have been shown to consume other sugars without induction lag (Lendenmann and Egli, 1995). Ecologically, although nutrient sources are diverse, they are scarce in supply making nutrient limitation one of the primary stresses experienced by cells in nature (Harder and

Dijkhuizen, 1983). Hence, by upregulating the expression of various catabolic proteins, cells can consume many substrates, perhaps simultaneously, and thus satisfy their nutrient and energy needs (Kovarova-Kovar and Egli, 1998). cAMP-CRP mediated relief from catabolite repression plays a primary role in this enhancement of cells' nutrient scavenging capability.

Transport and Binding Proteins

Carbohydrate transport proteins belonging to both phosphoenol pyruvate (*gatA*, *gatB*, *manX*), and binding protein (*rbsB*, *malK*, *mglA*, *mglB*) dependent nutrient uptake systems were upregulated in fed-batch phase (Table II, Fig. 2). These proteins are involved in the transport of galactitol (*gatA*, *gatB*), and various sugars [maltose (*malK*), galactose (*mglA*, *mglB*), ribose (*rbsB*), and mannose (*manX*)] and are known to be catabolite repressed in the presence of excess glucose (Busby and Kolb, 1996). Other researchers have also reported the upregulation of sugar transport genes in *E. coli* under glucose limitation in fed-batch (Yoon et al., 2003) and chemostat (Hua et al., 2004; Wick et al., 2001) cultures, however, mostly at the transcriptome level.

Maltose and galactose transport proteins are also known to play a direct role in high affinity scavenging of glucose, apart from transport of their respective sugars. *E. coli* uses the phospho-transferase system (PTS) for glucose transport during growth on millimolar glucose concentrations and switches to binding protein dependent *mgl*/*lamB* high affinity glucose uptake system when glucose levels drop to micromolar concentrations (Death and Ferenci, 1994). Under glucose limitation, the LamB glycoporin encoded in the *mal* operon (*malKlamBmalM*) increases the outer membrane permeability to glucose, which is then transported across the inner membrane via the high affinity *mgl* binding protein system (Death and Ferenci, 1993; Death et al., 1993). Induction of *mgl* and *mal* operons requires galactose and maltotriose, respectively, in addition to high cAMP levels (Ferenci, 1996), which is endogenously synthesized by cells under glucose-limited conditions (Death and Ferenci, 1994). Our results are consistent with this type of induction, as seen by the increased expression of *mal* and *mgl* operon genes in fed-batch phase. A similar method of endogenous ribose synthesis and a likely role of its transport proteins (e.g., *rbsB*) in glucose scavenging has been speculated (Death and Ferenci, 1994).

Many amino acid (*argT*) and oligopeptide (*oppA*, *oppF*, *mppA*) transport proteins were also upregulated in fed-batch phase. Wick et al. (2001) also reported the upregulation of *argT* during short-term physiological adaptation of *E. coli* to glucose limitation in continuous cultures. *E. coli* is known to release cell wall murein peptides into the culture medium during exponential growth (Goodell and Schwarz, 1985). Thus the upregulated peptide transport proteins may play a role in recycling these excreted peptides, and possibly in utilization of peptides from lysed cells, under glucose-limited conditions. MppA binds to murein tripeptides, which are then transported into the cytoplasm via the oligopeptide permease (*opp*) system (Park et al., 1998).

As is evident from our results, binding protein dependent transport systems are very important for cellular growth under nutrient-limited conditions, although they are energetically unfavorable and cost the cells ATP. Apart from transport of their respective substrates, binding proteins also function as chemotactic receptors when bound to their ligands (Stock and Surette, 1996), and hence may play a bigger role in sensing the overall quality of the cellular environment under glucose-limited conditions.

Central Intermediary Metabolism

Central intermediary metabolism proteins upregulated include those involved in metabolism of nucleotides (*cpdB*, *udp*, *add*) and glycolate/glyoxylate (*glcB*, *glcG*, *garR*), among others (Table II).

The RNA degrading enzyme–CpdB, and pyrimidine salvage pathway enzyme–Udp, are both catabolite repressed by glucose, and Add is involved in adenine salvage and recycling. These enzymes are likely upregulated for utilization of alternate carbon sources under glucose-limited conditions. *E. coli* is capable of utilizing purine and pyrimidine nucleosides and deoxynucleosides as sole sources of carbon and energy (Lin, 1996). Also, the cellular nucleic acid content is known to decrease with declining growth rates (Maaloe and Kjeldgaard, 1966). Hence, the proteins may also play a role in turnover of nucleic acids during slow growth conditions in fed-batch phase. Upregulation of nucleotide metabolism enzymes has been reported earlier under similar growth conditions (Yoon et al., 2003).

Glyoxylate is part of the anaplerotic shunt, required for replenishing the TCA cycle intermediates during growth on acetate or fatty acids (Cronan and Laporte, 1996). It is also a by-product of purine degradation (Vogels and Van der Drift, 1976) and glycolate metabolism. *E. coli* is capable of growing on glyoxylate as the sole carbon and energy source (Clark and Cronan, 1996). In this study, one enzyme (*glcB* and *garR*) in each of the glyoxylate metabolism pathways was upregulated. In one pathway, GlcB catalyzes the condensation of glyoxylate and acetyl-CoA to form malate, which enters the TCA cycle. The other pathway involves the condensation of two glyoxylate molecules to form tartronate semialdehyde, which is then reduced to glycerate by GarR and the pathway end product enters the glycolytic cycle (Pellicer et al., 1996). Earlier transcriptome studies have also reported the upregulation of *glc* operon genes (*glcB* and *glcG*) under carbon limitation (Hua et al., 2004; Yoon et al., 2003).

Upregulation of GlcB, coupled with the increased expression of TCA cycle enzymes (*gltA*, *sdhA*, *fumA*) and the gluconeogenic enzyme–PckA, suggests the potential functioning of the novel PEP-glyoxylate pathway, recently discovered in glucose-"hungry" *E. coli* (Fig. 2). This pathway, involving the glyoxylate shunt and PckA catalyzed conversion of oxaloacetate to PEP, was found to completely

oxidize glucose and satisfy the cellular anaplerotic needs in glucose-limited wild-type *E. coli* MG1655 (Fischer and Sauer, 2003). However, in case of wild-type *E. coli* W3110 strain (used in this study), the literature reports are contradictory as to the functioning of the glyoxylate bypass during glucose limitation. While Yang et al. (2003) found no activity of the glyoxylate shunt enzyme, isocitrate lyase (*aceA*), Yoon et al. (2003) reported the upregulation of the *aceBAK* operon under glucose limitation in chemostat and fed-batch cultures. In this study, while we didn't specifically observe the upregulation of *aceBAK* operon, overall results suggest the potential operation of the PEP-glyoxylate pathway under carbon-limited conditions.

Energy Metabolism

During growth on excess glucose, *E. coli* produces and excretes acetate via the reversible Pta-AckA pathway. Under glucose-limited conditions, acetate is reabsorbed via its conversion to acetyl-CoA by the catabolite repressed Acs enzyme (Kumari et al., 2000). The Acs enzyme was upregulated in this proteome study and has also been reported in other transcriptome studies (Hua et al., 2004; Yoon et al., 2003). Acetyl-CoA, which is also generated from fatty acid degradation, enters the TCA cycle or glyoxylate shunt. Hence, upregulation of the cycle is likely to aid in assimilation of acetyl-CoA generating substrates such as acetate and fatty acids under carbon-limiting conditions. In this study, we observed increased expression of TCA cycle enzymes (*gltA*, *sdhA*, *fumA*) in fed-batch phase (Table II, Fig. 2). Also, during growth on excess glucose, *E. coli* derives much of its energy from glycolysis, with repressed synthesis of TCA cycle enzymes (Lee et al., 1994). Hence, under carbon-limited conditions, the cycle is upregulated for satisfying the cellular energy needs and to supply intermediates for macromolecular biosynthesis. Transcriptome studies have also reported the upregulation of TCA cycle enzymes in glucose-limited cultures of *E. coli* (Hua et al., 2004; Yoon et al., 2003).

Cell Processes and Global Regulation

The ArcA global regulatory protein is part of the *arcAB* two-component signal transduction system which represses many aerobic metabolism enzymes during anaerobiosis (Iuchi and Lin, 1988) and aerobic stasis (Nystrom et al., 1996). Its increased expression in fed-batch phase is possibly due to stress from severe nutrient limitation, as DO concentration was non-limiting throughout the fermentation (Fig. 1b). In fact, the redox condition of the cell, rather than oxygen itself, has been argued to be the cellular signal for ArcA dependent gene regulation (Iuchi and Lin, 1993).

Many of the enzymes upregulated in this study (*gltA*, *sdhA*, *fumA*, *glcB*, *glcG*, *fadA*, *fadB*) are known to be both catabolite repressed by glucose and negatively regulated by ArcA (Iuchi and Lin, 1988; Lynch and Lin, 1996). We speculate that their relief from catabolite repression is greater than ArcA mediated repression, and hence the enzymes are upregulated in fed-batch phase. ArcA mediated global gene regulation also plays an integral role in the defense against oxidative damage and in controlling the drainage of endogenous reserves during aerobic carbon starvation (Nystrom et al., 1996).

The upregulated cell-process proteins are involved in stress protection and survival under glucose-limited conditions. PspA, a σ^s independent stationary phase protein, is important for *E. coli* survival under nutrient- and energy-limited conditions (Weiner and Model, 1994). Among the putative proteins upregulated, YnaF has been suggested to have a probable universal stress protein type function (Saveanu et al., 2002), and YggX is a SoxRS regulated protein involved in protection of iron-sulfur proteins against oxidative damage (Pomposiello et al., 2003).

Overall, the genes upregulated in fed-batch phase equip cells for (a) scavenging glucose present at low concentrations, (b) transporting and metabolizing a wide range of substrates, (c) tackling energy deficiency, and (d) coping with stressful conditions. Previous global studies on *E. coli* response to glucose limitation were primarily done at the transcriptome level, and limited data is available for the upregulation of some genes at the protein level, including GatY, MalK, MglA, MglB, RbsB, ArgT, SdhA, Udp (Ferenci, 1999; Wick et al., 2001; Yoon et al., 2003). Considering the modest correlation between mRNA and protein expression in *E. coli* (Lee et al., 2003), our study provides valuable proteomic evidence for the upregulation of many genes under glucose-limited conditions. Also, in this study, some protein spots (TnaA, MglA) appeared only on fed-batch gels and several proteins were differentially expressed by >4-fold, including DadX, FadB, GatB, RbsB, MalK, YtfQ, GlcG, and Acs. We suggest that the increased expression of these proteins may be used as sensory signals for detecting carbon limitation in bioprocesses.

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