DegU and Spo0A Jointly Control Transcription of Two Loci Required for Complex Colony Development by *Bacillus subtilis*[▽][†]

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Biofilm formation is an example of a multicellular process which depends on cooperative behavior and differentiation within a bacterial population. Our findings indicate that there is a complex feedback loop that maintains the stoichiometry of the extracellular matrix and other proteins required for complex colony development by *Bacillus subtilis*. Analysis of the transcriptional regulation of two DegU-activated genes that are required for complex colony development by *B. subtilis* revealed additional involvement of global regulators that are central to controlling biofilm formation. Activation of transcription from both the *yvcA* and *yuaB* promoters requires DegU~phosphate, but transcription is inhibited by direct AbrB binding to the promoter regions. Inhibition of transcription by AbrB is relieved when Spo0A~phosphate is generated due to its known role in inhibiting *abrB* expression. Deletion of SinR, a key coordinator of motility and biofilm formation, enhanced transcription from both loci; however, no evidence of a direct interaction with SinR for either the *yvcA* or *yuaB* promoter regions was observed. The enhanced transcription in the *sinR* mutant background was subsequently demonstrated to be dependent on biosynthesis of the polysaccharide component that forms the major constituent of the *B. subtilis* biofilm matrix. Together, these findings indicate that a genetic network dependent on activation of both DegU and Spo0A controls complex colony development by *B. subtilis*.

Bacteria are able to differentiate and coordinate activity in their offspring to accomplish complex multicellular processes, such as sporulation, biofilm formation, and swarming motility (1, 34, 44). Such multicellular behaviors are optimally exhibited by undomesticated species of bacteria, and consistent with this, wild isolates of the gram-positive soil bacterium *Bacillus subtilis* grow in sessile communities called biofilms that appear to be complex colonies or air-liquid interface pellicles, whose phenotypic characteristics are a result of cooperative behavior and differentiation (1, 43). Unraveling how *B. subtilis* integrates environmental and regulatory signals to coordinate the complex decision-making processes that precede and control its diverse multicellular behaviors has progressed substantially since the first reports of the biofilm-forming ability of this organism in 2001 (5, 17). To date, several transcriptional regulators have been demonstrated to be regulators involved in the formation of a sessile community, and they were recently examined systematically (reference 22 and references therein). The contributions of four pairs of global regulators, Spo0A/AbrB, DegS/DegU, SinI/SinR, and SlrR/SlrA, now form the working model for how biofilm formation by *B. subtilis* is controlled (9, 24).

Biofilm formation, the transition from a free-swimming state to an adhered state, coincides with the production of an exo-polymeric matrix that surrounds the sessile cells (6, 43). In *B. subtilis* two operons are essential for this process, an operon containing *epsA* to *epsO* (referred to below as the *eps* operon) and *yqxM-sipW-tasA* (referred to below as the *yqxM* operon) (4, 8, 18). The products of the *eps* operon direct the synthesis of the polysaccharide constituent of the extracellular matrix and encode a protein that disables the flagellum motor, rendering the cells nonmotile (3). TasA is a protein component of the extracellular matrix, and its correct localization depends on both SipW and YqxM activity (4, 36). SinR inhibits transcription of both the *eps* and *yqxM* operons by direct promoter binding (8, 20), an interaction that is disrupted by SinI, the antagonist of SinR (2, 20). The pleiotropic regulator AbrB is another negative regulator of biofilm formation (9, 18). AbrB represses transcription of the *eps* and *yqxM* operons via indirect and direct mechanisms, respectively (9, 18, 37). It additionally directly inhibits the transcription of the *yxaAB* operon, in which *yxaB* codes for a putative exopolysaccharide synthase that is able to restore biofilm formation by a *sigB* mutant when it is overexpressed (31). Both of these pathways are activated by Spo0A through its role in activating transcription of *sinI* and inhibiting transcription of *abrB* (9).

DegU is a response regulator which needs to be phosphorylated by its cognate sensor kinase, DegS, to activate biofilm formation (23, 42). Determination that DegU was required for production of the exopolymer poly-γ-glutamic acid, an extracellular polymer that can contribute to biofilm formation, indicated that DegU has further roles during biofilm formation (35). Recently, we and other workers have presented evidence demonstrating that DegU controls and discriminates between multicellular phenomena, including swarming motility, biofilm formation, and protease production, via a gradient in its phos-

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phorylation level. Two novel DegU-regulated genes were identified; in strain NCIB3610 *yvcA* is required for complex colony architecture, and in strain ATCC 6051 *yuaB* is required for pellicle formation (23, 42). These genes encode a putative membrane-bound lipoprotein (YvcA) and a small secreted protein (YuaB), but the exact contribution that these two proteins make to biofilm formation is not understood yet.

Here we describe the complex, but strikingly similar, regulatory control of *yvcA* transcription and *yuaB* transcription. For both genes we show that Spo0A~P exerts positive control through repressing *abrB* transcription, thus enabling DegU~P-dependent activation of transcription. Direct binding of AbrB to both the *yvcA* and *yuaB* promoter regions was confirmed in vitro. We ascribe an indirect regulatory role to SinR since we observed that transcription of *yvcA* and *yuaB* is elevated in the absence *sinR*, but we have shown that SinR does not bind to the *yvcA* and *yuaB* promoter regions. The increased level of *yvcA* and *yuaB* transcription in the *sinR* mutant was shown to be dependent on production of the exopolysaccharide synthesized by the products of the *eps* operon, and it is proposed that an intermediate in the intracellular sugar nucleotide cascade that is involved in exopolysaccharide synthesis has a positive effect on transcription of *yvcA* and *yuaB* via a mechanism that so far has not been identified.

MATERIALS AND METHODS

General strain construction and growth conditions. The *B. subtilis* strains used and constructed in this study are described in Table 1. *Escherichia coli* strain MC1061 [F′ *lacI*^q *lacZM15* Tn*10* (*tet*)] was used for construction and maintenance of plasmids (Table 2). *B. subtilis* JH642 derivatives were generated by transformation of competent cells with plasmids using standard protocols (19). SPP1 phage transduction for introduction of DNA into *B. subtilis* NCIB3610 was conducted as described previously (21). Both *E. coli* and *B. subtilis* strains were routinely grown in Luria-Bertani (LB) medium (10 g NaCl per liter, 5 g yeast extract per liter, 10 g tryptone per liter) or MSgg medium (5 mM potassium phosphate and 100 mM morpholinepropanesulfonic acid [pH 7.0] supplemented with 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, and appropriate amino acids at a final concentration of 50 μg ml^{-1} [5, 8]) at 37°C. When appropriate, antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}; erythromycin, 5 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; and spectinomycin, 100 μg ml^{-1}. Growth of complex colonies and observation of colony architecture were performed essentially as described previously (5, 42).

Plasmid construction. The 300-bp region upstream of the *yuaB* translational start site and the 240-bp region upstream of the *yvcA* translational start site were amplified from NCIB3610 genomic DNA using primer pairs NSW614/NSW615 and NSW408/NSW19, respectively (Table 3). The resulting PCR fragments were ligated into the BamHI and EcoRI sites located upstream of the *lacZ* gene in the *thrC* integration vector pDG1663 using the restriction sites incorporated in the primers to generate pNW500 (*yuaB*) and pNW209 (*yvcA*) (14) (Table 2). Plasmids pNW209 and pNW500 were introduced by selection for double-crossover recombination at the *thrC* locus into JH642 and transferred into the chromosome of NCIB3610 by phage transduction (Table 1).

Introduction of *sinR* into pET28a(+) to generate an N-terminal His$_6$ tag fusion was achieved by amplification of the *sinR* coding region from the chromosome of NCIB3610 using primers NSW368 and NSW369 (Table 3). The resulting PCR product was ligated into the NheI and XhoI sites of pET28a(+) to generate pNW90 (Table 2). The plasmid was sequenced to ensure that PCR-generated mutations were not present.

Plasmid pNW518 (Table 2) used to complement the *yuaB* mutation was generated by amplifying the *yuaB* coding sequence, including the ribosome binding site, from NCIB3610 genomic DNA using primers NSW626 and NSW645 (Table 3). The resulting PCR fragment was introduced into pDR111 using the HindIII and SphI restriction sites incorporated into the primers. The plasmid was sequenced to ensure that the sequence was correct and was subsequently introduced into JH642 at the *amyE* locus to generate strain NRS2283 (Table 1).

Strain construction. In order to delete *sinR*, long-flanking homology PCR based on a previously described method (27) was used. The region overlapping the translational start codon of *sinR* was amplified using primers NSW148 (5′-CAGGCGCTGAAAACCTTGTATCAACC-3′) and NSW149 (5′-**ATCACCTC AAATGGTTCGCTG**CCAATC<u>AA</u>TGTCATCACCTTCC-3′), and the region overlapping the termination stop codon of *sinR* was amplified using primers NSW150 (5′-**CGAGCGCCTACGAGGAATTTGTATCG**GGGAG<u>TAG</u>TGCCTGA GCAGAG-3′) and NSW151 (5′-GATGCAGCGGCTGCTGAAAAAATC-3′). The translational start is indicated by single underlining, and the translational termination codon is indicated by double underlining; bold type indicates sequences homologous to the primer sequences used to amplify the kanamycin resistance gene. The kanamycin resistance gene was amplified from plasmid pSac-Kan using primers NSW152 (5′-CAGCGAACCATTTGAGGTGATAGG-3′) and NSW153 (5′-CGATACAAATTCCTCGTAGGCGCTCGG-3′). The purified fragments were combined using a *sinR* 5′ PCR product/kanamycin resistance gene/*sinR* 3′ PCR product ratio of 1:2:1 in a PCR mixture containing primers NSW148 and NSW151 and LA *Taq* (TaKaRa). The resulting PCR fragment was transformed into competent JH642 cells, and the resulting colonies were screened by PCR to ensure that there was double recombination at the *sinR* locus (NRS1858). The mutated *sinR* gene was then transferred into NCIB3610 by phage transduction, with selection for kanamycin resistance (NRS1859).

β-Galactosidase assay. Biofilm growth medium (LB medium supplemented with 124 mM potassium phosphate [pH 7.0], 15 mM ammonium sulfate, 3.4 mM sodium citrate, 1 mM $MgSO_4$, and 0.1% glucose) was inoculated to obtain an optical density at 600 nm (OD$_{600}$) of 0.01 using a preculture grown to an OD$_{600}$ of ~0.5 in LB medium. Cultures were grown in 250-ml Erlenmeyer flasks or in glass tubes (1.5 by 15 cm). When required, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added after 60 min of growth. At the time points indicated, the OD$_{600}$ was determined and 500 μl of cell suspension was harvested by centrifugation. Each resulting cell pellet was stored at −20°C for subsequent enzymatic analysis, and the β-galactosidase assay was performed as described previously (29). On solid medium colonies were grown on MSgg medium plates containing 1.5% agar supplemented with threonine (40 μg/ml) for 18 h. The colonies were collected from each plate, suspended in 300 μl of Bug Buster Mastermix (Novagen), and incubated for 15 min at room temperature with gentle shaking. Subsequently, the cells were vortexed for 10 min after addition of glass beads to further disrupt the cells. The suspension was centrifuged at 16,000 × *g* for 20 min at 4°C to pellet the cell debris. The protein concentration of the cell lysate supernatant was determined using a Bradford protein assay (Pierce). β-Galactosidase activity (in Miller units) was calculated as described above relative to the protein concentration in the sample. The values presented below are the average β-galactosidase activities determined for six colonies isolated in three independent experiments and normalized to the protein concentration (in mg ml^{-1}); the standard errors of the means are also indicated.

Purification of AbrB and SinR. AbrB and His$_6$-SinR were purified basically by using previously described procedures (20, 38). The His$_6$ tag of His$_6$-SinR was removed using Novagen's thrombin cleavage capture kit.

Electrophoretic mobility shift assay. Protein-DNA gel shift assays were performed with purified SinR and AbrB. The promoter regions assessed were the region from bp −300 to 8 for *yuaB* (generated using primers NSW617 and NSW618), the regions from bp −304 to 50 (generated using primers NSW416 and NSW19) and from bp −121 to 50 (generated using primers NSW410 and NSW19) for *yvcA* (P$_{300}$ and P$_{120}$), the region from bp −302 to −1 (generated using primers NSW50 and NSW51) for *yqxM* as a positive control, and (for AbrB) the region from bp −388 to 47 bp (generated using primers NSW421 and NSW422) for *recA* as a negative control. The positions are relative to the translation start codon, and the A of the ATG codon represented bp 1. ^{32}P-labeled PCR fragments were generated by labeling one primer per primer pair (Table 3) prior to PCR amplification using [γ-^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). Binding reactions were conducted in 30-μl (total volume) mixtures for 20 min at room temperature. For SinR the reaction mixtures contained 10 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 50 ng μl^{-1} bovine serum albumin, 50 ng μl^{-1} poly(dI-dC), 0.5 to 1 ng ^{32}P-labeled DNA, and the amounts of SinR indicated below. For AbrB the reaction mixtures contained 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 5% glycerol, 0.05% Nonidet P-40, 0.5 mM dithiothreitol, 50 ng μl^{-1} bovine serum albumin, 50 ng μl^{-1} poly(dI-dC), 0.5 to 1 ng ^{32}P-labeled DNA, and the amounts of AbrB indicated below. Reaction samples (15 μl) were loaded onto a 5% polyacrylamide 0.5× Tris-borate-EDTA gel. Gels were run at 100 V, and after drying they were autoradiographed by exposure to a Fujifilm phosphorimaging plate or X-ray film.

TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype or description[a]	Reference, source, or construction[b]
JH642	*trpC2 pheA1*	32
NCIB3610	Prototroph	BGSC
BAL679	*trpC2 pheA1 spo0A*(D56N) (*spec*)	17
BAL373	*trpC2 pheA1 abrB*::*cat*	Grossman lab collection
BAL2340	*trpC2 pheA1 epsA*::pBL584(Φ[*Pspac-hy*-epsA-O]) (*cat*)	Lazazzera lab collection
BAL2423	*trpC2 pheA1 sinR*::*cat*	Lazazzera lab collection
BAL2428	*trpC2 pheA1 epsG*::pBL601 (*spec*)	Lazazzera lab collection
DS91	NCIB3610 *sinI*:*spec*	20
DS93	NCIB3610 *sinIR*::*spec*	20
W939	ATCC 6051 *yuaB*::*cat*	23
NRS1314	NCIB3610 *degU*::pBL204 (*cat*)	42
NRS1326	NCIB3610 *amyE*::*Pspank-hy-degU-lacI* (*spec*) *degU*::pBL204 (*cat*)	42
NRS1327	NCIB3610 *amyE*::*Pspank-hy-degU146-lacI* (*spec*) *degU*::pBL204 (*cat*)	42
NRS1390	NCIB3610 *yvcA*::pNW34 (*cat*)	42
NRS1502	NCIB3610 *epsG*::pBL601 (*spec*)	BAL2428 → 3610
NRS1603	*trpC2 pheA1 thrC*::*PyvcA-lacZ* (*erm*)	pNW209 → JH642
NRS1608	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*)	SPP1 NRS1603 → 3610
NRS1628	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *sinR*::*cat*	SPP1 BAL2423 → NRS1608
NRS1631	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *spo0A*(D56N) (*spec*)	SPP1 BAL679 → NRS1608
NRS1644	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *abrB*::*cat*	SPP1 BAL373 → NRS1608
NRS1685	NCIB3610 *epsA*::pBL584(Φ[*Pspac-hy-epsA-O*]) (*cat*)	SPP1 BAL2340 → 3610
NRS1858	*trpC2 pheA1 sinR*::*kan*	PCR → JH642
NRS1859	NCIB3610 *sinR*::*kan*	SPP1 NRS1858 → 3610
NRS2051	*trpC2 pheA1 thrC*::*PyuaB-lacZ* (*erm*)	pNW500 → JH642
NRS2052	NCIB3610 *thrC*::*PyuaB-lacZ* (*erm*)	SPP1 NRS2051 → 3610
NRS2053	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *spo0A*(D56N) (*spec*)	SPP1 NRS2051 → NRS1545
NRS2054	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *epsG*::*spec*	SPP1 NRS2051 → NRS1502
NRS2055	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *sinI*::*spec*	SPP1 NRS2051 → DS91
NRS2056	NCIB3610 *amyE*::*Pspank-hy-degU146-lacI* (*spec*) *degU*::pBL204 (*cat*) *thrC*::*yuaB-lacZ* (*erm*)	SPP1 NRS2051 → NRS1327
NRS2223	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *sinR*::*kan*	SPP1 NRS2051 → NRS1859
NRS2224	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *degU*::pBL204 (*cat*)	SPP1 NRS2051 → NRS1314
NRS2226	NCIB3610 *amyE*::*Pspank-hy-degU-lacI* (*spec*) *degU*::pBL204 (*cat*) *thrC*::*yuaB-lacZ* (*erm*)	SPP1 NRS2051 → NRS1326
NRS2228	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *abrB*::*cat*	SPP1 BAL373 → NRS2052
NRS2230	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *sinIR*::*spec*	SPP1 NRS2051 → DS93
NRS2231	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *epsG*::*spec sinR*::*kan*	SPP1 NRS1858 → NRS2054
NRS2232	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *epsA*::pBL584(Φ[*Pspac-hy-epsA-O*]) (*cat*)	SPP1 NRS2051 → NRS1685
NRS2233	NCIB3610 *thrC*::*yuaB-lacZ* (*erm*) *abrB*::*cat spo0A*(D56N) (*spec*)	SPP1 BAL679 → NRS2228
NRS2067	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *abrB*::*cat spo0A*(D56N) (*spec*)	SPP1 BAL679 → NRS1644
NRS2076	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *epsG*::*spec*	SPP1 BAL2428 → NRS1608
NRS2077	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *epsG*::*spec sinR*::*cat*	SPP1 BAL2423 → NRS2076
NRS2083	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *sinI*::*spec*	SPP1 DS91 → NRS1608
NRS2084	NCIB3610 *thrC*::*PyvcA-lacZ* (*erm*) *sinIR*::*spec*	SPP1 DS93 → NRS1608
NRS2097	NCIB3610 *yuaB*::*cat*	SPP1 W939 → 3610
NRS2283	*trpC2 pheA1 amyE*::*Phy-spank-yuaB* (*spec*)	pNW518 → JH642
NRS2299	NCIB3610 *amyE*::*Phy-spank-yuaB-lacI* (*spec*) *yuaB*::*cat*	NRS2283 → NRS2097

[a] Drug resistance cassettes are indicated as follows: *cat*, chloramphenicol resistance; *kan*, kanamycin resistance; *erm*, erythromycin resistance; *spec*, spectinomycin resistance.

[b] The direction of strain construction is indicated for DNA or the phage (SPP1) recipient strain.

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
pDG1663	*thrC* integration plasmid	14
pDR111	*amyE* integration plasmid	7
pQAB3W	P*tac abrB* (in JM109)	38
pET28a(+)	P_{T7} His$_6$ vector	Novagen
pSac-Kan	*sacA* integration plasmid	28
pMF502	P*spac-hy-gfp*	13
pNW90	$P_{IPTG(T7)}$ *sinR* in pET28a(+)	This study
pNW209	P*yvcA*$_{240}$-*lacZ* in pDG1663	This study
pNW500	P*yuaB-lacZ* in pDG1663	This study
pNW518	*amyE*::*Phy-spank-yuaB-lacI* in pDR111	This study

RESULTS AND DISCUSSION

YuaB is required for complex colony architecture. The DegU-regulated gene *yuaB* is required for pellicle formation by strain ATCC 6051 and is postulated to encode a small secreted protein (23). Transcription of *yuaB* was shown by Northern blot analysis to be enhanced in the presence of DegQ, a finding that indicates that *yuaB* is transcribed by DegU~P (23). However, deletion of *degS* from ATCC 6051 and NCIB3610 results in different swarming motility phenotypes, indicating that there are differences in the DegU regulons of these two strains (23, 42). Additionally, ATCC 6051 has recently been found to differ from strain NCIB3610 (used in

TABLE 3. Primers used in this study

Primer	Target	Sequence (5′ to 3′)[a]	Position[b]
NSW19	yvcA	CGAGGCGAATTCGGATCCCCTGTCAGGGCAAGT	36 to 50
NSW50	yqxM	TGGCGAATTCATAGACAAATCACACATTGTTTGATCA	−302 to −276
NSW51	yqxM	GCCAGAATTCGGATCCATCTTACCTCCTGTAAAACACTGTAA	−26 to −1
NSW74	yvcA	CGAGGCATGCTTATTTCTCCTTTTTATC	709 to 726
NSW368	sinR	AGGAGGCTAGCTTGATTGGCCAGCGTATTAAACAATA	1 to 26
NSW369	sinR	CTCCTCTCGAGCTACTCCTCTTTTTGGGATTTTCTCCAT	309 to 336
NSW408	yvcA	CGAGGCGAATTCAGCCGGATGATACGGCT	−243 to −227
NSW410	yvcA	CGAGGCGAATTCcCAAATTCCGCCATGA	−121 to −106
NSW416	yvcA	CGAGGCGAATTCTGCCGCTGGATGATGT	−304 to −289
NSW421	recA	AAACTCACTGGCAGCGATATCG	−388 to −367
NSW422	recA	TCTATTTGTTTAAGAGCCATATCTAAG	21 to 47
NSW614	yuaB	ATGCGAATTCTCAGCTGGAAAGCTCTAAAGC	−300 to −280
NSW615	yuaB	ATGCGGATCCGCGTTTCATAACAAAATTC	−8 to 8
NSW626	yuaB	AGCTAAGCTTCATTTTTTAGGGGGAATTTTGTTATG	3 to −22
NSW645	yuaB	AACTGCATGCTTAGTTGCAACCGCAAGGCTGA	543 to 525

[a] The restriction enzyme sites used for cloning are underlined.
[b] The primer annealing sites on the chromosome are relative to the A residue (position 1) of the ATG translational start codon.

this study) at at least two locations in its genome, at least one of which influences biofilm formation (24, 25). Therefore, it was necessary to establish whether YuaB activates complex colony development by strain NCIB3610 and to assess if, like *yvcA* transcription, *yuaB* transcription was activated by DegU~P, as DegU is known to have regulatory activity in both its nonphosphorylated and phosphorylated states (10). Highly consistent with the role of DegU and YuaB during pellicle formation in strain ATCC 6051, an NCIB3610-derived strain with a deletion in the single-gene operon *yuaB* (NRS2097) showed impaired complex colony architecture (Fig. 1A). Since the impact on complex colony architecture was greater than the impact on deletion of *yvcA* observed (42), this may indicate that *yuaB* encodes a protein required earlier in the complex colony development process than YvcA (Fig. 1A). The deletion phenotype was efficiently complemented by ectopic expression of the *yuaB* coding sequence under the control of a heterologous promoter from the *amyE* locus (Fig. 1B). While partial complementation was observed in the absence of IPTG, full complementation of the *yuaB* mutation was seen after addition of IPTG at concentrations of 10 and 100 μM. How YuaB functions is not known yet, but one hypothesis is that, like the secreted protein TasA (4), YuaB forms an additional structural component of the extracellular matrix. Alternatively, YuaB may form part of a feedback signaling cascade to regulate gene expression required for complex colony development by *B. subtilis*.

In line with previous findings for *yvcA* (42), transcription of *yuaB* was activated by DegU~P when *lacZ* was used as an in vivo reporter of transcription using a 300-bp *yuaB* promoter region. Deletion of *degU* resulted in an eightfold decrease in *yuaB* transcription which could not be complemented by ectopic expression of a mutant allele of *degU* that cannot be phosphorylated due to a point mutation in the amino acid that is phosphorylated by DegS (*degU*-D56N) (Fig. 1C) (42). In contrast, ectopic expression of the wild-type *degU* gene under the control of the IPTG-inducible promoter P$_{hy-spank}$ (42) resulted in wild-type levels of *yuaB* expression after addition of 5 μM IPTG and levels slightly higher than the wild-type levels after addition of 25 μM IPTG (Fig. 1C). These data indicate

that, like the overall complex colony development process, transcription of *yuaB* is activated by DegU~P (23, 42).

AbrB represses transcription of *yvcA* and *yuaB*. Only a limited number of genes that are known to be directly regulated by DegU have been characterized with respect to additional regulatory control, but what has become apparent from these studies is that multiple global regulators are involved (16, 33). For example, transcription of *aprE*, in addition to being activated by DegU, is controlled by SinR, AbrB, and Spo0A (2, 12). These regulators are all key players in the control of complex colony development by *B. subtilis* (5, 9, 17, 18, 22). Therefore, it was hypothesized that SinR, SinI, AbrB, and Spo0A may have a role in regulating the novel DegU-activated loci, *yvcA* and *yuaB*, that are also required for complex colony development (Fig. 1A). This possibility was addressed using mutant strains carrying *lacZ* as an in vivo reporter of either *yvcA* or *yuaB* transcription. For *yvcA* the 240-bp sequence upstream of the translational start site was used to drive *lacZ* transcription as this region of DNA has been defined as the optimal minimal *yvcA* promoter region (P$_{240}$) (see Fig. S1 in the supplemental material).

Introduction of a mutant allele of *spo0A* encoding a version of Spo0A that cannot be phosphorylated resulted in fivefold-decreased *yvcA* transcription during stationary phase ($P < 0.01$) (Fig. 2B). This finding is consistent with the role of Spo0A~P in activating biofilm formation (5, 17). It was subsequently determined that the lack of *yvcA* transcription observed in the *spo0A* mutant was mediated through its known role in inhibiting *abrB* expression (17, 39). Consistent with this, *yvcA* transcription was observed to be constitutive in an *abrB* mutant strain, and the inhibition of transcription observed in the *spo0A* mutant was bypassed in the *abrB spo0A* double mutant strain (Fig. 2A and 2B). The same phenomenon was observed when transcription from the *yuaB* promoter was examined. Deletion of *spo0A* resulted in an 11-fold ($P < 0.01$) decrease in transcription. In the *abrB* and *spoOA* mutants, the level of transcription was four- to fivefold higher ($P < 0.01$) than the wild-type level, respectively (Fig. 2C). These data indicate that both *yvcA* and *yuaB* are regulated indirectly by Spo0A~P through AbrB. Consistent with these findings, nei-
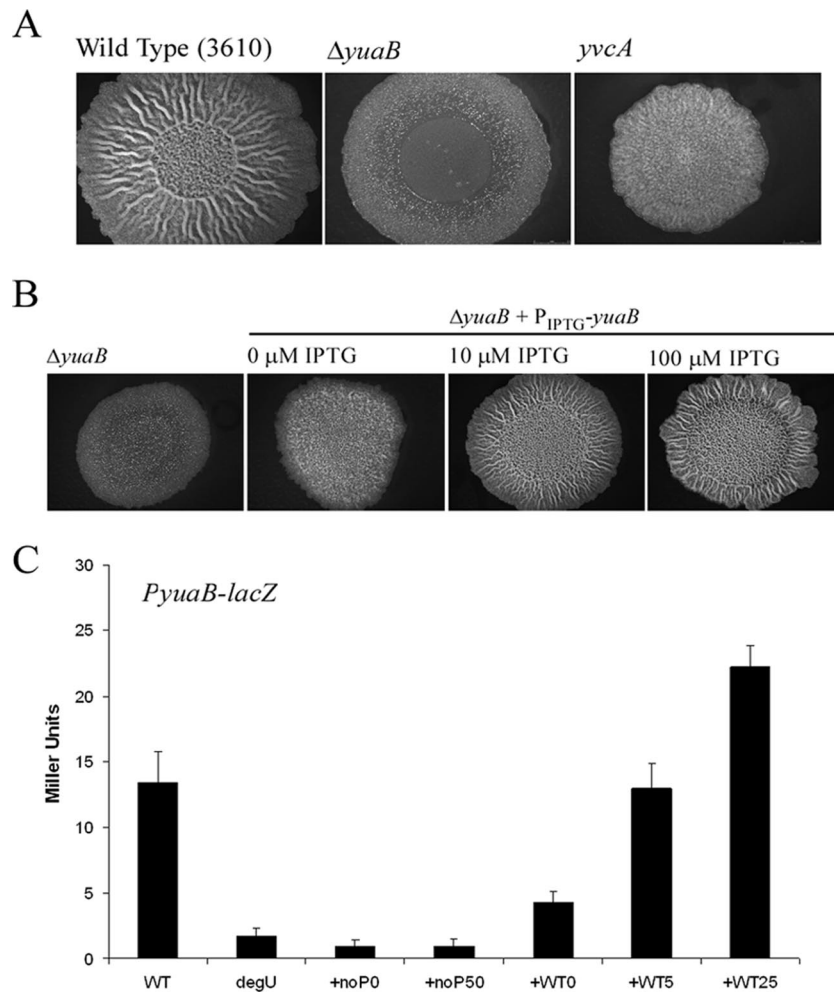
FIG. 1. Complex colony architecture requires DegU-dependent *yuaB* transcription. (A) Complex colony morphology of wild-type strain NCIB3610, *yuaB* mutant NRS2097, and *yvcA* mutant NRS1390 after 48 h of incubation at 37°C on MSgg agar. (B) Complementation of *yuaB* mutant NRS2097 through ectopic expression of *yuaB* under control of a heterologous promoter (NRS2299). Strains were grown in the presence of IPTG as indicated at the top for 48 h at 37°C on MSgg agar. (C) Transcription of *yuaB* (P*yuaB-lacZ*) in wild-type strain NRS2052 (WT), *degU* mutant NRS2224, and the *degU* mutant complemented with the P$_{IPTG}$-*degU*-D56N (NRS2056) and P$_{IPTG}$-*degU* (NRS2226) constructs. The IPTG concentrations used were 0, 5, and 25 μM for the P$_{IPTG}$-*degU* construct (+WT0, +WT5, and +WT25, respectively) and 0 and 50 μM for the P$_{IPTG}$-*degU*-D56N construct (+noP0 and +noP50, respectively). The bars indicate the β-galactosidase activities and the error bars indicate the standard errors of the means obtained for stationary-phase cells in three to five independent experiments.

ther gene was identified as a direct target of Spo0A using chromatin immunoprecipitation experiments (30), but both genes were determined by genome-wide transcriptome analysis to be members of the larger *spo0A* regulon (11).

AbrB binds to the promoter region of *yvcA* and *yuaB*. To establish that the AbrB-mediated repression of transcription was a direct regulatory event, DNA retardation assays with the *yvcA* and *yuaB* promoter regions and purified AbrB were performed. Using *yqxM* as a positive control (37) and the *recA* promoter region as a negative control (15), a direct interaction was demonstrated (Fig. 3). Addition of AbrB at a concentration of 150 nM was sufficient to initiate a promoter DNA-AbrB interaction, while complete *yvcA* and *yuaB* promoter binding was observed in the presence of 250 nM AbrB (Fig. 3). AbrB binding was not detected when only the 120-bp sequence upstream of the translational start site for *yvcA*, a region that also did not show any DegU~P-activated transcription when it was

fused with *lacZ* (see Fig. S1 in the supplemental material), was used. These data indicate that AbrB binds to the region between bp −120 and −240 upstream of the *yvcA* translational start site and binds within the 300-bp region upstream of the translation start site of *yuaB*, and they add *yvcA* and *yuaB* to the growing list of AbrB-inhibited targets that need to be derepressed for complex colony development to occur. These data highlight the key role that AbrB plays in controlling this multicellular behavior exhibited by *B. subtilis* (9, 17, 18, 31).

Deletion of *sinR* enhances transcription of *yvcA* and *yuaB*. Consistent with the role of SinR as a repressor of complex colony architecture (20), deletion of *sinR* resulted in a fourfold increase in transcription from the *yvcA* promoter during exponential growth (Fig. 4A and 4B) and a fourfold ($P < 0.01$) increase in transcription from the *yuaB* promoter throughout growth compared with the wild-type strain (Fig. 5A). SinI is the antagonist of SinR (2), and consistent with this, deletion of *sinI*
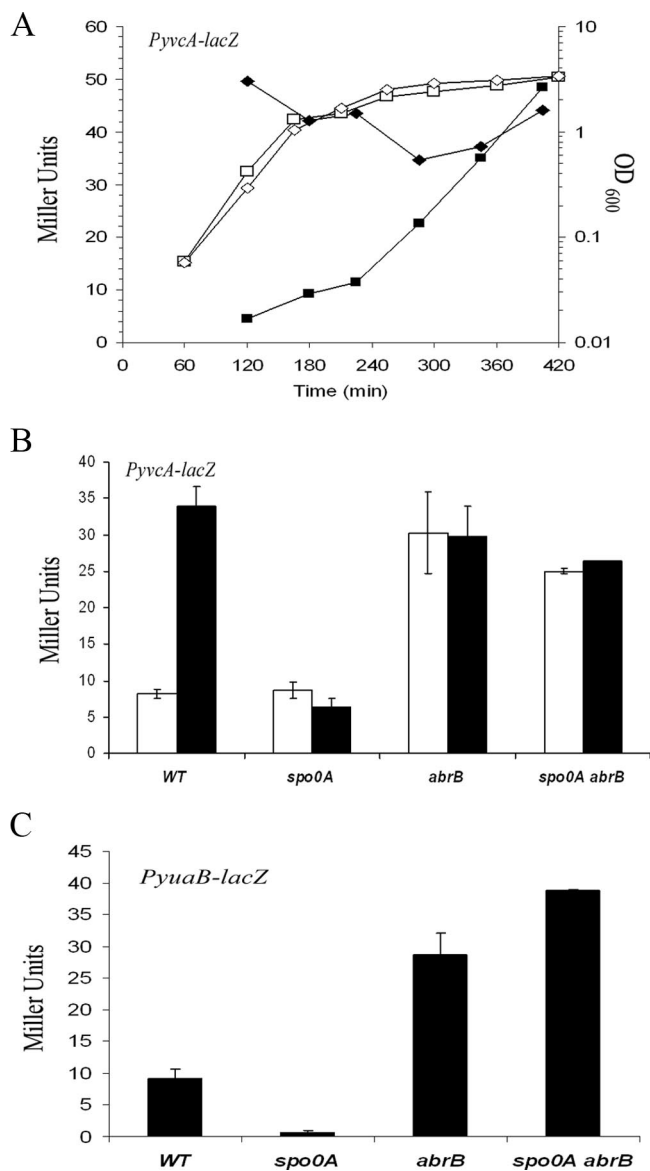
FIG. 2. Transcription of *yvcA* and *yuaB* is activated by Spo0A~P through *abrB* inhibition. (A) Transcription of *yvcA* (P*yvcA-lacZ*) in the wild-type (NRS1608) and *abrB* mutant (NRS1644) backgrounds. The symbols indicate the β-galactosidase activities (in Miller units) (filled symbols) and the growth (open symbols) expressed as OD$_{600}$ for the wild-type strain (squares) and *abrB* mutant (diamonds) strain at different times. (B) Transcription of *yvcA* (P*yvcA-lacZ*) in the wild-type strain (NRS1608) (*WT*), the *spo0A* mutant (NRS1631), the *abrB* mutant (NRS1644), and the *spo0A abrB* mutant (NRS2067). The bars indicate the β-galactosidase activities (in Miller units) and the error bars indicate the standard errors of the means obtained for exponential-phase cells (120 min) (open bars) and for stationary-phase cells (360 min) (filled bars). (C) Transcription of *yuaB* (P*yuaB-lacZ*) in the wild-type strain (NRS2052), the *spo0A* mutant (NRS2053), the *abrB* mutant (NRS2228), and the *spo0A abrB* mutant (NRS2233). The bars indicate the β-galactosidase activities (in Miller units) and the error bars indicate the standard errors of the means obtained for stationary-phase cells (360 min).

resulted in a twofold decrease in transcription for both the *yvcA* and *yuaB* promoters compared with the wild type (Fig. 4B and 5A). As expected, deletion of both *sinR* and its antagonist *sinI* in combination resulted in an increase in transcription

similar to that observed for the single *sinR* deletion (Fig. 4B and 5A). We wanted to ensure that the fourfold-enhanced transcription was not the result of inaccurate assessment of the cell number (and therefore gene transcription) in the *sinR* and *sinIR* mutant strains as shaking cultures of the *sinR* and *sinIR* mutant strains do not have a uniform optical density due to overexpression of components of the biofilm matrix (20). We did not think that this was likely as we used an LB medium-based growth medium for our experiments, in which the final transcriptional level of the *eps* operon in the absence of the repressor *sinR* was suppressed (data not shown). Thus, in this growth medium, the cells still clumped but at a lower level than in MSgg medium (data not shown), and therefore, the growth could be followed (Fig. 2A and 4A). However, in order to eliminate any doubt, the level of expression from the *yvcA* promoter in the presence and absence of *sinR* was calculated after preparation of soluble-protein cell lysates. This allowed normalization of the β-galactosidase activity to protein levels. Using this approach, a 4.5-fold-higher level of *yvcA* transcription in the *sinR* mutant was still observed, indicating that the recorded values were not the result of inaccurate assessment of the cell number (see Fig. S2 in the supplemental material). Our findings indicate that SinR inhibits transcription of both the *yvcA* and *yuaB* DegU-activated promoters. To establish that it was a direct interaction, we used electrophoretic mobility shift assays with purified SinR (Fig. 5C). Using radiolabeled *yvcA* and *yuaB* promoter regions, we were unable to demonstrate any interaction of SinR with either promoter region in the nanomolar range needed to bind to the *yqxM* promoter, a known SinR target (Fig. 5C) (8, 20). Therefore, in contrast to the known role of SinR in repressing *eps* and *yqxM* operon transcription (4, 8, 20), these findings do not support a model in which the inhibition of *yvcA* and *yuaB* transcription by SinR is directly mediated.

Synthesis of the exopolysaccharide is required for enhanced transcription. In the absence of a direct interaction of SinR with the *yvcA* and *yuaB* promoters, we postulated that the increase in transcription of *yvcA* and *yuaB* may be stimulated by the extracellular matrix or an intermediate in the biosynthetic process that is overproduced in both the *sinR* and *sinIR* mutants. To test this hypothesis, the level of transcription from the *yvcA* and *yuaB* promoters was measured in the presence of a mutation in the *eps* operon which prevents exopolysaccharide biosynthesis (20). In an *epsG sinR* mutant strain the *yvcA* transcription was equivalent to that of the wild-type strain (data not shown), and the *yuaB* transcription was 1.7-fold lower than that of the wild-type strain (Fig. 5A). These findings are consistent with the hypothesis that exopolysaccharide production stimulates transcription of these two DegU-activated loci. These experiments were conducted using cells grown in liquid LB medium-based growth medium, and we wanted to ensure that the same pattern of gene expression occurred under complex colony development conditions; therefore, we harvested colonies from solid MSgg medium after 18 h of growth. The level of β-galactosidase activity was normalized to the total amount of protein extracted from the colony. We observed a 2.5-fold increase ($P < 0.0001$) in *yuaB* transcription in the absence of *sinR* compared with the transcription in the wild-type strain (Fig. 5D) and confirmed that the increase in transcription was dependent on the production of the exo-
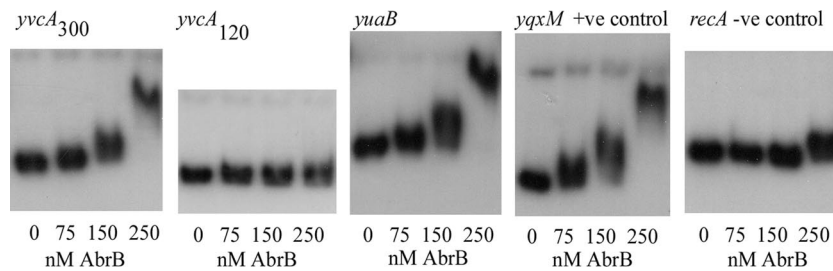
FIG. 3. AbrB is a direct repressor of *yvcA* and *yuaB* transcription. An electrophoretic mobility shift assay was performed using ^{32}P-labeled *yvcA*$_{300}$, *yvcA*$_{120}$, *yuaB*, *yqxM*, and *recA* promoter fragments incubated with AbrB at the concentrations indicated. +ve, positive; −ve, negative.

polymeric matrix since disruption of *epsG* in the *sinR* background resulted in loss of the enhanced level of transcription (Fig. 5D).

To further investigate the hypothesis that synthesis of the polysaccharide component of the biofilm matrix acted as a

FIG. 4. Deletion of *sinR* enhances transcription of *yvcA*. (A) Transcription of *yvcA* (P*yvcA-lacZ*) in the wild-type background (NRS1608) and the *sinR* mutant (NRS1628). The symbols indicate the β-galactosidase activities (in Miller units) (filled symbols) and the growth (open symbols) expressed as OD_{600} for the wild-type strain (squares) and *sinR* mutant strain (diamonds) at different times. (B) Transcription of *yvcA* (P*yvcA-lacZ*) in the wild-type background (NRS1608), the *sinR* mutant (NRS1628), the *sinI* mutant (NRS2083), and the *sinIR* mutant (NRS2084). The bars indicate the β-galactosidase activities (in Miller units) and the error bars indicate the standard errors of the means obtained for exponential-phase cells (120 min) (open bars) and stationary-phase cells (360 min) (filled bars) for three to five experiments.

signal responsible for enhancing *yvcA* and *yuaB* transcription, transcription of the *eps* operon was uncoupled from native regulation by replacing the *eps* operon promoter with a construct that enabled transcription of the *eps* operon from an IPTG-inducible promoter. Substitution of the promoter region allowed wild-type colony morphology to be exhibited in the presence of 30 μM IPTG (Fig. 5B). Using the P_{IPTG}-*eps* operon strain, the level of *yuaB* transcription was measured in the presence and absence of IPTG. Without addition of IPTG to cells carrying the P_{IPTG}-*eps* operon construct, the transcription of *yuaB* during early stationary phase was not significantly different from that measured for the wild-type strain, but addition of 10 μM IPTG resulted in a small but statistically significant increase in transcription (1.7-fold; $P < 0.01$ [data not shown]). Addition of 25 μM IPTG increased transcription of *yuaB* 3.5-fold ($P < 0.05$) during stationary phase to levels comparable to those observed for the *sinR* mutant strain (Fig. 5A). These findings are consistent with the DNA retardation data which indicated that SinR does not bind to the *yvcA* and *yuaB* promoters and could explain why *yvcA* and *yuaB* were not identified as SinR-regulated genes in genome-wide transcriptional profiling studies since those analyses were performed using a *sinR* mutant strain carrying a mutation in the *eps* operon (8). In conclusion, our data indicate that either a product of the *eps* operon, the biofilm matrix itself, or some unidentified regulatory step that requires matrix production enhances transcription of *yvcA* and *yuaB* in the *sinR* mutant strain.

The exopolysaccharide is not an extracellular signal triggering enhanced y*uaB* transcription. It is possible that the exopolysaccharide synthesized by the products of the *eps* operon could enhance transcription of *yvcA* and *yuaB* through recognition by a receptor on the cell surface, which in turn could trigger a signaling cascade within the cell. If this were the case, then overproduction of the exopolysaccharide would be detected for a strain that does not overproduce the exopolysaccharide when it is assessed in coculture experiments. To test this possibility, the level of transcription from the *yuaB* promoter fusion was measured for the wild-type strain that was cocultured at a 1:1 ratio with either the wild-type strain or the *sinIR* mutant strain that lacked the *yuaB* reporter fusion. It was established that the increased level of exopolysaccharide produced by the *sinIR* mutant did not result in enhanced transcription of *yuaB* in the wild-type strain, indicating that the exopolysaccharide did not function as an extracellular signaling molecule (data not shown). It is not known yet how overproduction of the extracellular polysaccharide enhances transcription of the *yvcA* and *yuaB* loci; however, one hypothesis favored by us is that car-
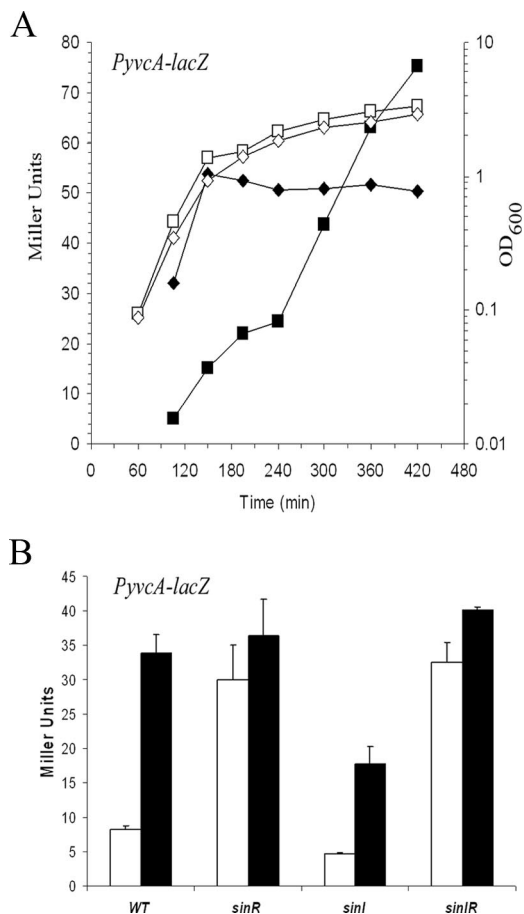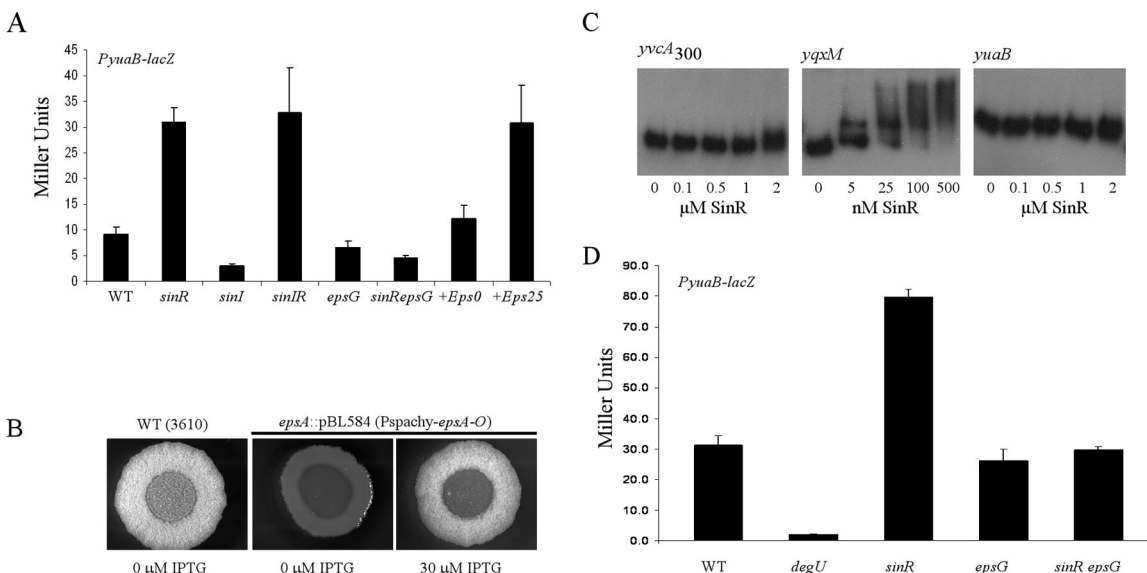
FIG. 5. SinR is not a direct repressor of *yuaB* transcription. (A) Transcription of *yuaB* (P*yuaB-lacZ*) in the wild-type background (NRS2052) (WT), the *sinR* mutant (NRS2223), the *sinI* mutant (NRS2055), the *sinIR* mutant (NRS2230), the *epsG* mutant (NRS2054), the *epsG sinR* mutant (NRS2231), and the P_{IPTG}-*eps* operon strain (NRS2232) with no IPTG (+*Eps0*) and 25 μM IPTG (+*Eps25*). The bars indicate the β-galactosidase activities (in Miller units) and the error bars indicate the standard errors of the means obtained for stationary-phase cells in three to five independent experiments. (B) Complex colony architecture can be restored to the *epsA*::pBL584(Φ[P*spachy-epsA-O*]) strain (P_{IPTG}-*epsA-O*) (NRS1685) in the presence of 30 μM IPTG after incubation for 24 h at 37°C on MSgg medium. (C) Electrophoretic mobility shift assay using ^{32}P-labeled *yvcA*$_{300}$, *yqxM*, and *yuaB* promoter fragments incubated with SinR at the concentrations indicated. (D) Transcription of *yuaB* (P*yuaB-lacZ*) in the wild-type (NRS2052), *degU* mutant (NRS2224), *sinR* mutant (NRS2223), *epsG* mutant (NRS2054), and *epsG sinR* mutant (NRS2231) backgrounds. The bars indicate the β-galactosidase activities (in Miller units) and the error bars indicate the standard errors of the means normalized to the total protein concentration (in mg ml^{-1}) determined using colonies grown on MSgg medium for 18 h in three independent experiments.

bohydrate intermediates in the polysaccharide biosynthetic process are detected intracellularly and used to mediate signal transduction processes. Detection of metabolic intermediates as a means to control gene expression can occur through the activity of *trans*-acting ribozymes, such as the glucosamine-6-phosphate-activated ribozyme encoded by *glmS* (26, 45).

Conclusion. AbrB and SinR are known for their role in inhibiting transcription of the *eps* and *yqxM* operons that are required for biosynthesis of the extracellular matrix of the *B. subtilis* biofilm (9, 20). Here we found that transcription of the DegU-activated genes *yvcA* and *yuaB* is also regulated by AbrB

and SinR. While we were able to clearly demonstrate that the inhibition exerted by AbrB was directly mediated (Fig. 3), we surprisingly also demonstrated that the inhibition exerted by SinR was indirectly mediated through the exopolysaccharide synthesized by the protein products of the *eps* operon (Fig. 5). These findings indicate that a complex feedback loop is established during complex colony development by *B. subtilis* to ensure that the stoichiometry of *yvcA* and *yuaB* transcription is kept in balance with that of the *eps* and *yqxM* operons (Fig. 6). The dual requirements of DegU and Spo0A activation prior to complex colony development are comparable to the regulatory network required to activate exoprotease production by *B. subtilis*. Exprotease production occurs in a small subpopulation of cells that reach threshold levels of DegU~P and Spo0A~P (40, 41). Therefore, our findings add complex colony development to the growing list of multicellular behavioral phenomena exhibited by *B. subtilis* that are subjected to an "AND-gating genetic circuit" that depends on the activation of both DegU and Spo0A (40, 41). Future work will investigate how this regulatory loop is maintained and whether fluctuations in the absolute levels of DegU and Spo0A activation are used as a mechanism to coordinate entry into the different multicellular behaviors that are exhibited by *B. subtilis*.
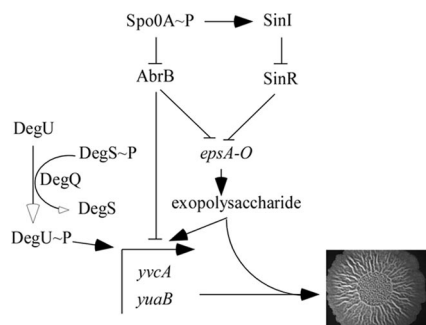

FIG. 6. A complex regulatory network controls biofilm formation. The diagram is a simplified diagram depicting the network that controls *yvcA* and *yuaB* transcription during complex colony development (5, 8, 9, 17, 20). Open arrowheads indicated changes in phosphorylation, filled arrowheads indicate activation of transcription, and T bars indicate inhibition of transcription.

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