Proteomic Analysis of Extracellular Proteins from *Escherichia coli* W3110

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While numerous proteomic analyses have been carried out on *Escherichia coli,* the vast majority have focused on expression of intracellular proteins. Yet, recent literature reports imply that even in laboratory strains, significant proteins may be found outside the cell. Here, we identify extracellular proteins associated with nonpathogenic *E. coli* strain W3110. Two-dimensional gel electrophoresis (2DE) revealed approximately 66 prominent protein spots during exponential growth (4 and 8 h shake flask culture) in minimal medium. The absence of detectable nucleic acids in the culture supernatant implies these proteins did not result from cell lysis. MALDI-TOF MS was used to identify 44 proteins, most of which have been previously identified as either outer membrane or extracellular proteins. In addition, 2DE protease zymogram analysis was carried out which facilitated identification of three extracellular proteases, one of which was not observed during standard 2DE. Our results are consistent with previous findings which imply outer membrane proteins are shed during growth.

Keywords: *Escherichia coli* • proteomics • mass spectrometry • zymogram

Introduction

Protein secretion into the growth environment plays an important role in the physiology and metabolism of both prokaryotic and eukaryotic microorganisms. While there are many reports in the literature regarding protein secretion from fungi[1] and Gram-positive bacteria (e.g., *Bacillus* sp.),[2] studies on protein secretion from Gram-negative bacteria, especially from *Escherichai coli*, are quite limited.[3] This is in spite of the fact that *E. coli* is widely used in the biotechnology industry for expression of recombinant proteins and as a model pathogen. The protein expression studies that do exist typically focus either on the identification, purification, or characterization of single secreted proteins, or on intracellular protein analysis.[4–8] As far as we are aware, there has not yet been a report on global analysis of extracellular protein associated with *E. coli* W3110.

This absence of data may be due to the fact that laboratory strains of *E. coli* are generally classified as nonsecretors of protein. However, in pathogenic *E. coli*, the secretory mechanism of a number of virulence factors has been studied in some detail.[9,10] In contrast, nonpathogenic *E. coil* are typically believed not to secrete protein. Recently, however, reports have shown that cryptic genes coding for secretion and pilation are also present in *E. coil* K12 laboratory strains.[11–13] These genes can be expressed during environmental variations and lead to the release of extracellular proteins.[14–16] This implies that analysis of extracellular proteins from both pathogenic and nonpathogenic *E. coli* strains can be important. This is espe-

cially true as nonpathogenic *E. coli* have been engineered to secrete recombinant proteins for the bioprocess industry.[17–19]

To date, proteome analysis employing the high resolving power of two-dimensional gel electrophoresis (2DE) coupled with highly sensitive mass spectrometry has proven to be the most powerful method for identification of proteins in complex mixtures and is suitable for the study of alteration of protein expression in an organism under varying environmental conditions.[20] With the use of this powerful approach, several recent studies have focused on analysis of extracellular proteins from Gram-positive bacteria used in the bioprocess industry (*Bacillus* sp.[11,21]) and pathogens (*Staphylococcus aureus*[12] and *Streptococcus pneumonia*[13]). Reports are also available on Gram-negative pathogens (*Helicobacter pylori*,[22] *Pseudomonas*,[23] and *Edwardsiella tarda*[24]).

Our objective in this study was to develop a better understanding of which cellular proteins are present in the extracellular environment when *E. coli* is grown in suspension culture. To accomplish this, we used a proteomic analysis approach employing 2DE to separate, and mass spectrometry to identify, extracellular proteins produced during shake flask cultivation of *E. coli* W3110.

Materials and Methods

Strain, Culture Conditions, and Analytical Methods. *E. coli* K12 wild-type strain W3110 (F-, IN (rrnD-rrnE) 1,λ-) was obtained from Coli Genetic Stock Center (CGSC). Storage, medium preparation, and growth conditions for this strain have been described previously.[25] The growth medium contained per liter: glucose, 10 g; KH$_2$PO$_4$, 13.3 g; (NH$_4$)$_2$HPO$_4$, 4 g; citric acid,

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1.7 g; EDTA, 8.4 mg; $MgSO_4 \cdot 7H_2O$, 1.2 g; $CoCl_2 \cdot 6H_2O$, 2.5 mg; $MnCl_2 \cdot 4H_2O$, 15 mg; $CuCl_2 \cdot 2H_2O$, 1.5 mg; H_3BO_3, 3 mg; $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg; $Zn(CH_3COO)_2 \cdot 2H_2O$, 13 mg; ferric citrate, 10 mg. The media were sterilized by autoclaving for 20 min at 121 °C. Cultivations were carried out in 850 mL of medium, at 250 rpm in a baffled 3 L Erlenmeyer flask, at 37 °C, for 24 h. Samples were withdrawn at regular intervals. Total extracellular proteins in the culture broth were measured by Bicinchonic acid method (Pierce, Rockford, IL). Total nucleic acids were measured fluorimetrically using the fluorescent dye bisBenzimide (Hoechst 33258; Sigma Chemical Co., St. Louis, MO).[26] Biomass was quantified by measuring OD at 600 nm. Glucose was measured by DNS (Dinitrosalicylic acid; Sigma Chemical Co., St. Louis, MO) method[27] using glucose as standard.

Protein Sample Preparation. Samples for proteome analysis were prepared in a manner similar to that used previously.[12,13,20,24,28] Briefly, cells were removed by centrifuging at 6000*g* for 15 min and discarded. The clear supernatant was collected, passed through a 0.22 μm filter, and proteins were precipitated by treating with 20% (v/v) TCA (trichloroacetic acid; Sigma Chemical Co., St. Louis, MO) in ice for 30 min. The precipitate was collected by centrifugation at 6000 × *g* for 10 min at 4 °C. The precipitated protein was further washed with acetone to remove traces of TCA, and finally, acetone was removed by speed vacuum treatment. TCA-precipitated protein pellets of microbial proteins were resolubilized in sample solubilization buffer (8 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT (Sigma Chemical Co., St. Louis, MO), and ampholytes 2% (v/v) 3–10 NL (Amersham Biosciences, San Francisco, CA)). After 2–3 h with intermittent vortexing, insoluble materials were removed by centrifugation at 6000*g* for 10 min. The supernatant was collected, and total soluble protein concentration was measured (PlusOne 2-D Quant Kit, Cat. No. 80-6483-56; Amersham Biosciences, Piscataway, NJ). The resulting solution was then stored at − 80 °C for later use in 2D electrophoresis.

2D Electrophoresis. Rehydration of Immobiline Dry Strips (IPG strip; Amersham Biosciences, Piscataway, NJ) with 22 μg protein sample was carried out with an Immobiline Dry Strip Re-swelling Tray (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions. IPG strips (pH 3–10 NL), 7 cm long, were used for the present study. The rehydrated strips were then subjected to IEF. Isoelectric focusing was performed using Multiphor II electrophoresis unit at 20 °C in gradient mode. Briefly, 7 cm strips were focused at 0–200 V for 1 min, 200–3500 V for 2.30 h, and 3500 V for 2.15 h, with a total of 8 kVh accumulated. After focusing, the strips were stored at − 80 °C for later use. Prior to the second dimension SDS-PAGE, IPG strips were equilibrated for 15 min in equilibration solution (5 mL) containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, and traces of bromophenol blue with 100 mg/10 mL (w/v) of DTT. A second step was carried out for 15 min by adding iodoacetamide (250 mg/10 mL) instead of DTT in equilibration solution. Five milliliters of equilibration solution was used for the 7 cm strip. Second dimension vertical SDS-PAGE was performed using precast mini-gels (Ready Gel, 12% Tris-HCl gel; Bio-Rad Laboratories, Hercules, CA), 1 mm in thickness. A mini-vertical electrophoresis system (Mini-Protean II) was employed according to the manufacturer's instructions. Briefly, electrophoresis was performed at a constant current of 5 mA/gel for 20 min followed by 12 mA/gel for 1.5 h until the bromophenol band had exited the gel. Gels were stained with neutral silver stain as described previously.[29] Electropherogram images were

obtained with an imaging densitometer (GS-800; Bio-Rad Laboratories, Hercules, CA) in gray-scale mode. Molecular weight and p*I* values were estimated using 2D electrophoresis SDS-PAGE markers (Cat. No. 6539; Sigma Chemical Co., St. Louis, MO and Cat. No. 161-0310; Bio-Rad Laboratories, Hercules, CA).

2D Protease Zymogram Analysis. Mini 2D zymogram gels were prepared using 10% Tris-HCl gel, 1 mm in thickness, having 0.9% gelatin (Sigma Chemical Co., St. Louis, MO) as the substrate for detecting protease activity. The gels were stored at 4 °C until use. The IEF was carried out as described above, and the focused strips were subjected for 2D electrophoresis also described above. After electrophoresis, the gels were preincubated for 3–5 h in 2.5% Triton X-100 for renaturing the protein. The gels were subsequently transferred to protease activity buffer (Bio-Rad Laboratories, Hercules, CA) and incubated at 37 °C overnight. Finally, the gels were stained with Coomassie blue for 3 h and destained several times in 50% methanol and 10% acetic acid in water until the clear zones for protease activity were visible. Electropherogram images were obtained with an imaging densitometer (GS-800; Bio-Rad Laboratories, Hercules, CA).

2D Gel Image Analysis. Image analyses of gels were performed using Z3 software (Compugen, Tel Aviv, Israel) as described previously.[25] Briefly, images of multiple gels were used to construct a composite or "raw master gel" (RMG) which eliminated noise and minor experimental discrepancies between gels. Spots on the gels were then identified and assigned a spot quantity (*q*) which characterizes spot size and intensity, is defined as the sum of the gray-level values of all pixels in a spot, and is expressed in terms of ppm (parts per million) of the total spot quantity on the gel. Thus, *q* is an approximate fractional representation of the amount of protein in a particular spot, and the ratio of *q* values, for the same spot on two different gels, gives an approximation of differential protein expression. Previous reproducibility studies in our laboratory have shown that differential expression differences between 2- and 3-fold are typically significant.[25]

Mass Spectrometry for Protein Identification. For mass spectrometric identification, gel spots were excised, destained, and digested with sequencing grade trypsin (Promega, Madison, WI) as described previously.[30] Peptides were extracted twice by adding 30 μL 0.1% trifluoroacetic acid in 30:70 water/acetonitrile. Desalting of the extract was carried out using ZipTip C 18 reverse-phase peptide separation matrix (Millipore, Billerica, MA). Samples were either used directly or concentrated under evaporative centrifugation and subjected to matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (Autoflex; Bruker Daltonics, Billerica, MA). Matrix solution was prepared fresh daily by dissolving 50 mM α-cyano-4-hydroxycinnamic acid in 30/70% acetonitrile/0.1% TFA (trifluroacetic acid) in water. To prepare samples, 0.3 μL of peptide solution was mixed with an equal volume of matrix solution, deposited on the instrument target plate, and air-dried at room temperature. Mass spectra of peptide digests were acquired as the average of the ion signals generated by the irradiation of the target with 50–100 laser pulses, in positive linear mode with an acceleration voltage of 20 kV. External calibration was performed using the standard proteins brady kinin (MH+ 1060.1) and insulin (MH+ 5734.6) and internally using trypsin autolytic peak (MH+ *m/z* 2212.42). The average peptide masses obtained were used for database search using ProFound (http://prowl.rockefeller.edu/). The search was car-
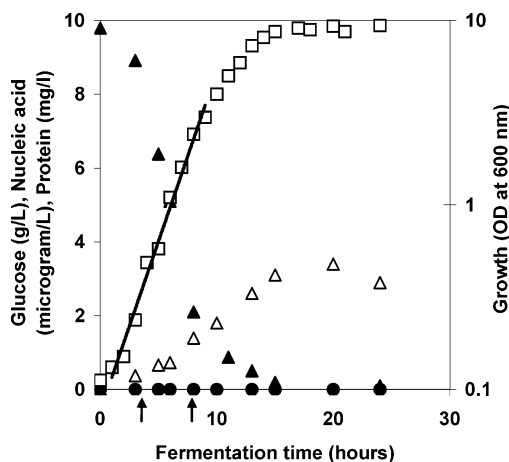
Figure 1. Typical growth curve of *E. coli* W 3110 during shake flask cultivation in minimal medium supplemented with glucose. Growth OD at 600 nm (□), glucose concentration (▲), total extracellular protein (△), and nucleic acid concentration (●). Arrows on time scale indicate proteome sample time (4 and 8 h). Straight line indicates exponential growth ($\mu = 0.42$ h^{-1}).

ried out between MW 10−100 and pI 3−10, allowing modification by iodoacetamide (Cys), with a maximum missed cut of 1. Identification was obtained by selecting proteins with a Z-score of 2.4 and a probability of at least 1.0e+000. Identifications obtained had at least 20% sequence coverage with 0.02 Da average mass tolerance or at least 4 peptides matched.

Results and Discussion

Cell Growth and Extracellular Protein Expression. Figure 1 shows time profiles for cell mass (absorbance at 600 nm) and glucose concentration during typical *E. coli* shake flask cultivations used in this study. Cells consumed glucose as sole carbon source, and grew exponentially for the first 10 h at a growth rate of 0.42 h^{-1}. At various time intervals, soluble, extracellular protein concentration was measured and found to reach a maximum of 3.5 mg L $^{-1}$. To determine whether extracellular protein resulted from cell lysis, we tested the culture supernatant as described previously,[26,28] and reproducibly found no detectable nucleic acids prior to 24 h (Figure 1). As a positive control, 36 and 48 h cultures were tested and showed a measurable amount of DNA (data not shown). In addition, we note that 2D gels (Figure 2) were significantly different from gels of *E. coli* lysate,[25,31] and (as described below) were devoid of specific cytosolic proteins that would likely be present in cell lysate (e.g., Hsp60 and Hsp70).

Our findings are in agreement with a recent study that employed the same *E. coli* strain.[28] However, these authors conducted high cell density fermentations and found that the extracellular protein concentration reached a value of 140 mg L $^{-1}$, in the absence of detectable nucleic acids. Additionally, they found that the concentration of extracellular membrane components (lipopolysacchrides and phospholipids) also increased. This led to the speculation that extracellular proteins resulted from outer membrane "shedding" versus cell lysis. We note that it is often assumed that protein secretion, without concomitant cell lysis, occurs primarily in Gram-positive bacteria[11] and in only a very few Gram-negative species (e.g., *Pseudomonas* sp.[23]). Thus, even though we found a relatively low concentration of extracellular protein in our culture supernatant, the identity of these proteins may provide valuable

information on the physiology and metabolic status of *E. coli* during cultivation.

Proteome Analysis. To initially separate and subsequently identify extracellular proteins, we used 2DE and mass spectrometry as described in the Materials and Methods. As *E. coli* physiology and metabolism have been reported to change during growth,[8] we selected samples from the early (4 h)- and late (8 h)-exponential phase for 2DE analysis (Figure 2). For each time point, image analysis of three gels, derived from three separate shake flask cultures, showed little pattern deviation and a high degree of reproducibility (data not shown). In addition, protein profiles of un-inoculated control flasks showed no protein spots. We note that in both 4- and 8-h gels, proteins are more concentrated in the acidic region (pI 4−7). The initial medium pH was 7.0 and reached a final value of pH 6.2. Proteins were also found distributed over a wide molecular weight range (10−110 kDa) with many high molecular weight proteins detected. A number of 8-h protein spots appear to be larger and darker than corresponding spots in the 4-h gels. This was confirmed by image analysis and quantification *(q* value). Results are presented in Figure 3. Of the 66 most significant protein spots, at least 18 showed significantly higher abundance in the 8-h gel than in the 4-h gel. This increase in protein concentration with time is consistent with the speculation that *E. coli* are shedding outer membrane or periplasmic proteins during growth.[28] We note, however, that at least three prominent spots (63, 64, and 65) are only present on the 4-h gel. The disappearance of these three spots may be due to low protein stability or proteolytic degradation in the later stages of growth.[22]

Of the 66 nominal protein species observed, we successfully identified 44 (Table 1). Previous studies imply that most are either membrane bound or secreted proteins. Many of these proteins are exported by specific secretory pathways and may aid in colonization, defense, or in providing nutrients for the cell. A number of these proteins have been reported as being growth phase-dependent and strictly regulated by environmental signals or under quorum sensing control.[9,32] Details follow.

Porin and Related Proteins. Porin and related proteins (spots 24, 25, 30, 31, 32, and 38) comprised the most abundant group detected in our culture supernatant and were present during both early (4 h)- and late (8 h)-exponential phase. These proteins generally form pore-like structures through which small hydrophilic molecules may passively diffuse and, thus, are typically located in the *E. coli* outer membrane.[33] Transport of these proteins to the outer membrane, or to the extracellular culture medium, is not well-understood. One theory is that outer membrane-anchored proteins have membrane-directed signals for secretion.[34] Alternatively, ompla (outer membrane phospholipase A) may be activated during growth and then converts the phospholipids to lysophospholipids, which may be responsible for the asymmetry of the outer membrane. The effect of this would be the release of membrane or periplasmic proteins to the culture medium.[35] Consistent with this is the increased abundance of phosphoporin (phoE), outer membrane protein F (Porin ompF), and outer membrane porin protein E (phoE) (spots 31, 30, and 32, respectively) in our late-exponential phase sample (8 h). Similar behavior was observed for ompF during high cell density fermentation[33,36] where increased cell density led to increased soluble ompF.

Hemolysin, Colicin, and Related Proteins. We also detected a number of known bacterial toxins in the extracellular
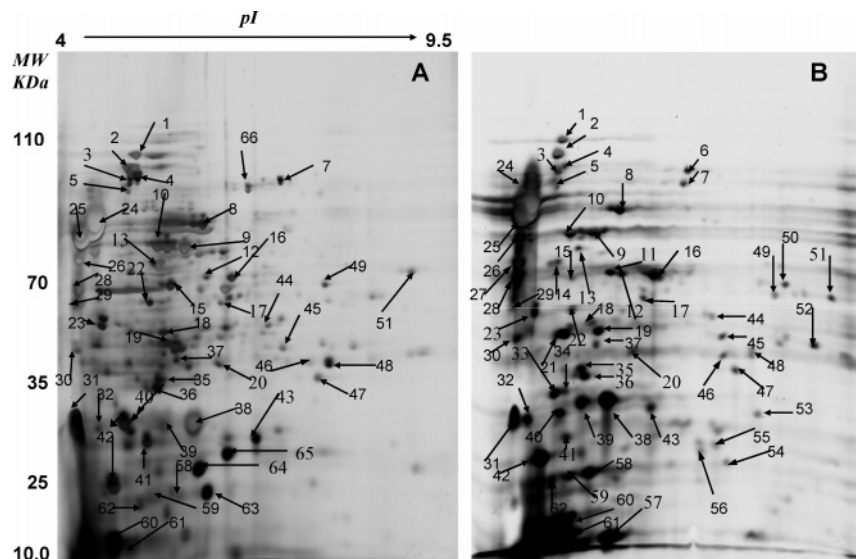
Figure 2. Two-dimensional electrophoresis (2DE) gel maps of extracellular proteins of *E. coli* W3110 cells grown in a shake flask on a glucose minimal medium. (A) Extracellular protein samples analyzed after 4 h of growth; (B) extracellular protein samples analyzed after 8 h of growth. Notations refer to the representative protein spots investigated in the present study (Table 1).
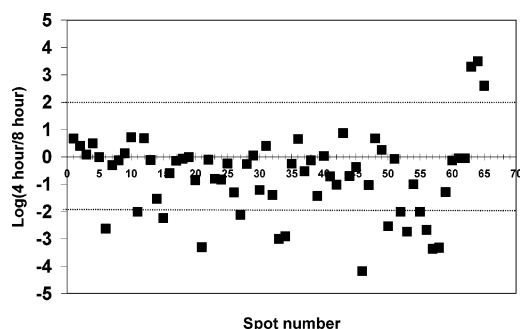

Figure 3. Expression level analysis of secreted proteins from 2D gel at two different sampling times (4 and 8 h). Image comparison was carried out with Z3 image analysis software as described in Materials and Methods.

medium, including hemolysin-related proteins (spots 1, 9, and 37). While pathogenic strains of *E. coli* are known to secrete exotoxins, such as hemolysin,[37] it has also been reported that nonpathogenic *E. coli* can release hemolysin due to the overproduction of several transcriptional regulators.[38] These appear to induce hemolytic activity absent under typical laboratory growth conditions.[38] It has also been reported that nonpathogenic *E. coli* contain a *gsp* gene cluster, homologous to the *esp* genes encoding the secretory machinery for toxins such as hemolysin.[38] Lack of expression of these genes under normal circumstances may be due to the transcription factor HNS, which has been shown to negatively regulate the GSP (general secretory pathway), as well as other virulence factors.[39−41] In addition to hemolysin, several colicin-related proteins were identified in the culture supernatant (spots 26, 34, and 57). Spot 34 was identified as colicin V (cvaA) and was present in culture supernatant from both growth samples. This protein has previously been reported to be produced by *E. coli* K-12 strains.[42] Spot 57 was identified as microcin, which is known to act against colicin, thus, protecting the cells themselves against colicin lethality.[43]

Interestingly, overexpression of *E. coli* lysis proteins, such as colicin and hemolysin, can activate otherwise dormant phosphophlipase, resulting in hydrolysis of phosphatidyletha-

nolamine (PE).[44] The result is the accumulation of lysophosphatidylethanolamine (LPE), which accumulates in the outer membrane. This presumably makes the membrane permeable to proteins and may help explain the accumulation of typically nonsecretory proteins in the extracellular medium.[45]

Transport/Export Proteins. We identified a number of proteins with transmembrane-related functions such as transport of proteins, sugars, or other components. Some of these proteins are found up-regulated in the 4-h sample. For example, protein spot 48, identified as Transport ATP binding protein (msbA), is up-regulated in 4-h samples. Spot number 65, which is unique and present only in the 4-h sample, has been identified as membrane-bound ATP synthase subunit (AtpB). While the reason it was present only in the 4-h sample is not clear, it suggests the protein may have been proteolytically degraded or unstable in the given extracellular conditions. Spot 39 was identified as putative transmembrane permease (YcjO) and was present in higher concentration in late-exponential phase samples. This protein is reported to be a putative membrane component and belongs to the uncharacterized members of the ABC super family of transporters (YcjO, YcjP, and YcjN).[46] On the basis of sequence similarity, these proteins may function together as an ATP-dependent sugar transport system.

Type II Secretory System and Other Proteins. Recently, a Type II secretion pathway was identified in nonpathogenic *E. coli*.[47] It has been reported that a subset of the Type II secretion system can form inner membrane pilus-like structures which act as pistons,[48] "pushing" secreted protein through the gated pores. In this study, we identified several extracellular proteins involved in the Type II pathway. One such protein appears to be Protein K (gspK; spot 19), of the general secretion pathway. We also identified extracellular enzymes whose transport is said to be controlled by the Type II secretion system, specifically lipase (spot 40) and proteases (spots 7 and 42). It has been reported that in many Gram-negative bacteria the secretion of proteases and lipases is under quorum-sensing control and is growth phase-dependent.[9] Consistent with this, we observed an up-regulation of metalloproteinase (spot 42) in late-exponential phase (8 h) culture. Similar results, in the extra-

Table 1. Extracellular Proteins from *E. coli* W3110 Identified via MALDI-TOF[a]

Spot No.	Protein ID	Sequence Coverage (%)	p*I*	MW	Accession Number	Gene
	Porin and Related Proteins					
24	Probable outer membrane porin involved in fimbrial assembly	50	4.9	95.82	Q8X2D8	ECS0
25	Pilin porin homologue htrE precursor	59	4.9	95.6	B53303	HTRE
31	Phosphoporin (Phoe)	32	4.8	36.82	EG10729	PHOE
30	OMPF outer membrane protein F precurser (porin OMPF) (outer membrane protein 1A or B)	43	4.8	39.32	P02931	OMPF
38	Chain A-Maltoporin Sucrose Complex	21	4.7	47.8	Q8X5W7	LAMB
32	Outer membrane porin protein E	33	4.9	38.9	P02932	PHOE
	Hemolysin, Colicin, and Related Proteins					
1	HLYI *E. coli* hemolysin chromosomal	30	5.5	109.55	P09983	HLYA
9	Putative outermembrane transporter ShiA/HecA/FhaA exoprotein	38	6	61.17	Q8XAN8	Z1543
37	Hemolysin Secretion protein D, Chromosomal	25	5.8	54.57	P09986	HLYD
26	CEAD *E. coli* Colicin D	60	5	74.67	P17998	CDA
34	Colicin V Secretory protein CVAA	36	6.9	47.33	P22519	CVAA
57	IMMD Colicin D immunity protein (Microcin D Immunity protein)	25	5.2	10.15	P11899	CDI
	Transport/Export/General Secretion Pathway Proteins					
3	Putative secretary effector protein	60	4.5	88.84	Q8X5G6	ECS1560
4	YFCU hypothetical outermembrane usher protein in AROC-FADC intergenic region precurser	20	4.7	97.19	P77196	YFCU
5	Hypothetical outer membrane protein yejo	37	4.7	91.24	P33924	YEJO
10	Secretion pathway related protein	39	6.6	51.94	O82884	ETPE
39	Putative transport system permease protein	28	4.8	31.319	Q8X8J2	YCJP
48	*E. coli* probable transport ATP biniding protein MSBA	27	8.8	64.56	Q8FJB1	MSBA
65	Membrane-bound ATP synthase subunit a AtpB	25	6.4	30.24	P00855	ATPB
19	Putative exoprotein J for general secretion pathway-protein K	24	5.6	37.34	P45762	GSPK
61	Putative transport Protein	21	4.7	25.78	A57723	YFJD
	Enzymes					
6	Putative amidase	34	8.9	68.02	Q8XDF5	YCBB
7	Proteinase II (EC 3.4.21.-)	46	5.8	82.18	P24555	PTRB
40	Putative lipase	28	4.9	36.47	Q8XD38	YBAC
42	Putative protease	30	4.8	27.1	Q8FJB5	YCAL
41	YBGC 25.8 kDa protein in PHRB−NEI intergenic region	54	5.8	26.07	P08999	YBGC
Z[b]	Putative protease	38	6.4	59.42	P37657	YHJS
	Adhesion/Receptor Proteins					
14	Translocated intimin receptor	30	4.8	56.32	P50190	TIR
21	UDP-*N*-acetyl glucosamine 2 epimerase (UDP-GlcNAc-2epimerase) Bacteriophage N4 adsorption protein C	23	5.8	42.46	Q8XAR8	WECB
28	Partial putative adhesion protein	66	4.8	55.5	NP287448	Z1963
58	Adhesin/virulence factor HeK	22	4.7	27.86	Q93LE3	HEK
	Lipoproteins					
44	NLPB *E. coli* lipoprotein 34 precursor	40	5.3	36.88	P21167	NLPB
8	Hypothetical lipoprotein yjbH precursor	48	6.1	78.64	P32689	YJBH
36	Outer membrane protein	28	5	34.888	Q8X8K6	OMPG
	Flagellar Proteins					
20	Flagellar motor switch protein FliG	27	4.7	36.76	P31067	FLIG
33	Flagellar bioynthesis protein FlgD	28	4.2	23.58	NP309480	FLGD
35	Flagellar hook associate protein (AAP1)	31	4.5	57.91	P33235	FLGK
	Other Membrane and Secreted Proteins					
15	YCGO *E. coli* putative Na(+)/H+ exchanger YCGO	28	5.2	62.44	P76007	CVRA
22	LeoA *E. coli*	31	5.1	65.09	Q9RFR9	LEOA
23	Hypothetical protein	24	4.7	39.23	NP415047	YFJK
43	Putative outer membrane usher protein precursor	29	4.9	40.39	Q7A9Y7	ECS4429
49	Putattive ATP binding component	26	9.4	46.20	NP 418649	YTFR
63	Hypothetical mambrane protein	21	5.4	28.98	P77307	YBBM
64	Putative integral membrane protein	27	5.2	27.29	Q7AAE6	ECS4139

[a] Proteins are classified according to function and location. [b] Protein identified from 2D zymogram (Figure 4), and not found in Figure 2.

cellular medium, have been observed during overproduction of serine proteases in *Aeromonas hydrophila*[49] and lipases in *Burkholderia cepacia*.[50]

To further assess extracellular protease activity, we used a 2D zymogram approach with gelatin as the substrate. The protease activities in the gelatin/polyacrylamide gel can be visualized after Coomassie staining as clear zones on a blue background. We detected and successfully identified three protease activities from our 8-h sample, as evidenced by the clear zones in Figure 4. These proteases were identified as

Proteinase II (PtrB), putative protease (YcaL), which were also found in previous 2D gels (spots 7 and 42), and putative protease (YhjS) which was not previously identified in Figure 2. As far as we are aware, this is the first 2D-zymogram of extracellular protease from *E. coli* W 3110 that has been reported. We note that an obvious step for improving industrial fermentations may be to delete the genes responsible for expression of these proteases. However, although proteases were present in the extracellular milieu, there appears to have been relatively little proteolysis as evidenced by the fact that
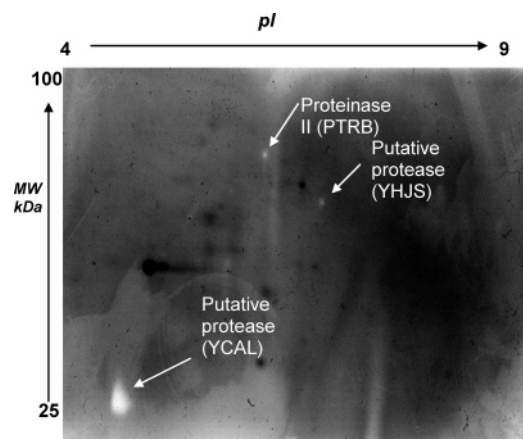
Figure 4. 2D electrophoresis zymogram analysis of *E. coli* W3110 extracellular proteases from 8-h sample. Clear zones indicate protease activity on Coomassie-stained gel. Identification of the proteases was carried out via MALDI-TOF mass spectrometry (as described in Materials and Methods). PTRB and YCAL correspond to spots 7 and 42 in Figure 2. YHJS was not identified in Figure 2.

most identified proteins migrated to their approximate theoretical molecular weight on the 2D gels (Figure 2).

Gram-negative bacterial lipoprotein is exported by the general secretion pathway and typically consists of a large group of proteins with many different functions. These proteins are normally anchored either at the inner or the outer membrane and have a signal sequence at the N-terminal end.[16] In our cultures, we identified several lipoproteins released to the medium (spots 44, 8, and 36).

We grouped other prominent identified proteins as adhesion/receptor proteins, flagellar proteins, and membrane-related proteins. We note that nearly all of these are typically associated with the outer membrane.

Conclusions

Using 2DE, mass spectrometry, and zymogram analysis, we report the extracellular proteome analysis for the *E. coli* K 12 laboratory strain W 3110. Out of 66 putative protein spots, we successfully identified 44 proteins. These cover a broad repertoire of proteins that are typically either secreted or belong to the outer membrane compartment of *E. coli.* Consistent with the speculation of others, the accumulation of soluble membrane proteins may be due to simple "leaking" or shedding of outer membrane components to the culture supernatant,[37] with little or no cell lysis as reported earlier during *E. coli* fermentations.[28] Thus, we conclude that the same phenomena is likely occurring here, that extra-cellular proteins are not the result of lysis, but of "leaking" or shedding outer membrane. We were able to detect three proteases using a 2D-zymogram approach, which gives direct evidence of secreted proteases in the culture broth. A change in environmental conditions during growth may have played a major role in the release or up-regulation of some proteins in these noncontrolled shake flask cultivations. The occurrence of some of the toxins may be due to the expression of some of the cryptic genes in *E. coli* K 12 strain W 3110 which are known to produce functional proteins due to changes in environmental conditions or stresses. Presence of these proteins may impact cellular behavior during fermentation or complicate recovery of beneficial products. Thus, our results imply further studies to broaden the secretory proteome,

of both pathogenic and nonpathogenic strains, may provide information on physiology applicable in the study of both pathogens and strains used for production of recombinant protein.

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