

## *Salmonella enterica* Serovar Typhimurium *rdoA* Is Growth Phase Regulated and Involved in Relaying Cpx-Induced Signals

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The disulfide oxidoreductase, DsbA, mediates disulfide bond formation in proteins as they enter or pass through the periplasm of gram-negative bacteria. Although DsbA function has been well characterized, less is known about the factors that control its expression. Previous studies with *Escherichia coli* demonstrated that *dsbA* is part of a two-gene operon that includes an uncharacterized, upstream gene, *yihE*, that is positively regulated via the Cpx stress response pathway. To clarify the role of the *yihE* homologue on *dsbA* expression in *Salmonella enterica* serovar Typhimurium, the effect of this gene (termed *rdoA*) on the regulation of *dsbA* expression was investigated. Transcriptional assays assessing *rdoA* promoter activity showed growth phase-dependent expression with maximal activity in stationary phase. Significant quantities of *rdoA* and *dsbA* transcripts exist in serovar Typhimurium, but only extremely low levels of *rdoA-dsbA* cotranscript were detected. Activation of the Cpx system in serovar Typhimurium increased synthesis of both *rdoA*- and *dsbA*-specific transcripts but did not significantly alter the levels of detectable cotranscript. These results indicate that Cpx-mediated induction of *dsbA* transcription in serovar Typhimurium does not occur through an *rdoA-dsbA* cotranscript. A deletion of the *rdoA* coding region was constructed to definitively test the relevance of the *rdoA-dsbA* cotranscript to *dsbA* expression. The absence of RdoA affects DsbA expression levels when the Cpx system is activated, and providing *rdoA* in *trans* complements this phenotype, supporting the hypothesis that a bicistronic mechanism is not involved in serovar Typhimurium *dsbA* regulation. The *rdoA* null strain was also shown to be altered in flagellar phase variation. First it was found that induction of the Cpx stress response pathway switched flagellar synthesis to primarily phase 2 flagellin, and this effect was then found to be abrogated in the *rdoA* null strain, suggesting the involvement of RdoA in mediating Cpx-related signaling.

Bacterial proteins located in or in transit through the periplasm undergo some degree of folding into their native conformations. Periplasmic foldases such as disulfide oxidoreductases and peptidyl-prolyl isomerases accelerate this folding process (4). The best-studied protein folding pathway is the Dsb system in *Escherichia coli* that catalyzes disulfide bond formation, reviewed in references 3, 4, 13, 18, 42, and 53). DsbA, a 21-kDa periplasmic disulfide oxidoreductase, plays a pivotal role in this system by transferring its disulfide bond to substrates as they are assembled (17, 20, 65). Homologues to DsbA have been identified throughout gram-negative and gram-positive bacteria (24, 29, 35, 49, 58–60, 63). The thiol-disulfide chemistry of the active site of DsbA, consisting of the sequence motif Cys<sup>30</sup>-Pro<sup>31</sup>-His<sup>32</sup>-Cys<sup>33</sup> with its donor intrapeptide disulfide bond, has been thoroughly investigated and is highly reactive, providing this enzyme with a high oxidizing potential (20, 22, 30, 42, 62, 65). *dsbA* mutants exhibit pleiotropic phenotypes, since they alter cellular activities depending on the presence of disulfide bond-containing enzymes. Processes such as motility, assembly of pili, resistance to reducing agents, and the production of virulence determinants are all severely affected, making DsbA important for growth and survival (4, 10, 31, 42, 59, 67).

Whereas the functional properties of DsbA have been investigated in detail, less is known about the factors that influ-

ence the expression of this catalyst. Most studies concerning *dsbA* regulation were carried out with *E. coli*, where *dsbA* is part of a two-gene operon that includes an uncharacterized (*yihE*) gene found immediately upstream of the *dsbA* coding region (7). Two promoters controlling *dsbA* transcription have been identified for *E. coli*. The proximal (*dsbA*) promoter is located in the 3' portion of the *yihE* coding region, and the distal (*yihE*) promoter is upstream of the *yihE* gene. Transcription from the proximal promoter is proposed to be relatively low and constitutive. Belin and Boquet constructed *yihE* null strains that showed a reduction in DsbA levels compared to the wild type, although this reduced level of DsbA expression showed no phenotype (7). When they examined a plasmid-borne *yihE-dsbA::TnphoA* translational fusion containing a frameshift mutation in the *yihE* gene in a *yihE* null background, the expression of the *TnphoA* fusion was reduced by one-third. The presence in *trans* of the wild-type *yihE* gene did not restore the wild-type levels of the fusion. The authors concluded that the frameshift mutation conferred a polar transcriptional defect on *dsbA* expression.

Pogliano et al. (51) showed that the activation of the two-component Cpx pathway, through a CpxR binding site located in the *yihE* promoter region, increased DsbA protein levels. This correlated with increased levels of transcripts originating from the *yihE* promoter as determined by nuclease protection assays (12, 51). Members of the Cpx regulon are part of a stress response system involved in sensing and responding to cell envelope protein folding defects (9, 52, 55). Cpx-activating signals include overproduction of the outer membrane lipopro-

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TABLE 1. *E. coli* or serovar Typhimurium strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Laboratory reference	Description	Source or reference
<i>E. coli</i>			
DH5 $\alpha$	NLM264	<i>supE44 <math>\Delta</math>lacU169 (<math>\phi</math>80lacZ<math>\Delta</math>M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	23
MG1655	NLM28	Wild type, derivative of K-12	Lab collection
MG1655 <i>dsbA</i>	NLM1	MG1655 <i>lacZ::Tn10(Tet<sup>r</sup>) dsbA::kan</i>	Lab collection
MC4100 <i>cpxR</i>	NLM367	MC4100 <i>cpxR::<math>\Omega</math>(spec)</i>	12
Serovar Typhimurium			
SL1344	NLM2217	<i>his</i> Str <sup>r</sup>	64
SL1344	NLM2218	NLM2217 containing pPrdoA	This study
SL1344 <i>dsbA</i>	NLM267	SL1344 <i>dsbA::kan</i>	59
SL1344 <i>dsbA</i>	NLM2219	NLM267 containing pPrdoA	This study
SL1344 <i>rdoA</i>	NLM2214	NLM2217 with a <i>rdoA</i> deletion of 766 bp containing pMP190	This study
SL1344 <i>rdoA</i>	NLM2239	NLM2214 cured of pMP190	This study
SL1344	NLM2244	NLM2217 containing pBAD18	This study
SL1344	NLM2245	NLM2217 containing pPrdoA, pND18	This study
SL1344	NLM2246	NLM2217 containing pPrdoA, pBAD18	This study
SL1344	NLM2247	NLM2217 containing pND18, pMEG2	This study
SL1344	NLM2248	NLM2217 containing pBAD18, pMEG2	This study
SL1344 <i>rdoA</i>	NLM2253	NLM2239 containing pPrdoA, pND18	This study
SL1344 <i>rdoA</i>	NLM2254	NLM2239 pPrdoA, pBAD18	This study
SL1344 <i>rdoA</i>	NLM2255	NLM2239 containing pND18, pMEG2	This study
SL1344 <i>rdoA</i>	NLM2256	NLM2239 containing pBAD18, pMEG2	This study
SL1344 <i>rdoA/rdoA+</i>	NLM2269	NLM2238 containing pLMN20	This study
SL1344 <i>cpxR</i>	NLM2274	NLM2217 transduced with <i>cpxR::<math>\Omega</math></i> from NLM367 containing pND18	This study
Plasmids			
pMEG2		pMP190 with a 258-bp fragment from immediately upstream of serovar Typhimurium <i>dsbA</i> SD sequence	19
pPrdoA		pMP190 with a 236-bp fragment from immediately upstream of serovar Typhimurium <i>rdoA</i> SD sequence	This study
pND18		pBAD18 which contains the <i>E. coli nlpE</i> gene under the control of the P <sub>BAD</sub> promoter of the <i>araBAD</i> (arabinose) operon	55
pMP190		15-kb transcriptional fusion vector containing a promoterless <i>lacZ</i> , Cm <sup>r</sup>	56
pMEG1		pBAD24 with 6.9-kb <i>NcoI</i> fragment from pLAFR2-31 containing <i>dsbA</i> and <i>rdoA</i>	19
pLMN20		pACYC184 containing a 1.5-kb <i>SacII</i> fragment from pMEG1 coding for <i>rdoA</i>	This study

<sup>a</sup> SD, Shine-Dalgarno.

tein NlpE (55), high-pH conditions (43), and an alteration in the phospholipid composition of the bacterial membrane (40).

In contrast to reports from *E. coli*, the proximal promoter region of *Salmonella enterica* serovar Typhimurium (herein referred to as serovar Typhimurium) *dsbA* showed more complex regulation of expression. Two *dsbA*-specific transcripts were identified for this organism and mapped to two separate promoters proximal to the *dsbA* coding region (19). Growth phase-dependent promoter activity, with high levels of activity in stationary phase, was demonstrated using a *dsbA* transcriptional fusion containing both *dsbA*-proximal promoters, and growth conditions, such as lowered pH and oxygen levels, were shown to affect *dsbA* transcription (19). In its natural habitats, serovar Typhimurium often encounters a variety of stressful conditions, including fluctuations in pH, hypoxia, oxidative stress, and nutrient limitation (6, 48, 57). The organism can actively adapt to survive under these conditions by inducing systems (for example, the acid tolerance response) and/or specific genes, including virulence determinants, that enhance cellular resistance and provide cross-protection to additional stresses (36, 57).

To study the possible effect of the upstream gene on the regulation of *dsbA* expression, the DNA sequence further upstream of serovar Typhimurium *dsbA* was determined and the transcripts encoded by this region were analyzed. While the

sequence corresponding to serovar Typhimurium *dsbA* and the upstream open reading frame (ORF), termed *rdoA* (regulator of disulfide oxidoreductase A), is homologous to the *E. coli yihE-dsbA* operon, differences were identified in their respective control of *dsbA* expression. Our results indicate that the *rdoA-dsbA* cotranscript does not play a significant role in Cpx-mediated induction of *dsbA* transcription, unlike the situation proposed to occur with *E. coli*, although RdoA does influence DsbA protein levels. This study also showed that RdoA also affects flagellin phase variation in a Cpx-dependent manner, suggesting a role for RdoA in relaying Cpx signals.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** Table 1 describes all bacterial strains and plasmids used in this study. For  $\beta$ -galactosidase assays, overnight cultures were diluted 1:100 either in fresh Luria-Bertaini (LB) broth or in minimal E-media (pH 7.6 or 4.6) (61) supplemented with 0.4% glucose and amino acids (to a final concentration of 50  $\mu$ g/ml). Cells were grown at 37°C with aeration. For  $\beta$ -galactosidase assays and RNA preparation of strains containing pND18 or pBAD18 (control) vectors, overnight cultures were diluted 1:100 and grown to an optical density at 600 nm (OD<sub>600</sub>) of approximately 0.3 in LB with 0.2% glucose (repressing the arabinose promoter in pND18 and pBAD18 vectors). Cultures were centrifuged, and the supernatant was aspirated off. Cell pellets were resuspended in fresh LB media containing either 0.4% arabinose (inducing the arabinose promoter in the pBAD18-based vectors) or 0.2% glucose (repressing the arabinose promoter). Cultures were subsequently assayed for  $\beta$ -galactosidase activity at various time points or, in the case of RNA extraction,

grown for 1 h before harvesting of RNA. When antibiotics were required, the medium was supplemented with 30 µg of chloramphenicol/ml or 50 µg of ampicillin/ml. The growth of the bacterial cultures was monitored by measuring the OD<sub>600</sub>.

**Construction of the *rdoA::lacZ* transcriptional fusion (pPrdoA).** The primers Nm64 (5' TAACTCGAGCAAATCGGAAAGATTAC3') containing a *Xho*I restriction site (underlined) and Nm57 (5' TTAAGATCTATACTACGAATGATTCAG3') containing a *Bgl*II restriction site (underlined) were used to PCR amplify a 236-bp *rdoA* putative promoter fragment (position, 228 bp upstream to 15 bp upstream of the *rdoA* start codon) using the template pMEG1. This *rdoA* promoter region was cloned into pMP190, predigested with *Sal*I (compatible to the *Xho*I site of the PCR amplicon) and *Bgl*II. This construct, called pPrdoA, containing the putative promoter, transcriptional start site, and sequence up to but not including the ribosome binding site is similar to the *dsbA::lacZ* transcriptional fusion vector, pMEG2.

**Assays.** Transcriptional activity from the *rdoA::lacZ* promoter fusion construct was measured in all serovar Typhimurium strains by assaying β-galactosidase activity as described by Miller (41). Aliquots of cultures were assayed at time intervals throughout the growth of the culture or at specific growth phases. In addition, assays were performed on one in five dilutions of aliquots, once the OD<sub>600</sub> values of cultures reached a value of 1. Each experiment was performed at least twice.

**RNA preparation and RT-PCR.** Total RNA was isolated using Trizol (Life Technologies, Gibco BRL) from 1 ml of culture of *S. enterica* serovar Typhimurium SL1344 and *E. coli* MG1655 strains containing pND18 (inducible NlpE-overproducing plasmid) or control vector (pBAD18) after 1 h of induction with 0.4% arabinose or repression with 0.2% glucose. Approximately 20 µg of total RNA was subjected to DNase treatment (RQ1 RNase-free DNase; Promega) and subsequent purification using RNeasy columns (RNeasy Mini kit; Qiagen). Multiplex reverse transcription (RT) reactions (Retroscript kit; Ambion) using 100 pmol of internal control primer and the serovar Typhimurium-specific target primers Nm78 (5' CGCATCAACGAACACTTTACGG 3') and Nm88 (5' CCAACGACGAATCAACCAGG 3') or the *E. coli* target primers Nm103 (5' CCTGCGTTGATAAATACATCGC 3') and Nm105 (5' CCAACGCCGATTAGCAGG 3') were performed on approximately 1 to 3 µg of purified RNA with minor changes to the manufacturer's instructions. Changes to the protocol include longer heat denaturation of the RNA template (6 min at 80°C) and elevated incubation temperatures (56°C). In addition, RT negative control reactions were performed where water was added instead of the RT enzyme (data not shown). Multiplex PCR amplification was performed using an internal control primer pair and specific target primer pairs: for serovar Typhimurium, Nm88 and Nm89 (5' GGCAACATTCTATGGCGTG 3'); and for *E. coli*, Nm104 (5' GGAATATTCTCTGGCGCGATGGTCC 3') and Nm105 (5' CCAACGCCGATTAGCAGG 3'). Fifty-microliter PCRs were carried out using 2.5 U of *Taq* DNA polymerase (Life Technologies, Gibco BRL), 5 µl of RT reaction sample as a template, 50 pmol of each PCR primer pair, 1× PCR buffer, 0.2 mM (each) deoxynucleoside triphosphate, and 1.5 mM MgCl<sub>2</sub>. Template cDNA was denatured for 2 min at 94°C before *Taq* DNA polymerase was added. A 25- to 30-cycle PCR (94°C for 45 s, 60°C for 30 s, and 72°C for 1 min with a final extension reaction of 72°C for 7 min) was carried out. To aid in qualitative analysis, specific target RT-PCR products were normalized to an established endogenous internal control transcript (*tsf* encoding the elongation factor EF-Tsf), the expression of which is relatively constant in serovar Typhimurium at the growth phases studied in this work (26). RT-PCR primers used in the detection of *tsf* transcript are described by Holmstrom et al. (26). PCR products of both internal control transcript (563 bp) and target (246 bp for serovar Typhimurium *rdoA-dsbA* cotranscript only or *rdoA* monocistronic and cotranscript; 241 bp for *E. coli yihE-dsbA* cotranscript only or *yihE* specific and cotranscript) were visualized by agarose gel electrophoresis on a 1.2% agarose gel containing 1 µg of ethidium bromide/ml.

**Northern blot analysis.** Approximately 30 µg of total RNA from serovar Typhimurium SL1344 or *E. coli* MG1655 strains were separated on a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane (positively charged; Boehringer Mannheim) as described by Fourney et al. (16). Membranes were initially probed with a serovar Typhimurium *rdoA* DNA probe or an *E. coli yihE* DNA probe and subsequently stripped and reprobed with a serovar Typhimurium *dsbA* probe or an *E. coli dsbA* DNA probe. Each of the probes was generated by PCR and gel purified using a Qiaquick gel purification kit (Qiagen) the N-terminus-specific serovar Typhimurium *dsbA* probe (position, 107 to 400 bp downstream of the *dsbA* ATG start site) using primers Nm86 (5' CTGGCGAACCCAGGTACTG 3') and Nm78; a full-length *rdoA* probe (position, 25 to 855 bp downstream of the *rdoA* ATG start site) using primers Nm99 (5' ACGCTACACCCGGAAACCATC 3') and Nm89; an N-terminus-specific *E. coli*

*dsbA* probe (position, 107 to 404 bp downstream of the *dsbA* ATG start site) using primers Nm102 (5' CTGGCGCGCCGAAGTGCTG 3') and Nm103; and a C terminus *E. coli yihE* probe (position, 614 to 855 bp downstream of the *yihE* ATG start site) using primers Nm104 (5' GGAATATTCTCTGGCGCGATGGTCC 3') and Nm105 (5' CCAACGCCGATTAGCCAGG 3'). Fifty nanograms of all probes were random prime labeled with an oligolabeling kit and [α-<sup>32</sup>P] dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) according to the manufacturer's instructions. The efficiency of labeling was monitored following procedure C of the manufacturer's protocol, and labeled probes were separated from unincorporated <sup>32</sup>P-labeled nucleotides using a QIAquick Spin column and the QIAquick nucleotide removal protocol (Qiagen). All prehybridizations, hybridizations, Northern washes, and removal of labeled probes from membranes were performed according to the basic protocol described by Kingston (33). Washed membranes were subsequently exposed to a storage phosphor-imaging screen (Kodak). Phosphorescence signals were captured and quantitated using the Personal Molecular Imager FX (Bio-Rad) and Quantity One (version 4.0; Bio-Rad) quantitation software. For comparisons, the Northern signals were normalized to the 16S rRNA band of the specific RNA preparations loaded.

**Gel electrophoresis and Western blotting.** Periplasmic protein preparations were separated using a sodium dodecyl sulfate–12% polyacrylamide gel. Briefly, periplasmic protein isolation involved growing overnight serovar Typhimurium SL1344 (pND18) or control vector cells in 0.2% glucose (repressed) or 0.4% arabinose (induced) to an OD<sub>600</sub> of 1. One milliliter of culture was centrifuged and resuspended in 250 µl of Tris-HCl (0.2 M). Subsequently, a solution containing 1 M sucrose in 0.2 M Tris-HCl, 2.5 µl of EDTA (0.1 M), 7.5 µl of lysozyme (4 mg/ml), and 500 µl of distilled water was added and incubated on ice for 2 min. A 30-min incubation was carried out after adding 20 µl of MgCl<sub>2</sub> (1 M), and proteins were isolated from the supernatant by trichloroacetic acid extraction.

Two-dimensional (2D) gel electrophoresis was carried out following the manufacturer's suggested conditions using immobilized pH gradient (Bio-Rad) strips of pH ranges 3 to 10, 3 to 6, or 4 to 7 in the first dimension. The samples were run in the second dimension on 12% acrylamide gels using the Protean II system (Bio-Rad). Gels were silver stained (66) and digitized using a transmission flatbed scanner. All data analysis was carried out using the BioImage software package (Genomic Solutions). A minimum of three sample replicates were carried out for each strain or growth condition examined.

Western immunoblots were carried out as previously described by Klapper et al. (34) using polyclonal anti-DsbA sera, peroxidase-conjugated secondary antibody, and the SuperSignal West chemiluminescence detection system (Pierce).

**Peptide mass fingerprinting.** Protein spots of interest were excised from the acrylamide gels using a Bio-Rad spot cutter, digested with trypsin, and extracted from the gel pieces using a MicroMass MassPrep digestion robot. Mass spectrometric analysis was carried out using a MicroMass M@LDI mass spectrometer. The spectrum data were analyzed using the MASCOT software (50).

**Creation of an *rdoA* deletion strain.** Primer Nm114 (5' GGAATTCGTCAA CTGGGGCTCAACG 3') containing an *Eco*RI restriction site (underlined) and primer Nm115 (5' CGGGATCCTTGTCGTTTCATCCCATCCGG 3') containing a *Bam*HI restriction site (underlined) were used to PCR amplify an 881-bp DNA fragment (position, 855 bp upstream of the *rdoA* start codon to 11 bp downstream of the *rdoA* start codon) using the template pMEG1. This fragment (left flank) includes the first 11 nucleotides of the *rdoA* coding region and enough upstream sequence to allow for homologous recombination. The primers Nm116 (5' CGGGATCCACGGCTGAAATTGGTCTCA 3') containing a *Bam*HI restriction site (underlined) and Nm117 (5' GGAATTCGTCTGCTCGTATCCATGC 3') containing an *Eco*RI restriction site (underlined) were used to PCR amplify an 806-bp fragment (position, 778 to 1,568 bp downstream of the *rdoA* start codon) using the template pMEG1. This fragment (right flank) includes the last 70 codons of the *rdoA* coding region and enough downstream sequence to allow for homologous recombination. The left and right flank amplicons were gel purified using the QIAquick gel purification kit (Qiagen Inc.), digested with *Bam*HI, and ligated together. PCR amplification using 5 µl of the ligation reaction as a template and the outer *Eco*RI restriction site containing primers Nm114 and Nm117 was performed to amplify a 1.69-kb DNA fragment containing 11 bp of the N terminus and 210 bp of the C terminus of the *rdoA* ORF but lacking the intervening 766 bp of the *rdoA* coding region. This 1.69-kb *rdoA* deletion fragment was gel purified using the QIAquick spin column (Qiagen Inc.), digested with *Eco*RI, and subcloned into the ampicillin-resistant and streptomycin-sensitive counter-selectable suicide vector pKAS32 (21). Ligands were transformed into *E. coli* MC4100λpir strains and selected on ampicillin plates. Plasmid purified from these colonies was then electroporated into the conjugative *E. coli* Sm10λpir strain. This donor strain was conjugated with the streptomycin- and chloramphenicol-resistant serovar Typhimurium SL1344 re-

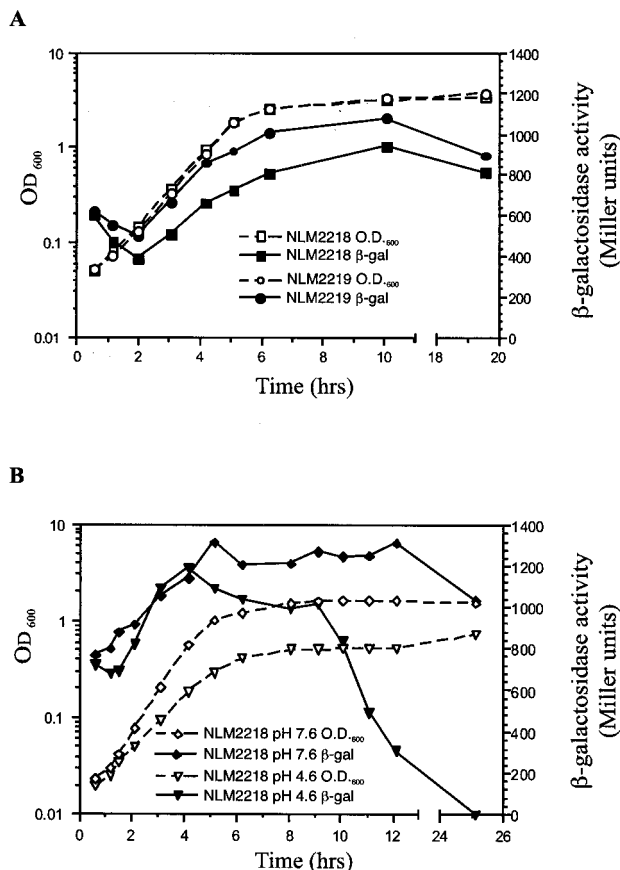


FIG. 1. Expression of the *rdoA::lacZ* promoter (pPrdoA) in wild-type (NLM2218) and *dsbA* null (NLM2219) serovar Typhimurium SL1344 background strains throughout the growth curve in rich medium (A) or wild-type (NLM2218) serovar Typhimurium growth in minimal E-glucose medium at pH 7.6 or 4.6 (B). Each point in a curve is the average activity of two samples, and the standard deviation for all data points is less than 5% of the mean. The  $\beta$ -galactosidase activity curves of pPrdoA are representative of two to three separate trials.

ipient strain containing the plasmid pMP190. Transconjugants were selected on LB plates containing chloramphenicol (to select against the donor *E. coli* strain) and ampicillin (to select for the plasmids). Twenty colonies from each of the conjugations were then restreaked on streptomycin plates, permitting selection of single-crossover clones of the *rdoA* deletion fragment. Approximately 250 individual colonies were picked and patched onto streptomycin and ampicillin plates. Colonies in which a double crossover event occurred by homologous recombination of the *rdoA* deletion fragment grew on streptomycin plates but did not grow on ampicillin plates. One such colony (designated NLM 2214) was discovered, and colony PCR amplification confirmed the presence of the *rdoA* deletion fragment in the chromosome of serovar Typhimurium SL1344. Colonies were then screened for loss of the plasmid pMP190 by repeated subculturing in the absence of the selective antibiotic chloramphenicol followed by screening for lack of growth on chloramphenicol-containing plates generating NLM2239.

**Cloning the *rdoA* gene.** The coding region of the *rdoA* gene was cloned into the *Sac*II site of pACYC184 by isolation of a *Sac*II *rdoA*-containing fragment from pMEG1. This construct (pLMN20) includes the native *rdoA* promoter region and the first 60 bases of the *dsbA* coding region downstream of *rdoA* but does not encode functional DsbA.

**RESULTS**

**The *rdoA* promoter activity is growth phase dependent, and its activity changes in response to the lack of DsbA, minimal medium, and acid shock conditions.** A 236-bp *rdoA* putative promoter region, immediately upstream of the ribosome bind-

ing site of *rdoA*, was fused in front of the reporter *lacZ* gene in the low-copy-number vector pMP190 to form the transcriptional fusion plasmid pPrdoA. The behavior of this putative promoter region in rich medium was examined by measuring  $\beta$ -galactosidase activity for wild-type serovar Typhimurium SL1344 and a *dsbA* null background strain (Fig. 1). In rich medium, the putative *rdoA* promoter region was functional and exhibited growth-phase-dependent expression, with maximum activity occurring in stationary phase. Initially, after 1:100 subculturing from overnight culture, *rdoA* promoter activity was quite high (approximately 600 Miller units) and declined rapidly (Fig. 1A). Promoter activity then increased to a maximum of 900 Miller units. After 20 h of growth, activity remained elevated and comparable to earlier stationary phase expression levels. An increase in *rdoA* transcript levels in stationary phase was also detected using RT-PCR (data not shown). An elevated expression of the *rdoA* promoter (approximately 200 Miller units) was observed in a *dsbA* null background at every stage of growth (Fig. 1A), indicating that a lack of disulfide bond-forming ability (provided by DsbA) in the periplasm has a positive feedback effect on *rdoA* transcription.

The effect of minimal medium and acid shock conditions on *rdoA* expression (situations that serovar Typhimurium is likely to encounter in the environment of the host [57]) was measured in wild-type SL1344 cells containing pPrdoA that were grown in minimal E glucose media (pH 7.6 and pH 4.6) (Fig. 1B). Growth-phase-dependent expression of the *rdoA* promoter was again displayed in minimal medium; however, in comparison to rich medium conditions, activity was elevated (by 300 to 400 Miller units) at all stages of growth. The *rdoA* promoter activity fell sharply in stationary phase SL1344 cells grown in acidic-pH media. Plating of cells to assess viability showed no significant difference in viable cell counts from 9 to 14 h (data not shown). This result indicated that the *rdoA* promoter is turned off in stationary phase under acidic growth conditions.

**The *rdoA* and *dsbA* promoters are induced at different points in the growth phase.** It has been previously shown that a *dsbA* transcriptional fusion (pMEG2) is growth phase dependent

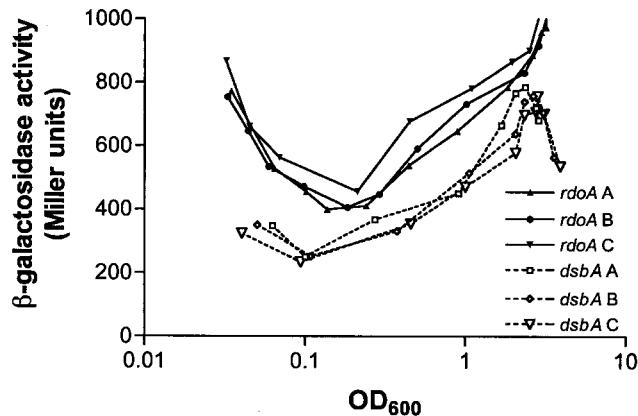


FIG. 2. A comparison of *rdoA::lacZ* (pPrdoA) and *dsbA::lacZ* (pMEG2) expression in the wild-type serovar Typhimurium SL1344 strain throughout the growth curve in rich medium. The  $\beta$ -galactosidase activities of pPrdoA (*rdoA*) and pMEG2 (*dsbA*) from three separate assays are plotted (A, B, and C). Each point in a curve represents the average activity of two samples.

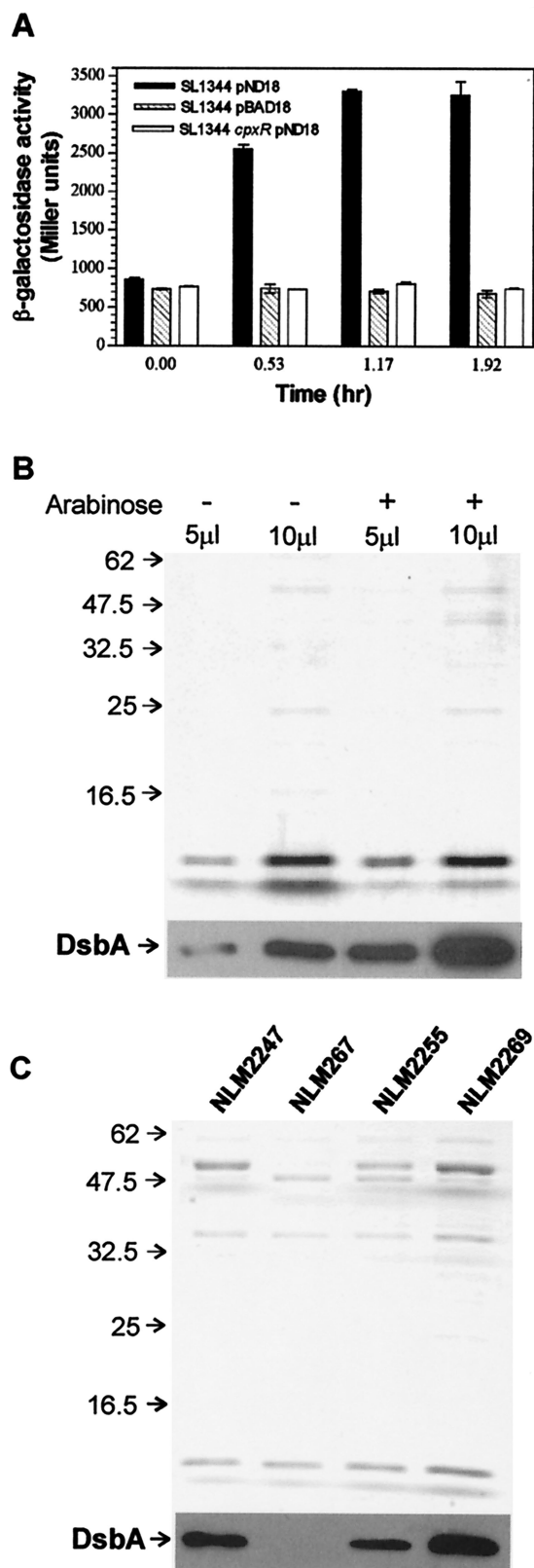


FIG. 3. Effects of inducing NlpE on the *rdoA* promoter and DsbA protein levels. (A) A comparison of promoter activity of *rdoA* with or without induction of the Cpx response via expression of NlpE by comparing *rdoA::lacZ* (pPrdoA) in cells containing an arabinose-inducible *nlpE* (pND18) or control (pBAD18) vector. The *cpxR::* $\Omega$

with maximal expression in stationary phase (19). The activity of the *rdoA* promoter fusion was compared to that of the *dsbA::lacZ* promoter fusion vector to identify similarities and variations in the expression patterns of these two growth-phase-dependent promoters. The magnitude of *rdoA* promoter expression was higher than that of the *dsbA* promoter at all stages of growth (Fig. 2). For both promoters,  $\beta$ -galactosidase activity decreases initially, indicating that  $\beta$ -galactosidase turnover exceeds synthesis. At an  $OD_{600}$  of 0.1, the *dsbA* promoter drives a net increase in activity while the *rdoA* promoter activity does not surpass the  $\beta$ -galactosidase turnover rate until the  $OD_{600} = 0.25$ . The  $\beta$ -galactosidase levels then increase at a slightly higher rate with the *rdoA* promoter than the *dsbA* promoter until stationary phase, where the *dsbA* promoter-driven activity falls off somewhat. This variation in terms of the growth-phase-dependent activity of these promoters suggests that different regulatory mechanisms influence the transcription of the *rdoA* and *dsbA* promoters.

*E. coli* NlpE overexpression stimulates serovar Typhimurium *rdoA* promoter activity. The sequence of the serovar Typhimurium *rdoA* ORF and that of the corresponding *E. coli* *yihE* ORF are very similar (78% nucleotide sequence identity, and the deduced amino acid sequences are 86% identical for both ORFs). Comparing the promoter insert region of pPrdoA to the same region in *E. coli* revealed high sequence identity, indicating that the most likely promoter in the insert contains a putative CpxR-P-responsive promoter box as characterized for *yihE* (51). Overexpression of *E. coli* outer membrane lipoprotein, NlpE, has been shown to stimulate the homologous *yihE* promoter fivefold by activating the Cpx pathway (12, 51, 55). To ascertain if the *rdoA* promoter is also inducible by the Cpx pathway in wild-type serovar Typhimurium, SL1344 cells containing pPrdoA reporter plasmid were transformed with an inducible *E. coli* NlpE overproducing plasmid (pND18) or control vector (pBAD18).  $\beta$ -galactosidase assay results indicated that *rdoA* promoter activity increased approximately threefold over that of the control vector after induction of NlpE expression (Fig. 3A). This correlated with increased levels of DsbA protein when NlpE was overexpressed (Fig. 3B) and did not occur when *rdoA* promoter activity was measured

strain is included to demonstrate that the effect on the *rdoA* promoter is mediated by the Cpx pathway. Cells were grown to an  $OD_{600}$  of 0.3 in the presence of 0.2% glucose. The cultures were then split, and 0.4% arabinose was added to one. Cultures were assayed for  $\beta$ -galactosidase activity at the time of adding arabinose ( $t = 0$ ) and several times later. (B) Upper panel, protein gel of periplasmic extracts from cells grown under NlpE-uninduced (-) and induced (+) conditions at two loading concentrations (5 and 10  $\mu$ l) stained with Coomassie blue to indicate total protein levels (DsbA is not visible on this gel); lower panel, Western immunoblot developed with anti-DsbA antiserum. DsbA is more abundant when NlpE is overexpressed. (C) Upper panel, protein gel of periplasmic extracts from the wild-type strain (NLM2247), *dsbA* null strain (NLM267), *rdoA* null strain (NLM2255), and *rdoA* null strain containing a copy of *rdoA* in *trans* (NLM2269) grown under NlpE-inducing conditions (all strains contain pND18 and were grown in the presence of arabinose) stained with Coomassie blue to indicate total protein levels (DsbA is not visible); lower panel, Western immunoblot developed with anti-DsbA antiserum. Inducing NlpE does not increase the DsbA levels to that of the wild type (NLM2247) in the *rdoA* null (NLM2255) strain. The wild-type phenotype is regained when *rdoA* is provided in *trans* (NLM2269).

in a *cpxR* null background (Fig. 3A). These findings are similar to the *yihE* promoter expression pattern, as measured by a nuclease protection assay, and increased DsbA protein levels, measured by pulse-chase immunoprecipitation, reported by Pogliano et al. (51) during the activation of the Cpx pathway in *E. coli*. Henceforth, the *E. coli* NlpE-overproducing plasmid, pND18, was utilized as a tool to potently stimulate serovar Typhimurium *rdoA* promoter expression.

***E. coli* NlpE overexpression induces serovar Typhimurium *rdoA* and *dsbA* transcript levels but does not significantly alter the levels of *rdoA-dsbA* cotranscript.** Reports from studies with *E. coli* have established that the *yihE-dsbA* cotranscript is necessary for *dsbA* expression (7, 51). We evaluated the DsbA protein in a *rdoA* null background and found that DsbA levels decreased under conditions of NlpE overexpression compared to the wild type and that normal DsbA levels could be restored by providing a copy of *rdoA* in *trans* (Fig. 3C). DsbA was also quantitated from 2D gels from at least three separately prepared samples for each growth condition and found to be twofold reduced in the *rdoA* null strain compared with the wild type under NlpE-induced conditions. These protein expression results suggested that the contribution of the *rdoA* transcript to *dsbA* expression needed to be clarified, and this question was evaluated with serovar Typhimurium and with *E. coli*.

To identify the transcripts originating from the serovar Typhimurium *rdoA-dsbA* region and clarify their possible effect on *dsbA* regulation, Northern hybridization using serovar Typhimurium *dsbA* and *rdoA* internal probes was employed on total RNA harvested from wild-type SL1344 strains containing either the NlpE-overexpressing plasmid (pND18) or control vector (pBAD18) (Fig. 4B). Northern blot analysis of membranes probed with a *dsbA*-specific fragment detected two distinct *dsbA* transcripts in wild-type serovar Typhimurium (of approximately 0.8 and 0.7 kb), with a 2.5-fold increase in the level of the smaller transcript under NlpE-overproducing conditions compared to the level for "induced" cells containing the control vector. The two *dsbA*-encoding transcripts described here and by Goecke et al. (19) have been mapped to two distinct *dsbA* promoters located in the 3' end of the RdoA coding region.

A putative *rdoA-dsbA* cotranscript (size, approximately 1.8 kb) was rarely clearly identified using a serovar Typhimurium *dsbA* probe (Fig. 4B) despite numerous attempts. This faint 1.8-kb signal matched the expected length of a cotranscript and could also be detected using an *rdoA* probe and long exposure times (data not shown). Additional transcripts detected of approximately 1.38 kb are too small to encode functional cotranscripts. The most prominent 1-kb transcript detected with the *rdoA* probe matches the expected length of *rdoA* mRNA. Densitometric quantitation revealed an approximately 1.5-fold increase of the 1.0-kb *rdoA* signal from wild-type cells overexpressing NlpE versus control vector. This result agrees with the *rdoA* transcriptional fusion reporter assays, in which an increase in *rdoA* promoter expression were observed under the same conditions. In a *dsbA* null strain overexpressing NlpE, considerably higher levels of the 1-kb transcript (an additional threefold increase) was observed than was found with wild-type cells overexpressing NlpE (data not shown).

To discern transcriptional similarities or differences that may exist in the same region in *E. coli*, Northern blot analysis

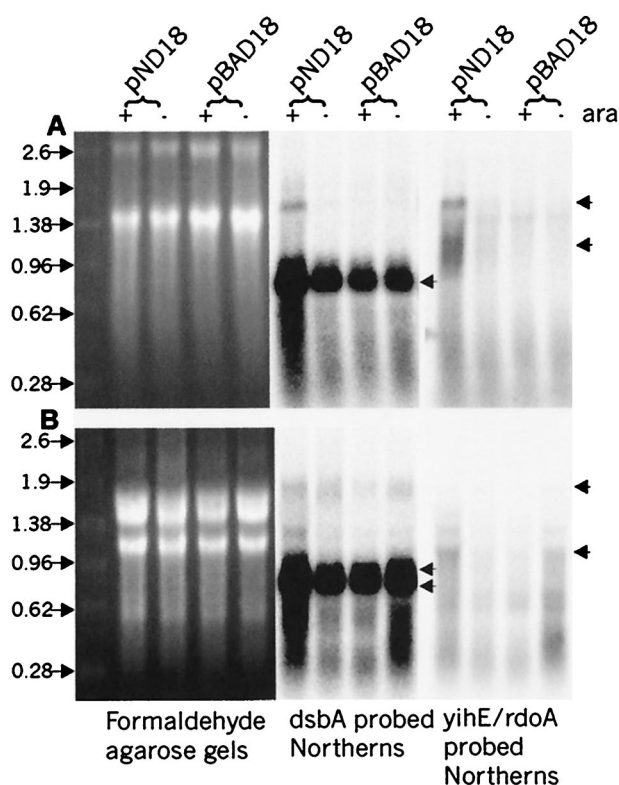


FIG. 4. Transcript analysis of *yihE*, *rdoA*, and *dsbA* in serovar Typhimurium and *E. coli*. RNA formaldehyde agarose gels show total RNA amounts loaded in each lane. *E. coli* (A) and *S. typhimurium* (B) cells containing pBAD18 (control) vector or pND18 (NlpE-overexpressing plasmid) induced (+) with 0.4% arabinose or uninduced/repressed (-) with 0.2% glucose are analyzed. The left panels show the agarose gels, while the right panels show the corresponding Northern blots of a single membrane probed with either a *dsbA* probe or a *yihE/rdoA* probe. Cotranscript (approximately 1.8 kb) was detected using an *E. coli dsbA* probe or a *yihE* probe (upper arrow, panel A) and a serovar Typhimurium *dsbA* probe (upper arrow, panel B). The most abundant transcript in both organisms is *dsbA*, with one transcript of approximately 0.8 kb in *E. coli* and two transcripts of approximately 0.7 and 0.8 in serovar Typhimurium (double arrows, panel B). The *yihE* or *rdoA* transcripts of approximately 1.0 kb are indicated with arrows for both organisms. In serovar Typhimurium, fragmentation of the 23S rRNA occurs, which results in a different rRNA banding pattern than is seen with *E. coli* rRNA (27).

was also performed with *E. coli* MG1655 strains that carried NlpE-overproducing or control plasmid (Fig. 4A). A striking difference between the two organisms was clearly demonstrated in which a *yihE-dsbA* cotranscript (of approximately 1.8 kb) was identified by using both *E. coli dsbA* and *yihE* probes (Fig. 4A). In contrast to serovar Typhimurium, a distinct increase of this transcript was distinguished between pND18-induced RNA samples and uninduced RNA samples, although the signal still appeared to be faint (approximately a 3-day exposure to phosphorimager screen) compared to the more abundant 0.7-kb transcript (an approximately 4-h exposure to phosphorimager screen). The *E. coli* RNA membrane probed for *yihE* displayed two separate bands of approximately 1.8 and 1 kb (Fig. 4A). The signals matched predicted sizes of cotranscript and monocistronic *yihE* mRNA. Distinct increases of both bands in cells overexpressing NlpE versus the control

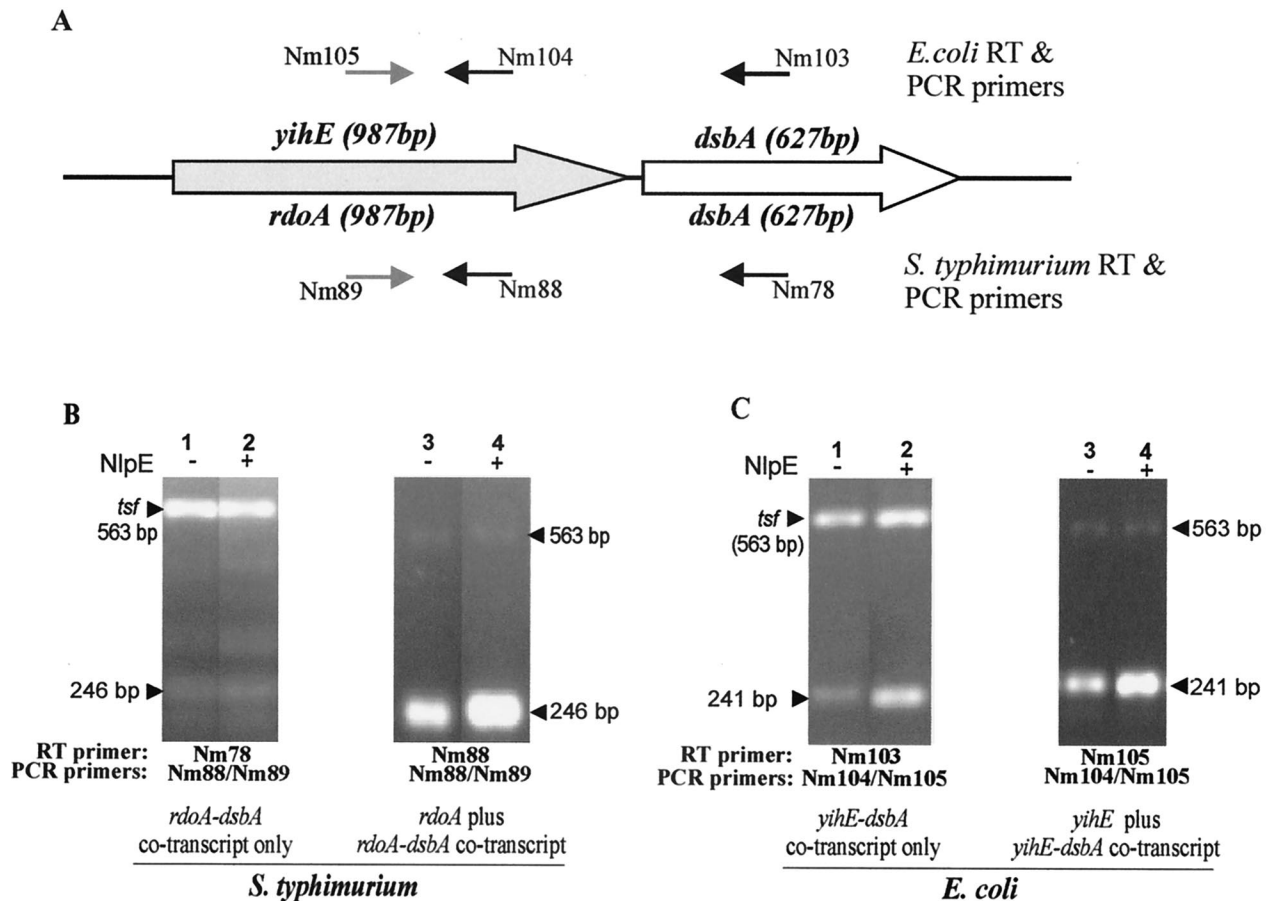


FIG. 5. Transcript analysis of *yihE*, *rdoA*, *rdoA-dsbA*, and *yihE-dsbA* by RT-PCR. The primers utilized in the RT-PCR are illustrated in panel A, which shows the organization of the *rdoA* (*yihE*)-*dsbA* region in serovar Typhimurium and *E. coli*. Serovar Typhimurium primer Nm78 binds 400 bp downstream of the *dsbA* start codon, while primers Nm88 and Nm89 bind 855 and 612 bp downstream of the *rdoA* start codon. *E. coli* primer Nm103 binds 404 bp downstream of the *dsbA* start codon, while primers Nm105 and Nm104 bind 855 and 614 bp downstream of the *yihE* start codon. In panels B and C the lower amplicons are the target amplicons, while the upper bands are the *tsf* (encoding the elongation factor EF-Tsf) internal control amplicon used as a standard in the qualitative assessment of pND18 (NlpE)-induced (+) or pBAD18 (control vector)-induced (-) samples of serovar Typhimurium (B) and *E. coli* (C) RNA preparations. The size of the EF-*tsf* amplicon is 563 bp, while the serovar Typhimurium target amplicon for the *rdoA-dsbA*- and *rdoA*-specific transcript is 246 bp. The *E. coli* target amplicon for the *yihE-dsbA*- or *yihE*-specific transcript is 241 bp.

vector were also observed. Furthermore, strains containing the control vector and the glucose-repressed NlpE plasmid did not reveal detectable levels of either of these transcript signals.

Since the *rdoA* transcript was not easily detected using Northern analysis, RT-PCR was also performed to compare *rdoA* monocistronic and *rdoA-dsbA* cotranscript amounts in pND18 (*nlpE*)- and pBAD18 (control vector)-overexpressing conditions (see Fig. 5A for primers and Materials and Methods for strategy). Figure 5B shows RT-PCR amplicons of *rdoA-dsbA* cotranscript or cotranscript plus *rdoA* monocistronic transcript from SL1344 cells overexpressing NlpE and controls. The RT-PCR products were compared to measure differential cotranscript or *rdoA* monocistronic transcript expression, based upon an internal control transcript, *tsf*, encoding the elongation factor EF-Tsf (26). The expression of the internal control is constant in serovar Typhimurium at the growth phase studied and appears in similar amounts in SL1344 cells overexpressing NlpE or pBAD18 control vector cells for the specific RT-PCR amplicon examined. Figure 5B shows that

there is very little difference in the amount of cotranscript in serovar Typhimurium cells under NlpE-overproducing conditions compared to the control vector. However, judged against the internal control, a definite increase in the quantity of the *rdoA* monocistronic mRNA plus cotranscript was observed in NlpE-overexpressing cells versus results for control vector-containing cells. Since we know from the RT-PCR analysis of the cotranscript alone that its expression level is comparatively equal between strains overexpressing NlpE and the control vector, this must mean there is a significant increase in the quantity of the *rdoA* monocistronic transcript in NlpE-overexpressing cells. This result confirms Northern blotting data on the increase in the amounts of *rdoA* monocistronic transcripts under the same conditions.

Comparative RT-PCR analysis was also extended to *E. coli* MG1655 strains that carried NlpE-overproducing or control plasmid. Figure 5C illustrates an increase in the *yihE-dsbA* cotranscript in strains overproducing NlpE versus results with the control vector. This result agrees with Northern blot data

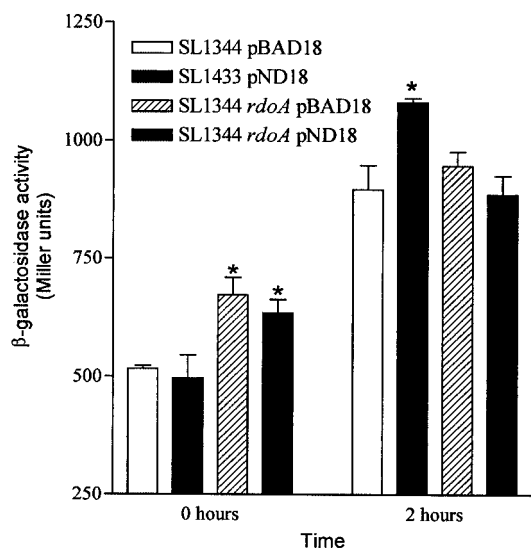


FIG. 6. Overexpression of NlpE increases *dsbA* promoter activity. A comparison of *dsbA::lacZ* expression using strains containing pMEG2 and either the vector control (pBAD18) or the NlpE expressing vector (pND18) at  $t = 0$  and 2 h after induction of NlpE expression with 0.2% arabinose. Cells were grown to an  $OD_{600}$  of 0.6 prior to induction. The asterisks indicate that the mean is statistically significantly different from the mean with SL1344 pBAD18 at either time point ( $P < 0.05$ ).

using *dsbA* and *yihE* probes. Moreover, an increase in the amount of cotranscript plus *yihE* monocistronic RT-PCR amplicon was detected in the *E. coli* strain overproducing NlpE versus results with the control vector. This is due only in part to an increase in cotranscript and also involves an increase in the level of *yihE* monocistronic mRNA.

**Phenotypes associated with RdoA.** Although the data presented here point to different transcripts contributing to *rdoA* and *dsbA* expression levels, still nothing is known about the function of RdoA. It was thought that RdoA could play a role as an intermediate in the signaling pathways initiated by activating the Cpx system. The effect of *rdoA* expression on *dsbA* transcription was examined (Fig. 6), and it was determined that an increase in *dsbA* promoter activity occurred in response to NlpE expression. Although this change was modest (20% above uninduced level), this increase was not seen in the *rdoA* null strain. There was also a notable difference between wild-type and *rdoA* null strains in mid-log-phase cells without NlpE expression (Fig. 6,  $t = 0$ ), indicating that the absence of *rdoA* increases *dsbA* transcription. This difference was not seen in stationary phase cells. It may be that lack of *rdoA* indirectly triggers *dsbA* transcription during exponential phase growth through other, as yet undefined, pathways.

To further define the role of RdoA in serovar Typhimurium, 2D gel electrophoresis analysis was carried out in an effort to identify proteins affected by the presence or absence of RdoA. If RdoA is part of a signaling pathway, it would be likely to have an effect on more than one target protein. The 2D gel analysis was carried out by comparing the protein profiles of serovar Typhimurium SL1344 pBAD18, SL1344 pND18, SL1344 *rdoA* pBAD18, and SL1344 *rdoA* pND18 all grown in the presence of arabinose. DsbA levels were observed to decrease by twofold in the *rdoA* null strain in an RdoA/NlpE

overexpression-dependent manner via 2D gel electrophoresis (data not shown). Many other proteins were noted that also changed in abundance under NlpE overexpression conditions (N. Martin, unpublished observations). One of the more intriguing changes seen was an NlpE/RdoA-dependent shift in flagellin production. Figure 7 shows a portion of the 2D gels containing four different proteins that were identified by peptide mass fingerprinting via mass spectrometry, FliC, FljB, GroEL, and Trigger factor. In control cells (Fig. 7A), the flagellar protein type predominantly expressed is FliC. There is also a predominant spot identified as GroEL, with a much less abundant spot corresponding to Trigger factor. The overexpression of NlpE results in a switch to the predominant expression of FljB and also results in enhanced production of Trigger factor (Fig. 7B). When NlpE is overexpressed in the *rdoA* null strain, flagellar protein expression switches back to predominantly FliC and, in this case, Trigger factor levels stay approximately the same as when NlpE is overexpressed in wild-type cells. The protein profile (ratio of FljB to FliC) in the *rdoA* null strain with pBAD18 is very similar to that for the wild type without NlpE expression (Fig. 7D). The results show that the absence of RdoA alters the response to NlpE overproduction, presumably via the Cpx pathway, suggesting a role for RdoA in the transduction of Cpx-related signals to a subset of the Cpx-responsive targets.

## DISCUSSION

This work sought to clarify the nature of transcripts originating from the *rdoA* promoter, namely the *rdoA* monocistronic and *rdoA-dsbA* cotranscripts, in the regulation of *dsbA* expression and also to examine the role of RdoA in serovar Typhimurium. The results clearly establish that *rdoA* promoter activity is growth phase dependent. This expression pattern occurred in wild-type and *dsbA* null background strains as well as in wild-type cells grown in rich LB media and minimal media at neutral pH. The growth-phase-dependent expression of the *rdoA* promoter was found to be independent of RpoS (data not shown). RpoS or  $\sigma^s$  is the stationary phase sigma factor that regulates the expression of a number of genes that are involved in the adaptive changes required for stress management during stationary phase (36, 39). Other promoters in *E. coli*, such as the P1 promoter of *osmC* gene (8), the promoter of the *glgCAP* operon (25), and the *trxA* thioredoxin gene promoter (38), have been reported to be growth phase regulated, also independently of RpoS.

Expression of the *rdoA* promoter is sensitive to the media conditions. For instance, minimal medium growth conditions increased promoter activity (approximately 300 to 400 Miller units) throughout growth. The *dsbA* promoter is also more highly active in minimal medium, (19) although the reason is not clear. In the case of acidic minimal medium, the *rdoA* promoter was turned off during stationary phase. While the factors responsible for such a decrease are unknown, the results point out that *rdoA* expression is not essential for viability of serovar Typhimurium during stationary phase under acid-stressed conditions. Studies of gene expression under low-pH conditions have focused mainly on inducible gene expression, although clearly some uncharacterized genes are turned off (15). Two major low-pH-inducible systems or acid tolerance



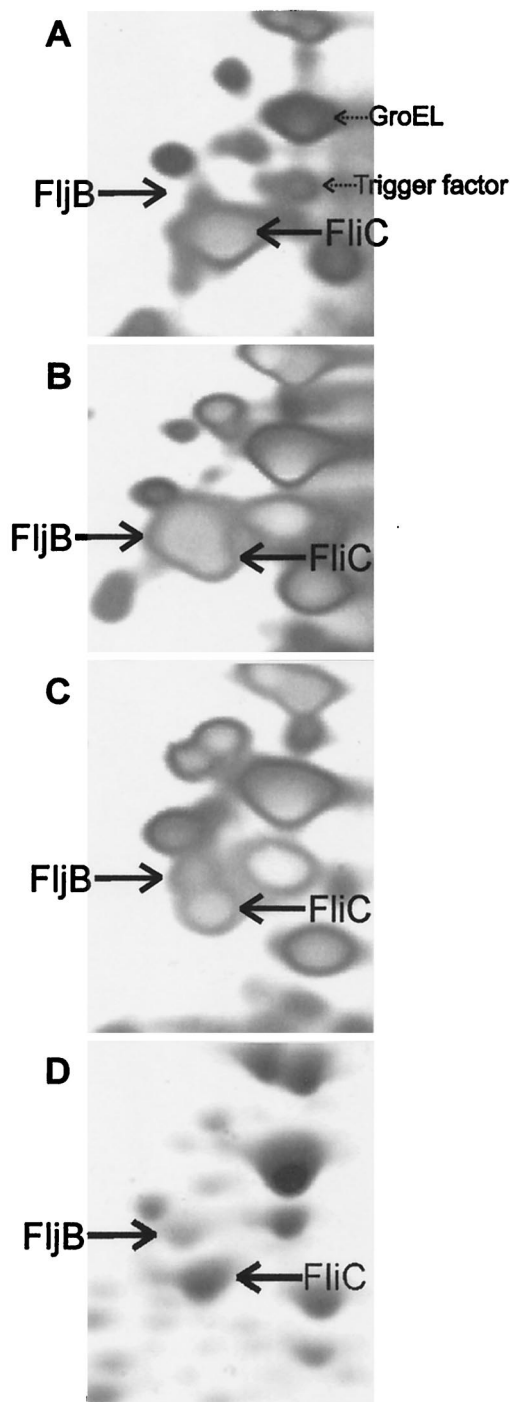


FIG. 7. Inducing the Cpx pathway alters flagellin expression (phase variation) in a Cpx/RdoA-dependent fashion. 2D gel analysis of the flagellins expressed in serovar Typhimurium under NlpE-inducing and noninducing conditions shows that the primary flagellin produced in wild-type cells containing pBAD18 is FljC (A). In wild-type cells containing pND18 (expressing NlpE), the flagellin switches to primarily FljB (B). In the absence of *rdoA*, cells containing pND18 revert to producing much less FljB (C) while cells containing pBAD18 also express predominately FljC (D). All cultures were grown in the presence of 0.2% arabinose for 2 h after addition at an  $OD_{600}$  of 0.6. Two additional protein spots, GroEL and Trigger factor, identified by mass spectrometry, are also indicated for comparison. Because small variations in total protein loading and staining can affect the overall spot intensity, it is important to compare the ratios of FljB to FljC in each gel rather than spot intensity.

responses (ATR) have been described for serovar Typhimurium, based on the growth phase at which each becomes induced (14). The log phase ATR system, induced when exponentially growing cells are adapted to pH 5.8 and the pH is lowered further to pH 4.5, results in the induction of 50 acid shock proteins, a subset of them members of the RpoS, PhoPQ, or *fur* regulons (5, 14). The second, a distinct RpoS- and *fur*-independent, stationary-phase ATR, induces more than 10 stationary phase acid shock proteins (2, 14). Few genes have been identified that are repressed by low-pH conditions, two examples being serovar Typhimurium *OmpF*, a major outer membrane porin (15), and *Shigella sonnei* *VirF*, a transcriptional activator of the IpaBCD host cell invasion apparatus (43).

Although the *rdoA* (this work) and *dsbA* (19) promoters both show strong growth phase dependence, different properties were revealed in terms of growth-stage-dependent increases in promoter activity. In the case of the *dsbA* promoter, the global regulator H-NS, a "histone-like" protein, has been demonstrated to modulate *dsbA* promoter expression during mid-exponential phase and plays a role in the repression of *dsbA* transcription in early-exponential-phase growth (C. V. Gallant, T. V. Ponnampalam, H. Spencer, and N. L. Martin, unpublished results). Although the exact regulatory mechanism behind the induction in *rdoA* expression remains to be elucidated, the continued high level of expression of the *rdoA* promoter during stationary phase suggests that the *rdoA* gene product may be important for stationary phase processes for serovar Typhimurium.

For a serovar Typhimurium *dsbA* null strain, an elevated response of the *rdoA* promoter (an approximately 200-Miller-unit increase) is observed compared with results for a wild-type strain at every stage of growth. These results indicate that a lack of disulfide bond-forming ability (provided by DsbA) in the periplasm has a positive feedback effect on *rdoA* transcription for serovar Typhimurium. This implies that a signal transduction mechanism exists linking the presence of DsbA (in the periplasm) to *rdoA* transcription (in the cytoplasm). Such a system is most likely the Cpx pathway, responding to a higher incidence of misfolded proteins in the periplasm as described for *E. coli* (11, 51). Although the *cpxRA* genes have been sequenced for serovar Typhimurium, this two-component signal transduction pathway has not been characterized for this organism. However, support for the Cpx pathway being involved in stimulating *rdoA* transcription comes from the following: (i) the information available for *E. coli* on the Cpx up-regulation of the homologous *yihE* gene (12, 51); (ii) the presence of a CpxR-P recognition sequence in the promoter of *rdoA* and the increase of *rdoA* promoter activity under conditions of *E. coli* NlpE overproduction, which is not seen in a *cpxR* null background; and (iii) the increase in the levels of the 1-kb *rdoA* monocistronic transcript for both wild-type and *dsbA* null strains with NlpE overexpression.

The increase in serovar Typhimurium *rdoA* promoter activity under NlpE-overproducing conditions, coupled to the parallel increases of the 1-kb *rdoA* transcript and the 0.7-kb *dsbA* transcript under similar conditions, suggests that Cpx-mediated signals affect *dsbA* expression, either directly at the *dsbA* promoter or indirectly through an *rdoA*-related mechanism. The presence of a conserved CpxR binding site in the *rdoA*

promoter suggests that the Cpx response is mediated through *rdoA*; however, Northern blot analysis and RT-PCR of NlpE induced and uninduced serovar Typhimurium RNA preparations showed very minor variations in *rdoA-dsbA* cotranscript production, far less than expected if the cotranscript serves as a source for functional *rdoA* and *dsbA* transcripts. Homologues of *yihE/rdoA* identified in *Pseudomonas* (*P. stutzeri*, 45% protein identity; *P. aeruginosa*, 43% protein identity; *P. fluorescens*, 44% protein identity) and *Shewanella putrefaciens* (52% protein identity), as well as the *Bacillus subtilis* YerI protein (25% protein identity), are not found immediately upstream of these organisms' *dsbA* genes. In these bacteria, *yihE* and *dsbA* may be coregulated via a regulon, but in each of these cases the contribution of a *yihE-dsbA* cotranscript to DsbA expression levels is impossible. In serovar Typhimurium, if a cotranscript is synthesized, its processing must be extremely rapid and complete, such that no detectable difference in the levels of the bicistronic mRNA is observed between NlpE-overexpressed RNA preparations and uninduced RNA preparations, even in the sensitive RT-PCR based assay system used here. Evidence from other studies suggests that such rapid processing is unlikely. For instance, the well-characterized RNase E processing of the *E. coli* Pap operon, *papBA* transcript, clearly shows differential amounts of the polycistronic *papBA* mRNA under different promoter-inducing conditions (45, 46). In another example, substitution of the native promoter of the *E. coli* *rph-pyrE* operon with an inducible promoter and analysis of the transcripts synthesized under induced and uninduced conditions showed obvious variations in the levels of the dicistronic *rph-pyrE* mRNA (1). Rapid processing of an *rdoA-dsbA* cotranscript could be up-regulated by the Cpx pathway as an alternative explanation for the lack of significant difference in the levels of the bicistronic mRNA between NlpE-overexpressed and uninduced RNA preparations. However, such a mechanism that links the activation of the Cpx pathway and increased RNase activity or mRNA turnover has not been described to date in the literature. Preliminary RNase protection assays have failed to detect any additional evidence for *rdoA-dsbA* cotranscription products (data not shown). Transcriptional regulation of this *rdoA/dsbA* region is therefore unique in serovar Typhimurium, since Pogliano et al. (51) and Danese and Silhavy (11) established that the cotranscript induced via the Cpx pathway in *E. coli* plays a significant role in the regulation of *dsbA* expression. In our hands, a clear increase in the *yihE-dsbA* cotranscript and the *yihE*-specific transcript was detected by both Northern hybridization and RT-PCR, confirming the view that the cotranscript contributes to *dsbA* expression in *E. coli*. The presence of extremely limited amounts of larger-than-1-kb *rdoA*-containing transcripts in serovar Typhimurium therefore seems more likely to be due to leaky transcriptional termination between the *rdoA* and *dsbA* coding regions.

Although it is clear that *rdoA* has an effect on *dsbA* levels, the precise mechanism of *rdoA* influence remains to be elucidated for serovar Typhimurium. One potential posttranscriptional mechanism would involve *rdoA* mediating an increase in stability of the 0.7-kb *dsbA* transcript, such that this transcript accumulates under Cpx-induced conditions. An analogous mode of regulation occurs in the posttranscriptional control of polynucleotide phosphorylase (*pnp*) during cold acclimation

for *E. coli* (68). The increase of *pnp* transcript during cold shock is mainly due to the increased stability of the *pnp* mRNA brought about by a protein-RNA interaction and less, if at all, to the activation of the two upstream promoters (68).

Alternatively, RdoA could be a transcriptional regulator involved in relaying or modulating Cpx-initiated signals. There is an NlpE/RdoA-dependent effect at the level of transcription of the *dsbA* promoter; however, it is still unknown if RdoA is directly mediating this effect or if RdoA acts via another component. By using 2D gel electrophoresis, it was also established that RdoA is involved in flagellar phase variation under NlpE-overproducing conditions. Flagellar phase variation has been under investigation for a number of years, but the environmental signals initiating phase variation are poorly understood. It is known that phase variation is mediated by the reversible inversion of a 996-bp portion of the chromosome that either positions a promoter allowing expression of FljB along with a repressor of FliC (FljA) or displaces this promoter so that FljB and FljA are not expressed and the FliC gene, which is at a separate chromosomal location, is expressed (54, 69, 70). This DNA inversion is mediated by the Hin recombinase (44). At another level of transcriptional control, the abundance of functional FliA, a flagellar gene-specific sigma factor, influences flagellar filament gene expression (32), but since both the *fliC* and *fljB* promoters are responsive to FliA, it is not likely to be involved with Cpx-mediated phase variation. The nature of the actual signaling pathways influencing phase variation will be the subject of further study.

It is also interesting to speculate on the reason that the Cpx pathway is involved in phase variation. Otto and Silhavy (47) recently presented convincing evidence that the *E. coli* Cpx pathway and NlpE are involved in sensing and responding to bacterial cell adhesion to environmental surfaces. They hypothesize that NlpE may be involved in relaying an adhesion-specific signal to the Cpx system, as they showed that NlpE is required for normal adhesion to occur and NlpE is unlikely to be able to act directly as an adhesin. A change in flagellar type may also be important in an initial interaction with a surface or some additional virulence aspect in serovar Typhimurium. Using "phase-locked" mutants of serovar Typhimurium, it has been demonstrated that FliC-expressing strains have a selective advantage over FljB-expressing strains in a murine model for typhoid fever, although no difference in the ability to invade epithelial cells or in the induction of enteropathogenesis was found between FliC- and FljB-expressing strains (28).

Although the *E. coli yihE-dsbA* and serovar Typhimurium *rdoA-dsbA* regions share more than 79% homology at the DNA level and both organisms are closely related, they differ significantly in terms of their pathogenicity and the types of environment encountered. Therefore, differences in the transcriptional regulation of *dsbA* and *rdoA* as a response to the need for these proteins, important for growth and survival in specific conditions, is not unexpected. One poorly understood issue is the mechanism of *rdoA* influence over *dsbA* expression and the ultimate role of RdoA in the bacterial cell. A DNA array study of *yihE* and *dsbA* null strains of *Shigella flexneri* showed significant alterations in transcription levels of more than 100 different genes involved in diverse processes such as glycolysis, central intermediary metabolism, structural cell proteins, and transport proteins (37), lending little clarity to the

role of *yihE*. Curiously, in the same study *dsbA* mutants caused a slight decrease in *cpxA/R* transcription, contrary to previous studies showing induction of the Cpx pathway in the absence of disulfide oxidoreductase activity (51). The work described here has identified two proteins, DsbA and FljB, whose expression is at least partially dependent upon RdoA. Further studies aimed at elucidating the function of RdoA and its relationship to DsbA, flagellar phase variation, and the Cpx pathway for serovar Typhimurium are currently under way.

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