Research Article

Identification of IbeR as a Stationary-Phase Regulator in Meningitic *Escherichia coli* K1 that Carries a Loss-of-Function Mutation in *rpoS*

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IbeR is a regulator present in meningitic *Escherichia coli* strain E44 that carries a loss-of-function mutation in the stationary-phase (SP) regulatory gene *rpoS*. In order to determine whether *IbeR* is an SP regulator in E44, two-dimensional gel electrophoresis and LC-MS were used to compare the proteomes of a noninvasive *ibeR* deletion mutant BR2 and its parent strain E44 in the SP. Four up-regulated (*TufB*, *GapA*, *OmpA*, *AhpC*) and three down-regulated (*LpdA*, *TnaA*, *OpmC*) proteins in BR2 were identified when compared to E44. All these proteins contribute to energy metabolism or stress resistance, which is related to SP regulation. One of the down-regulated proteins, tryptophanase (*TnaA*), which is regulated by *RpoS* in other *E. coli* strains, is associated with SP regulation via production of a signal molecule indole. Our studies demonstrated that *TnaA* was required for E44 invasion, and that indole was able to restore the noninvasive phenotype of the *tnaA* mutant. The production of indole was significantly reduced in BR2, indicating that *ibeR* is required for the indole production via *tnaA*. Survival studies under different stress conditions indicated that *IbeR* contributed to bacteria stress resistance in the SP. Taken together, *IbeR* is a novel regulator contributing to the SP regulation.

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1. Introduction

Neonatal bacterial meningitis continues to be the most common serious infection of the central nervous system (CNS) in newborns with high morbidity and mortality despite the availability of effective bactericidal antibiotics over the last sixty years [1, 2]. This high morbidity and mortality are due to inadequate knowledge of the pathogenesis of this disease.

E. coli is the most common gram-negative bacterium causing neonatal sepsis and meningitis [1]. Bacterial meningitis in newborns is due to hematogenous spread of the pathogen to the meninges. The most important issue in the pathogenesis of *E. coli* meningitis is how circulating pathogens cross the blood-brain barrier (BBB), which is mainly

composed of brain microvascular endothelial cells (BMECs) [3, 4]. Our previous studies showed that *E. coli* K1 invasion of human BMEC was significantly greater with stationary-phase (SP) cultures than with exponential-phase cultures, suggesting that expression of *E. coli* K1 invasion-associated virulence genes is strongly regulated in a growth-phase-dependent manner [5]. A nonsense mutation in the SP regulatory gene *rpoS* was identified in *E. coli* K1 strains E44 and IHE3034 [5]. Complementation with the wild type *E. coli* K12 *rpoS* gene significantly enhanced IHE3034 invasion of BMEC, but failed to improve the invasion activity of another *E. coli* K1 strain E44. These studies suggest that the growth-phase-dependent invasion of BMEC by IHE3034 is affected by *RpoS* and that E44 carries a loss-of-function

TABLE 1: *E. coli* K1 (meningitic) or K12 (nonpathogenic) strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference(s)
Strains		
RS218	O18 : K1 : H7 (CSF)	[6–9]
E44	RS218, Rif ^r	[6–9]
DH5 α (λ pir)	K 12 strain	[6, 7]
SM10 (λ pir)	K 12 strain	[6, 7]
MC4100	K 12 strain	[5]
RH90	K 12 strain, <i>rpoS</i> deletion mutant	[5]
BR2	<i>ibeR</i> deletion mutant of E44	This study
TNA44	<i>tnaA</i> deletion mutant of E44	This study
Plasmids		
pCVD442	Amp ^r , oiRr6K, <i>sacB</i> , mobRP4	[10, 11]
pCBR2	pCVD442 carrying an <i>ibeR</i> deletion, Amp ^r	This study
pGEM-T	Amp ^r , <i>lacZ</i>	Promega
pCTNA2	pCVD442 carrying a <i>tnaA</i> deletion, Amp ^r	This study
pGTNA	pGEM-T carrying a 3.7 kb fragment containing <i>tnaA</i> gene	This study
pStyABB	containing the gene of monooxygenase for indole assay, Amp ^r	[12]
pWKS30	Amp ^r , <i>lacZ</i>	[6]
pWKS1030	pWKS30 carrying an 18 Kb <i>ibeR</i> locus	[6]

Amp^r, ampicillin resistant; *lacZ*, a partial gene coding for the N-terminal fragment of β -galactosidase; Kan^r, kanamycin resistant; Rif^r, rifampin resistant.

mutation in the *rpoS* gene. However, the SP gene regulation in E44 has remained an unanswered question.

Several virulence factors, including Ibe (termed after invasion of brain endothelial cells) proteins [6, 7], OmpA [8], YijP [9], FimH [13], AslA [14], and TraJ [15], have been identified in various strains of *E. coli* in the in vitro and in vivo models of the BBB as invasins. Most of those invasion genes are present in the *E. coli* K-12 genome [4, 16]. However, the *ibeA* gene encoding a 50 kDa protein has been found to be unique to some pathogenic *E. coli* K1 strains (e.g., C5 and RS218), while laboratory strains of *E. coli* K-12 (e.g., DH5 and HB101), as well as noninvasive *E. coli* (e.g., E412), lack *ibeA* [4]. Recently, vimentin has been identified as an IbeA-binding protein on the surface of human BMEC [17].

Using the *ibeA* gene as a probe, we have identified a 20.3 kb genomic locus as a *genetic island of meningitic E. coli* containing *ibeA* (GimA) [16]. This locus is situated between *yjiD* and *yjiE*, adjacent to the *fim* operon, and absent in nonpathogenic *E. coli* K12 strains. GimA consists of 15 genes that form 4 operons. The first three operons (*ptnIPKC*, *cglDTEC*, *gcxKRCI*) may be involved in energy metabolism and the last operon (*ibeRAT*) contributes to *E. coli* K1 invasion of BMEC. Our previous work showed that GimA-mediated invasion of human endothelial cells is regulated by carbon source [16]. This is consistent with the observations by others that carbon source modulates expression of virulence factors in several pathogenic bacteria [4]. The *ibeRAT* operon encodes IbeR, IbeA, and IbeT. IbeA and IbeT contribute to *E. coli* K1 invasion and adhesion [18, 19]. Our previous studies suggest that IbeR is a novel regulatory protein that is present in pathogenic *E. coli* K1 [16]. It belongs to the NtrC/NifA family of transcriptional activators, carrying a sigma 54-interaction domain and

showing significant sequence homology to various regulatory proteins for glycerol metabolism operon in *Citrobacter freundii* (P45512), acetoacetate metabolism in *E. coli* K12, sigma L-dependent transcription in *B. subtilis* (P54529), NIF-specific regulation in *Herbaspirillum seropedicae*, *dhaR* transcription in *E. coli* K12, and globe signal transduction in *Clostridium beijerinckii* [4, 20]. However, it is unknown how IbeR contributes to the pathogenesis of meningitic infection by modulating the virulence of the pathogen. As E44 carries a nonsense mutation in the *rpoS* gene and exhibits strong invasion activity in the SP, there must be alternative regulatory mechanisms responsible for the SP gene expression. We speculated that *ibeR* is an *rpoS*-like regulator in SP gene expression in E44. In order to dissect the regulation of SP gene expression in E44 that is associated with the pathogenesis of *E. coli* meningitis, a comparative proteomic analysis of an *ibeR* deletion mutant (BR2) and its parent strain E44 was carried out in this study. Our studies suggested that the *ibeR* gene was involved in regulating SP gene expression related to stress resistance and pathogenesis.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Medium. The bacterial strain and plasmid vectors and their relevant characteristics are shown in Table 1. The mutant strains used in this study were derived from E44, which is a rifampin-resistant strain derived from a neonatal meningitis isolate, *E. coli* K1 RS218 (O18:K1:H7) [4, 21]. *E. coli* DH5 α and pGEM-T easy vector were used for subcloning. SM10 (λ pir), DH5 α (λ pir), and pCVD442 were used for making isogenic deletion mutants of *ibeR* and *tnaA* [6, 10, 11]. *E. coli* K12 strain

TABLE 2: Oligonucleotides used for cloning, sequencing, and making the deletion mutants of *ibeR* and *tnaA* genes.

Primers	Sequences of primers	Retained amino acids
primers for <i>ibeR</i> deletion		
IbeR-S1 (S = SalI)	5'-GATGTCGACGGGCTTTTCGGCGTCA-3'	Total 56 residues
IbeR-B1 (B = BamHI)	5'-CGGGATCCAGTGGCGAGGGTCA-3'	52 N-terminal residues
IbeR-B2 (B = BamHI)	5'-CAGGATCCAAATGTTGAGCATGCAG-3'	(MDIIMNKES...)
IbeR-X2 (X = XbaI)	5'-CGTCTAGATAAGGGCTAAACATATCG-3'	4 C-terminal residues
		(...GSKC)
primers for <i>tnaA</i> deletion		
TN-S1 (S = SalI)	5'-GGGTCGACCAGAGATCTGGCCGAAT T-3'	Total 56 residues
TN-B1 (B = BamHI)	5'-ACGGATCCAATAACACGAATGCGGAACGGTTC-3'	21 N-terminal residues
TN-B2 (B = BamHI)	5'-TTAGATCTTTTAAACATGTGAAAGAGAACGCG-3'	(MKDYVMENFK...)
TN-X2 (X = XbaI)	5'-CCTCTAGATTAGCCAAATTTAGGTAACAC G-3'	35 C-terminal residues
		(...RHFTAKLKEV)

MC4100 [22] and its *rpoS* insertion mutation Tn10 mutant RH90 [23] were used as positive and negative controls for RpoS, respectively. Plasmid pStyABB, which carries the gene for monooxygenase, was used for indole assay [12]. *E. coli* strains were grown at 37°C in Luria broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) or brain heart infusion (BHI, Difco Laboratories, Detroit, Mich, USA) broth and were stored at -70°C in LB plus 20% glycerol. When it was necessary, the medium was supplemented with ampicillin (100 µg/mL) and rifampin (100 µg/mL) for the positive selection of plasmids or bacterial strains (Table 1).

2.2. Extraction and Manipulation of Plasmids and Subcloning. All genetic manipulations were done by using standard methods, as described elsewhere [24]. Plasmid DNA was extracted by using a plasmid mini kit (Qiagen, Calif, USA). DNA fragments were purified and were extracted from agarose gel slices, using QIAquick Gel Extraction Kit (Qiagen). Competent *E. coli* cells were made in 10% glycerol and were transformed by electroporation as described previously [6, 7].

2.3. Construction of Isogenic in-Frame Deletion Mutants of *ibeR* and *tnaA*. To determine the role of the *ibeR* gene in the growth-phase-dependent *E. coli* K1 invasion of BMEC, an isogenic deletion mutant of *ibeR* was generated as follows. Two PCR DNA fragments, B (1.2 kb) and R (1.0 kb), flanking a 1.8-kb region to be deleted were produced from two pairs of primers (IbeR-S1/IbeR-B1 for B and IbeR-B2/IbeR-X2 for R, see Table 2). The two fragments were ligated to make a 2.2 kb fragment (BR) that carries an *ibeR* internal deletion. The BR fragment was subcloned between SalI and XbaI sites on pCVD442 [10], and the resulting recombinant plasmid was named pCBR2. The mutants named BR2 were obtained by mating E44 with SM10 λpir that carries pCBR2 as described previously [6]. We used PCR and DNA sequencing to confirm the internal deletion in the *ibeR* deletion mutant BR2 and the desired chromosomal gene *ibeR* of the mutant with primers IbeR-S1 and IbeR-X2 (Table 2). Amplification was done by using the following cycle profile: 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 70°C for 1.5 minutes.

For the *tnaA* in-frame deletion mutant, the same method was used as *ibeR* deletion. Briefly, 2 PCR DNA fragments, FTN5 (1.0-kb) and FTN3 (1.4-kb), were made to flank a 1.3 kb region containing *tnaA* to be deleted, by using 2 primer pairs (TN-S1/TN-B1 for FTN5 and TN-B2/TN-X2 for FTN3, see Table 2). Then the two fragments were ligated to make a 2.4 kb fragment (FTN53) that carried a *tnaA* internal deletion. The FTN53 fragment was subcloned into pCVD442 between SalI and XbaI sites to get the suicide plasmid pCTNA2. To get the *tnaA* in-frame deletion mutant, TNA44, conjugation and screening were carried out as described above. The *tnaA* gene deletion in the mutant TNA44 was confirmed by PCR using the primers TN-S1 and TN-X2 (Table 2).

2.4. Invasion Assay. Human BMECs were routinely cultured in RPMI 1640 medium (Mediatech, Herndon, Va, USA) containing 10% heat inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM sodium pyruvate, essential amino acids, vitamins, penicillin G (50 µg/mL), and streptomycin (100 µg/mL) at 37°C in 5% CO₂ [7]. Invasion assays were performed as previously described [6, 7, 18]. The number of intracellular bacteria was determined on blood agar plates after the extracellular bacteria were killed by incubation of the monolayers with experimental medium containing gentamicin (100 µg/mL) for 1 hour. Results were expressed as percent invasion ($100 \times (\text{number of intracellular bacteria recovered})/(\text{number of bacteria inoculated})$).

2.5. Protein Extraction and 2DE. Protein extraction and 2DE were carried out as described [25] with minor modifications. Briefly, *E. coli* strain E44 and the *ibeR* deletion mutant BR2 were cultured in BHI medium overnight without agitation. The bacterial cells were harvested at the stationary-phase (OD = 2.5–3.0) by centrifugation at 6000 × g for 10 minutes. Denaturing protein extraction (phenol extraction procedure) was performed according to Saravanan and Rose [26]. The lyophilized pellets were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% pH 3–10 Biolytes, 1% DTT; 1 mg dry pellets for 200 µL buffer) and shaken on vortex for 1 hour at room temperature. The first and second dimensions of the PAGE were performed at least

in triplicate (to reduce the likelihood of differences based solely upon gel-to-gel variability) according to the standard protocols developed and defined by Bio-Rad. Solubilized total *E. coli* protein samples (200 μ L each) were loaded on 11 cm immobilized pH gradient (IPG) strips (pH 4–7). Rehydration/loading was done passively (no voltage) for 1 hour, followed immediately by 14 hours of active rehydration (50 V) at 20°C. Isoelectric focusing of the IPG strips was performed at 20°C using a 50 μ A current limit per strip to prevent damage to the strip and the instrument. Electrophoresis was carried out as follows: step 1, 250 V for 20 minutes; step 2, a rapid ramp to 8000 V; step 3, focusing at 8000 V for 55 000 Vhr; step 4, hold at 400 V. Due to latent ionic components in the sample the actual running voltage was only approximately 6500 V. After the first dimensional run was completed, the IPG strips were equilibrated in buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, and 2% DTT) for 15 minutes and then in buffer II (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol, and 2% DTT) for additional 15 minutes. The second dimension SDS-PAGE was performed with 15% resolving gels and 5% stacking gels (160 $\times$ 180 $\times$ 0.5 mm). The gels were stained with 0.1% coomassie brilliant blue (CBB) R-250. The 2DE gels were scanned at a 200 bpi resolution with Typhoon scanner (Amersham Biosciences, NJ, USA), and analyzed with ImageMaster 2D Platinum version 6.0 (GE Healthcare BIO-Science, NJ, USA). Only those protein spots having differences in density of 1.5-fold or greater between the groups were chosen. Moreover, all protein spots selected for analysis were shown to have significant difference in protein density (mean $\pm$ SEM, $P < .01$) by software of SPSS 10.0.

2.6. In-Gel Protein Digestion. Protein bands were excised from preparative coomassie blue-stained gels and washed several times with destaining solutions (25 mM NH_4HCO_3 for 15 minutes and then with 50% acetonitrile containing 25 mM NH_4HCO_3 for 15 minutes). Gel pieces were then dehydrated with 100% acetonitrile, dried, and then incubated with a reducing solution (25 mM NH_4HCO_3 containing 10 mM dithiothreitol) for 1 hour at 56°C and subsequently with an alkylating solution (25 mM NH_4HCO_3 containing 55 mM iodoacetamide) for 45 minutes at room temperature. After reduction and alkylation, gels were washed several times with the destaining solutions and finally with pure water for 15 minutes before being treated again with 100% acetonitrile. Depending on the protein content, 2–3 μ L of 0.1 μ g/ μ L modified trypsin (Promega, Wiss, USA, sequencing grade) in 25 mM NH_4HCO_3 was added over the gel spots and incubated for 30 minutes. About 7–10 μ L of 25 mM NH_4HCO_3 was then added to cover the gel spots and incubated at 37°C overnight. The in-gel digestion products were extracted with formic acid/acetonitrile solutions followed by evaporation. Samples were desalted using mZipTip C18 pipette tips (Millipore, Mass, USA) before MS/MS analysis.

2.7. Protein Identification by LC-MS/MS. The sample was resuspended in 10 μ L of 0.1% formic acid, injected via

an autosampler (Surveyor, ThermoFinnigan, Calif, USA) and subjected to reverse phase liquid chromatography using ThermoFinnigan Surveyor MS-Pump in conjunction with a BioBasic 18 100 $\times$ 0.18 mm reverse-phase capillary column (ThermoFinnigan, Calif, USA). Mass analysis was done using a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with a nanospray ion source (ThermoFinnigan, Calif, USA) employing a 4 cm metal emitter (Proxeon, Odense, Denmark). Spray voltage of the mass spectrometer was set to 2.9 kV and capillary temperature was set at 190°C. The column equilibrated for 5 minutes at 1.5 μ L/min with 95% Solution A and 5% Solution B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) followed by a linear gradient was initiated 5 minutes after sample injection ramping to 65% Solution A over 45 minutes. Solution A was increased to 80% over the subsequent 5 minutes and held at 80% for 5 minutes, after which the column was reequilibrated back to 5% Solution A (aqueous). Mass spectra were acquired in the m/z 400–1800 range. A data-dependent acquisition mode was used where each of the top five ions for a given scan was subjected to MS/MS analysis. The protein identification was conducted with the MS/MS search software Mascot 1.9 with confirmatory or complementary analyses using TurboSequest as implemented in the Bioworks 3.2. *E. coli* genome sequences at the National Center for Biotechnology Information (NCBI) were used as the primary search databases and searches were complemented with the NCBI nonredundant protein database.

2.8. Indole Assay. For determination of indole production, we followed the method of indole conversion into indigo as described previously [12, 27], with minor modifications. All strains E44, BR2, TNA44, MG1655, and RH90 were transformed with the pStyABB plasmid, which constitutively expresses the StyAB protein converting indole to indigo. The bacteria were incubated in M9 medium containing 0.4% glucose and 100 μ g/mL ampicillin overnight with shaking. Then the bacteria were collected by centrifugation and resuspended to $\text{OD}_{600} = 0.2$ in BHI medium supplied with 100 μ g/mL ampicillin, and incubated at 37°C without shaking. To determine the indigo formation at different time points, batch-grown cells were harvested every 2 hours by centrifugation. Then the bacteria were lysed in DMSO for 30 minutes. The samples were read at 600 nm to determine the indigo concentration by comparison to a standard curve.

2.9. Determination of Resistance to Environmental Stress. Bacteria were grown in BHI broth at 37°C overnight without shaking, and collected by centrifugation. The number of cells was measured on the basis of their OD at 600 nm. Bacteria were suspended and diluted to 10^7 cells/mL in PBS for the following assays. For heat shock, 100 μ L of bacteria was heated at 54°C for 3 minutes. For alkali endurance, the bacterial suspension was mixed with equal volume of Tris buffer (1 M, pH = 10.0) and 8 volumes of water (final concentration, 100 mM, pH 10.0) and incubated at 37°C for 30 minutes. For acid endurance, 1/10 volume of the bacterial

FIGURE 1: Generation and characterization of *ibeR* deletion mutant from the meningitic strain E44. (a) Generation of the *ibeR* deletion mutant (BR2). Two DNA fragments flanking *ibeR* were amplified and ligated into the suicide vector pCVR2. The *ibeR* deletion mutants were generated through gene allele exchanges and suicide vector loss. The arrowheads indicate primer locations. (b) Verification of the *ibeR* deletion by PCR. The parent strain E44 and the *ibeR* deletion mutant BR2 were verified through colony PCR with primers listed in Table 2. (c) Invasion of HBMEC with the *E. coli* parent E44 carrying pWKS30, the *ibeR* mutant BR2 with pWKS30, and the complemented BR2 carrying the *ibeR* locus in pWKS30. E44/pWKS, BR2/pWKS, and BR2/pWKS1030 were incubated with HBMEC monolayers and the standard invasion assay was carried out as described in Section 2. The results are expressed as relative invasion%. Columns marked with ** are significantly different ($P < .01$).

suspension was mixed with LB containing acetic acid (final concentration, 90 mM, pH 2.8) and incubated at 37°C for 20 minutes. For high osmolarity challenge, bacteria were mixed with an equal volume of 4.8 M NaCl (final concentration, 2.4 M) and incubated at 37°C for 1 hour. For oxidative stress, bacteria were harvested and resuspended in an equal volume of PBS containing 10 μ M H₂O₂ incubated at 37°C for 30 minutes. After exposure to these stresses, bacteria were diluted in 0.9% saline and plated in duplicate on LB agar plates. The surviving rate with stress was calculated from the ratio of the bacterial number under stress condition to the bacterial number under nonstress condition. The surviving rate without stress was calculated from the bacterial number grown on plates.

3. Results and Discussions

3.1. The *ibeR* Regulatory Gene is Required for Invasion of Human BMEC by Meningitic *E. coli* K1 Strain E44. The gene *ibeR* in *E. coli* K1 E44 is predicted as the only regulatory protein present in the *ibeRAT* operon in GimA by bioinformatics approaches [16]. To determine the role

of *ibeR* gene in the growth-phase-dependent invasion of BMEC by meningitic *E. coli* K1, an isogenic in-frame deletion mutant of *ibeR* was made by chromosomal gene replacement with the recombinant suicide plasmid pCVR2 carrying a 2.2 kb DNA fragment with *ibeR* internal deletion (Figure 1(a)). The 2.2 kb DNA fragment was generated by ligation of two PCR amplicons (1.2 and 1.0 kbs) flanking the 1.8 kb *ibeR* coding region. The *ibeR* deletion mutant was obtained by mating E44 with SM10 λ pir carrying pCVR2. The mutant colony morphology on LB agar plates and growth rate in LB broth were the same as the parent strain E44. The deletion of *ibeR* was confirmed by colony PCR and DNA sequencing (Figure 1(b)). In order to examine the virulence phenotype of the *ibeR* deletion mutant, a comparative study of the invasiveness of E44 (parent strain), BR2, and the complemented BR2 was carried out. As shown in Figure 1(c), the relative invasion rate of BR2 was significantly reduced as compared to that of E44 and the plasmid pWKS1030 carrying the *ibeR* gene was able to complement the noninvasive phenotype of BR2, suggesting that the *ibeR* gene contributes to the *E. coli* E44 invasion process.

TABLE 3: Identification of differentially displayed proteins in 2D maps.

Spot number	Protein ID	Access number	Mass(KD)/PI (theriol)	Mass(KD)/PI (analytic)	Peptides matched	Sequence coverage	Function category
downregulated							
D1	dihydrolipoamide dehydrogenase (LpdA)	gi 15799800	50.9/5.79	51.0/6.05	249	47%	Central metabolism (CM): E3 component of pyruvate dehydrogenase complex
D2	tryptophanase (TnaA)	gi 15804305	53.8/5.88	49.0/6.10	92	34%	Response to environmental modifications (REMs), and CM: initiation of indole signaling
D3	outer membrane protein C (OmpC)	gi 15802768	40.5/4.55	40.5/4.55	53	50%	REM: osmotically regulated porin
D4	outer membrane protein C (OmpC)	gi 15802768	40.5/4.55	40.5/4.60	7	20%	REM: osmotically regulated porin
upregulated							
U1	elongation factor EF-Tu (TufB)	gi 15803852	43.4/5.3	43.5/5.30	55	45%	REM: binding and transport of aminoacyl-tRNA
U2	glyceraldehyde-3-phosphate (G-3P) dehydrogenase A (GapA)	gi 15802193	35.7/6.61	36.0/6.20	94	32%	CM: oxidation and phosphorylation of G-3P to 1, 3-bisphosphoglycerate
U3	outer membrane protein 3a (OmpA)	gi 15800816	37.3/5.99	30.0/5.80	35	48%	REM: maintaining cell envelope integrity
U4	alkyl hydroperoxide reductase (AhpC)	gi 15800320	20.9/5.03	22.0/5.15	57	49%	REM: a primary scavenger of endogenous H ₂ O ₂ at a low (10 ⁻⁵ M) concentration

3.2. 2D Proteomic Analyses of *IbeR*-Regulated SP Protein Expression in Meningitic Strain E44. To determine the role of *ibeR* in regulating SP gene expression of meningitic *E. coli* K1, the wild type *E. coli* E44 and its *ibeR* deletion mutant BR2 were cultured in BHI broth overnight. The total proteins of each strain were extracted from the cells as described in Section 2. The whole cell extracts were analyzed on the 2D protein gels. Approximately 800 spots were detected on a gel image. The experiment was performed three times with two sets of independently grown cultures. Only spots showing the same pattern in three independent runs were retained and quantified using the software ImageMaster 2D Platinum version 6.0. Figures 2(a) and 2(b) showed the protein patterns of E44 and BR2, respectively. All the upregulated spots and downregulated spots satisfying the criteria as mentioned above were marked on both the 2D maps. They were excised and identified by LC-MS/MS (Table 3). Eight protein spots were found to be differentially expressed in BR2 as compared to its parent strain E44. Among them, 4 protein spots were significantly upregulated in BR2 including elongation factor EF-Tu (TufB, spot U1), glyceraldehyde-3-phosphate dehydrogenase A (GapA, spot U2), outer membrane protein 3a (OmpA, spot U3), and alkyl hydroperoxide reductase (AhpC, spot U4), while 4 protein spots were of decreased abundance in BR2, including dihydrolipoamide dehydrogenase (LpdA, spot D1), tryptophanase (TnaA, spot D2), and two isoforms of outer membrane protein C (OmpC, spot D3, and D4). Figure 3(a)

showed the enlargements of each changed protein marked with black arrows and spot numbers. The relative ratios of each downregulated protein and upregulated protein were shown in Figures 3(b) and 3(c).

We classified the proteins into two main categories on the basis of their roles in the SP growth of *E. coli* cells: (a) response to environmental modifications (including TnaA, TufB, OmpC, OmpA, and AhpC) and (b) central metabolism (including LpdA and GapA). Since *ibeR* was hypothesized as an SP-regulator contributing to the growth regulation and virulence of E44, its role in the invasion process and resistance to stress conditions should be further characterized. TnaA, a tryptophanase, degrades tryptophan, resulting in the formation of indole, which has been proposed to act as an extracellular signal in stationary phase cells of *E. coli* [28, 29]. Production of indole, via the enzymatic activity of TnaA, is also induced during biofilm formation [30]. TnaA, which was controlled by RpoS in other *E. coli* strains [27], is one of the most important transcriptional regulators for the gene expressions in SP cells [31]. TufB (EF-Tu) is responsible for binding and transporting the appropriate codon-specified aminoacyl-tRNA to the aminoacyl (A) site of the ribosome [32, 33]. In addition to its function in translation elongation, elongation factor Tu is implicated in protein folding and protection from stress like a chaperone molecule [34].

OmpC, as well as OmpF, is a porin protein present on the outer membrane of *E. coli*, responding to the osmotic challenge. OmpR, as a regulator, activates transcription of

FIGURE 2: 2DE maps of *E. coli* strains with stationary-phase cultures in BHI medium. (a) E44 (wild type strain). (b) BR2 (the *ibeR* deletion mutant). The upregulated proteins were circled with a solid line and marked with U1–U4; the downregulated proteins were circled with a broken line and marked D1–D4.

FIGURE 3: Comparative analysis of the protein spots showing significant changes. (a) Enlargement of differentially expressed protein spots (indicated with arrows) from Figure 2. (b) The relative level of down-regulated proteins. (c) The relative level of up-regulated proteins. The protein spot intensities of E44 were showed as black columns, and the protein spot intensities of BR2 were showed as white columns. Columns marked with ** are significantly different ($P < 0.01$).

ompF and *ompC* [35], and changes the ratio of these two, so that the total level of porin proteins remains approximately constant [36, 37]. OmpF, which produces slightly wider pores (1.2 nm) than does OmpC, predominates at low osmotic strength, whereas OmpC (1.1 nm) predominates at high osmotic strength [38]. In *E. coli*, the expression of OmpC is repressed at low osmolarity and induced at high osmolarity. It has been proposed that the smaller pores formed by OmpC could reduce the diffusion of larger hydrophobic and negatively charged molecules when bacteria encounter high osmolarity conditions as in the host compartments. Presumably, this protein is very important for the stress resistance of *E. coli* in the stationary phase. In this study, the downregulation of OmpC resulting from the *ibeR* deletion might decrease resistance to high osmolarity. In addition, it had been reported that OmpC is involved in invasion of epithelial cells by Crohn's disease-associated *E. coli* strain LF82 and *Shigella flexneri* [39, 40], suggesting that OmpC might be involved in E44 invasion of the host tissue barriers. OmpA is a major protein in the outer membrane of both pathogenic and nonpathogenic *E. coli* [41]. As shown in our previous study, the *ompA*-deletion mutant of E44 was significantly more sensitive than that of its parent strain to SDS, cholate, acidic environment, and high osmolarity [41]. OmpA is downregulated upon entry into SP by sigmaE, which plays a central role in maintaining cell envelope integrity both under stress conditions and during normal growth [42, 43]. We demonstrated here that OmpA was upregulated in the *ibeR* mutant BR2 (Figure 3), suggesting that OmpA expression is suppressed upon entry into SP by IbeR in a manner similar to sigmaE.

Alkyl hydroperoxidase (AhpC) functions as a primary scavenger of endogenous H₂O₂ at a low (10⁻⁵ M) concentration [44]. All of *ahpC*, *katG*, and *katE* genes are known to participate in the antioxidant defense mechanism against H₂O₂-induced stress in *E. coli*. It has been reported that SP-inducible RpoS regulates *katE* gene expression and OxyR regulates *ahpC* and *katG* genes [45, 46]. Our previous study has demonstrated that *E. coli* K1 RS218 had a nonsense mutation in its *rpoS* gene, resulting in a negligible *katE* activity, but no obvious difference in *katG* [5]. In this study, the increase in *ahpC* expression indicated that the *ibeR* deletion led to an increased oxidative stress in SP compared with the wild type strain, suggesting that *ibeR* is involved in the resistance to oxidative stress upon entry into SP.

Lipoamide dehydrogenase (LpdA), which is the same as dihydrolipoamide dehydrogenase (DLDH), makes up the E3 component of pyruvate dehydrogenase complex, 2-oxo glutarate dehydrogenase, and branched-chain 2-oxo acid dehydrogenase complexes. DLDH has been identified as virulence factors contributing to the pathogenesis of bacterial infections caused by *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* because it enhances their survival within the host cells [47, 48]. As shown in Figure 3, LpdA was downregulated in the *ibeR* deletion mutant BR2, suggesting that this enzyme might be involved in the virulence of meningitic *E. coli* K1 via enhancing the pathogen survival within the host. Recently, GapA in *E. coli* has been identified as one of a few proteins, which harbors

functionally important thiol groups against oxidative stress [49]. As GapA is upregulated in BR2 (Figure 3), IbeR may be involved in the negative control of *gapA* in SP.

In summary, all these proteins contribute to growth-related carbon source metabolism or stress resistance. They are associated with the SP regulation. Among these proteins, TnaA is the most important one as it produces the signal molecule indole and is regulated by RpoS [27]. Since E44 carries a loss-of-function mutation in its *rpoS* gene [5], there should be alternative signaling pathway(s) to complement the functional deficiency of RpoS in this pathogenic *E. coli* strain. Our proteomic analyses showed that the TnaA expression was significantly affected by IbeR, which might be functionally equivalent to RpoS. Therefore, our focus for further studies was placed on how TnaA is regulated by IbeR.

3.3. Indole Production is Controlled by IbeR via Regulation of TnaA. To test our hypothesis that IbeR is an RpoS-like regulator, the *tnaA* in-frame deletion mutant TNA44 was generated with the same gene replacement approach that was used for the *ibeR* deletion mutant. TNA44 was obtained by mating E44 with SM10 λ pir carrying the recombinant suicide plasmid pCTNA2 which contains the truncated *tnaA* gene. The virulence phenotype of TNA44 was examined with invasion assays. Although overall growth rates did not differ between the mutants (BR2 and TNA44) and their parent strain E44 (Figure 4(a)), the invasive capability of TNA44 (19%) and BR2 (35%) was significantly reduced as compared to that of E44 (100%) (Figures 4(c)-4(d)). These data suggest that TnaA is an important downstream regulator that is required for IbeR-modulated *E. coli* K1 invasion.

As our proteomics analysis had shown that *tnaA* expression was induced by IbeR in SP and the *tnaA* and *ibeR* deletion resulted in a deficiency in indole production in SP-cultures (Figure 4(b)), we further tested whether the TnaA product indole, as an SP extracellular signal molecule, played a role in the process of invasion. Indole is converted to indigo (which is not further degraded in *E. coli*) by several monooxygenases, thus providing an easy method for its determination [12]. A plasmid pStyABB, carrying the gene for styrene monooxygenase, was used to monitor the indole production through its conversion to indigo. The plasmid was transformed into *E. coli* strains E44, BR2, and TNA44, and the indole production was measured at different time points for these strains in BHI media (Figure 4(b)). The production of indole in E44 was revealed by indigo accumulation. By contrast, the indole production was almost abolished in the *tnaA* deletion mutant and severely reduced in the *ibeR* deletion mutant (Figure 4(b)). These results demonstrated that the indole production was controlled by *ibeR* through *tnaA*. To examine whether indole could complement the noninvasive phenotype of *tnaA* and *ibeR* deletion mutants, indole was supplied in the BHI medium at 100 μ M to the TNA44 and BR2 SP cultures. The result showed that indole was able to significantly enhance the relative invasion rate of TNA44 (19% to 69%) and BR2 (35% to 65%) as compared to that of E44, suggesting that indole could partly complement the noninvasive phenotype

FIGURE 4: Role of TnaA in indole production and bacterial invasion. (a) Growth curves of E44, TNA44, and BR2, (b) indole production (IP) of *E. coli* strains E44, BR2, and TNA44, (c) the invasion phenotypes of E44, TNA44, and indole(100 μM)-complemented mutant TNA44, and (d) the invasion phenotypes of E44, BR2, and indole(100 μM)-complemented mutant BR2. Columns marked with * are significantly different ($P < .05$), ** are significantly different ($P < .01$).

of TNA44 (Figure 4(c)) and BR2 (Figure 4(d)). Lacour and Landini have shown that the *rpoS* gene in *E. coli* K12 strain MG1655 controls the production of indole, which acts as a signal molecule in SP cells, via regulation of TnaA, the indole-producing enzyme [27]. As TnaA is regulated by IbeR in E44, it is most likely that IbeR is a novel regulator to complement the functional deficiency of RpoS in E44.

3.4. The Role of *ibeR* in Stress Conditions. It has been reported that RpoS is able to positively and negatively control expression of a large set of genes when bacteria enter into the SP [46, 50, 51]. During such transition, bacteria undergo physiological changes that allow their SP organisms to survive better in such insults as heat, high-osmotic environment, starvation, UV radiation, H₂O₂, and

acid than their exponential counterpart [50, 52]. The loss of RpoS resulted in the decrease of stress resistance and cell survival in the SP [5, 53]. Although our study showed that the loss of *ibeR* did not affect the growth rate in BHI medium, the survival rates of the *ibeR* deletion mutant BR2 in the SP significantly decreased as compared to that of the wild type strain E44 even without any stress treatment (Figure 5(a)). Our proteomics analysis also revealed that the most significant proteomic changes in the *ibeR* deletion mutant were related to bacterial response to environmental modifications. For example, AhpC, as a primary scavenger of endogenous H₂O₂, was upregulated in the *ibeR* deletion mutant, implying that the loss of *ibeR* resulted in the decreased survival rates of bacterial cells under an oxidative stress in the SP. OmpC, as a porin protein, was downregulated in BR2, perhaps resulting

FIGURE 5: The relative survival rate of the BR2 mutant comparing with E44. (a) Both strains in stationary phase without any stress treatment, (b) both strains were tested with different environmental stress including heat shock (54°C for 3 minutes), alkali (Tris pH = 10.0 for 30 minutes), acid endurance (acetic acid, pH = 2.8 for 20 minutes), high osmolarity challenge (2.4 M NaCl for 1 hour), and oxidative stress (10 μ M H₂O₂ for 30 minutes). Gray columns: E44; white columns: BR2. Columns marked with ** are significantly different ($P < .01$).

in the decreased resistance to osmolarity stress. These results suggested that *ibeR* plays a regulatory role in response to stress conditions in E44 that carries a nonsense mutation in *rpoS*. To examine the function of *ibeR* in response to stress environments, we performed several survival assays under different stress conditions, including heat shock (54°C for 3 minutes), alkali endurance (Tris, pH = 10 for 30 minutes), acid endurance (acetic acid, pH = 2.8 for 20 minutes), high osmolarity challenge (2.5 M NaCl for 1 hour), and oxidative stress (10 μ M H₂O₂ for 30 minutes). In all the survival experiments, the wild type strain E44 showed higher survival rates than the *ibeR* deletion mutant, indicating that the *ibeR* gene is required for all these stress resistances (Figure 5(b)). Especially in the heat shock assay, the loss of *ibeR* resulted in over 95% cells death, indicating that *ibeR* played a vital role in temperature sensitivity in this strain. In the other stress treatments, the *ibeR* deletion also significantly reduced the survival rates (more than 60% cell death) of BR2 as compared to that of the wild type strain E44, suggesting that IbeR had a global regulatory role in the resistance to acid, alkali, high osmolarity and oxidative stress. In the survival assays for the *E. coli* control strain MG1655, the *rpoS* deletion mutant RH90 also decreased the survival levels in these five stress conditions, showing the similar patterns like *ibeR* in response to stress environments (data not shown). Combining the proteomics analysis and the stress survival studies, we conclude that IbeR is an RpoS-like regulator to control gene expression of proteins that are critical for stress-resistance and cell survival in the SP in E44, which carries a loss-of-function mutation in the *rpoS* gene.

4. Concluding Remarks

Currently, most *E. coli* meningitis studies are done with SP cultures in which the pathogen invasion of human BMEC is significantly greater than the log phase cultures. In most strains of *E. coli*, RpoS plays a central role in regulating the SP regulatory genes for protecting cells against starvation

and stress damage. RpoS, the major SP regulator, has also been shown to regulate the expression of microbial virulence genes in various bacteria including *E. coli* K1 (O157:H7), *Salmonella typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica*, *Vibrio cholera*, and *Borrelia burgdorferi*, [5, 53, 54]. Surprisingly, however, RpoS was found to be inactive in meningitic strain E44 [5]. The current proteomic studies may provide an answer to the long-standing question regarding the SP gene regulation in E44. Combining the proteomics analysis, virulence determination, and the stress survival studies of the *ibeR* mutant BR2, we have demonstrated for the first time that IbeR serves as an RpoS-like regulator to control gene expression that is critical for stress-resistance and cell survival in the SP of E44.

IbeR is not a structural homologue of RpoS as IbeR and RpoS do not share any significant sequence homology. RpoS (also known as σ^{38} , σ^s , or KatF) is a global regulator in *E. coli*, which is the second principal σ subunit after the major σ^{70} factor [5]. In E44, however, IbeR appears not to be a master regulator on the basis of its genomic prevalence and functional spectrum. The prevalence of the GimA locus carrying *ibeR* is highly dependent on the origin of the strain and on the subgroup it belongs to (A, B1, B2, and D) (4). In all the studies, where the presence of this locus has been analyzed, GimA was found to be restricted to the B2 subgroup, a subgroup that includes strains with the highest virulence in mice and the highest level of virulence determinants (4). Our proteomic studies showed that a limited number of genes were regulated by IbeR, suggesting that IbeR is a regulator with a narrow functional spectrum. In other *E. coli* strains, either pathogenic or probiotic strains, functional heterogeneity of RpoS in stress tolerance was widely observed [5, 53, 55]. Those studies have shown that some *E. coli* strains can maintain their stress tolerance capability or significantly modulate their stress resistance phenotype independent of their *rpoS* genotypes. Such adaptation processes compromising the RpoS-dependent stress responses may have significant impact on bacterial survival

in environments, as well as in the host's stomach and intestine [53, 55]. IbeR, a regulator in the GimA regulon, may contribute to bacterial virulence adaptation process in E44 to complement the functional deficiency of RpoS.

Another significant finding of our proteomic studies is that IbeR in meningitic strain E44 is able to upregulate TnaA, which is controlled by RpoS in other *E. coli* strains [56]. The virulence determination of the *tnaA* mutant showed that TnaA and its product indole were required for E44 invasion of human BMEC. The generation of indole, via the tryptophanase activity of TnaA, was also observed during the formation of biofilm in *E. coli* and other bacteria [28, 56]. In addition to the initiation of indole-mediated signaling, TnaA (tryptophanase) is able to catabolize tryptophan, cysteine, and serine to pyruvate [29, 56]. The Three proteins significantly upregulated by IbeR are TnaA, LpdA, and OmpC, all of which are directly or indirectly involved in pyruvate metabolism. LpdA (dihydrolipoamide dehydrogenase) is the E3 component of pyruvate dehydrogenase complex. OmpC, an osmotically regulated porin, may facilitate nutrient uptake [56]. On the other hand, the three operons (*ptnIPKC*, *cglDTEC*, and *gxcKRCI*) in the GimA regulon may also directly or indirectly contribute to pyruvate metabolism by converting dihydroxyacetone, glycerol, and glycerate to pyruvate [16]. It has been shown that the capability to catabolize carbon source is an important parameter in the ability to persist and compete in stationary phase [29]. This raises the possibility that signaling by indole, which is regulated by IbeR via TnaA, may play a critical role in the pathways that prepare the pathogens for a nutrient-poor environment (e.g., CNS) when the carbon source becomes limited for energy production.

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