Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	Canadian Journal of Microbiology
Manuscript ID	cjm-2016-0158.R1
Manuscript Type:	Article
Date Submitted by the Author:	30-Apr-2016
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Keyword:	Edwardsiella tarda, Sigma factor, Comparative proteomic analysis

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Comparative analysis of sigma factors RpoS, FliA, and RpoN in *Edwardsiella tarda*

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Abstract

Sigma factors are important regulators that bacteria employ to cope with environmental changes. Studies on the functions of sigma factors have uncovered their roles in many important cellular activities, such as growth, stress tolerance, motility, biofilm formation, and virulence. However, comparative analyses of sigma factors, which examine their common and unique features or elucidate their cross-regulatory relationships, have rarely been conducted for *Edwardsiella tarda*. Here, we characterized and compared motility and resistance to oxidative stress of *E. tarda* strains complemented with *rpoS*, *fliA*, and *rpoN* mutants. The results suggest that the sigma factors, FliA and RpoN, regulated motility, whereas RpoS exhibited no such function. RpoS and RpoN were essential for oxidative stress resistance, whereas FliA had no obvious impact under oxidative stress conditions. Furthermore, 2-dimensional gel electrophoresis (2-DE) based proteomics analysis, combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS/MS), revealed 12 differentially expressed protein spots that represented 11 proteins between the mutant and wild-type strains. Quantification of the expression of target genes by quantitative reverse transcription PCR (qRT-PCR) confirmed the results of our proteomics analysis. Collectively, these results suggest that these sigma factors are multi-functional mediators involved in controlling the expression of many metabolic pathway genes.

Keywords *Edwardsiella tarda*, Sigma factor, Comparative proteomic analysis

Introduction

Edwardsiella tarda, a member of the family Enterobacteriaceae, is a gram-negative bacterium and major aquaculture pathogen that infects a wide range of hosts, including marine and fresh-water fish species (Slaven et al. 2001). Infection by *E. tarda* results in an enterohemorrhagic septicemic disease (i.e. edwardsiellosis), which causes severe economic losses in the aquaculture industry (Mohanty and Sahoo 2007). In addition, as an opportunistic pathogen, *E. tarda* has developed a complex mechanism for intestinal and extra-intestinal infection in fish, animals, and human beings (Helmann and Chamberlin 1988; Nelson et al. 2009; Tsuji et al. 2008).

There are five alternative sigma factors in *E. tarda*, namely σ^{24} (*rpoE*), σ^{32} (*rpoH*), σ^{38} (*rpoS*), σ^{54} (*rpoN*), and σ^{28} (*fliA*), each of which is encoded by only one gene copy. These sigma factors are involved in maintaining cellular homeostasis in response to various environmental stressors. RpoS is key for the survival of many pathogens, and has been demonstrated to play an essential role in survival under acute food shortage conditions and in protection against acidic, osmotic, and oxidative stress (Landini et al. 2014; Schellhorn 2014). The flagellum mediates bacteria motility and is a potential factor in bacterial virulence. Therefore, FliA is a potential virulence factor due to its essential roles in flagellar filament structure and biofilm formation. RpoN was originally found in some enteric bacteria, such as *Escherichia*, but is widely distributed among the bacteria (Buck et al. 2000; Merrick and Coppard 1989). It was reported that RpoN plays important roles in nitrogen utilization (Riordan et al. 2010). In *Escherichia coli*, RpoN controls the expression of approximately 30 operons, half of which involved in nitrogen assimilation (Riordan et al. 2010). Moreover, RpoN is also responsible for virulence, stress responses (Gruber and Gross 2003), and flagellum biosynthesis in *Xanthomonas campestris* (Yang et al. 2009).

In our previous studies, an *rpoN* deletion mutant was constructed from the *E. tarda* wild-type strain EIB202 and investigated in comparison to the wild-type strain (Wang et al. 2012).

RpoN plays an important role in the control of virulence genes and is essential for resistance to H₂O₂, starvation, high osmotic pressure, and acid (Wang et al. 2012). In this study, we constructed *rpoS* and *fliA* deletion mutants and complemented strains. Through a set of physiology experiments, we characterized and compared motility and resistance to oxidative stress for these *E. tarda* strains. Our results suggested that FliA and RpoN regulated motility, whereas RpoS had no such function. RpoS was essential for resistance to oxidative stress, whereas FliA had no obvious impact. Furthermore, 2-DE based proteomics approaches were applied to investigate differentially expressed proteins among the *E. tarda* strains, using the whole-cell protein fraction of *E. tarda* EIB202, *rpoS*, and *fliA* and *rpoN* deletion mutants. Comparative proteomic analysis, combined with MALDI-TOF MS/MS, revealed 12 identified protein spots that represented 11 proteins between the mutant strains and the wild-type strain. These results support the conclusion that RpoS, FliA, and RpoN are multi-functional mediators involved in regulating the expression of various metabolic pathway genes.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table S1. *E. tarda* strains were grown in TSB (tryptone, 15 g L⁻¹; soya peptone, 5 g L⁻¹; NaCl, 5 g L⁻¹) or TSA (tryptone, 15 g L⁻¹; soya peptone, 5 g L⁻¹; NaCl, 5 g L⁻¹; agar, 18 g L⁻¹) at 28 °C. When necessary, an appropriate amount of antibiotics (Sigma-Aldrich, St. Louis, MO) were supplemented to the medium at the following final concentrations: 15 µg mL⁻¹ colistin (Col), 30 µg mL⁻¹ chloramphenicol (Cm), 50 µg mL⁻¹ ampicillin (Amp), and 50 µg mL⁻¹ kanamycin (Kan). *E. coli* strains were grown in LB (tryptone, 10 g L⁻¹; yeast extract, 5 g L⁻¹; NaCl, 10 g L⁻¹) at 37 °C. Plasmids used in this study were constructed in *E. coli* strains and then transferred into *E. tarda* strains through electroporation or mating with *E. coli* SM10 λ pir.

Construction of *rpoS* and *fliA* in-frame deletion mutants and complemented strains

Primers used in this study are listed in Table S2. PCR amplified DNA fragments used for constructing the in-frame deletion mutations of *rpoS* and *fliA* were generated respectively by overlap PCR. Two PCR fragments were obtained from *E. tarda* EIB202 genomic DNA with the primer pairs of *rpoSU*-H1-F plus *rpoSU*-H2-R (a 596-bp fragment containing the upstream segment of *rpoS*) and *rpoSD*-H1-F plus *rpoSD*-H2-R (a 833-bp fragment containing the downstream segment of *rpoS*) using Pfu DNA polymerase (Fermentas, Vilnius, Lithuania). A 1421-bp product was obtained by overlap PCR with the primers *rpoSU*-H1-F and *rpoSD*-H2-R. The overlap fragment containing a deletion from nucleotides 1–977 was sequenced and ligated into the suicide vector, pRE112, with *SacI* and *KpnI* restriction sites. The resulting plasmid, pRED-*rpoS*, was mated from *E. coli* SM10 λ pir into *E. tarda* EIB202. The markerless in-frame deletion *rpoS* mutant in EIB202 was constructed using a double-crossover markerless gene deletion system (Lan et al. 2007). A similar method was used to obtain the Δ *fliA* mutant. The selected mutants were verified by PCR and DNA sequencing.

To construct the complemented strains, *rpoS*⁺ and *fliA*⁺, an intact *rpoS* and *fliA* gene containing the putative promoter region was amplified with primers RpoS-c-sen/RpoS-c-ant and FliAF-c-P1/FliA-c-P2, and then cloned into the *Hind*III-/*Bam*HI-digested low-copy plasmid, pACYC184, to create pACYC-*rpoS* and pACYC-*fliA*. The plasmids were respectively transformed into the *rpoS* and *fliA* mutants by electroporation. The desired complemented strains were screened and verified by PCR.

Motility assay

E. tarda wild-type (wt) and mutant strains were streaked out on TSA-medium plates and incubated at 28 °C for 24 h. For the cell motility test, a single colony of each strain was spotted onto a TSA-medium plate (0.4 % agar) and incubated for 48 h at 28 °C.

Physiological experiments

Growth of *E. tarda* strains was measured in LB at 28 °C. The oxidative challenges (3% and 1.5% H₂O₂) were carried out as described previously (Tian et al. 2008; Xiao et al. 2009) with some modification. *E. tarda* wt, Δ *fliA*, *fliA*⁺, as well as the Δ *rpoS* and *rpoS*⁺ strains were evenly streaked on LB media and incubated at 25 °C for 2 h, then treated with 10 μ L of either 3% or 1.5% H₂O₂, which was spotted on the agar with 6 mm sterile filter paper. The inhibition zones were measured after culturing for 48 h.

Preparation of soluble whole-cell extracts

The protocol for protein extraction from *E. tarda* was based on the method described previously with modification (Daware et al. 2012). *E. tarda* strains were grown in DEME (KCell, Beijing, China) for 24 h. Cells were adjusted to an OD value of 0.5 at 600 nm using fresh DEME and inoculated into fresh DEME at 1:50 dilutions. Protein samples were isolated after 24 hours. Bacterial cultures were disrupted with a micro-sonicator on ice. TCA was added to precipitate proteins at a final concentration of 10%, and the mixture was kept for 1 hour in an ice bath. The protein was collected by centrifugation at 15,000 $\times$ g for 5 min at

4 °C, followed by three washes with ice-cold acetone. The pellet was air dried and dissolved with lysis buffer (9 M urea, 2% CHAPS, 1% DTT, 1% ampholyte 3-10 NL). Insoluble materials were removed by centrifugation at $15,000 \times g$ for 10 min at room temperature. The concentration of each protein sample was determined by the Bradford method (Bradford 1976).

DIGE

Experimental samples were compared in one experiment using 2D DIGE (Ettan DIGE, GE Healthcare, UK). All proteins were solubilized to a final concentration of 5 mg/mL with DIGE lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS). The pH of protein lysates was adjusted to pH 8.5 with 50 mM NaOH. A pool, to be used as an internal standard sample, was generated from an equal amount (25 µg) of all 12 samples. CyDye was reconstituted in anhydrous DMF to a working solution of 0.4 mM. Protein samples were then labeled according to the standard protocol using the CyDye DIGE minimal labeling kit (GE Healthcare). In brief, 1 µL of Cy3 or Cy5 dye DIGE fluors was added to the corresponding protein lysate (see Table S3) so that 50 µg of each sample was labeled with 400 pmol of fluor. Cy2 (6 µL) was added to the internal standard protein lysate. The reactions were incubated on ice in the dark for thirty minutes and then terminated by adding 1 µL of 10 mM lysine (Sigma-Aldrich, St. Louis, MO) for every 400 pmol of fluor. For each gel, 50 µg of internal standard sample protein was mixed together with two experimental samples according to Table S3, and the volume was adjusted to 450 µL with rehydration buffer (9 M Urea, 2% CHAPS, 1% DTT, 1% pharmalyte pH3-10 NL, trace bromophenol blue). The procedure for the separation of protein samples was the same as normal 2-DE, except that all the processes were performed in the dark. When the second dimension separation was finished, the gels were scanned using a TyphoonTM FLA 9000 biomolecular imager (GE Healthcare, Uppsala, Sweden).

2-DE and silver staining

The samples were separated using a 24 cm Immobiline DryStrip in the pH range of 3–10 NL

according to the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). First-dimension isoelectric focusing was performed with the Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare, Uppsala, Sweden). Proteins were loaded by passive in-gel rehydration for 12 hours. IEF was performed under the following conditions: 100 V for 1 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, gradient 10,000 V for 3 h, and a total focusing of 100 kVh at 10,000 V. After IEF, the IPG strips were equilibrated for 15 min in reducing equilibration buffer (6 M Urea, 75 mM Tris-HCl, pH 8.8, 34.5% (v/v) Glycerol, 2% (w/v) SDS, 1% (w/v) DDT) and subsequently alkylated for 15 min in alkylation equilibration buffer (6 M Urea, 75 mM TrisHCl pH 8.8, 34.5% (v/v) Glycerol, 2% (w/v) SDS, 4% (w/v) acrylamide (Sigma, St. Louis, USA)). The second dimension separation, SDS-PAGE, was performed in 11% polyacrylamide gels using an Ettan™ 2-D DIGE system (GE Healthcare, Uppsala, Sweden) in Tris-glycine buffer.

Silver staining was performed according to a previous report (Rao et al. 2004). The gels were fixed in 30% ethanol, 10% acetic acid for 30 min, followed by two rinses in 20% ethanol for 10 minutes each and then two rinses in water for 10 minutes each. The gels were sensitized by soaking in 0.8 mM sodium thiosulfate for one minute, followed by two rinses in water for one minute each. 12 mM silver nitrate was used to impregnate the gel for 20 minutes, followed by two rinses in water for 30 seconds each. The gel was then transferred into the developer solution (2% (w/v) sodium carbonate plus 400 µl 37% methanal per liter). When bands appeared, the developer was replaced with stop solution (4% (w/v) Tris and 2% (v/v) acetic acid), in which the gel was soaked for 30 minutes. After two 30 minute washes in water, the gel was scanned on a scanner (EPSON 11000XL, Nagano-ken, Japan).

Protein identification by MALDI-TOF/TOF analysis

The reproducible spots of interest were excised and identified by a plus 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) analyzer (Applied Biosystems, Foster City, CA, USA). The acquired MS spectra were submitted to the MASCOT search engine for protein/peptide sequence identification using the NCBI and

MSDB protein databases. The parameters were as follows: fragment mass tolerance, ± 0.8 Da; one missed cleavage per peptide; fixed modifications of carbamidomethyl; peptide mass tolerance, 100 ppm; peptide charge state, 1+. A protein score of more than 45 was considered statistically significant, $p < 0.05$, (Perkins et al. 1999).

RNA preparation, reverse transcription, and quantitative PCR

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) following the recommendations of the manufacturer. cDNA was synthesized from 1 μ g of total RNA using reverse transcriptase (Roche, Mannheim, Germany) following the manufacturer's instructions and amplified by PCR. Quantitative PCR experiments were done in triplicate in a qPCR Bio-Rad CFX96 System using SYBR Premix (Takara Bio, Shiga, Japan). The relative expression of each gene was quantified by the Log2 RQ method (relative quantity, $RQ = 2^{-\Delta\Delta CT}$), using *rpoD* as an endogenous control. Sequences for the primers used are listed in Supplemental Table S2.

Results

Construction of the *rpoS* and *fliA* deletion mutants and complemented strains of *E. tarda* EIB202

Using a double-crossover markerless gene deletion system with the suicide vector, pRE112, a 977-bp (from 1 to 977 bp) core portion of the 987-bp *rpoS* gene, and a 692-bp (from 26 to 717 bp) core portion of the 723-bp *fliA* gene were deleted in *E. tarda* EIB202. For each gene, three Cm-sensitive (Cm^s) second-crossover recombinants were selected as candidate gene deletion strains and confirmed by PCR, DNA sequencing, and Southern blot (Fig. S1 and Fig. S2). The *rpoS* and *fliA* deletion mutants were named $\Delta rpoS$ and $\Delta fliA$, respectively.

The full ORFs of *rpoS* and *fliA* were amplified by PCR and ligated into the plasmid, pACYC184, and the resulting complementation plasmids were named pACYC-*rpoS* and pACYC-*fliA*, which were subsequently introduced into $\Delta rpoS$ and $\Delta fliA$, respectively, and verified by PCR analysis and sequencing (data not shown). The resulting complementation strains were named *rpoS*⁺ and *fliA*⁺.

FliA, but not *RpoS*, is essential for motility of *E. tarda*

To investigate the effect of sigma factors on motility, we compared motility among the *E. tarda* strains. *E. tarda* wt, $\Delta rpoS$, and $\Delta fliA$ were inoculated vertically in 0.4% LB agar media. After being cultured for 24 h, motility was fully lost for $\Delta fliA$, but was restored when $\Delta fliA$ was complemented with pACYC184-*fliA* (Fig. 1). Moreover, the motility of $\Delta fliA$ was not enhanced after prolonged growth for 48 h (data not shown). This was also observed for $\Delta rpoN$ in our previous research (Wang et al. 2012). In contrast, the *rpoS* mutant showed normal motility, which was highly similar to the wild-type strain (Fig. 1). These results demonstrated that *fliA* and *rpoN*, but not *rpoS*, are important regulators that are required for motility in *E. tarda*.

RpoS, but not FliA, is essential for oxidative stress resistance

In our previous work, we found that *rpoN* was an essential regulatory gene for protection against H₂O₂ stress (Wang et al. 2012). Furthermore, an earlier report suggested that RpoS, a sigma factor involved in general stress response, was important for responding to oxidative stress by controlling the transcriptions of *katB* and *sodB* in *E. tarda* (Xiao et al. 2009). However, the role of *fliA* in oxidative stress is unknown. In order to evaluate the function of FliA in stress adaptation of *E. tarda*, we investigated the growth sensitivity of *E. tarda* strains when exposed to H₂O₂. *E. tarda* wt, $\Delta fliA$ and *fliA*⁺, $\Delta rpoS$ and *rpoS*⁺, and $\Delta rpoN$ and *rpoN*⁺ (positive control) were treated with different concentrations (3% or 1.5%) of H₂O₂, and the inhibition zones were measured after culturing for 48 h. As shown in Fig. 2, $\Delta rpoS$ was about 50% less resistant to H₂O₂ than the wild-type was, whereas the *rpoS* complemented strain, *rpoS*⁺, had a similar sensitivity to oxidative stress as that of the wild-type strain. Similarly, $\Delta rpoN$ was slightly more sensitive compared to wild-type, showing about a 16% decrease in growth in response to H₂O₂, whereas *rpoN*⁺ restored sensitivity to wild-type levels. These results are similar to our previous work (Wang et al. 2012). Conversely, the *fliA* deletion mutant did not exhibit the same sensitivity to oxidative stress as $\Delta rpoS$. These results indicate that RpoS and RpoN played important roles in oxidative stress resistance, whereas FliA had no such regulatory role.

Identification of proteins differentially expressed amongst *E. tarda* wild-type, $\Delta rpoS$, $\Delta fliA$, and $\Delta rpoN$ strains

In order to establish an in-depth understanding of the function of the sigma factors in terms of mediating the levels of protein expression, we applied a comparative proteomic analysis to identify differentially expressed proteins among the wt, $\Delta rpoS$, $\Delta fliA$, and $\Delta rpoN$ strains. Total soluble proteins were extracted from the four *E. tarda* strains, which were cultured in DEME media at 25 °C for 24 h. Soluble whole-cell protein extracts were then subjected to 2D-DIGE (Fig. 3A). A total of 723 unique spots were identified from all the images, and differences between wt/ $\Delta rpoS$, wt/ $\Delta fliA$, wt/ $\Delta rpoN$, $\Delta rpoS/\Delta fliA$, $\Delta rpoS/\Delta rpoN$ and $\Delta fliA/\Delta rpoN$ were

analyzed with DeCyder software (GE Healthcare, Uppsala, Sweden) using default parameters. In parallel, the same protein samples were also subjected to 2-DE, stained with silver, and analyzed by imagemaster software (GE Healthcare, Uppsala, Sweden). Thirty protein spots that showed similar differential trends (>1.1 times, $p < 0.05$) between 2D-DIGE and 2-DE were selected and further analyzed by MALDI-TOF-MS/MS. Mass spectrometry analysis successfully identified 10 differentially expressed proteins (Table 1). Moreover, the same soluble whole-cell protein extracts were also resolved by 2-DE. Protein spots were visualized following silver staining (Fig. 3B). Two new differential proteins were further identified (>2 -fold, $p < 0.05$) (Table 1). The relative abundance levels of the 12 identified protein spots between the mutant strain and the wild-type strain are shown in Fig. 4 and Table S4, respectively.

Among the identified proteins spots, we found 4 spots involved in nitrogen metabolism, namely spots 4721, 5708, 6601, and 2055, corresponding to *gadB1*, *gadB2*, and *glnA*, which encode two glutamate decarboxylases and one glutamine synthetase, respectively. Interestingly, mass spectrometric analysis revealed that spots 4721 and 5708 represented the same protein, although they separated with an apparently different isoelectric point during 2D-DIGE. In addition, strain-dependent similarity in relative expression levels of spots 4721 and 5708 was observed (Fig. 4). Spots 4721 and 5708 expression were downregulated by 1.72- and 2.11-fold, respectively, in the *rpoS* deletion mutant, but were upregulated by 1.86- and 2.12-fold, respectively, in the *fliA* deletion mutant and by 1.36- and 1.52-fold, respectively, in the *rpoN* deletion mutant (Fig. 4). Spot 6601 represented a glutamate decarboxylase encoded by *gadB2*, whose expression was similar with the expression profile of *gadB1*-dramatically downregulated by 16.67-fold in $\Delta rpoS$ and upregulated by 2.19- and 1.14-fold in $\Delta fliA$ and $\Delta rpoN$ strains, respectively (Fig. 4). Furthermore, another important nitrogen assimilation protein, the glutamine synthetase encoded by *glnA* (Spot ID 2055), was found to have no obvious expression difference in $\Delta rpoS$ and $\Delta fliA$, but was notably downregulated by 78% in $\Delta rpoN$. These results indicate that these sigma factors are important nitrogen metabolism regulators.

Notably, the only catalase/oxidase (encoded by ETAE_0889, spot ID 1521), which is the vital enzyme in protecting the cell from oxidative damage, was identified by the comparative proteomic analysis (Table. 1). Expression of catalase/oxidase was downregulated by 1.32-fold in $\Delta rpoS$, whereas no obvious difference was observed in $\Delta fliA$ (1.11-fold) and it was slightly upregulated in $\Delta rpoN$ (1.23-fold) (Fig. 4). These results corroborate the findings presented in Fig. 2, and further demonstrate that RpoS is the essential factor responsible for the oxidative stress response.

Spot 2615 was the function-uncharacterized protein, ETAE_1939. When *rpoS* was absent, its protein abundance decreased over 2-fold ($p < 0.05$) compared to wt, $\Delta fliA$, and $\Delta rpoN$, whose *rpoS* was not deleted and between which there were no obvious differences in the protein's relative abundances (Fig. S3).

To further demonstrate that the transcription profiles of the genes that encode these identified proteins are differentially regulated, quantitative reverse transcription PCR (qRT-PCR) was performed to quantify the transcript levels of several genes (*gadB1*, *gadB2*, *evpH*, and ETAE_0889) between the three mutant strains and the wild-type strain. As shown in Fig. 5, transcription of the two glutamate decarboxylase encoding genes, *gadB1* and *gadB2*, was respectively 2.0- and 3.2-fold downregulated in $\Delta rpoS$, 2.4- and 1.9-fold upregulated in $\Delta fliA$, and 1.1- and 0.9-fold in $\Delta rpoN$. These results were similar to the 2-D gel results (Fig. 4 and Table S4). In addition, transcript levels of ETAE_0889 and *evpH* were investigated. Most of the qRT-PCR results were in accordance with protein expression, except that the expression of ETAE_0889 in $\Delta rpoS$ showed an opposite trend compared to the 2-D gel results.

Discussion

In the genome of *E. tarda*, 6 sigma factor encoding genes were found, namely *rpoD*, *rpoS*, *fliA*, *rpoN*, *rpoE*, and *rpoH*. RpoD is known to be the primary housekeeping sigma factor and is essential to fundamental cellular activities, thus it exists in all known bacteria (Gruber and Bryant 1997). The other 5 are alternative sigma factors, and each one has specific functions and are involved in different regulatory profiles (Helmann and Chamberlin 1988). Typically, alternative sigma factors regulate the expression of genes involved in specific physiological or developmental processes (Helmann and Chamberlin 1988). The role of RpoS, RpoN, and FliA have been investigated recently in *E. tarda* (Liu et al. 2014; Wang et al. 2012; Xiao et al. 2009; Xu et al. 2014), while the other two alternative sigma factors, RpoH and RpoE, have been demonstrated to play an important role in heat shock response and extracytoplasmic stress, respectively, in *E. coli* (Ishihama 2000). In the present and our previous studies (Wang et al. 2012), we successfully constructed $\Delta rpoN$, $\Delta rpoS$, and $\Delta fliA$ mutants, which allowed us to characterize and compare the function of these sigma factors simultaneously. In addition, we also attempted to construct *rpoH* and *rpoE* deletion strains, which were unsuccessful. However, an *rpoE* overexpression strain was successfully obtained, and some preliminary data has shown that the transcription of *degP* and another 5 genes are significantly affected when *rpoE* is overexpressed (data not shown). This indicates an important role for RpoE, which is currently under investigation.

Through physiology experiments, significant roles of the three sigma factors (RpoN, RpoS, and FliA) were elucidated and interplays among them could be concluded. The alternative sigma gene, *fliA*, encodes a flagellar sigma factor, which was initially found to direct transcription of flagellar genes in *E. coli* (Ishihama 2000; Ohnishi et al. 1990). Similarly, FliA was shown to play an important role in the formation of flagella in *E. tarda* (Xu et al. 2014). Here, the $\Delta fliA$ mutant presented a flagellum-impaired phenotype, which suggests that FliA was the direct sigma factor controlling motility in *E. tarda*. The transcription level of *fliA* was

reduced significantly in an *rpoN* mutant (Dong et al. 2011; Liu et al. 2014), indicating a positive regulation of RpoN on *fliA* transcription. RpoN was also reported to positively control the expression of other flagella-related genes (Zhao et al. 2010). The deletion of *rpoN* and consequent loss of motility in *E. tarda* (Wang et al. 2012) could be explained by the mechanism of regulatory interactions among these sigma factors. Therefore, RpoN could be a crucial motility regulator. In *E. coli*, *rpoS* mutants were highly motile in comparison to wild-type (Dong and Schellhorn 2009; Makinoshima et al. 2003), and the transcription level of *fliA* was increased in the both *rpoS* mutants of *E. coli* (Dong et al. 2011) and *E. tarda* ((Dong et al. 2011; Liu et al. 2014), so it was surprising that *rpoS* had no impact on motility in *E. tarda* in the present study. Notably, recent studies have revealed that the regulation of gene expression by RpoS and RpoN proceeds by a substantial antagonistic regulatory manner (Dong et al. 2011), and that the transcriptional level of *fliA* was decreased in a *rpoN* plus *rpoS* double deletion mutant (Liu et al. 2014), which indicated that the regulatory effect of RpoS on motility was weaker than RpoN in *E. tarda*. In the present study, it is possible that the weak effect of the single deletion of *rpoS* on *E. tarda* motility was not detectable. In general, a complex regulatory interaction of the three sigma factors, RpoN, RpoS, and FliA, was observed in *E. tarda*.

To further elucidate the roles of the sigma factors in this study, we conducted a comparative proteomic analysis to determine the differentially expressed proteins by comparing proteomic profiles among wt, $\Delta rpoS$, $\Delta fliA$, and $\Delta rpoN$. Consequently, 11 unique proteins representing 12 protein spots were identified. Interestingly, GadB1 was represented by two spots, 4721 and 5708, that had the same molecular mass but different isoelectric points. This was further validated because the expression tendency of the two spots was highly similar among the strains. These results indicated that at least two forms of GadB1 exist in *E. tarda*. Notably, expression of the two glutamate decarboxylase encoding genes, *gadB1* and *gadB2*, was decreased in the $\Delta rpoS$ mutant but increased in the $\Delta fliA$ and the $\Delta rpoN$ mutants, suggesting that glutamate decarboxylases expression was positively regulated by RpoS but negatively by RpoN and FliA. It is well known that RpoN is mainly responsible for transcription of nitrogen

utilization genes (Reitzer and Schneider 2001; Riordan et al. 2010). However, the observation that RpoS and FliA regulate nitrogen related genes like RpoN is unexpected, which further indicates that the target genes of the sigma factors are interwoven. Moreover, there is another possible mechanism that may exist, such as cross-regulation among the sigma factors. Given that limited experimental data is currently available regarding the direct interaction between these sigma factors and the identification of genes that are affected upon their deletion, further investigation is necessary.

Understanding the virulence mechanisms of the pathogenic bacteria, *E. tarda*, is globally important. The relationship between sigma factors and virulence is being studied by our team. Here, using blast analysis, we found that gene homologues for the ETAE_1939 protein with unknown function (Spot ID 2615), which was identified through comparative proteomic analysis, might only exist in pathogenic bacteria, like *Salmonella enterica*, *Serratia marcescens*, and *Cronobacter sakazakii*. In addition, the abundance of ETAE_1939 was significantly decreased (Table S4 and Fig. S3) and transcription was 2.4-fold downregulated (data not shown) in $\Delta rpoS$. Given that RpoS played essential roles in the virulence network, the newly identified protein, ETAE_1939, may also be involved in the pathogenicity of *E. tarda*.

Acknowledgements

The authors thank Dr. Zhaolan Mo (Institute of Oceanology, Chinese Academy of Sciences, China) for kindly sending the strains and plasmids used for mutant construction in this study. This work was supported by the National Special Fund for State Key Laboratory of Bioreactor Engineering.

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Legends

Fig. 1 The motility ability of *E. tarda* wild-type and sigma mutant. The *E. tarda* strains were inoculated vertically in 0.4% LB agar media. Cultures were grown at 25°C for 24h.

Fig.2 Histogram of inhibition zones of the *E. tarda* strains by H₂O₂. The *E. tarda* strains were streaked on LB agar and incubated at 25°C for 2h. Then 10 µL of the indicated concentrations of H₂O₂ were spotted on the agar with a 6 mm sterile filter paper. The inhibition zones were measured after 48h incubation. Error bars indicate standard deviations from 3 independent biological replicates.

Fig. 3 Analysis of differential proteins of *E. tarda* strains using 2-DE based proteomics. 19 Images of 2D DIGE and 2DE. Arrows indicate the corresponding proteins spots indicated in Table 3 (see below) identified by MALDI-TOF-MS/MS. **A**, image of 2D DIGE of Gel 1 internal Standard; **B**, image of wt by silver staining.

Fig. 4 The relative abundance ratios of each identified protein spots between the *E. tarda* strains.

Fig. 5 qRT-PCR analysis of transcription profiles of the encoding genes of several differential proteins. RNAs were extracted from *E. tarda* wild-type and mutant strains cultured in DEME media at 25°C for 24h. Relative fold changes represent the level of expression in the each mutant strain compared to the level of expression in the wild-type. Error bars indicate standard deviations from 3 independent biological replicates. ND: not detected.

Table 1 Annotations and MS information of the identified differential proteins

Spot ID	Protein Description	Gene Name	gi in NCBI	Locus_tag	Mr	pI	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%	Pep. Count
6601	glutamate decarboxylase	<i>gadB2</i>	gi 269140211	ETAE_2868	52395	5.97	109	100	36	97.561	12
1419	GTP-binding protein	<i>yihK</i>	gi 269140833	ETAE_3492	67298	5.18	151	100	107	100	10
2055	glutamine synthetase	<i>glnA</i>	gi 269140834	ETAE_3493	51877	5.15	129	100	66	99.998	11
1894	transcription elongation factor	<i>nusA</i>	gi 269137762	ETAE_0404	54855	4.57	353	100	220	100	19
1521	catalase/peroxidase		gi 269138245	ETAE_0889	79916	5.28	352	100	272	100	15
1036	type VI secretion system protein EvpH	<i>evpH</i>	gi 269139781	ETAE_2436	95833	5.49	384	100	223	100	24
2556	serine endoprotease		gi 269140114	ETAE_2771	47077	7.64	117	100	76	100	8
4721	glutamate decarboxylase	<i>gadB1</i>	gi 269138142	ETAE_0786	64480.4	5.78	195	100	154	100	9
5708	glutamate decarboxylase	<i>gadB1</i>	gi 269138142	ETAE_0786	64480.4	5.78	213	100	156	100	11
2615	hypothetical protein ETAE_1939		gi 269139286	ETAE_1939	58023.9	5.55	201	100	103	100	15
5406	iron-containing alcohol dehydrogenase		gi 269137598	ETAE_0240	42610.7	5.73	189	100	86	100	14
5812	outer membrane protein (porin)		gi 269139173	ETAE_1826	42331.2	4.68	305	100	245	100	11

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Fig. 2 Histogram of inhibition zones of the *E. tarda* strains by H₂O₂. The *E. tarda* strains were streaked on LB agar and incubated at 25°C for 2h. Then 10 μ L of the indicated concentrations of H₂O₂ were spotted on the agar with a 6 mm sterile filter paper. The inhibition zones were measured after 48h incubation. Error bars indicate standard deviations from 3 independent biological replicates.

360x249mm (96 x 96 DPI)

Fig. 3 Analysis of differential proteins of *E. tarda* strains using 2-DE based proteomics. Images of 2D DIGE and 2DE. Arrows indicate the corresponding proteins spots indicated in Table 1 (see below) identified by MALDI-TOF-MS/MS. A, image of 2D DIGE of Gel 1 internal Standard; B, image of wt by silver staining.

133x207mm (150 x 150 DPI)

Fig. 4 The relative abundance ratios of each identified protein spots between the *E. tarda* strains.

379x242mm (96 x 96 DPI)

Fig. 5 qRT-PCR analysis of transcription profiles of the encoding genes of several differential proteins. RNAs were extracted from *E. tarda* wild-type and mutant strains cultured in DEME media at 25°C for 24h. Relative fold changes represent the level of expression in the each mutant strain compared to the level of expression in the wild-type. Error bars indicate standard deviations from 3 independent biological replicates. ND: not detected.

289x263mm (96 x 96 DPI)