Proteomic Profiling of Recombinant *Escherichia coli* in High-Cell-Density Fermentations for Improved Production of an Antibody Fragment Biopharmaceutical

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By using two-dimensional polyacrylamide gel electrophoresis, a proteomic analysis over time was conducted with high-cell-density, industrial, phosphate-limited *Escherichia coli* fermentations at the 10-liter scale. During production, a recombinant, humanized antibody fragment was secreted and assembled in a soluble form in the periplasm. *E. coli* protein changes associated with culture conditions were distinguished from protein changes associated with heterologous protein expression. Protein spots were monitored quantitatively and qualitatively. Differentially expressed proteins were quantitatively assessed by using a *t*-test method with a 1% false discovery rate as a significance criterion. As determined by this criterion, 81 protein spots changed significantly between 14 and 72 h (final time) of the control fermentations (vector only). Qualitative (on-off) comparisons indicated that 20 more protein spots were present only at 14 or 72 h in the control fermentations. These changes reflected physiological responses to the culture conditions. In control and production fermentations at 72 h, 25 protein spots were significantly differentially expressed. In addition, 19 protein spots were present only in control or production fermentations at this time. The quantitative and qualitative changes were attributable to overexpression of recombinant protein. The physiological changes observed during the fermentations included the up-regulation of phosphate starvation proteins and the down-regulation of ribosomal proteins and nucleotide biosynthesis proteins. Synthesis of the stress protein phage shock protein A (PspA) was strongly correlated with synthesis of a recombinant product. This suggested that manipulation of PspA levels might improve the soluble recombinant protein yield in the periplasm for this bioprocess. Indeed, controlled coexpression of PspA during production led to a moderate, but statistically significant, improvement in the yield.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is an established analytical technique for studying microbial physiology (24, 40). While many 2-D PAGE studies have been performed with cells cultured in shake flasks, there is also a need to study high-cell-density, fed-batch fermentations of *Escherichia coli*, which are widely used in industry to obtain high productivity (9, 26). Results of 2-D PAGE analyses can identify targets for bioprocess improvement, such as genes to delete from the host cell chromosome (9) or to coexpress with a product (17, 18). Ultimately, proteome analysis may improve the design and control of industrial fermentation processes.

There have been previous 2-D PAGE analyses of high-cell-density fermentations of *E. coli* (7–9, 16, 17, 19–21, 38), in many cases involving the production of recombinant protein. Studies of fed-batch fermentations conducted in Genentech laboratories have focused on end-of-run analyses to assess multiproduct host cell protein immunoassays (7, 8) or product proteolysis (9). The latter studies (9) involved production of the same antibody fragment, as discussed in this paper. The same fermentation process was utilized in both cases, and the host

strains were similar. The recombinant antibody fragment, anti-CD18 F(ab')₂, was developed as a biopharmaceutical for the treatment of acute myocardial infarction (i.e., heart attacks). The bioprocess for anti-CD18 F(ab')₂ production is a good model system for the manufacture of secreted antibody fragments by use of *E. coli*.

In the present study, the dynamics of the *E. coli* proteome were recorded during an industrial fermentation process with and without induction of recombinant antibody synthesis. One major objective was to distinguish protein changes due to culture conditions, as the cells grew to a high cell density, from protein changes due to recombinant product expression. In the statistical analysis of protein spot quantities, a *t*-test method was utilized to identify changes by using criteria for significance that take multiple hypothesis tests into account. Another important goal was to understand the observed proteome dynamics in the context of *E. coli* physiology. Ultimately, the information obtained in the proteomic profiling analysis was used to obtain a moderate improvement in the bioprocess for the production of anti-CD18 F(ab')₂.

MATERIALS AND METHODS

Bacterial strain and plasmids. The host used in this study was *E. coli* K-12 strain 59A7 (9), with the following genotype: IN(rmD-rmE)1 Δ*fhuA* Δ*phoA* Δ*E15* Δ(*argF-lac*)169 *deoC2* *ilvG2096* *degP41* Δ*prc* *sprW148R*. In the case of the production expression system, 59A7 harbored the pBR322-based plasmid pS1130 (9). This plasmid contains a dicistronic mRNA for the anti-CD18 F(ab')₂ heavy chain and light chain behind the phosphate-starvation-inducible *phoA* promoter.

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The two open reading frames encode N-terminal STII signal peptides for secretion. The heavy chain open reading frame includes a sequence that encodes a 33-residue, C-terminal leucine zipper (LZ) domain (this domain facilitates dimerization and is later cleaved in the recovery process). For the control expression system, 59A7 harbored the control plasmid pBR322 (3), the vector backbone for pS1130 without the *phoA* promoter region or the product genes.

To construct pPspA, the gene encoding phage shock protein A (PspA) was amplified by PCR by using *E. coli* W3110 genomic DNA and was cloned into pMMB206 (31), a vector harboring *lacI^R* and compatible with pS1130. This placed *pspA* under the tight control of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *taclacUV5* promoter (*P_{taclacUV5}*). A strong ribosome binding site and optimal spacer (translation initiation region; GGA GGA AAA ACA AC) were included in the forward PCR primer to engineer this region in front of *pspA*.

Culture conditions. High-cell-density fermentations (10 liters) were performed essentially as described previously (8). When the initial glucose was consumed, a concentrated glucose solution was added to maintain the dissolved oxygen level near zero. Glucose was limited at regular intervals, leading to sudden increases in the dissolved oxygen level, at which time glucose feeding was increased. The growth medium was designed to be phosphate limited to induce recombinant product expression from the *phoA* promoter. Ammonium hydroxide and sulfuric acid were added as needed to maintain the pH at 7.0. The temperature was maintained at 30°C, and the culture was incubated for 72 h.

Duplicate control and production fermentations were conducted. Cell culture samples were collected every 2 h after the culture reached an optical density at 550 nm (OD_{550}) of 20. Samples were diluted to an OD_{550} of 20, and 1 ml was harvested by centrifugation to obtain 20 OD-ml pellets, which were stored at -20°C or below. Cell pellets were acquired at approximately 14, 27, 43, 59, and 72 h.

For PspA coexpression studies, strain 59A7 harboring plasmids pS1130 and pPspA was grown by using the process described above. Addition of 30 μ g chloramphenicol per ml to the medium selected for pPspA. IPTG was added at 29 h, when the culture had reached an OD_{550} of 150 (just before product induction occurred). Three replicate fermentations were performed with no IPTG and 0.02 mM IPTG, and a single fermentation was performed with 2 mM IPTG.

Recombinant protein yield assays. Recombinant anti-CD18 was measured in two ways. First, total light chain and total heavy chain were measured by reversed-phase chromatography following denaturation with guanidine-HCl and dithiothreitol as previously described (9). Second, anti-CD18 was measured in its intact, soluble form by using affinity chromatography. To do this, soluble protein was prepared by mixing 100- μ l cell culture samples from the fermentors in a solution of Tris-EDTA and lysozyme as previously described (9). Each mixture was incubated on ice for 15 min, vortexed for 5 s, sonicated (one round of 10 pulses), and centrifuged for 20 min at 10,000 $\times$ g. The supernatant was analyzed by using an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, Calif.) with protein G affinity chromatography on a POROS G/M column (2.1 by 30 mm; PerSeptive Biosystems, Framingham, Mass.). The step gradient program was as follows: 1.3 min of phosphate-buffered saline (pH 7.2), followed by 3 min of 4 M urea-HCl (pH 2.6) and finally 1.7 min of phosphate-buffered saline. The column was maintained under ambient conditions, the injection volume was 50 μ l, and protein was detected at a wavelength of 280 nm.

In both HPLC assays, purified anti-CD18 F(ab')₂ served as a standard. A cell culture aliquot from a control run was added to standard samples to account for background *E. coli* proteins and to determine appropriate baselines. Peak area integration was performed by using the ChemStation 1100 software.

2-D PAGE. 2-D PAGE was performed as previously described (8). Eighteen-centimeter pH 3 to 10 nonlinear immobilized pH gradient gel strips (Amersham Pharmacia Biotech, Piscataway, N.J.) were rehydrated overnight with samples, and isoelectric focusing was conducted for a total of 50,000 V·h for separation in the first dimension. For separation in the second dimension, the DALT system (Amersham Pharmacia Biotech) was used to perform sodium dodecyl sulfate-PAGE. Gels were fixed and stained with SyproRuby as previously described (34).

Image analysis of 2-D gels. Gels generated for all five times from each of the four fermentations were run in duplicate, resulting in a total of 40 gels for image analysis. Digitized images of 2-D gels were obtained with a charge-coupled device camera as previously described (8). Images containing no pixel saturation (obtained with 4-s exposures) were analyzed further. Spot detection and 2-D pattern matching were performed by using PDQuest 7.1 (Bio-Rad, Hercules, Calif.). Classic extended matching was conducted such that following a primary autolandmarking phase, lower-confidence matches were made. Matchsets were normalized based upon the total quantity in all valid spots. A 16-gel matchset,

consisting of endpoints of the fermentation time course (14 and 72 h for both control and production gels), was generated. This matchset was then edited extensively by correcting mismatched protein spots between gels, defining spot boundaries in certain cases, and removing smears from the analysis.

Statistical analyses of spot quantities. Statistical analyses were conducted by using data exported from the 16-gel matchset as an Excel (Microsoft, Seattle, Wash.) spreadsheet and performed in Splus, version 3.4 for Sun SPARC (Mathsoft, Cambridge, Mass.).

Protein changes associated with high-cell-density fermentation (i.e., physiological changes) were identified by comparing 2-D protein patterns from control fermentations at 14 and 72 h. Protein changes related to overexpression of the recombinant antibody fragment were identified by comparing 72-h control gels to 72-h production gels (final sample time).

Approximately 800 protein spots were detected in seven of the eight gels and were analyzed further statistically. Protein spots detected in fewer than seven gels were eliminated from further statistical analysis. Protein spot intensity values were log transformed to ensure that the error variability was more constant about the average signal for a wide range of intensities and that the errors more closely followed the Gaussian distribution. Log transformation of raw signal intensities is commonly performed during analysis of cDNA microarray data for similar reasons (10).

For each protein spot, the hypothesis of equivalent protein expression in the two groups was tested by using a two-sample Welch *t* test (27) and the resultant *P* value. The Welch *t* test is a robust variant of the classic two-sample *t* test; it does not assume a common underlying level of data variance between the data sets. For each protein spot, a small *P* value provides evidence against the null hypothesis of equal protein expression in the two groups. When *P* values from many *t* tests (i.e., one value for each protein spot included in the analysis) are considered, however, adjustments to the usual criteria for statistical significance must be made in order to avoid large numbers of false-positive findings (see reference 15 for a review of this issue in the context of DNA microarray experiments).

The primary criterion for determining statistical significance was control of the false discovery rate (FDR) (4). Controlling the FDR at a fixed percentage provides a list of statistically significantly changed protein spots such that no more than a small percentage of the proteins listed, determined by the FDR chosen (such as 1%), is expected to be falsely positive. We calculated the corresponding fold change for the proteins in our lists using geometric means, which ensured that an outlying value did not have undue influence on the calculated ratio.

In addition to the quantitative analysis described above, a list of qualitatively changed protein spots was compiled for both comparisons (control fermentations at 14 h versus control fermentations at 72 h and control fermentations at 72 h versus production fermentations at 72 h). Protein spots were considered to have undergone a qualitative (on-off) change when all four signals were present for one group but the other group showed no signal in any gel. For example, if a protein spot was detected in all four gels from a production run but in none of the gels from a control run for the 72-h endpoint, the change was considered an on-off change for this comparison.

Peptide mass fingerprinting. Protein spots of interest were cored from the 2-D gels and digested in situ, and the peptides were extracted as described previously (7). In addition to the protein spots for which there were changes in abundance, many spots adjacent to these proteins were cored and subjected to peptide mass fingerprint analysis. This facilitated the identification of isoforms (i.e., multiple protein spots representing a single gene product, such as charge variants), which are common in *E. coli* proteome maps (28).

Mass spectra were acquired in reflectron mode by using a Voyager DE STR matrix-assisted laser desorption/ionization—time of flight instrument (PE Biosystems, Foster City, Calif.) equipped for delayed extraction. Peptide samples and the 2,4,6-trihydroxyacetophenone matrix were applied to a matrix-assisted laser desorption/ionization plate as previously described (7).

Protein spots that were identified as significantly changed from the 1% FDR and the on-off lists, as well as spots that showed a large change (threefold or more) in the comparisons, were selected for coring. Only spots whose abundance was deemed sufficient for identification (and the potential corresponding local isoforms) were cored. This eliminated a number of protein spots from further analysis.

By using tryptic peptide molecular masses, proteins were identified by searching a protein sequence database that is a compilation of the National Center for Biotechnology Information, PIR, and Swiss-Prot databases with ProFound (Proteometrics, New York, N.Y.), as previously described (7). Once proteins of interest were identified, they were classified into families according to function by using the ExPASy (www.expasy.ch) and EcoCyc (www.ecocyc.org) databases.

Determination of protein quantity dynamics. A 40-gel matchset, consisting of all gels for the time courses for both control and production fermentations, was created to generate kinetic profiles of protein spots discovered in the quantitative and qualitative analyses of the 16-gel matchset. Following spot editing for valid normalization, extensive editing was performed for protein spots of interest to ensure correct matching.

Graphs of spot quantities over time were drawn by exporting the 40-gel matchset data into an Excel (Microsoft) spreadsheet and adding the isoform values. Geometric mean quantities were plotted on a linear scale. In a separate calculation, one standard deviation was added and subtracted from the mean on the log scale to generate error bars. If we plotted time course graphs on a log scale, certain effects were unclear, and small differences at low quantities were falsely emphasized. Therefore, all time course graphs were plotted on a linear scale. The conversion from the log scale to the linear scale produced asymmetric error bars.

Western blot analysis. Western blot analysis was performed essentially as previously described (7). The primary antibody was rabbit anti-PspA antiserum (a generous gift from Peter Model, Rockefeller University) diluted 1:10,000. Cell culture aliquots were added to 2× Tricine sample buffer (Invitrogen, Carlsbad, Calif.) and normalized by using OD₅₅₀ values so that equivalent cell numbers were loaded. A Ponceau S (Sigma-Aldrich, St. Louis, Mo.) staining solution (0.1% Ponceau S in 5% acetic acid) was used to verify equivalent loading. Whole-cell lysates (5 µl) and See-Blue Plus prestained molecular weight marker were analyzed in a 12% polyacrylamide bis-Tris gel with morpholinepropanesulfonic acid (MOPS)-sodium dodecyl sulfate running buffer (Invitrogen). Chemiluminescent detection was conducted with an ECL Plus Western blot detection kit (Amersham Biosciences) by using an exposure time of 2 s. Samples for immunoblot analysis were taken 6 h after IPTG induction.

RESULTS

Growth and yield curves. The protocol used for production fermentation and sampling timing is shown in Fig. 1a. In this figure, the cell density, dissolved oxygen level, specific growth rate, and product yield are shown together in order to visualize all relevant factors at once. The fermentation proceeded for 72 h. At 14 h, the first time sampled, increasing oxygen demand had reduced the dissolved oxygen level to near zero, marking the start of linear cell growth. After this, the level of dissolved oxygen was near zero most of the time. Phosphate limitation occurred at approximately 30 h, leading to induction of product expression.

The actual growth curves for control and production fermentations were quite similar (Fig. 1b). Light and heavy chain expression was induced by phosphate limitation in the production run, as expected (Fig. 1c).

2-D PAGE and protein spot analysis. Samples were collected during the fermentations for 2-D PAGE analysis. Representative 2-D gels for control and production fermentations at 72 h are shown in Fig. 2. An obvious difference between the control and production proteomes is the presence of recombinant antibody-related protein spots in the production gel. Other protein changes, such as PspA up-regulation, could also be observed by visual inspection. While these changes were obvious, alterations in lower-level proteins were less obvious, as were less dramatic changes. To further investigate on-off changes and more subtle quantitative changes, digitized gel images were analyzed carefully with the PDQuest software (Bio-Rad), and statistical analyses were performed.

Multiple hypothesis-testing criteria were applied to define cutoffs for significant *P* values (Table 1). A comparison of the first and last times (14 and 72 h) for the control fermentations by using *t* tests with an FDR criterion of 1% showed that 81 protein spots underwent significant changes in abundance. Thirty-three of the protein spots were down-regulated, and 48

FIG. 1. Process for manufacture of anti-CD18 F(ab')₂-LZ. (a) Idealized fermentation profiles for the process. The arrows indicate sampling times. (b) Growth curves for control and production runs. Symbols: □ and ■, replicate control fermentations of 59A7 harboring pBR322; △ and ▲, replicate production fermentations of 59A7 harboring pS1130. (c) Total amounts of light chain (LC) and heavy chain (HC) expressed over time in a production fermentation. The results were derived from a single reversed-phase HPLC assay, but the trends and values are representative of the results of duplicate assays. Symbols: ■, recombinant light chain; ▲, heavy chain.

FIG. 2. Two-dimensional protein profiles for end-of-run (72-h) samples for control (A) and production (B) gels. Qualitative (on-off) protein spot changes are indicated by circles. Green crosses indicate protein spots detected in production fermentations but not in control fermentations. Blue triangles indicate protein spots detected in control fermentations but not in production fermentations. Red plus signs indicate protein spots that were up-regulated in production fermentations compared to control fermentations, and red circles indicate protein spots that were up-regulated in control fermentations compared to production fermentations. Prominent protein spots that were up-regulated in the production gel and which were identified as anti-CD18 light chain (anti-CD18 LC) and PspA are labeled. IEF, isoelectric focusing; SDS, sodium dodecyl sulfate.

TABLE 1. Number of quantitative protein spot changes, as determined by using different criteria, and number of on-off changes^a

Comparison	No. of changes			
	1% FDR	5% FDR	On-off	Total for 1% FDR and on-off ^b
14 h versus 72 h for control fermentations	81	239	20	101
Control versus production fermentations at 72 h	25	127	19	44

^a The primary criterion used for statistical analysis was an FDR of 1%. The less stringent criterion for declaring significance was an FDR of 5%; in this case more false positives were expected.

^b Total number of protein spots that underwent quantitative (as determined by using the 1% FDR criterion) and qualitative (on-off) changes.

were up-regulated. Twenty protein spots displayed on-off changes. Based on this group of quantitative and qualitative changes, we identified 73 protein spots by peptide mass mapping (Tables 2 and 3). These analyses identified physiological changes associated with a high-cell-density culture.

Applying the 1% FDR criterion to compare 72-h control and 72-h production gels, we observed 25 protein spots that underwent significant changes in abundance. Seven of the protein spots were down-regulated, and 18 were up-regulated. Nineteen protein spots displayed on-off changes. By using peptide mass mapping, 21 of these quantitative changes (Table 4) and qualitative changes (light chain isoforms and heavy chain as discussed below) were identified. These analyses identified changes associated with recombinant protein expression.

When the FDR was increased from 1 to 5%, the number of significant protein spot changes increased from 81 to 239 for control fermentations between 14 and 72 h and from 25 to 127 for control fermentations compared with production fermentations at 72 h (Table 1). These differences illustrate the fact that the criteria used for determining significant protein quantity changes strongly affect the number of changes recognized.

There were two additional means for verifying whether a change in the protein spot quantity was real. First, previously published information about *E. coli* physiology could be used to interpret protein changes, particularly coordinate changes in proteins belonging to the same regulon or stimulon. Second, the protein levels observed in the time course analysis provided trends that could further validate changes that appeared in endpoint analyses. In general, the proteins discussed below changed according to the *t* tests for data from the 16-gel matchset (endpoint analysis) with a 1% FDR. The changes were verified by using the entire time course analysis of the 40-gel matchset, with isoform quantities summed. In certain instances, 5% FDR changes are discussed below if other proteins within the same regulon or stimulon also changed with a 1 or 5% FDR or if the changes were supported by the time course analysis. In these cases, the change was often large (threefold or more).

Proteome characterization. (i) Antibody light chain and heavy chain. As expected, the light and heavy chains of the antibody fragment were highly visible at late times in the production fermentations (Fig. 2, 3a, and 4a) but not in the control fermentations. Their appearance corresponded with induction

of phosphate starvation proteins, as described below. The light chain appeared as two dominant isoforms and as two minor, acidic isoforms. The heavy chain is rather hydrophobic and poorly soluble; it migrated as a smear in the gel and was difficult to quantify. Therefore, we relied upon reversed-phase HPLC to monitor the increases in the light chain and the heavy chain over time (Fig. 1c).

(ii) Phosphate starvation proteins. Several proteins known to be involved in the phosphate starvation transcriptional response and the transport and metabolism of inorganic and organic sources of phosphate (PhnD, PhnI, PhoB, PhoU, PstB, PstS, UgpB, UgpC) were up-regulated over time in both control and production runs (Fig. 3b and 4b). Representative protein spots are shown in endpoint gel images in Fig. 3b. Profiles of recombinant antibody (Fig. 1c and 4a) and phosphate starvation proteins (Fig. 4b) showed that there was marked up-regulation between 27 and 43 h, when phosphate starvation was expected to occur.

(iii) Ribosomal proteins. Proteins from the large and small ribosomal subunits (RplI and RpsA) were down-regulated over time during control and production runs (Fig. 4c). Ribosomal proteins RplJ and RpsJ were down-regulated, as observed by visual inspection, but they were not easily quantified because of poor spot resolution (data not shown). In addition, decreases in the dominant isoforms of the small ribosomal subunit protein RpsF were observed in the gels over time in the control fermentations (Table 2).

(iv) Nucleotide biosynthesis and degradation proteins. Sharp down-regulation of several nucleotide biosynthetic proteins (Adk, Ndk, PrsA, PurC, PurD, PurH, PurM, PyrA, PyrB, PyrC, PyrG, PyrI) was seen between 14 and 27 h in control and production runs. The trends for representative members of this class are shown in Fig. 4d. The protein quantities remained low for the remainder of the fermentations. These proteins are involved in purine and pyrimidine biosynthesis, as well as synthesis of nucleoside triphosphates and regulation of their concentrations.

While nucleotide biosynthesis proteins decreased in abundance, nucleotide degradation proteins increased in abundance between 14 and 27 h (Fig. 4e). The pyrimidine salvage enzymes cytidine deaminase (Cdd) and uridine phosphorylase (Udp) were up-regulated in control and production runs (Fig. 4e). Another protein in this category, DeoD (purine nucleoside phosphorylase), showed an unusual behavior. DeoD initially increased in abundance in both control and production fermentations, but then the level decreased to nearly the original level in control runs, which was significantly lower than the 72-h level in production runs (Fig. 4e).

(v) Oxidative damage defense proteins. In the control runs, SodA (manganese superoxide dismutase), which destroys toxic, oxidative free radicals, was down-regulated in the middle of the fermentations, and then the abundance returned to a high level between 43 and 72 h (Fig. 4f). In the production runs, however, the SodA levels remained low after the initial decrease. SodB (iron superoxide dismutase) was strongly up-regulated in both control and production fermentations between the first and second times, and the level remained high in all runs (Fig. 4f). Subunits of alkyl hydroperoxide reductase (AhpC and AhpF), which are involved in the protection of DNA from oxidative damage, were up-regulated more dramati-

TABLE 2. Protein spots identified by peptide mass fingerprinting after 2-D gel comparisons of control fermentations at 14 and 72 h: quantitative changes with 1% FDR criterion^a

Function	Protein	Fold change (72 h/14 h) ^b	Description
Phosphate starvation inducible	PhoB	4.4	Phosphate regulation transcriptional regulatory protein
	PstS	23	Phosphate ABC transporter subunit
	PstS (clip)	3.4	Phosphate ABC transporter subunit
Ribosomal	UgpB (isoform)	9.4	Glycerol-3-phosphate ABC transporter subunit
	RpsA	0.3	30S ribosomal subunit protein S1
	RpsF (isoform I)	0.1	30S ribosomal subunit protein S6
	RpsF (isoform I)	0.1	30S ribosomal subunit protein S6
	RpII	0.3	50S ribosomal subunit protein L9
Nucleotide biosynthesis	Adk	0.8	Adenylate kinase
	Ndk	0.3	Nucleoside diphosphate kinase
	PrsA	0.6	Phosphoribosylpyrophosphate synthase
	PurB	0.5	5'-Phosphoribosyl-4-(N-succinocarboxamide)-5-Aminoimidazole lyase/adenylosuccinate lyase
	PurC	0.4	Phosphoribosylaminoimidazole-succinocarboxamide synthase monomer
	PurD	0.2	Phosphoribosylamine-glycine ligase
	PurH (isoform I)	0.3	AICAR transformylase/IMP cyclohydrolase
	PurH (isoform II)	0.3	AICAR transformylase/IMP cyclohydrolase
	PurM	0.2	Phosphoribosylformylglycinamide cyclo-ligase monomer
	PyrA	0.2	Carbamoyl phosphate synthase subunit
	PyrB (isoform I)	0.1	Aspartate-carbamoyltransferase subunit
	PyrB (isoform II)	0.1	Aspartate-carbamoyltransferase subunit
	PyrC (isoform)	0.4	Dihydroorotase monomer
	PyrI	0.1	Aspartate-carbamoyltransferase subunit
Oxidative damage defense	Tpx (isoform)	7.1	Thiol peroxidase II
	SodB	2.4	Iron superoxide dismutase subunit
	AhpF	4.1	Alkylhydroperoxide reductase subunit
Stress	PspA	2.5	Phage shock protein A
Amino acid biosynthesis	YbdQ or UP12	6.9	Universal stress protein of the UspA family
	Asd	0.5	Aspartate semialdehyde dehydrogenase
	DapA	1.6	Dihydrodipicolinate synthase
	GltD	0.3	Glutamate synthase subunit
	HisJ	0.6	Histidine ABC transporter
Fatty acid biosynthesis	IlvI	5.1	Acetolactate synthase III/acetohydroxybutanoate synthase III subunit
	FabI	1.9	Enoyl-acyl carrier protein reductase (NADH)
	TesB	2.7	Thioesterase II
Carbon and energy metabolism	AldH	1.9	Aldehyde dehydrogenase
	GAPDH-A	2.3	Glyceraldehyde-3-phosphate dehydrogenase A monomer
	GldA	11	Glycerol dehydrogenase monomer
	MasZ	2.0	Malate synthase G
	PepC	0.5	Phosphoenolpyruvate carboxylase monomer
Transport and binding	TpiA	0.5	Triose phosphate isomerase monomer
	GatY	4.4	Tagatose-1,6-bisphosphate aldolase 2
	ModA	0.2	Molybdate ABC transporter
	OppA	2.3	Oligopeptide ABC transporter subunit
	RbsB	2.8	Ribose ABC transporter
Synthesis and modification of macromolecules	FliY (isoform)	0.6	Periplasmic cystine-binding protein
	ParE	0.2	DNA topoisomerase IV subunit
	LysS	0.2	Lysyl tRNA synthetase subunit
	FkbA	0.5	FKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase
	Rrf	1.2	Ribosome recycling factor
Transcriptional regulator	NifU	0.4	Scaffold protein involved in iron-sulfur cluster assembly
	LpcA	1.7	Phosphoheptose isomerase for lipopolysaccharide core biosynthesis
	SspA	0.7	Stringent starvation protein A transcriptional regulator
	NusG	0.7	Transcription antitermination protein
	CspC	0.3	Cold shock-like protein CspC
Other	Homologue of <i>Bacillus subtilis</i> RibH	0.5	
	MdaB	4.4	NADPH quinone reductase
	OrdL	0.1	Probable oxidoreductase
	Unknown protein from 2-D PAGE	0.7	
	YajQ	1.8	Nucleotide binding protein
	YchF	1.7	Putative GTP-binding protein
	YdjA	2.2	Unknown conserved protein
	YhgI	0.6	Hypothetical protein

^a In certain cases the spot identified was a clip or an isoform of another protein spot on the gel.

^b Fold changes were calculated by using geometric means.

TABLE 3. Protein spots identified by peptide mass fingerprinting after 2-D gel comparisons of control fermentations at 14 and 72 h: on-off changes

Function	Protein	Time at which spot was present (h)	Description
Phosphate starvation inducible	PstB	72	Phosphate ABC transporter subunit
	PhnD (minor isoform)	72	Alkylphosphonate ABC transporter subunit
	PhnI	72	Cryptic protein involved in phosphonate metabolism
	PhoU	72	Phosphate transport system transcriptional regulator
	UgpB (isoform)	72	Glycerol-3-phosphate ABC transporter subunit
	UgpC	72	Glycerol-3-phosphate ABC transporter subunit
	Udg	72	UDP-glucose 6-dehydrogenase
Macromolecule biosynthesis of colanic acid extracellular polysaccharide			
Amino acid metabolism	MetE	72	Cobalamin-independent homocysteine transmethylese
	YcaC	72	Putative cysteine hydrolase monomer
Outer membrane porin	FecA	14	Iron(III) dicitrate transport protein
Transcriptional regulator	H-NS (minor isoform)	14	DNA-binding protein

ically in control fermentations than in production fermentations (Fig. 4f). One of the three AhpC isoforms was difficult to quantify due to comigration with inorganic pyrophosphatase. Thiol peroxide reductase (Tpx), a periplasmic redox modulator that is part of the AhpC family, was also up-regulated in both control and production fermentations (Fig. 4f).

(vi) Stress proteins. Of all the *E. coli* proteins, PspA was the most highly up-regulated protein in production fermentations (Fig. 4g). When the levels of PspA isoforms were summed, we determined that there was a 49-fold increase between 14 and 72 h in production fermentations (Fig. 4g). Furthermore, the level of PspA at 72 h was 15-fold higher in production fermentations than in control fermentations (Fig. 4g).

Another stress protein, YbdQ (also known as UP12), was noticeably up-regulated in the control fermentations, although quantification from the production fermentations was difficult (Fig. 4g). This protein belongs to the family of universal stress proteins (6).

PspA manipulation for bioprocess development. The strong correlation between increased levels of PspA and recombinant antibody suggested that PspA could be a good target for bio-

process improvement. To determine whether controlled expression of *pspA* could enhance the soluble, recombinant antibody yield in *E. coli*, fermentations were performed with strain 59A7 harboring the production plasmid (pS1130) and pPspA (encoding PspA under the tightly controlled $P_{\text{tac}^{\text{lacUV5}}}$ promoter). IPTG was added to the culture medium at several concentrations (0, 0.02, and 2 mM) prior to product induction.

The results of Western blot analysis (Fig. 5) confirmed that the PspA level increased with an increase in the IPTG concentration. Growth was not affected by addition of IPTG at the levels tested, as the cell density profiles were comparable to those obtained in the absence of inducer (data not shown). The final soluble recombinant antibody yield obtained with 2 mM IPTG was low (0.53 g/liter), suggesting that there was possible competition for translational machinery when PspA expression was high. Therefore, an additional study was conducted with a lower IPTG concentration (0.02 mM instead of 2 mM).

Replicate fermentations were performed with and without 0.02 mM IPTG. Soluble antibody profiles were measured by protein G affinity chromatography. The results showed that addition of 0.02 mM IPTG had a positive effect on the final

TABLE 4. Protein spots identified by peptide mass fingerprinting after 2-D gel comparisons of control fermentations and production fermentations at 72 h: quantitative changes with 1% FDR criterion^a

Function	Protein	Fold change (production/control) ^b	Description
Stress	PspA (dominant isoform)	9.9	Phage shock protein A
	PspA (minor isoform)	16	Phage shock protein A
Nucleotide degradation	DeoB (minor isoform)	2.1	Phosphopentomutase
	DeoD (dominant isoform)	2.6	Purine nucleoside phosphorylase
	DeoD (minor isoform)	5.0	Purine nucleoside phosphorylase
Protein degradation	PepD (isoform)	0.6	Peptidase D
Incorporation of metal ions	Fes	0.6	Enterochelin esterase
Fatty acid biosynthesis	FabI	0.6	Enoyl-ACP reductase
Transcriptional regulator	OmpR (isoform)	0.5	Transcriptional regulatory protein of Omp expression
Lipopolysaccharide biosynthesis	LpcA	0.6	Phosphoheptose isomerase
Other	Gst	0.6	Glutathione transferase monomer
	YbIS	0.5	Conserved hypothetical protein
	YcaC	0.7	Putative cysteine hydrolase subunit
	YgfZ or UP14	0.6	Putative enzyme
			Putative phosphotransferase system
	Usg	0.5	Enzyme II A component
	YhgI	0.7	Putative uncharacterized transport protein

^a In certain cases, the spot identified was an isoform of another protein spot on the gel.

^b Fold changes were calculated by using geometric means.

FIG. 3. Zoomed-in views of 2-D gels for control and production fermentations at 14 and 72 h. (a) Recombinant antibody expression in production fermentations and corresponding regions of the gels for control fermentations. Dominant and minor light chain (LC) isoforms and heavy chain (HC) are indicated. The heavy chain was not highly soluble in the 2-D gel system, so it migrated as a smear. (b) Phosphate starvation proteins UgpB and PstS, which were quantified, and PhnD, which was not easily quantified in the gels.

soluble antibody yield (Fig. 6). The yield plateaued toward the end of the fermentation, so the values were averaged for the last three times (65, 69, and 72 h). The yield variability for the three replicate fermentations was higher when IPTG was added than when IPTG was not added, as indicated by the error bars in Fig. 6. Therefore, a one-tailed Welch t test (assuming unequal variances between the two data sets) was used to determine whether PspA induction improved soluble antibody yields significantly. The t test indicated that the mean increase in the yield (50%, from 0.71 to 1.1 g/liter) upon PspA overexpression (Fig. 6) was statistically significant ($P = 0.029$).

DISCUSSION

These studies were undertaken to better understand the physiological changes that occur in *E. coli* over the course of an industrial fermentation process and to possibly identify strategies for bioprocess improvement. For the quantitative analyses, a statistical approach was sought that would allow the recognition of significant protein spot quantity changes by using criteria that were strict enough to eliminate most false

positives without overlooking real protein changes. It is common in proteome studies to set a fold change criterion for a significant spot quantity change, which has ranged in previous studies from 1.5-fold (17) to 6-fold (37). Creating an arbitrary cutoff for protein spots of all intensities does not take into account effects of protein spot resolution or intensity. The importance of characterizing the significance level for a given change in protein expression as a function of spot intensity level was recently noted (2). In general, well-resolved protein spots are more reproducible than poorly resolved spots, and very-low-intensity spots are more variable than high-intensity spots. Because spot variability depends upon the properties of the protein (e.g., molecular weight and pI), we applied protein-specific t tests that treated the estimated variability of each protein spot independently. As part of a novel approach to dealing with quantitative analysis of 2-D gel data, FDR criteria were applied to account for the multiple hypothesis tests in our analyses when significant spot quantity changes were determined. This general technique is gaining popularity in statistical analysis of microarray experiments for transcriptomics (15,

FIG. 4. Protein quantity dynamics over the course of the fermentations. Time course graphs are grouped into the following categories (reading from left to right): anti-CD18 light chain (LC) isoforms (a), phosphate starvation proteins (b), ribosomal proteins (c), nucleotide biosynthesis proteins (d), nucleotide degradation proteins (e), oxidative damage defense proteins (f), and stress proteins (g). The red circles are data for samples from the production fermentations. The blue triangles are data for samples from the control fermentations. The data are the geometric mean spot quantities for the four gels corresponding to each sample point. The error bars were generated as described in Materials and Methods. The protein quantities represent the sum of isoforms in many cases.

39) but to our knowledge has not previously been applied to 2-D gel data.

Antibody light chain and heavy chain. As expected, overexpression of recombinant antibody light chain and heavy chain was observed in production fermentations but not in control

fermentations (Fig. 2, 3a, and 4a). As observed in this study, the light chain typically appears as two dominant isoforms (9). It may be deamidated, although detailed characterization of potential sites of deamidation was not performed. Major charge variants of recombinant proteins expressed in *E. coli* have been

FIG. 5. Western blot analysis conducted with anti-PspA antisera to show differential PspA induction from pPspA. IPTG was added at different levels in replicate fermentations at 29 h. Samples for immunoblot analysis were taken 6 h postinduction.

observed in other proteome studies. For example, in recombinant human growth hormone (Nutropin) production, the major acidic isoforms of the product were consistent with deamidated species, based upon detailed characterization of the purified recombinant protein (7). In the present study, in addition to the major anti-CD18 light chain isoforms, two minor, acidic isoforms were observed at a molecular weight slightly greater than that of the main forms (Fig. 3a and 4a). These minor isoforms may have been SsrA tagged (i.e., they may have harbored an 11-amino-acid tag that targeted them for proteolysis), a hypothesis which is consistent with their migration in the gels and with their absence from the proteomes of protease-positive host strains (9, 42). Truncated light chain was not observed in production fermentations, in contrast to observations in a previous study (9), because the host strain used here contained mutations in genes encoding the DegP and Prc proteases.

Phosphate starvation proteins. The appearance of recombinant antibody coincided with the up-regulation of phosphate starvation proteins at about 30 h (Fig. 4b). This up-regulation was expected because the growth medium was designed to become phosphate limited to induce recombinant antibody expression from the *phoA* promoter.

In general, phosphate starvation protein levels were lower in the production fermentations than in the corresponding control runs (Fig. 3b and 4b). This was not detected in the statistical analysis of 72-h samples with a 1% FDR criterion. However, some differences were recognized as significant when a 5% FDR criterion was applied. We speculated that because pS1130, the production plasmid, contained a *phoB* box (binding site), it competed for PhoB, the transcriptional activator of the expression of the phosphate starvation genes. Since the control plasmid, pBR322, did not contain a *phoB* box, cells

FIG. 6. Effects of manipulating PspA expression with various levels of IPTG prior to product induction on recombinant antibody yield. IPTG was added at 29 h. The bars indicate averages of three independent fermentations for addition of no IPTG or 0.02 mM IPTG, and the error bars indicate one standard deviation. Soluble, assembled F(ab')₂-LZ was measured by protein G affinity chromatography.

harboring this plasmid could have had higher levels of phosphate starvation proteins than cells harboring the production plasmid.

Ribosomal proteins. During the fermentations, the cell density increased linearly and then gradually plateaued with a decrease in the growth rate (Fig. 1). The number of ribosomes per cell (ribosome density) is growth rate controlled and decreases as the growth rate declines (29). Therefore, a decrease in ribosomal protein levels could be anticipated and was observed (Fig. 4c). Furthermore, phosphate-starved cells can obtain phosphate from ribosome degradation (11), and this likely contributed to the ribosomal protein down-regulation when growth rates leveled off.

Previous work has shown that synthesis rates (35, 38) and steady-state levels (17) of ribosomal proteins, as well as rRNA levels (36), decrease during recombinant protein synthesis in fed-batch fermentations. In the present study, the ribosomal protein decrease seemed to occur in both control and production runs. This decrease was not more prominent in the production runs (Fig. 4c), suggesting that recombinant protein expression was not responsible for the decrease in ribosomal protein.

RpsF occurs in several posttranslationally modified forms that differ in the length of the polyglutamate side chain added at the carboxy terminus (22), and these isoforms can be discerned on 2-D gels (41), which is consistent with our observations.

Nucleotide biosynthesis and degradation proteins. There was sharp down-regulation of nucleotide biosynthetic proteins between 14 and 27 h (Fig. 4d). One possible explanation for this is that a small decrease in the cellular ribosome content repressed (ribo)nucleotide biosynthesis early in the fermentations. The *E. coli* 59A7 host strain harbors a *deoC* mutation, which disrupts the pyrimidine salvage pathway. We speculated that this mutation may have contributed to the high levels of proteins involved in nucleotide biosynthesis observed at 14 h since cells cannot recycle pyrimidines; pyrimidine metabolism influences purine metabolism as well (33, 44). However, the *deoC* mutation does not explain the sharp decrease in nucleotide biosynthesis protein abundance.

Concerning nucleotide degradation, up-regulation of Udp and Cdd, two pyrimidine salvage enzymes, was recognized when a 5% FDR criterion was applied in our analysis of the control runs. This change was supported by the corresponding time course graphs (Fig. 4e). We postulated that ribonucleotides released by ribosome degradation may have induced these enzymes. There was a sharp increase in the levels of these proteins between 14 and 27 h, before phosphate starvation occurred, so they do not seem to be induced specifically to recover phosphate from nucleotides. The unexpected down-regulation of DeoD late in the control fermentations (Fig. 4e) is difficult to explain.

Oxidative damage defense proteins. Shortly after the fermentors were inoculated, the dissolved oxygen level decreased rapidly and remained low for the rest of the fermentations (Fig. 1a). The air sparged throughout the fermentations allowed the cells to continue respiratory metabolism. Over time, oxidative damage defense proteins changed in a variety of ways (Fig. 4).

It is not entirely clear why some of these proteins were

up-regulated. AhpCF is reportedly required to protect cells incubated under aerobic, phosphate-limited conditions (32), and the up-regulation of the subunits of this enzyme complex is consistent with the coincident up-regulation of the phosphate starvation proteins (Fig. 4b and 4f).

Certain members of the class containing the oxidative damage defense enzymes were down-regulated at the end of production runs compared to the control runs. For AhpC and SodA, this down-regulation was significant when a 5% FDR criterion was applied. The changes in protein levels followed trends over time (Fig. 4f), suggesting that they were real. However, the underlying induction mechanism is unknown. The difference between control and production fermentations with respect to phosphate starvation-associated up-regulation of AhpC and AhpF (Fig. 4f) may have been related to the absence of the *phoB* box from the control plasmid, as discussed above for phosphate starvation proteins.

Stress proteins. One of the most interesting and dramatic changes observed was the up-regulation of PspA in anti-CD18 production fermentations (Fig. 2 and 4g), as previously reported by Chen et al. (9). PspA is a stress protein known to be induced under a variety of conditions, including filamentous phage infection, blocked protein secretion, and proton motive force uncoupler addition (30, 40). This peripheral membrane protein plays a role in maintaining inner membrane integrity and the proton motive force.

One possible reason for the PspA up-regulation in production fermentations was that the Sec-dependent secretion system might have been partially blocked during overexpression of recombinant light chain and heavy chain. However, secretory protein precursors (40) were not detected for host or recombinant proteins. Previously, we described a 2-D PAGE analysis which revealed PspA up-regulation in a control fermentation for recombinant human growth hormone production in *E. coli* (7). In that case, PspA up-regulation was associated with the synthesis of a C-terminal β -lactamase fragment from a control plasmid (7). As in the present study, there was no evidence for secretion system blockage.

Up-regulation of DnaK and GroEL was not observed in our control fermentations, in contrast to previous 2-D PAGE studies performed with nonproducing cells (38, 43). There were several differences between the culture conditions employed in our studies and the culture conditions used in the other studies, including temperature. Our fermentation was conducted at 30°C (to provide enhanced soluble protein production), while the other fermentations were conducted at 35°C (38) or 37°C (43). Interestingly, while up-regulation of the stress proteins GroEL and DnaK was not observed, up-regulation of YbdQ, a stress protein induced under a variety of conditions (6), was seen in our control fermentations (Fig. 4g). DnaK and GroEL, which are cytoplasmic chaperones, were not necessarily expected to be up-regulated in production runs because the light chain and heavy chain were secreted into the periplasm and did not accumulate in the cytoplasm.

Few dramatic product-related changes upon proteome characterization. Overall, few dramatic changes were observed in the proteome of *E. coli* cells overexpressing recombinant anti-CD18. This observation is in contrast to previous reports (5, 14, 17, 23) of how gratuitous overexpression of genes in *E. coli* can lead to considerable physiological changes, including growth

inhibition and ribosome destruction. We did not observe growth inhibition in cells overexpressing anti-CD18 light and heavy chains (Fig. 1b). Furthermore, the protein changes in our control fermentations generally correlated well with the changes in production fermentations (Fig. 4), suggesting that the physiological responses were similar in the two cases. These similarities could be expected given our previous qualitative 2-D PAGE analysis of recombinant human growth hormone-producing fermentations (7). For several low-abundance protein spots, we observed on-off changes and statistically significant quantitative changes. These changes were not readily analyzed by peptide mass fingerprinting and remain unidentified.

Manipulation of PspA expression in production runs. To assess whether manipulation of PspA synthesis could elevate $F(ab')_2$ yields in *E. coli*, PspA synthesis was induced by adding various levels of IPTG to cells harboring pPspA (Fig. 5) prior to recombinant antibody expression. This was a preventive maintenance approach in which plasmid-encoded PspA was synthesized before chromosome-encoded PspA was induced. A moderate increase in the soluble antibody fragment yield was observed when 0.02 mM IPTG was added (Fig. 6). The levels of total and soluble light and heavy chains, as measured by reversed-phase HPLC, also increased with addition of 0.02 mM IPTG compared to no IPTG addition (data not shown).

Despite the accumulation of about 3 g of heavy chain per liter and 8 g of light chain per liter in these experiments, the amount of properly assembled divalent antibody fragment $[F(ab')_2]$ was only around 1 g/liter (Fig. 1c and 6). If the discrepancy were simply due to limited heavy chain, then almost 6 g/liter would have been expected. Obviously, light and heavy chains were not efficiently assembled into $F(ab')_2$. Several experiments have been performed to address this inefficiency of assembly, such as expressing the light and heavy chain genes from independent promoters and adjusting the timing of expression. These studies are important for determining the rate-limiting steps in proper assembly of secreted, recombinant proteins and will be described elsewhere (D. Andersen, unpublished data).

Stress protein coexpression has been used to improve the cytoplasmic solubility (1, 13) and/or secretion (25) of aggregation-prone recombinant proteins in *E. coli*. PspA coexpression was recently shown to increase transport efficiency through the Tat translocation pathway (12), a secretion pathway separate from the Sec system. However, the mechanism by which PspA improved product yield in our study is not yet known.

As noted in another study (17), a number of interesting protein spot changes are observed in characterizations of fed-batch fermentations by 2-D PAGE, but they are difficult to explain. Proteomic analysis of fermentation processes can nonetheless be useful for suggesting potential targets for bioprocess improvement (18), especially targets that are not obvious a priori. In this work, PspA was such a protein. By manipulating the coexpression of PspA with a recombinant antibody fragment in *E. coli*, the yield of a secreted biopharmaceutical in an industrial process was improved.

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