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The YvrI Alternative σ Factor Is Essential for Acid Stress Induction of Oxalate Decarboxylase in *Bacillus subtilis*▽†

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YvrI is a recently identified alternative σ factor in *Bacillus subtilis* that requires the coactivator YvrHa to activate transcription. Previously, a strain engineered to overproduce YvrI was found to overproduce oxalate decarboxylase (OxdC), and further analysis identified three YvrI-activated promoters preceding the *yvrI-yvrHa*, *yvrJ*, and *oxdC-yvrL* operons. Independently, proteome analyses identified OxdC as a highly abundant, cell wall-associated protein that accumulated under acidic growth conditions. We show here that the accumulation of OxdC in the cell wall proteome under acidic growth conditions is absolutely dependent on YvrI and is correlated with enhanced transcription of both the *yvrI-yvrHa* and the *oxdC-yvrL* operons. Conversely, OxdC accumulates to a high level even under nonacidic growth conditions in cells lacking YvrL, a negative regulator of YvrI/YvrHa-dependent transcription. These results indicate that YvrI and its associated coregulators YvrHa and YvrL are required for the regulation of OxdC expression by acid stress. The high-level accumulation of OxdC depends, in part, on a strong *oxdC* promoter. A regulatory sequence with similarity to an upstream promoter element (UP) was identified upstream of the *oxdC* promoter and is required for high-level promoter activity. Conservation of the YvrI/YvrHa/YvrL regulatory system among related species allowed us to deduce an expanded consensus sequence for the compositionally unusual promoters recognized by this new σ factor.

The *Bacillus subtilis* genome encodes at least 18 σ factors (15), including a recently identified and significantly divergent σ^{70} family protein named YvrI (25). Whole-genome microarray analyses suggest that YvrI is expressed in response to certain cell wall-acting antibiotics, such as vancomycin. Since YvrI has similarities to σ factors, we speculated that YvrI was a regulatory protein. We therefore constructed a strain in which YvrI expression could be induced in order to identify likely target genes. These studies led to the finding that the artificial induction of YvrI resulted in a concomitant upregulation of OxdC, an oxalate decarboxylase (2, 33) encoded by the divergent gene. OxdC was sufficiently abundant in these studies to be easily visible by Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) analysis of whole-cell lysates.

Further biochemical studies indicated that, as expected for a σ-like protein, YvrI binds to RNA polymerase (25). When it is overexpressed from a heterologous inducible promoter, YvrI and its coactivator YvrHa activate transcription of at least three operons (five genes) in *B. subtilis*, including *yvrI-yvrHa*, *yvrJ*, and *oxdC-yvrL*. Surprisingly, three of these five genes encode regulatory proteins: YvrI is a σ factor, YvrHa is a required coactivator for YvrI, and YvrL is a predicted membrane protein that negatively regulates YvrI activity. The function of YvrJ is unknown, and OxdC is postulated to help protect cells against low-pH stress by consuming protons upon decarboxylation of oxalate (33).

Treatment with cell wall-active antibiotics upregulated expression of the *yvrI* operon and of *yvrL*, but the same conditions did not lead to an apparent upregulation of *oxdC*. Since OxdC appears to be the primary target of this regulatory network, this raised questions about the physiological role of the YvrI-YvrHa regulatory system. OxdC accumulates as a highly abundant protein in the cell wall proteome in cells grown under acidic conditions (generated either by addition of 1% acidic phytate to LB medium or simply by adjustment of the medium with HCl to pH 5.4), and we speculated that the function of the YvrI-mediated regulatory system might be to control the acid induction of *oxdC*.

Here, we demonstrate that the YvrI σ factor is required for the acid induction of OxdC. At the RNA level, both the *yvrI-yvrHa* and the *oxdC* operons are highly induced during growth under acidic conditions, and this transcriptional induction is reflected in the protein composition of the cell wall: OxdC is highly abundant only in acid-grown cells. Moreover, OxdC is absent from *yvrI* mutant cells even under acidic growth conditions. YvrL has previously been suggested to negatively regulate YvrI-YvrHa activity, but its influence under acidic and neutral pH conditions and its overall impact on OxdC accumulation were unclear (25). Here we show that a *yvrL* mutant accumulates OxdC protein in the cell wall even in the absence of acid stress. Thus, YvrL appears to prevent expression and activity of the YvrI σ factor under nonstress conditions. Genome sequence comparisons indicate that the YvrI-YvrHa regulatory system is

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conserved in several different bacilli, although the gene composition and arrangement differ among species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* wild-type strain CU1065 and its derivatives were used in this work and are described in Table 1. Integrative plasmids and oligomers used in this work are also described in Table 1. *B. subtilis* strains were cultivated with vigorous agitation in complete Luria Bertani (LB) medium. Phytate was purchased as phytic acid sodium salt from Sigma-Aldrich (product number P8810). Conditional expression of YvrI was accomplished by inducing cells of *B. subtilis* strain HB7709 with 2% xylose for 1 h before harvesting, unless otherwise noted. For acid induction experiments, bacteria were cultivated in LB medium, and the pH was adjusted to 7.5 with HCl before sterilization. Acid stress was provoked by supplementing 1 liter of LB medium with 1% phytic acid sodium salts (phytate) just before inoculation with the overnight culture to a starting optical density at 540 nm (OD_{540}) of 0.07. The pH of the LB medium was decreased to 5.4 by the addition of 1% phytate at the beginning of cultivation and increased during the growth curve, which was monitored extensively in the previous study (4). Samples were taken at OD_{540}s of 2.0, 3.0, 5.0, and 8.0 (see reference 4) (see Fig. 2A), since in the previous study, OxdC secretion was observed at OD_{540}s of 2.0 and 3.0. Also, in the previous study, we showed that OxdC was induced in the cell wall proteome at OD_{540}s of 2.0 and 3.0 in LB medium that was adjusted to pH 5.4 by HCl before inoculation with overnight culture (4) (see Fig. 3C). Specific effects of phytate on the cell wall proteome are shown in the previous study and include the redistribution of cell wall proteins such as WprA and the WapA processing products. These WprA and WapA effects were not observed with cells grown in HCl-acidified medium. Thus, although phytate has non-acid-related effects on cell physiology, our previous studies suggest that induction of OxdC is primarily a function of acid stress, similar to the effects of acidification to the same pH with HCl. In this study, we have used phytate-acidified medium to provoke acid stress since the quality of the two-dimensional (2D) gel separation was decreased when cells were grown in HCl-acidified medium.

Genetic techniques. Standard genetic techniques were used throughout this work. Unmarked deletions in *yvrL*, *oxdC*, *yvrJ*, *yvrI* and *yvrHa*, and *yvrGHb* were generated using the plasmid pMAD (6), as described previously (25). Primers used that have not already been described are as follows: Δ*oxdC* (oligonucleotide 3580-3581/3582-3583), Δ*yvrJ* (3626-3627/3628-3629), and Δ*yvrGHb* (2857-2858/2859-2860). Conditional YvrI induction was accomplished by cloning a FLAG-tagged derivative of the *yvrI* gene into the xylose induction plasmid pSWEET (9) and integrating the construct into the *B. subtilis amyE* locus. Transcriptional fusions to promoterless *lacZ* genes were constructed using the plasmid pDG1663 (16) with subsequent integration into the *thrC* locus of *B. subtilis*. Beta-galactosidase assays were conducted using standard procedures as previously described (25).

Preparation of cell wall proteins. *B. subtilis* cells were grown in 1 liter LB medium with and without 1% phytate, and samples of 250 ml were harvested at OD_{540}s of 2.0, 3.0, 5.0, and 8.0. To prepare cell wall proteins, cells were centrifuged and washed, and the cell wall proteins were extracted with 1.5 M LiCl, 25 mM Tris-HCl, pH 8.0, as described previously (5).

Proteome analyses and image analysis. The protein pellets were resolved in a solution containing 2 M thiourea and 8 M urea, and insoluble material was removed by centrifugation. The protein content was determined using the Bradford assay (10). For preparation of 2D PAGE, 200 μg of the protein extracts were separated using nonlinear immobilized pH gradients in the pH range of 3 to 10 (Amersham Biosciences) and a Multiphor II apparatus (Amersham Pharmacia Biotech) as described previously (3). The resulting 2D gels were fixed in 40% (vol/vol) ethanol and 10% (vol/vol) acidic acid and stained with colloidal Coomassie brilliant blue (Amersham Biosciences). The image analysis was performed with Decodon Delta 2D software (Greifswald, Germany).

Northern blot experiments. Total RNA of *B. subtilis* strains was isolated from cells along the growth curve by the acid phenol method as described previously (26). Northern blot analyses were performed as described previously (35). Hybridization specific for *yvrI* and *oxdC* were conducted with digoxigenin-labeled RNA probes synthesized in vitro with T7 RNA polymerase from a T7 promoter containing internal PCR products of *yvrI* and *oxdC* using the following primers: oxdCfor, 5′ GAGGAGACAAAGGAGCAACG3′, and oxdCT7rev, 5′CTAATACGACTCACTATAGGGAGAGTCAGATGCAAAAACGGTCA3′; and yvrIfor, 5′AGCAATCCTCAGCATTACCG3′, and yvrIT7rev, 5′CTAATACGACTCACTATAGGGAGAGTCTCCAGCCCCTTTTTATGA3′.

Bioinformatic techniques. To determine the distribution of the *yvrI-yvrHa* operon (and other associated genes) in bacterial genomes, we used the BLASTp program to search the nonredundant protein database. A separate BLASTp analysis of all finished and unfinished bacterial genomes yielded similar, but less-complete, results.

Since the sequence of YvrI is similar to that of other σ factors, differentiating between true YvrI homologs and incidentally similar σ factors in genomes is problematic. Therefore, we determined the distribution of YvrI by determining the distribution of YvrHa, a more distinct protein (in terms of primary amino acid sequence) that is encoded in the *yvrI* operon and is required as a coactivator of transcription from YvrI-dependent promoters. We infer here that the incidence of a putative YvrHa homolog preceded by a σ factor gene with high similarity to YvrI constitutes a homolog of the *yvrI-yvrHa* operon that we previously characterized in *B. subtilis* (25). Promoter sequence prediction was conducted using Virtual Footprint (http://prodoric.tu-bs.de/vfp/) software with a position weight matrix generated from known YvrI promoters and by manual sequence scanning in the relevant regions upstream of genes. Alignments were conducted using ClustalW and WebLogo software.

RESULTS

YvrI is essential for acid stress induction of *oxdC* transcription. Previously, we showed that overexpression of YvrI in *B. subtilis* led to high-level activation of the *oxdC-yvrL* promoter and accumulation of OxdC as visualized on Coomassie-stained protein gels (25). Independently, it was observed by using proteome analyses that OxdC is maximally expressed under acidic growth conditions (LB medium acidified to pH 5.4 with either phytate or HCl), under which it accumulates as a highly abundant, cell wall-associated protein (4). We therefore hypothesized that a primary response to low-pH stress might be an increase in the activity of the YvrI σ which, by virtue of autoinduction, would positively regulate its own expression and that of the *oxdC-yvrL* operon.

To monitor transcription of the *yvrI-yvrHa* operon, we isolated RNA from cells grown in LB medium acidified with 1% phytate (pH 5.4) or unamended (pH 7.5), as previously described for proteome analyses (4). Northern blot analyses indicated a strong, growth phase-dependent expression of the *yvrI-yvrHa* transcript in the acidified medium but not in the unamended LB (Fig. 1A). Interestingly, mRNA levels for the *yvrI-yvrHa* operon were maximal after transition to higher cell densities (from OD_{540}s of 2 to 3) under acidic conditions and decreased at later times during the stationary phase (Fig. 1A). The lack of expression in unamended LB medium is due to negative regulation by YvrL, since expression in the *yvrL* mutant strain in LB medium was very similar to that observed with wild-type cells in the phytate-amended medium. Expression of the YvrI-YvrHa-positive regulators was correlated with the transcription of *oxdC* (Fig. 1B). Our previous studies (25) left open the possibility that other σ factors could play a role in activating *oxdC* transcription under certain conditions, but the present analysis demonstrates that expression of the *oxdC-yvrL* operon is completely dependent on *yvrI*. These results also extend the interpretation from previous studies in which we reported a very modest (two- to threefold) induction of an *oxdC-lacZ* fusion in cells grown at pH 5.2 compared to those grown at pH 7.5. These studies probably underestimated the true magnitude of the induction due to poor expression or activity of β-galactosidase under low-pH conditions.

YvrI and YvrL regulate OxdC accumulation in response to acid stress. To directly monitor the roles of the YvrI-YvrHa and YvrL regulators in OxdC production at the protein level, we used 2D PAGE analyses of cell wall-associated proteins (Fig. 2). Consistent with the results of the Northern blot anal-

TABLE 1. Strains, plasmids, and oligomers used in this study

Strain, plasmid, or oligonucleotide	Parental strain (integration), relevant genotype, or oligonucleotide sequence[a]	Source
Strains		
CU1065	W168	Laboratory collection
HB7709	CU1065 (pSM002) *amyE*::$P_{xyl\text{-}yvrI\text{-}FLAG}$	25
HB7720	CU1065 (pSM007) Δ*yvrI*	25
HB7723	CU1065 (pSM010) Δ*yvrHb-yvrG*	This work
HB7725	HB7709 (pSM008) Δ*yvrHa*	25
HB7867	CU1065 (pSM073) Δ*oxdC*	This work
HB7868	CU1065 (pSM074) Δ*yvrL*	25
HB7870	CU1065 (pSM022) Δ*yvrJ*	This work
HB7911	HB7709 (pSM106) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bsu)	This work
HB7938	HB7709 (pSM128) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bpu)	This work
HB7939	HB7709 (pSM129) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bli)	This work
HB7940	HB7709 (pSM130) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bam)	This work
HB7941	HB7725 (pSM128) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bpu)	This work
HB7942	HB7725 (pSM129) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bli)	This work
HB7943	HB7725 (pSM130) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bam)	This work
HB7944	HB7725 (pSM106) *thrC*::$P_{oxdC\text{-}lacZ}$ (Bsu)	This work
HB7945	HB7725 (pSM121) *thrC*::$P_{oxdC\text{-}lacZ}$ (wt UP element)	This work
HB7946	HB7725 (pSM122) *thrC*::$P_{oxdC\text{-}lacZ}$ (mut1 UP element)	This work
HB7947	HB7725 (pSM123) *thrC*::$P_{oxdC\text{-}lacZ}$ (mut2 UP element)	This work
HB7948	HB7725 (pSM124) *thrC*::$P_{oxdC\text{-}lacZ}$ (mut3 UP element)	This work
Plasmids		
pDG1663	Promoterless *lacZ* reporter vector (*thrC* integration)	16
pSWEET	Xylose-inducible expression vector (*amyE* integration)	9
pMAD	Generates unmarked in-frame deletions	6
pSM002	(pSWEET) 0.6-kb *yvrI*-FLAG gene PCR product (oligonucleotides 2749–2750) used for conditional YvrI-FLAG expression vector in *B. subtilis*	25
pSM007	(pMAD) 1.5-kb PCR product used to generate 474-nt in-frame deletion in *yvrI**	25
pSM008	(pMAD) 1.5-kb PCR product used to generate 270-nt in-frame deletion in *yvrHa**	25
pSM010	(pMAD) 1.5-kb PCR product used to generate deletion of the *yvrHb-yvrG* operon*	This work
pSM022	(pMAD) 1.5-kb PCR product used to generate 102-nt in-frame deletion in *yvrJ**	This work
pSM073	(pMAD) 1.5-kb PCR product used to generate 1,077-nt in-frame deletion in *oxdC**	This work
pSM074	(pMAD) 1.5-kb PCR product used to generate 366-nt in-frame deletion in *yvrL**	25
pSM106	(pDG1663) 0.18-kbp PCR product carrying only the *oxdC* promoter (oligonucleotides 3808–3809), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	25
pSM121	(pDG1663) 0.18-kbp PCR product carrying wt *oxdC* promoter (oligonucleotides 4147–3809), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM122	(pDG1663) 0.18-kbp PCR product carrying A-tract mutant *oxdC* promoter (oligonucleotides 4148–3809), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM123	(pDG1663) 0.18-kbp PCR product carrying T-tract mutant *oxdC* promoter (oligonucleotides 4149–3809), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM124	(pDG1663) 0.18-kbp PCR product carrying combined A/T-tract mutant *oxdC* promoter (oligonucleotides 4150–3809), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM128	(pDG1663) 0.18-kbp PCR product carrying *B. pumilis oxdC* promoter (oligonucleotides 4203–4204), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM129	(pDG1663) 0.18-kbp PCR product carrying *B. licheniformis oxdC* promoter (oligonucleotides 4205–4206), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
pSM130	(pDG1663) 0.18-kbp PCR product carrying *B. amyloliquefaciens oxdC* promoter (oligonucleotides 4207–4208), generates $P_{oxdC\text{-}lacZ}$ transcriptional fusion	This work
Oligonucleotides		
2857	ATAGGATCCCACTGACGGTTGATCATCC	
2858	TATGTCGACGAACGCGTTTGATCATGTCC	
2859	ATAGTCGACGCAGAACGAACATTGGAA	
2860	ACACCATGGCAGCACGTGACATATTTAGC	
3580	ATAGGATCCAAGATGCGAATCATTGAATTC	
3581	ATAGTCGACCGTTGCTCCTTTGTCTCCTC	
3582	ATAGTCGACGTAGTGAAAAAGAAATGCAG	
3583	TATCCATGGCATTATGCTCGTTTCCGTAACG	
3626	ATAGGATCCGGACTCCCCTAATGAATCTGC	
3627	ATAGTCGACCTGATCCATAGGAAAATACCTC	
3628	ATAGTCGACATAGAACTAATGACAGAACTG	
3629	ATACCATGGGACTTCTTTTGGAGTGTGGG	
3808	CGTGAATTCGTTTGATCAACTAATAGAAC	
3809	ATAAAGCTTCATGAAATGTTTCCTCC	
4147	CGTGGAATTCAAAAAAATAATTTTTCAATCGAAGTTGACTTTTCACTGGT	
4148	CGTGGAATTCAACCCAATAATTTTTCAATCGAAGTTGACTTTTCACTGGT	
4149	CGTGGAATTCAAAAAAATAATGGGTCAATCGAAGTTGACTTTTCACTGGT	
4150	CGTGGAATTCAACCCAATAATGGGTCAATCGAAGTTGACTTTTCACTGGT	
4203	CGTGAATTCTTTTGACAGTTTACTTGCTG	
4204	ATAAAGCTTCATGATAAAATCCCCTTTC	
4205	CGTGAATTCGCAATGTCGGCTTTCCAGCTG	
4206	ATAAAGCTTCATGTGATATCCCCTCTTTC	
4207	CGTGAATTCCCATGTATCTTCTCACACGG	
4208	ATAGGATCCCATGTAAATCGTCTCCTCTC	

[a] *, see Materials and Methods. Bsu, *B. subtilis*; Bpu, *B. pumilis*; Bli, *B. licheniformis*; and Bam, *B. amyloliquefaciens*. wt, wild type; mut, mutant; nt, nucleotide.

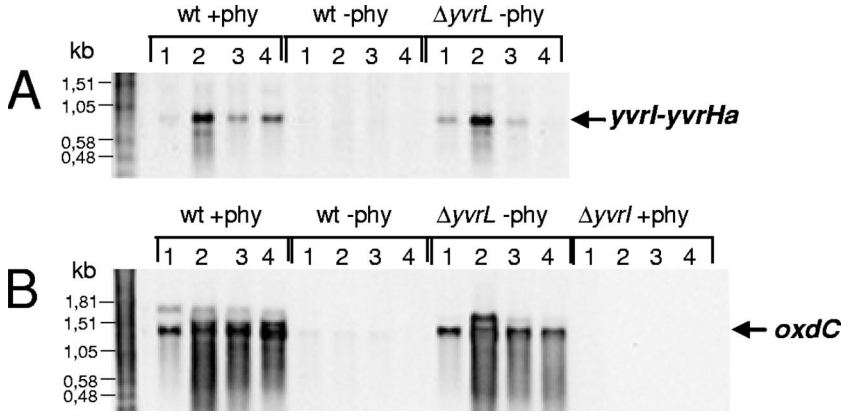

FIG. 1. Northern blot analysis of the *yvrI-yvrHa* (A) and *oxdC-yvrL* (B) transcripts. The *B. subtilis* wild-type (wt), *yvrI*, and *yvrL* mutant strains (as indicated) were grown in LB medium with 1% phytate (+phy) or without phytate (−phy). RNA samples were harvested after transition to stationary phase at OD_{540}s of 2 (lane 1), 3 (lane 2), 5 (lane 3), and 8 (lane 4). RNA blots were hybridized with mRNA probes specific for *yvrI* (A) or *oxdC* (B). Arrows (right) indicate sizes of the *yvrI-yvrHa*- and *oxdC*-specific transcripts.

ysis, OxdC accumulated at high levels in post-exponential-phase wild-type cells grown in LB medium with 1% phytate (Fig. 2A). OxdC colocalized with other known cell wall-associated proteins (4, 5) such as WapA, LytBCDE, YoeB, and Hag (flagellin) and eventually became the most abundant cell wall protein under these conditions (see Fig. S1 and S2 in the supplemental material). Expression was absent from a strain with an unmarked in-frame deletion in *yvrI* (Fig. 2B), and this strain appeared virtually identical to the *oxdC* deletion strain (data not shown) in the cell wall proteome analysis. A *yvrL* mutation did not have a dramatic effect under these acidic growth conditions (Fig. 2C), although there was a slight increase in the amount of OxdC protein noted at the two earliest time points. In contrast, when cells were grown in LB medium

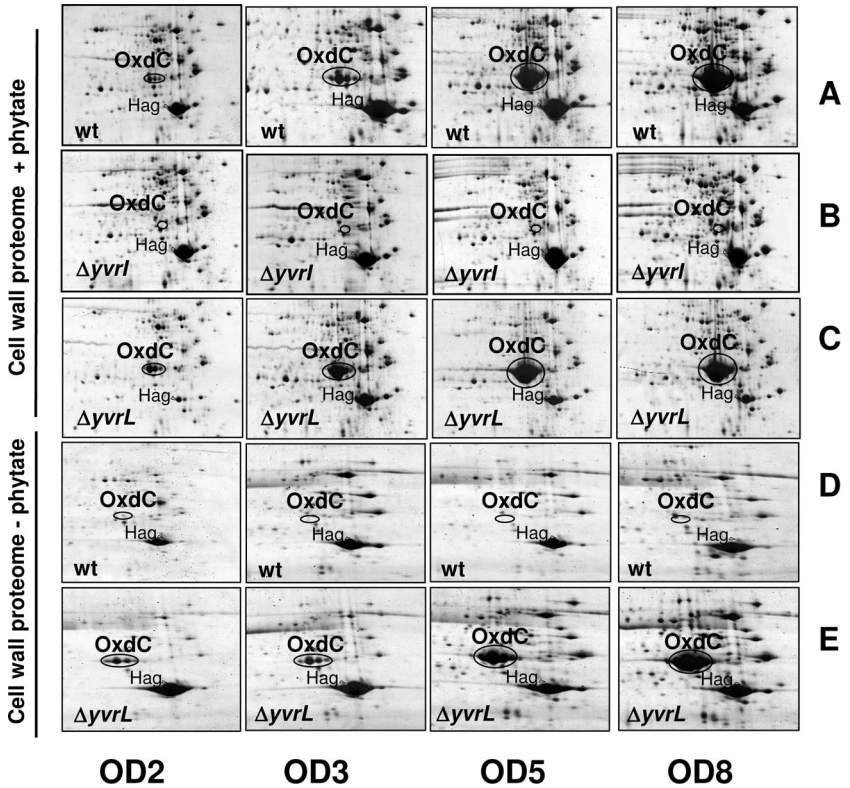

FIG. 2. Cell wall proteome analysis of *B. subtilis* wild-type (wt), Δ*yvrI*, and Δ*yvrL* mutant strains in the presence (rows A to C, +phytate) and absence (rows D to E, −phytate) of 1% phytate. Each row shows proteins from cells harvested during early exponential-phase (OD_{540}, 2), mid-exponential-phase (OD_{540}, 3), late exponential-phase (OD_{540}, 5), and stationary-phase (OD_{540}, 8) growth (as monitored by OD_{540} measurements). Positions of the OxdC and Hag (flagellin) protein are indicated. (A) Wild type; (B) *yvrI* deletion; (C) *yvrL* deletion with phytate; (D) wild type; (E) *yvrL* deletion without phytate.

TABLE 2. Distribution of the YvrI-YvrHa regulatory system among bacteria

Organism	*yvrI-yvrHa*	*oxdC*	*yvrL*	*yvrJ*	Conserved gene context
B. subtilis 168	+	+	+	+	+
B. amyloliquefaciens FzB42	+	+	+	+	+
B. licheniformis ATCC 14580	+	+	+	+	+
B. pumilis SAFR-032	+	+	+	+	+
B. thuringiensis 97-27	+	+	−	−	−
B. cereus G9241	+	+	−	+	−
B. clausii KSM-K16	+	+	−	+	−
Lysinibacillus sphaericus C3-41	+	+	−	+	Partial
Bacillus sp. strain B14905	+	+	−	+	Partial
B. cereus AH1134	+	+	−	+	Partial
Paenibacillus larvae BRL-230010	+	+	−	−	−
Bacillus sp. strain SG-1	+	+	−	−	−
B. cereus W	+	+	−	+	−
B. megaterium P73	+	+	−	−	−
Bacillus sp. strain NRRLMB-14911	+	+	−	+	Partial
Oceanobacillus iheyensis HTE831	+	+	−	+	−

at pH 7.5, OxdC accumulation was dramatic in the *yvrL* mutant (Fig. 2E) but not apparent in the wild type (Fig. 2D). These results demonstrate that YvrI is required for the acid stress-dependent induction of OxdC and suggest that YvrL prevents the expression or secretion of OxdC under nonstress conditions.

Distribution of YvrI in *Firmicutes*. YvrI is somewhat divergent from other σ^{70} family factors as it lacks a predicted region 2, a segment that is usually the most highly conserved region in σ^{70}-related factors (24) and is involved in interactions with core RNA polymerase (7, 12, 21, 31, 36). It may be that for this reason, YvrI escaped definition as a *B. subtilis* σ factor until recently. In *B. subtilis*, YvrI seems focused on the transcription of the *oxdC* gene. The approximately 3-kb *yvrI-yvrHa-oxdC-yvrL* gene region represents a self-contained transcription cassette encoding a devoted σ factor gene (*yvrI*) and the only three known target promoters for YvrI ($P_{oxdC\text{-}yvrL}$, P_{yvrJ}, and $P_{yvrI\text{-}yvrHa}$). This gene cluster includes two genes (*yvrHa* and *yvrL*) whose products act as regulators of YvrI-dependent transcription.

Since YvrI seems to be an important component of the acid stress response in *B. subtilis*, we wished to determine how broadly distributed this YvrI-based transcription locus is in other bacterial genomes. A BLASTp analysis of the nonredundant protein database revealed 16 instances of the *yvrI-yvrHa* operon, all within the bacilli (Table 2). In only four of these species (*B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilis*) is the full genetic context conserved (Fig. 3A). All 16 species carry *oxdC* homologs, but only in the above-named four instances is this target gene found adjacent to the *yvrI-yvrHa* operon. *yvrL*, a negative regulator of YvrI, was identified only in these four species (Table 2) and is always downstream of (and probably transcriptionally linked to) *oxdC*, as was previously shown for *B. subtilis* (25). After *oxdC*, *yvrJ* is the most broadly distributed gene from this region in bacteria and is found throughout the bacilli and in many clostridial species. *yvrJ* is found in 12 of the 16 genomes listed in Table 2 but, in many cases, at dispersed positions on the chromosome. *Lysinibacillus sphaericus* and *Bacillus* sp. strain 14905 retain a *yvrJ* gene in the same position as that in *B. subtilis*, but this open reading frame (ORF) is not followed by the *oxdC-yvrL* operon (Fig. 3B). In *B. cereus* AH1134, *yvrJ* is on the opposite strand (as it is in *B. subtilis*) but is immediately downstream of the *yvrI-yvrHa* operon rather than upstream (Fig. 3C). Interestingly, in *Bacillus* sp. strain NRRLB-14911, *yvrJ* is embedded

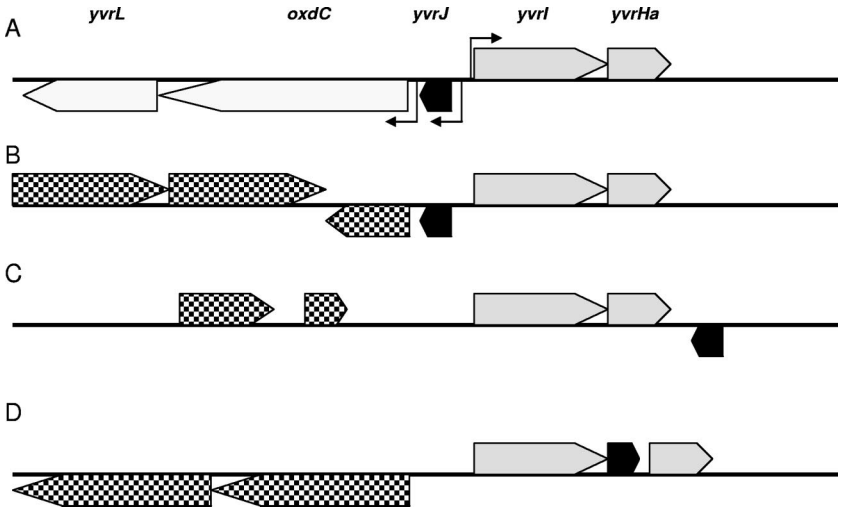

FIG. 3. Genetic context of *yvrI* in bacilli. (A) The gene order is shown as originally described for *B. subtilis* but also conserved in *B. pumilis*, *B. licheniformis*, *and B. amyloliquefaciens*. Positions of experimentally verified YvrI-dependent promoters in *B. subtilis* (angled arrows) are positionally conserved in all four species. Genetic contexts are also shown for *L. sphaericus* and *Bacillus* sp. strain 14905 (B), *B. cereus* AH1134 (C), and *Bacillus* sp. strain NRRLB-14911 (D). All other species listed in Table 1 either lack or have alternative genomic positions for *yvrJ* (black ORFs), *oxdC*, and *yvrL*. Genes upstream of the *yvrI-yvrHa* operon (grey ORFs) shown in panels B, C, and D (checkered ORFs) are not positionally conserved among these species. Gene position in panel B is for *Bacillus* sp. strain 14905.

```
BsuYvrI     GAAAGTAGTAGTTTACTTTTTTTTGCTTTC-CTTCATTTAATTTAGTAG--- 48
BamYvrI     GAAAGGAGTAGTTTACTTTTTTTTGCTTTC-TTTCATTTAATTTAAATA--- 48
BliYvrI     GAAGAGATGTGTTTACTTTTTTTTCATTTC-TTTCATCTAAATAAATAG--- 48
BpuYvrI     GTCTCACAAAGTTTACTTTTTTTAACTTTT-GTCCACTTAATGCAAGTA--- 48
BsuYvrJ     CTTTGCAGCAGTTTACGTTTTATCGGTTTT-CCTCACTTACATACATGA--- 48
BamYvrJ     GGGATATCAGGTTTACAGGAGCCGCTCTTTCTTTCACTTATATACATGA--- 49
BliYvrJ     ATAGTTCTTTGCTTACATTTTTCAAACTTT-CGTCACTTCTTAACATAG--- 48
BpuYvrJ     CTCCCCCTTTGATTTCCTATTTCTTCTTTCATACCACTTATAGA-GAAG--- 48
BsuOxdC     TTCAATCGAAGTTGACTTTTCACTGGTTTT-TTTCACTTAACAAAACAG--- 48
BamOxdC     TTCACACGAAGTTGACTTTTCATCGGAACG-CATCACTTAACAAAGCAG--- 48
BliOxdC     GAAACCGATGATTGACATCGGCCTCACTTT-TTTCACTTAACATGACAG--- 48
BpuOxdC     GATGAATCAGATTGACATCTTCTTGATTCG-TTTCACCTTATGAAAACG--- 48
cons                  gtT aC t t       tt    tCActTa     a
```

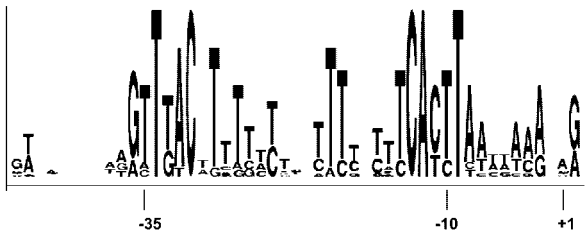

FIG. 4. ClustalW alignment of known *B. subtilis* (Bsu) YvrI-dependent promoters and predicted promoters from *B. pumilis* (Bpu), *B. licheniformis* (Bli), and *B. amyloliquefaciens* (Bam). The consensus sequence is based upon 100% conservation (uppercase letters) and ≥75% conservation (lowercase letters). A WebLogo depiction of sequence conservation in sequences as aligned with ClustalW is shown. Nucleotide positions are indicated.

between *yvrI* and *yvrHa* on the same strand and appears to be part of the *yvrI-yvrHa* operon in this species (Fig. 3D). The remaining eight genomes have vastly different gene organizations around the *yvrI-yvrHa* locus, indicating that considerable genetic reorganization has occurred.

We next searched the genomes of these bacteria for candidate YvrI-dependent promoters by using either a position weight matrix approach or by manual inspection. The putative YvrI-dependent promoter sequences were recognizable and positionally conserved only for the four species with conserved genetic context (Table 2; Fig. 3A) compared with those previously identified in the *B. subtilis* genome (Fig. 4). We were unable to identify obvious candidate promoters in the remaining genomes, raising the possibility that in addition to diversity in gene synteny, sequences acting as YvrI-dependent promoters have also undergone divergence.

Broadened consensus sequence for YvrI-dependent promoters. Using the established promoter consensus deduced from *B. subtilis*, we identified probable promoter elements (P_{oxdC}, P_{yvrJ}, and P_{yvrI}) upstream of the *oxdC*, *yvrJ*, and *yvrI* homologs in *B. licheniformis*, *B. amyloliquefaciens*, and *B. pumilis* (Fig. 4). A sequence alignment of these predicted and experimentally verified promoters (as visualized using WebLogo) reveals strongly conserved residues around the −10 and −35 regions of these sequences (Fig. 4) and additionally reveals that the spacer region is thymidine (or, more generally, pyrimidine) rich, as was originally noted for the *B. subtilis* promoters (25).

To ascertain whether these candidate promoters are active, we generated *lacZ* transcriptional fusions to each of the four *oxdC* promoters (the most active of the three YvrI-dependent promoters in *B. subtilis*) and integrated these constructs into a *B. subtilis* host that could conditionally overexpress *B. subtilis* YvrI. In each of these cases, xylose induction of YvrI resulted in the activation of transcription (Fig. 5, left panel). Under these same conditions but with a host carrying a deletion in the *yvrHa* gene, transcription was not observed (Fig. 5, right panel). Therefore, as in *B. subtilis*, transcription from heterologous P_{oxdC} elements is activated by YvrI and depends on the coregulator of transcription, YvrHa. A broadened consensus for YvrI-dependent promoters will assist in the analysis of nucleotide recognition and topology requirements for the activation from these unusual promoters.

The high activity of the *oxdC* promoter requires an upstream activating sequence. In *B. subtilis*, P_{oxdC} is the most

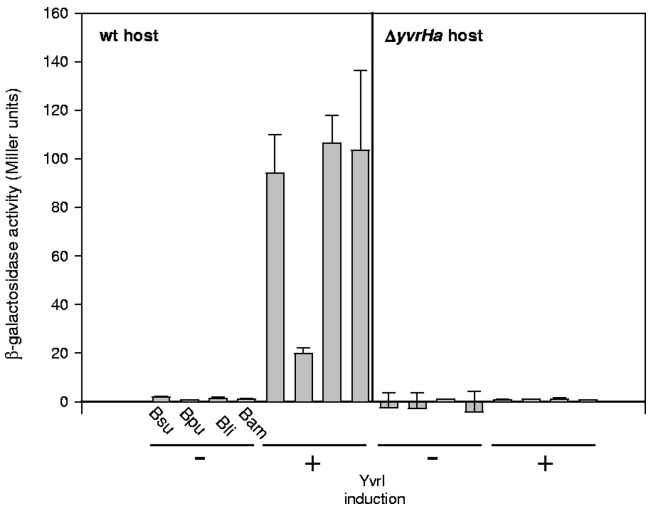

FIG. 5. Activity of heterologous $P_{oxdC\text{-}lacZ}$ fusions in *B. subtilis*. Fusions to the *B. subtilis* (Bsu), *B. pumilis* (Bpu), *B. licheniformis* (Bli), and *B. amyloliquefaciens* (Bam) promoter P_{oxdC} were assessed in the absence (−) and presence (+) of YvrI induction from an ectopic location in wild-type (wt) host cells (left panel) and in a host carrying a *yvrHa* deletion (right panel). Cells were grown to an OD_{540} of 0.4 before induction for 1 h with 2% xylose.

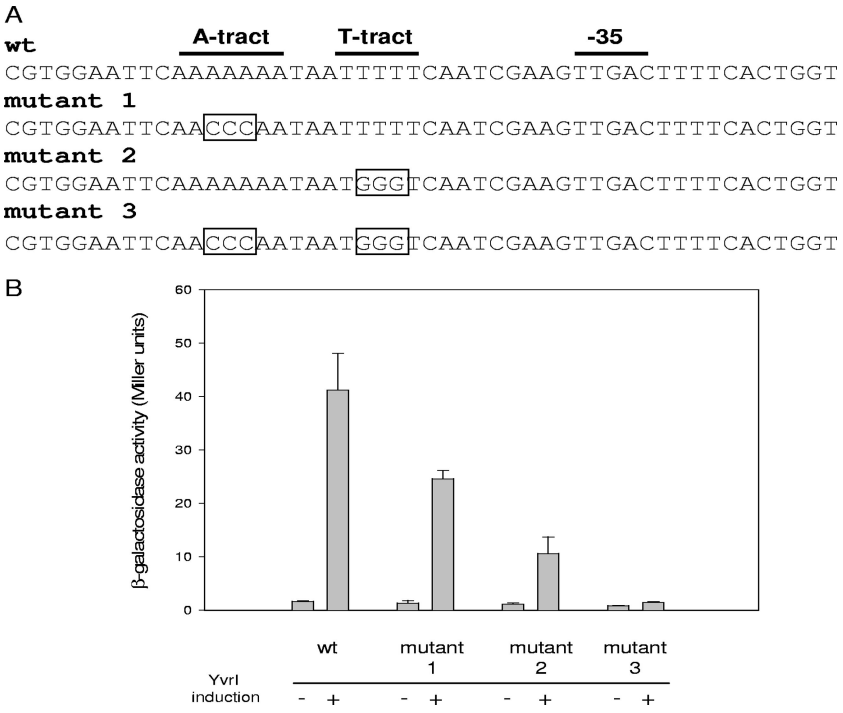

FIG. 6. Influence of the upstream regulatory sequence on *B. subtilis* P_{oxdC} activity. (A) Partial sequence of the P_{oxdC} promoter region showing the −35 region, the T tract and A tract, and the mutant derivatives of these tracts. Each promoter region is transcriptionally fused to a promoterless *lacZ* gene and integrated into *B. subtilis* carrying an ectopically integrated *yvrI* gene that can be conditionally induced in the presence of xylose. (B) Activity of the wild type (wt) and the mutant derivatives of P_{oxdC} in the absence (−) and presence (+) of YvrI induction. Cells were grown to an OD_{540} of 0.4 before induction for 1 h with 2% xylose.

active YvrI-dependent promoter and is approximately 100-fold more active than the autoregulated *yvrI* promoter (25). As noted previously, the three YvrI-YvrHa-activated promoters share similar sequences in their −35 and −10 regions. Promoter strength is a complex reflection of the numerous interactions between RNA polymerase and the promoter recognition region, and, in general, it is difficult to discern those features that contribute to promoter strength by simple inspection. As a general trend, promoters that closely match the consensus (presumed optimal) sequences in the −35 and −10 elements are stronger than those that do not. However, for some very strong σ^{70}-dependent promoters, if the −35 and −10 elements are changed so they are identical to the consensus, promoter activity decreases as transcription initiation becomes limited by promoter clearance. Since the consensus elements for YvrI-dependent promoters have not yet been experimentally investigated, the relationship between these elements and promoter strength is not yet clear.

One factor that can strongly increase the activity of promoters recognized by several different classes of holoenzyme is the upstream promoter (UP) element (28). UP elements are characterized by phased A- and T-rich sequences upstream of the −35 element, and they interact directly with the carboxyl-terminal domains of the alpha subunits to increase RNA polymerase binding (1, 13, 29). We observed that P_{oxdC} from *B. subtilis* and *B. amyloliquefaciens* (and to some extent from *B. licheniformis* and *B. pumilis*) carries a candidate UP element that is not present upstream of the weaker *yvrI* and *yvrJ* promoters (see Fig. S3 in the supplemental material). We therefore hypothesized that this sequence motif might contribute to the high transcriptional activity of this promoter and therefore ultimately to the high-level expression of the OxdC protein.

Using site-directed mutagenesis, we introduced transversion mutations into either the A or the T tract, or both, and monitored promoter strength, using a *lacZ* reporter fusion. Individually, these mutations reduced promoter activity to 60% and 29% of the wild type, respectively (Fig. 6B). When these mutations were combined, they further reduced activity to about 5% of that observed for the wild-type sequence. Therefore, the high level of transcription from P_{oxdC} appears to depend, in large part, upon this upstream element.

DISCUSSION

We have established that the YvrI alternative σ factor and its coregulators YvrHa and YvrL are critical for the regulation of OxdC in response to acidic growth conditions. OxdC has been proposed to play a role in pH homeostasis since the decarboxylation of oxalate to formate results in proton consumption (33). In *Escherichia coli*, there are three rather well-characterized amino acid-dependent acid resistance systems that contribute to survival after exposure to low-pH conditions (14). In one of the systems, the decarboxylases GadA and GadB are expressed in stationary phase or can be induced by acidic conditions in minimal medium. These enzymes catalyze the proton-consuming conversion of glutamate into γ-aminobutyric acid. This activity contributes to survival at a pH level as low as 2.5 by maintaining a considerably higher cyto-

plasmic pH (22, 23). An antiporter, GadC, imports glutamate while it exports the decarboxylation product γ-aminobutyric acid (14). An extracytoplasmic location for OxdC in *B. subtilis* could suggest that these cells are modulating extracytoplasmic pH in the local environment of the cell wall. An extracytoplasmic location may also position this enzyme for a detoxifying role in a soil environment replete with oxalate-rich plant biomass. There are other bacteria, such as *Oxalobacter formigenes*, which activate oxalate to oxalyl-coenzyme A, which is then degraded by the oxalyl-coenzyme A decarboxylase (27). Whether OxdC is involved in pH homeostasis or other processes is not yet known, and, to date, we have not detected a phenotype for the *oxdC* mutant strain. However, it is clear that the expression of OxdC is heavily regulated by a devoted σ factor-based transcriptional circuit, and ultimately, it becomes the most abundant protein in the cell wall proteome under acid stress conditions. A previous proteomic analysis using *Synechocystis* sp. strain PCC 6803 showed that its OxdC homolog was induced and secreted into the periplasm when cells were grown under acidic conditions (20).

The mechanisms linking induction of OxdC to growth under low-pH conditions are not yet clear but likely involve YvrL. YvrL is a predicted membrane protein postulated (25) to function as an anti-σ factor (17, 19) for YvrI. If that is correct, then this protein will be a pivotal component of the signal transduction pathway (11) leading from an external signal (low pH) to an internal response (YvrI activity) in *B. subtilis*.

We have investigated the distribution of this newest member of the σ factor family in *B. subtilis*. Out of 84 finished and unfinished genomes of the order *Bacillales*, the *yvrI-yvrHa* operon is rather narrowly restricted to sixteen genomes. In *B. subtilis*, the *yvrI* gene is the central component of this transcription cassette, composed of *yvrI*, two accessory genes (*yvrHa* and *yvrL*) whose products regulate the activity of YvrI, and two additional target genes, *oxdC* and *yvrJ*. This transcription cassette is also conserved in the genomes of *B. pumilis*, *B. licheniformis*, and *B. amyloliquefaciens*. Many of the other 16 genomes carrying the *yvrI-yvrHa* operon have lost the proximal association between this operon and the *oxdC-yvrL* operon. A rearranged but enduring association between *yvrJ* and the *yvrI-yvrHa* operon in most of these species, however, may indicate a role for YvrJ in the regulation or manifestation of YvrI activity, but we have yet to identify a phenotype for the *yvrJ* mutant.

Sequence comparisons identified nine additional candidate YvrI-dependent promoters from three of these species, and the activity of those elements preceding the *oxdC* orthologs was confirmed experimentally. Alignment of these known and putative YvrI-dependent promoters confirmed the previously assigned −35 and −10 recognition elements and additionally identified two other notable features. First, these known and candidate YvrI-dependent promoters typically contain spacer regions very rich in pyrimidine. Second, those promoters preceding the highly active *oxdC* promoter are associated with sequences predicted to function as UP elements (1, 18, 28, 29). Indeed, mutation analyses indicate that this UP element-like sequence strongly enhances the activity of the *B. subtilis oxdC* promoter element.

Since OxdC accumulates most abundantly in the cell wall proteome fraction, secretion and cell wall targeting of the enzyme might represent another point of regulation. A secreted oxalate decarboxylase has also been described for the fungus *Collybia velutipes* (8). Furthermore, there are two recent studies about periplasmic oxalate decarboxylase homologs in *Agrobacterium tumefaciens* (32) and *Synechocystis* sp. strain PCC 6803, which are exported via the twin arginine translocation (Tat) pathway. The oxalate decarboxylase MncA was identified as the most abundant Mn^{2+}-containing periplasmic protein in *Synechocystis* that folds and entraps Mn^{2+} in the cytoplasm before it is exported via the Tat pathway (34). However, the OxdC protein of *B. subtilis* completely lacks a cleavable N-terminal signal peptide. Thus, the export route by which OxdC reaches the extracytoplasmic compartment is presently unknown. Genetic analyses have, to date, also failed to identify any additional factors needed for the secretion of OxdC. However, our results show that overexpression of the YvrI σ factor in the absence of acid stress in the *yvrL* deletion mutant resulted in the accumulation of OxdC in the cell wall proteome. These results could suggest that OxdC targeting to the cell wall depends either on genes controlled by the YvrI σ or on a constitutively expressed unknown export mechanism. Since the role of YvrJ is presently unknown, we tested the effect of a *yvrJ* deletion on accumulation of OxdC in the cell wall fraction. However, OxdC accumulates normally in the cell wall proteome of the *yvrJ* deletion mutant (see Fig. S1 in the supplemental material). Finally, it was previously reported that a deletion in the two-component regulatory system YvrGHb abolished transcription of the *yvrI-yvrHa* operon and of operons encoding the major cell surface proteins WapA and WprA (30). Our results showed that the *yvrGHb* deletion did not affect accumulation of OxdC in the cell wall proteome (see Fig. S2 in the supplemental material). However, it did abolish accumulation of WapA, confirming findings of previous studies (30).

We are unaware of another case in which a positive regulator of promoter activation is encoded in the same operon as a σ factor, as is the case for *yvrI* and *yvrHa*. It is also unusual that the gene for YvrL, a predicted membrane protein that is a candidate anti-σ factor cognate for YvrI, is located downstream of and transcriptionally linked to *oxdC*. We previously showed that while *oxdC* and *yvrL* are transcribed as an operon, an attenuator sequence between the genes reduces transcription of *yvrL* relative to that of *oxdC* (25). Although the physiological role of OxdC remains cryptic, the expression of this highly abundant protein is heavily regulated and the YvrI-YvrHa-YvrL regulatory system seems devoted to its conditional expression under low-pH conditions.

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