The atypical two-subunit $\sigma$ factor from *Bacillus subtilis* is regulated by an integral membrane protein and acid stress

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Extracytoplasmic function (ECF) $\sigma$ factors constitute a major component of the physicochemical sensory apparatus in bacteria. Most ECF $\sigma$ factors are co-expressed with a negative regulator called an anti-$\sigma$ factor that binds to its cognate $\sigma$ factor and sequesters it from productive association with core RNA polymerase (RNAP). Anti-$\sigma$ factors constitute an important element of signal transduction pathways that mediate an appropriate transcriptional response to changing environmental conditions. The *Bacillus subtilis* genome encodes seven canonical ECF $\sigma$ factors and six of these are co-expressed with experimentally verified anti-$\sigma$ factors. *B. subtilis* also expresses an ECF-like atypical two-subunit $\sigma$ factor composed of subunits SigO and RsoA that becomes active after exposure to certain cell-wall-acting antibiotics and to growth under acidic conditions. This work describes the identification and preliminary characterization of a protein (RsiO, formerly YvrL) that constitutes the anti-$\sigma$ factor cognate to SigO–RsoA. Synthesis of RsiO represses SigO–RsoA-dependent transcription initiation by binding the N-terminus of SigO under neutral (pH 7) conditions. Reconstitution of the SigO–RsoA–RsiO regulatory system into a heterologous host reveals that the imposition of acid stress (pH 5.4) abolishes the ability of RsiO to repress SigO–RsoA-dependent transcription and this correlates with loss of RsiO binding affinity for SigO. A current model for RsiO function indicates that RsiO responds, either directly or indirectly, to increased extracytoplasmic hydrogen ion concentration and becomes inactivated. This results in the release of SigO into the cytoplasm, where it productively associates with RsoA and core RNAP to initiate transcription from target promoters in the cell.
of anti-σ factor activity can occur in one of several ways: a conformational change in the structure of the anti-σ factor, regulated intramembrane proteolysis (RIP) of the anti-σ factor, or phosphorylation and partner switching by an anti-anti-σ factor (Ellermeier & Losick, 2006; Francez-Charlot et al., 2009; Heinrich & Wiegert, 2009; Kang et al., 1999; Paget et al., 2001).

The Gram-positive bacterium B. subtilis possesses eight ECF-like σ factors (including SigO–RsoA). The regulation of B. subtilis ECF σ factor SigW (σW) by its cognate anti-σ factor has been investigated in detail. The σW stress response confers resistance to cell envelope perturbing agents including antibiotics and detergents (Butcher & Helmann, 2006; Ellermeier & Losick, 2006; Mascher et al., 2007), and is also activated after overproduction of membrane proteins (Zweers et al., 2009). σW is released into the cytoplasm when its anti-σ factor RsiW undergoes RIP through a cascade of proteolytic events catalyzed by PrsW, TSP, RasP and ClpXP or ClpEP (Heinrich et al., 2009; Ho & Ellermeier, 2012; Schöbel et al., 2004). It has been suggested that PrsW, the site-1 protease, is the sensor of the environmental stressor (Ellermeier & Losick, 2006), but further research into the mechanism suggests the involvement of a negative regulator of proteolysis in sensing the stress signal (Heinrich et al., 2009). More recently, the regulation of σV by its anti-σ factor RsiV has been investigated (Hastie et al., 2013, 2014). Like the σW stress response, the σV stress response chemically remodels cell envelope structure providing resistance to its inducer, lysozyme (Guariglia-Oropeza & Helmann, 2011; Ho et al., 2011). RsiV is a receptor for lysozyme, and when bound to lysozyme, the site-1 cleavage site on RsiV is exposed to the activity of the peptidase SipS (Hastie et al., 2014).

SigO–RsoA is an atypical two-subunit σ factor from B. subtilis (MacLellan et al., 2008, 2009a). Both proteins possess sequence similarity to the ECF σ70 family of proteins, interact with each other and with RNAP subunits, and are required for the initiation of transcription from a set of related promoters in the B. subtilis genome (MacLellan et al., 2009a, b). SigO–RsoA is required for the transcription of three operons, including the sigO–rsoA operon and the oxdC–rsoI operon (Fig. 1a). ECF σ factors regulate the expression of target genes in response to changing physicochemical conditions in the cellular environment (Helmann, 2002). In the case of SigO and RsoA, the two-subunit σ factor is expressed in the cell after exposure to certain cell-wall-acting antibiotics and acid stress conditions (Cao et al., 2002; Hackmann et al., 2009; MacLellan et al., 2009b). The SigO–RsoA regulon member gene rsiO (formerly known as yvrL) (see Fig. 1a, b) is predicted to encode a membrane spanning protein. It was previously shown that the deletion of rsiO increased basal activity of SigO–RsoA activity in B. subtilis under non-inducing conditions (MacLellan et al., 2008), and also resulted in the increased accumulation of another regulon member protein, OxdC (MacLellan et al., 2009b), suggesting that rsiO encoded a negative regulator of SigO–RsoA activity.

In the present study, we carried out preliminary characterization of RsiO function by reconstituting (and isolating) all three regulatory proteins (SigO–RsoA–RsiO) in a heterologous host. In this genetic background, we found that co-expression of RsiO inhibits SigO–RsoA transcription initiation activity at neutral pH by specifically interacting with the N-terminal half of SigO. Furthermore, the inhibitory activity of RsiO is lost under acidic growth conditions and this loss of function correlates with a loss in its ability to bind SigO. On this basis, we identified RsiO as the cognate anti-σ factor for the two-subunit σ factor SigO–RsoA. Loss of function of RsiO in a heterologous genetic background raises the interesting possibility that this protein may play an important role in sensing hydrogen ion concentration, in addition to being a respondent to low pH conditions.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this work are listed in Tables S1 and S2 (available in the online Supplementary Material). Cells were grown in LB broth (Fisher) or M9 minimal salts medium (Sigma-Aldrich) or these media solidified with 1.6% agar. Plasmids were maintained with 100 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹, 10 μg gentamicin ml⁻¹ or 25 μg chloramphenicol ml⁻¹. Bacteria were grown at 37 or 30 °C for bacterial two-hybrid (BACTH) assays or at 20 °C when protein expression was induced using 0.7 mM IPTG. Acid response assays were conducted using M9 minimal broth, no additional buffer (pH 6.97 ± 0.07) or M9 supplemented with 0.1 M MES poised at pH 5.40 ± 0.02.

**Promoter activity assays.** A 1% volume of an overnight culture was used to inoculate a volume of fresh medium, followed by growth for 2 h at 37 °C prior to induction of protein expression. Induction of protein expression from pET-DUET (Novagen) derivatives using 0.7 mM IPTG (final concentration) occurred over 16 h during growth at 20 °C with shaking. In most cases, the sigO–rsoA operon was cloned into MCS1 (multiple cloning site 1) of pET-DUET, while rsoI was cloned into MCS2. Cells were harvested at an optical density (OD₆₀₀) of 0.7–0.8. Promoter (PoxdB) activation was monitored using β-galactosidase activity assays when using PoxdB-lacZ (pSAD00) or fluorometry when using PoxdB-gfp (pSRM9) promoter fusion constructs. All readings were conducted on a Paradigm multimodal spectrophotometer (Molecular Devices).

**Immunoblot analysis.** To monitor the abundance of RsiO during promoter activity assays, cells were collected by centrifugation and disrupted via two freeze–thaw cycles. Lysis was promoted by the maintenance of pLysS in cells. Cell lysate was resuspended in 10 mM Tris/HCl (pH 8.0) and liberated nucleic acids were digested using micrococcal nuclease (NEB). Fractions enriched for cytoplasmic membrane was isolated by subjecting crude whole-cell lysate to high-speed centrifugation (15 870 g or Eppendorf fixed angle rotor FA-45-24-11) for 20 min. The resultant supernatant was then subjected to ultracentrifugation (100 000 g) for 2 h. Pellets recovered after ultracentrifugation were considered to be enriched for membrane constituents. Culture volume equivalents of crude (whole-cell) lysate or soluble protein and membrane-enriched samples were suspended in Laemmli buffer. A 25 μl aliquot of each sample was
separated using 14 % SDS-PAGE gels and electrophoresis at 120 V. Separated proteins were transferred to PVDF membrane and blocked with 3 % non-fat milk dissolved in TTBS [20 mM Tris/HCl (pH 7.5), 500 mM NaCl, 0.05 % Tween 20]. Membranes were probed for the presence of the FLAG epitope using anti-FLAG rabbit derived antibodies (Sigma-Aldrich), and visualized using goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Sigma-Aldrich).

**BACTH assays.** Protein–protein interactions were detected using an adenylate cyclase-based BACTH assay (Euromedex). In this system, test genes are fused in-frame to T25 or T18 cyaA gene fragments and proteins are, therefore, produced as fusion proteins. A unique feature of this system is that the pUT18-based plasmid has a high copy number and the pKT25-based plasmid has a low copy number (Karimova et al., 1998). Since the pUT18-based plasmid is derived from pUC19 it is likely to have a copy number of from 500 to 700 replications per cell (Lin-Chao et al., 1992). The pKT25-based plasmid is a derivative of pSU40 and carries a p15A origin; therefore, is likely to have a copy number of approximately 10 replications per cell (Bartolomé et al., 1991). We exploited the resultant gene dosage effects toward providing high or low expression of the cloned fusion genes. Recombinant plasmids were produced by cloning the sigO, rsoA and rsiO genes into plasmids pKT25, pKNT25, pUT18 or pUT18C (see Table S2). In some cases, SigO was bisected to produce N-terminal and C-terminal fusions to CyaA. The full-length SigO protein is 176 amino acids in length and the N-terminal fragment tested consisted of the first 93 amino acids, a moiety we have previously shown specifically interacts with RsoA in vivo and in vitro (MacLellan et al., 2009a). The C-terminal SigO fragment tested consisted of amino acids 94–176. Additional constructs containing rsiO or rsoA not translationally fused to adenylate cyclase domains were also generated to characterize the effects of introducing a third protein into the cell that might potentially compete with a test protein–protein interaction (Table S2). For BACTH vectors co-expressing SigO and RsoA, the native sigO–rsoA operon was inserted into the vector where only the upstream sigO gene product would become fused via its N-terminus to the C-terminus of CyaA fragments (either T18 or T25). In vectors where RsiO was co-expressed with either SigO or RsoA, a DNA fragment containing rsiO was fused to a lac promoter (derived from PET16b) and was inserted immediately downstream of the cyaA–sigO/rsoA locus using the KpnI/EcoR I restriction sites that occur in this part of the plasmids.

Test plasmid pairs were used to co-transform E. coli BTH101. An interaction between test fusion proteins produces β-galactosidase activity, which was monitored on plates supplemented with X-Gal or

![Fig. 1. Regulon of two-subunit σ (SigO–RsoA) from B. subtilis and predicted topology of its anti-σ factor (RsiO).](image-url)

(a) Genetic map depicting the protein encoding regions in the SigO–RsoA regulon and their associated promoters. The stem–loop structure indicates a transcription attenuator located between genes oxdC and rsiO (MacLellan et al. 2008). Vertical grey bars in the rsiO ORF indicate predicted membrane spanning regions. Former gene names are included in parentheses. (b) Predicted structure of anti-σ factor RsiO. Topology prediction generated using TM-Coffee (Chang et al. 2012) and rendered using the program Proter (Omasits et al. 2014).
in liquid assays using ONPG. Assays were performed in 96-well microtitre plates. Experimental cultures were grown in 200 µl M9 minimal medium supplemented with 100 µg ampicillin ml⁻¹ and 50 µg kanamycin ml⁻¹ for 2 h at 37 °C pre-induction. Following supplementation with 0.7 mM IPTG, cultures were incubated at 20 °C for 16 h. Cells were harvested and β-galactosidase activity was determined using standard assay conditions as described by Miller (1972). Activity is reported as the mean and SD of at least three independent assays and normalized to the fusion protein pair showing maximum (100 %) interaction.

**Genetic techniques.** Site-directed mutagenesis was performed with Phusion DNA Polymerase (NEB) based upon the QuickChange site-directed mutagenesis protocol (Stratagene). All recombinant plasmids were constructed using standard cloning strategies and were confirmed by sequencing.

**Computational analyses.** Protein sequences were obtained from *B. subtilis* genome using either SubtiList website (http://genolist.pasteur.fr/SubtiList/), National Center for Biotechnology Information BLAST search tool (http://www.ncbi.nlm.nih.gov/blast/) or BSORF *B. subtilis* Genome Database (http://bacillus.genome.jp/). Sequences extracted were aligned using CLUSTALO (Sievers et al., 2011) or TM-Coffee (Chang et al., 2012). Protein localization predictions and transmembrane domain structural predictions were performed using TM-Coffee. Sequence similarities and secondary-structure information from aligned sequences are rendered in Fig. 1 using Procter (Omasits et al., 2014).

**RESULTS**

**Genetic organization of the SigO–RsoA regulon**

In *B. subtilis*, SigO–RsoA initiates transcription from three related promoters driving the expression of three operons (sigO–rsoA, yvrJ and oxdC–rsiO) (Fig. 1a) (MacLellan et al., 2008, 2009a, b). Previous work demonstrated that deletion of the gene downstream of oxdC (rsiO, formerly yvrL) resulted in an increase in basal SigO–RsoA activity (MacLellan et al., 2008) and increased OxdC accumulation in *B. subtilis* cells (MacLellan et al., 2009b) suggesting that RsiO, a predicted membrane protein (Fig. 1b), negatively regulates the SigO–RsoA system. *rsiO* expression is driven from a promoter upstream of oxdC, but an attenuator sequence located between the ORFs (Fig. 1a) reduces *rsiO* transcription relative to oxdC transcription (MacLellan et al., 2008). In this work, we have explored the molecular basis for RsiO inhibitory activity on SigO–RsoA function by reconstituting the SigO–RsoA–RsiO regulatory system into *E. coli*.

**RsiO represses the activity of SigO–RsoA in *E. coli***

To isolate the SigO–RsoA–RsiO system from other *B. subtilis* factors, we reconstituted the system in *E. coli*. Previously, we showed that SigO and RsoA are able to productively associate with *E. coli* RNAP and drive expression from a target promoter (P_oxdC) in this genetic background (Davis et al., 2015).

Consistent with previous results using *E. coli* as a host and with activity in the native host *B. subtilis* (MacLellan et al., 2009a), co-expression of SigO and RsoA is required to initiate transcription from a P_oxdC–gfp fusion (Fig. 2a). When epitope-tagged RsiO (RsiOFLAG) was co-expressed with SigO–RsoA, promoter activity declined by about 86 %. An immunoblot analysis using anti-FLAG antibodies confirmed RsiOFLAG expression, including a strong signal from the sample enriched in cytoplasmic membrane (Fig. 2b). These data suggest that at least some fraction of RsiO inserts into the cytoplasmic membrane and that its presence in the cell strongly reduces SigO–RsoA-dependent transcription.

**RsiO interacts with the N-terminus of SigO**

Anti-σ factors typically interact with cognate σ factors thereby sequestering the σ factor from the RNAP core.

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**Fig. 2.** RsiO negatively regulates the ability of SigO–RsoA to activate transcription in heterologous host *E. coli*. (a) Transcription activation by SigO and RsoA from P_oxdC–gfp fusion co-expressed with RsiO in *E. coli*. SigO, RsoA and RsiO-FLAG expression was induced with 0.7 mM IPTG from pET-DUET. The percentage GFP fluorescence is reported as the mean ± SD (n=6), normalized to activation in the absence of RsiO at pH 7 (7.1 × 10⁻⁶ arbitrary units). (b) Coomassie stained SDS-PAGE gel and immunoblot using anti-FLAG antibodies to detect RsiOFLAG accumulation in the whole-cell lysate and the membrane-enriched fraction of each strain after induction with IPTG. M, Molecular mass marker; MP, membrane fraction; WC, whole-cell lysate.
We used an adenylate cyclase-based BACTH assay (Euro-medex) to determine whether RsiO interacts with either of the two-subunit σ factor subunits. This BACTH system consists of two base plasmids, pT18 (a high-copy-number plasmid) and pT25 (a low-copy-number plasmid), from which T18 or T25 subfragments of CyaA fused to the test proteins are expressed. When SigO or RsoA fusion proteins were co-expressed with an RsiO fusion protein, only SigO interacted with RsiO (Fig. 3a). When SigO was expressed as either an N-terminal fragment (amino acids 1–93) or C-terminal fragment (amino acids 94–176), RsiO interacted with the N-terminal segment of SigO, but not the C-terminal segment (Fig. 3a). This parallels the interaction between SigO and RsoA, as RsoA also specifically interacts with the N-terminal half of SigO (MacLellan et al., 2009a). To check whether the SigO–RsoA interaction would be antagonistic to the SigO–RsiO interaction, we co-expressed all three proteins in the cell and found that so long as RsiO was expressed from the high-copy-number plasmid (pT18), the co-expression of RsoA had no impact on the SigO–RsiO interaction (Fig. 3a). However, when SigO and RsoA were expressed from the high-copy-number pT18 plasmid and RsiO was expressed from the low-copy-number pT25 plasmid (Fig. 3b), co-expression of RsoA reduced the SigO–RsiO interaction to near negative control levels. When two mutations (F67S and E69V) that completely or partially (respectively) impair the ability of RsoA to interact with SigO (Davis et al., 2015) were introduced into RsoA, the interaction between RsiO and SigO was restored (Fig. 3b). Taken together, the data suggest that RsiO specifically interacts with the N-terminus of SigO and negatively regulates

**Fig. 3.** N-terminus of SigO is required and sufficient for interaction with RsiO. BACTH protein–protein interaction assay of full-length RsiO–CyaA fusion protein co-expressed with full-length, N-terminal (N.) and C-terminal (C.) SigO, or full-length RsoA–CyaA fusion proteins. (a) Full-length RsiO fused to T18 CyaA (high-copy-number plasmid); the interaction between SigO and RsiO was salvaged in the presence of RsoA (MacLellan et al., 2009a). To check whether the SigO–RsoA interaction would be antagonistic to the SigO–RsiO interaction, we co-expressed all three proteins in the cell and found that so long as RsiO was expressed from the high-copy-number plasmid (pT18), the co-expression of RsoA had no impact on the SigO–RsiO interaction (Fig. 3a). However, when SigO and RsoA were expressed from the high-copy-number pT18 plasmid and RsiO was expressed from the low-copy-number pT25 plasmid (Fig. 3b), co-expression of RsoA reduced the SigO–RsiO interaction to near negative control levels. When two mutations (F67S and E69V) that completely or partially (respectively) impair the ability of RsoA to interact with SigO (Davis et al., 2015) were introduced into RsoA, the interaction between RsiO and SigO was restored (Fig. 3b). Taken together, the data suggest that RsiO specifically interacts with the N-terminus of SigO and negatively regulates
SigO–RsoA activity, presumably by sequestering the SigO from RsoA and core RNAP. RsoA expression (when expressed from a high-copy-number plasmid) is antagonistic to the SigO–RsiO interaction suggesting that the SigO–RsoA and SigO–RsiO interaction interfaces may, in part, overlap.

**RsiO competes with RsoA for interaction with SigO**

We also tested the effect of RsiO on the interaction between SigO and RsoA (Fig. 4). Consistent with previously analyses (MacLellan et al., 2009a), SigO and the N-terminal half of SigO interact with RsoA (Fig. 4a). So long as RsoA was expressed from the low-copy-number pT25 plasmid (Fig. 4a), expression from the rsiO gene carried on either the high- or low-copy-number plasmid was sufficient to significantly reduce the interaction between RsoA and full-length SigO, and nearly abolish the interaction between RsoA and the N-terminal half of SigO (Fig. 4a). However, when RsoA was expressed from the high-copy-number pT18 plasmid (Fig. 4b), only expression from the rsiO gene carried on the high-copy-number plasmid (pT18) impaired the RsoA and SigO interaction. Expression from rsiO carried on the low-copy-number plasmid (pT25) had no detectable effect on the interaction. The expression of rsiO from the high-copy-number pT18 plasmid had no effect on the interaction between two control leucine zipper fusion proteins (Fig. 4a inset). These data again suggest that the binding of RsoA and RsiO to the N-terminus of SigO is competitive and implies a sharing or overlap of binding interfaces on the surface of SigO. Because of problems stemming from the use of immunogenic tags on SigO and RsoA (see Discussion), we are

![Fig. 4](http://mic.microbiologyresearch.org)
unable to directly determine protein levels during experiments. However, the results suggest that expression from high- or low-copy-number plasmids (essentially gene dosage effects) can be considered to some degree a basis for inferring resulting protein levels in the cell, and can explain the differential effects stemming from expressing the sigO, rsoA and rsiO genes from plasmids of differing copy numbers.

**Acid induction of SigO–RsoA activity in E. coli**

SigO–RsoA activity is induced during growth in acidified culture medium (MacLellan et al., 2009b). Since Rsio appears to sequester SigO under non-inducing neutral pH conditions in *E. coli*, we wished to explore the effects of imposing acid stress on the SigO–RsoA–Rsio regulatory system in this genetic background. Under neutral (pH 7) growth conditions (Fig. 5a), co-expression of SigO and RsoA activates transcription of a * PxodC–lacZ* fusion. Co-expression with Rsio, however, inhibits this activity to about 30% of the activity in the absence of Rsio (consistent with the data shown in Fig. 2). Surprisingly, when the same assay is conducted with cells exposed to acid (pH 5.4) stress during growth, Rsio loses the ability to inhibit SigO–RsoA transcription activity (Fig. 5b) despite the accumulation of Rsio under both pH conditions (Fig. 5c).

The loss of the Rsio ability to inhibit SigO–RsoA activity could be related to a loss in its ability to bind SigO and sequester it from the core RNAP. To check this, we used the BACTH system to examine the SigO–Rsio interaction after imposition of acid stress. The apparent interaction of Rsio with either the N-terminal segment of SigO or full-length SigO is significantly reduced under acidic conditions, relative to neutral conditions (Fig. 6a). We found that acidified medium had no effect on two control interactions (an interaction between a pair of leucine zipper domain proteins and the SigO–RsoA interaction) (Fig. 6b). These results indicate that lowering the pH of the expression culture medium results in the specific deactivation of Rsio. Presumably, this deactivation releases SigO, allowing it to interact with RsoA, form holoenzyme and initiate transcription from the target promoter; thus, explaining how SigO–RsoA activity becomes manifest under acidic conditions.

Finally, we demonstrated that Rsio synthesis, when Rsio is expressed from the high-copy-number plasmid pT18, impairs the interaction between SigO and RsoA at pH 7.
DISCUSSION

The *B. subtilis* SigO–RsoA proteins constitute an atypical two-subunit σ factor (MacLellan *et al.*, 2008, 2009a) that is activated in response to certain cell-wall-acting antibiotics and to growth under acidic conditions (Cao *et al.*, 2002; Hachmann *et al.*, 2009; MacLellan *et al.*, 2009b). In this communication, we identify the protein encoded by gene *rsiO* as the cognate anti-σ factor to SigO–RsoA. RsiO is a predicted membrane protein. Unlike the six other anti-σ factors encoded in the *B. subtilis* genome, rsiO is not encoded in the same operon as its cognate σ factor (see Fig. 1a). With four predicted transmembrane domains, it is also more structurally complex than many other integral membrane regulators that usually consist of a single transmembrane domain (see Fig. 1b).

Stemming from observations in *B. subtilis* that RsiO appears to negatively regulate SigO–RsoA activity (MacLellan *et al.*, 2008, 2009b), we decided to isolate the regulatory system (sigO–rsoA–rsiO) in a heterologous host genetic background to remove any possible influences of other *B. subtilis* regulatory proteins. This strategy was successful but posed some complications. Overexpression of σ factor proteins in *E. coli* often leads to predominantly insoluble inclusion bodies. The accumulation of appreciable levels of active SigO and RsoA during our experiments required that all expression experiments be conducted at 20 °C on a minimal medium, as previously described (Davis *et al.*, 2015). Also, we would have wished to monitor the abundance of all proteins during these experiments. However, we observed (not shown) that imparting immunogenic tags to all three proteins (SigO, RsoA and RsiO) dramatically reduced the activity of the system. Therefore, we opted to leave SigO and RsoA untagged and monitored their presence phenotypically (e.g. the initiation of transcription from a target promoter–reporter gene fusion demonstrates that active SigO–RsoA is accumulating in the cell). We did, however, impart an immunogenic FLAG tag to RsiO to qualitatively detect its presence in the cell when it was included in experiments.

In this study, we show that RsiO binds the N-terminus of SigO and inhibits SigO–RsoA–dependent promoter activation, presumably by sequestering SigO at the membrane and preventing its association with RsoA and core RNAP. We previously showed using BACTH assays and pull-down assays with purified proteins that RsoA also interacts with SigO via its N-terminus (MacLellan *et al.*, 2009a). Here, we show that RsoA expressed from a high-copy-number plasmid impairs the interaction between SigO and RsiO, and that RsiO expression from a high-copy-number plasmid impairs the interaction between SigO and RsoA. We conclude that both RsoA and RsiO are unable to simultaneously bind the N-terminus of SigO, perhaps reflecting a partially or wholly shared interaction interface on the surface of SigO.

Interestingly, we were also able to establish that the SigO–RsoA–RsiO system responds to acid stress in *E. coli* as it does in the native host *B. subtilis*, in the sense that SigO–RsoA becomes active under acidic growth conditions. Specifically, we found that in the *E. coli* genetic background, the imposition of acidic growth conditions abolished the inhibitory effect of RsiO on SigO–RsoA transcription initiation activity (Fig. 5) and that this effect correlated with the inability of RsiO to bind SigO at pH 5.4 (Fig. 6). The mechanism by which RsiO inhibitory activity is abolished under acidic conditions is currently being investigated.

Under environmental conditions that elevate ECF σ factor activity, many integral membrane anti-σ factors undergo RIP, as is the case for anti-σ factors regulating σ^E^ from *E. coli* and σ^W^ and σ^V^ from *B. subtilis* (Chaba *et al.*, 2007; Grigorova *et al.*, 2004; Hirstie *et al.*, 2014; Ho & Ellermeier, 2012). RIP is initiated by a highly specific site-1-protease (S1P). Only following cleavage of the anti-σ factor by S1P can site-2 proteolysis, by general proteases such as RseP and RasP, occur. Cleavage by site-2-protease produces a proteolytic tag (VAA) that results in further degradation of the anti-σ factor by cytoplasmic ClpXP or ClpEP and release of the σ factor (Heinrich *et al.*, 2009; Ho & Ellermeier, 2012). RsiO does possess the amino acid motif VAA, partially conserved amongst orthologous proteins (Fig. S2) in its fourth transmembrane domain, suggesting a possible location for site-2 proteolysis. Thus, acidic conditions may activate a proteolytic cascade in *E. coli* that can recognize the *B. subtilis* protein RsiO as a substrate, ultimately resulting in the release of SigO into the cytoplasm. Arguing against this scenario, however, is the observation that under acidic conditions where the inhibitory effect of RsiO is largely abolished, RsiO remains quite abundant in cells (Fig. 6c). Nevertheless, our present experiments only qualitatively indicated the presence of RsiO and were not expressly designed to detect possible proteolysis of RsiO. More specific experiments will be required to determine whether RsiO is catalytically degraded under acidic conditions in *E. coli* and in *B. subtilis*.

A second intriguing possibility that explains the loss of RsiO activity in *E. coli* under acidic conditions is that RsiO is directly inactivated at a certain threshold hydrogen (hydronium) ion concentration by a mechanism that does not involve a host proteolytic cascade, or any other *E. coli*-specific mechanism. In other words, RsiO may be the sensor of, as well as a respondent to, acidic conditions in the extracytoplasmic environment.

To further characterize the function of RsiO and the mechanism by which it becomes inactive under acidic
conditions, our current efforts are focused on developing a robust experimental system where we can reliably monitor the cellular abundance of all key proteins (SigO, RsoA and RsiO) under neutral and acidic pH growth conditions. Such a system will promote the design of experiments to monitor the fate of RsiO after imposition of acidic conditions in the E. coli genetic background, as well as in B. subtilis cells. We are particularly interested in exploring the possibility that direct sensing of hydrogen ion concentration by RsiO may, at least in part, play a role in its inactivation under low pH conditions as this would be, to our knowledge, an unprecedented sensory activity by an anti-σ factor. It has not escaped our notice that the predicted structure of RsiO (Fig. 1b) and alignment of RsiO orthologues (Fig. S2) indicate an aspartate residue in the second extracytoplasmic loop that is strictly conserved amongst orthologous proteins. The possibility that protonation of the aspartyl carboxylate (perhaps possessing an elevated pKₐ due to the local environment) could provide a hydrogen ion sensing mechanism is one scenario that is being investigated.

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