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Autoregulation of *swrAA* and Motility in *Bacillus subtilis*▽

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We demonstrate that transcription of the gene *swrAA*, required for swarming migration in *Bacillus subtilis*, is driven by two promoters: a *sigD*-dependent promoter and a putative *sigA*-dependent promoter, which is inactive during growth in liquid Luria-Bertani medium and becomes active in the presence of the phosphorylated form of the response regulator DegU or on semisolid surfaces. Since *sigD* transcription is enhanced by SwrAA, this finding reveals that *swrA* expression is controlled by a positive feedback loop. We also demonstrate that the positive action of SwrAA in swimming and swarming motility is prevented in strains carrying a deletion of the two-component system *degS-degU* and that this effect is independent of *swrAA* transcription. Therefore, both DegU and SwrAA must be present to achieve full motility in *B. subtilis*.

A wild-type copy of the *swrAA* gene is necessary for swarming motility in both undomesticated and laboratory strains of *Bacillus subtilis* (3, 12). Laboratory strains (e.g., 168) that carry an sfp^0 allele and a frameshift mutation in the *swrAA* gene have a nonswarmer (Swr^-) phenotype (3, 12, 13, 14, 31). The role played by *swrAA* in swarming is to enhance transcription of the operon *fla/che*, which contains *sigD*, the gene coding for the alternative sigma factor σ^D, as well as genes necessary for flagellum biosynthesis and chemotaxis (14). This notwithstanding, SwrAA does not resemble a DNA binding protein and does not show any particular feature by in silico analysis, nor does it display any similarity to characterized entries in protein databases, hampering the elucidation of its mechanism of action.

In order to gain insights into the biological role that it plays in the activation of the swarming behavior, our efforts were concentrated on the expression profile of the *swrA* dicistronic operon which contains *swrAA* (3).

The pleiotropic effects on the synthesis of degradative enzymes, competence, sporulation, and motility of mutations in the two-component system DegS-DegU have been extensively described previously (16, 23). In particular, motility is negatively affected by *degS*(Hy) and *degU*(Hy) mutations. These mutations increase the half-life of the phosphorylated form of DegU (DegU~P) (1, 6, 23). In contrast, the necessity of a low level of DegU~P, for motility in general (36) and for swarming in particular (15, 37), has been pointed out for undomesticated and laboratory strains, but its role has yet to be established. Albeit in previous reports *swrAA* was never identified as a DegU-regulated gene (18, 26), it has been recently shown that *swrAA* transcription is negatively affected by deletions of *degU* or *degS* (15).

Here we demonstrate that *swrAA* has two promoters: a σ^D-dependent promoter, active in planktonic growth, and a putative σ^A-dependent promoter triggered by DegU~P, in concordance with published results (15). Furthermore, we show that in the absence of the functional alleles of either DegU or SwrAA *B. subtilis* is unable to fully swim as well as to swarm (12, 37). Although DegU activates *swrAA* transcription, SwrAA overexpression per se does not compensate for the loss of DegU, suggesting that DegU cooperates with SwrAA to achieve complete motility in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* strains used in this study are listed in Table 1. All strains were grown at 37°C in Luria-Bertani broth (LB broth; tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g per liter) (30). Media were routinely solidified with 1.5% agar, unless otherwise indicated. The *Escherichia coli* strains DH5α (*supE44 lacU169* [Δ80*lacZ*ΔM15] *hsdR17*[r_K^- m_K^+] *recA1 endA1 gyrA96 thi-1 relA1*), used for molecular cloning, and BL21(DE3) (F^- *ompT* $hsdS_B[r_B^-$ $m_B^-]$ *gal dcm* [DE3]), used for protein purification, were grown at 37°C in LB broth. When required, media were supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (100 μg/ml), ampicillin (100 μg/ml), kanamycin (2 μg/ml), chloramphenicol (5 μg/ml), phleomycin (5 μg/ml), spectinomycin (60 μg/ml), and erythromycin (1 and 50 μg/ml). When appropriate, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the media.

Genetic techniques. *B. subtilis* strains were transformed with chromosomal or plasmid DNA by the procedure previously described (17). *E. coli* transformation was performed according to standard protocols (30).

Strain construction. All primers used are listed in Table 2. To construct a strain carrying the *swrAA* mutant gene (PB5370), a PCR fragment from PB1831 (31), amplified using primers yvjB-F and yvjD2661, was cotransformed with plasmid pDG148 (35) in PB5249 ($swrAA^+$) and selected for phleomycin resistance. Colonies were screened according to the *swrAA* mutant phenotype (loss of swarming motility): the Swr mutant clones obtained were kept on antibiotic-free medium to facilitate plasmid loss, and the *swrA* locus was sequenced.

For the construction of strains with the in-locus *swrA* promoter mutations, a DNA segment corresponding to the 3′ region of *ctpB* and part of the intergenic region between *ctpB* and *swrAA* was amplified from PB5249 DNA using primers yvjB-F (KpnI site) and yvjB.rev.X (XhoI site). The fragment was inserted between the KpnI/XhoI sites of pJM114 (27), downstream of the kanamycin resistance gene, thus generating pCC0. The region upstream of the *swrAA* open reading frame and the *swrAA* gene itself were amplified using primers UpPromA (EcoRI site) and yvjD3220 (EcoRI site). The amplified product was cloned upstream of the kanamycin resistance gene of pCC0, thus producing $pCCP_{swrA}WT$. Mutations were introduced in $pCCP_{swrA}WT$ by site-directed mutagenesis PCR, using primers DPSigD F and DPSigD R, deleting 3 nucleotides (nt) from the σ^D consensus sequence, and/or primers DPSigA F and DPSigA R, deleting 3 nt from the σ^A consensus sequence. The plasmids obtained are listed in Table 3. $swrAA^+$ strains containing the promoter mutations, PB5392 to PB5395, were produced by transformation of PB5249 with these plasmids linearized with XmnI and by selection for kanamycin resistance. Strains were checked by phenotypic assays and verified by diagnostic restriction digestion with

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

Strain	Genotype	Reference or source
MH5636	*trpC2 pheA1 cat* His_6-*rpoC*; Cm	28
PB5094	*trpC2 swrAA* mutant Δ(*degS degU*); Km	QB4238 (23)
PB5249	*trpC2 pheA1 swrAA*$^+$	31
PB5342	*trpC2 pheA1 swrAA* mutant Δ(*degS degU*); Km	PB5094 → PB5370
PB5343	*trpC2 pheA1 swrAA*$^+$ Δ(*degS degU*); Km	PB5094 → PB5249
PB5370	*trpC2 pheA1 swrAA* mutant	This study
PB5383	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy); Sp	Amati et al., unpublished
PB5392	*trpC2 pheA1* P_{swrA}WT-*swrAA*$^+$; Km	pCCP$_{swrA}$WT → PB5249
PB5393	*trpC2 pheA1* $P_{swrA}A^-$-*swrAA*$^+$; Km	pCCP$_{swrA}A^-$ → PB5249
PB5394	*trpC2 pheA1* $P_{swrA}D^-$-*swrAA*$^+$; Km	pCCP$_{swrA}D^-$ → PB5249
PB5395	*trpC2 pheA1* $P_{swrA}DA^-$-*swrAA*$^+$; Km	pCCP$_{swrA}DA^-$ → PB5249
PB5396	*trpC2 pheA1* P_{swrA}WT-*swrAA* mutant; Km	pCCP$_{swrA}$WT → PB5370
PB5397	*trpC2 pheA1* $P_{swrA}A^-$-*swrAA* mutant; Km	pCCP$_{swrA}A^-$ → PB5370
PB5398	*trpC2 pheA1* $P_{swrA}D^-$-*swrAA* mutant; Km	pCCP$_{swrA}D^-$ → PB5370
PB5399	*trpC2 pheA1* $P_{swrA}DA^-$-*swrAA* mutant; Km	pCCP$_{swrA}DA^-$ → PB5370
PB5400	*trpC2 pheA1 swrAA*$^+$ *pks*::P_{swrA}*WT-lacZ*; Cm	pJM783P$_{swrA}$WT → PB5249
PB5401	*trpC2 pheA1 swrAA*$^+$ *pks*::$P_{swrA}A^-$*-lacZ*; Cm	pJM783P$s_{wrA}A^-$ → PB5249
PB5402	*trpC2 pheA1 swrAA*$^+$ *pks*::$P_{swrA}D^-$*-lacZ*; Cm	pJM783P$_{swrA}D^-$ → PB5249
PB5403	*trpC2 pheA1 swrAA*$^+$ *pks*::$P_{swrA}DA^-$*-lacZ*; Cm	pJM783P$_{swrA}DA^-$ → PB5249
PB5404	*trpC2 pheA1 swrAA* mutant *pks*::P_{swrA}*WT-lacZ*; Cm	pJM783P$_{swrA}$WT → PB5370
PB5405	*trpC2 pheA1 swrAA* mutant *pks*::$P_{swrA}A^-$*-lacZ*; Cm	pJM783P$s_{wrA}A^-$ → PB5370
PB5406	*trpC2 pheA1 swrAA* mutant *pks*::$P_{swrA}D^-$*-lacZ*; Cm	pJM783P$_{swrA}D^-$ → PB5370
PB5407	*trpC2 pheA1 swrAA* mutant *pks*::$P_{swrA}DA^-$*-lacZ*; Cm	pJM783P$_{swrA}DA^-$ → PB5370
PB5408	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy) *pks*::P_{swrA}WT-*lacZ*; Cm Sp	PB5383 → PB5400
PB5409	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy) *pks*::$P_{swrA}A^-$*-lacZ*; Cm Sp	PB5383 → PB5401
PB5410	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy) *pks*::$P_{swrA}D^-$*-lacZ*; Cm Sp	PB5383 → PB5402
PB5411	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy) *pks*::$P_{swrA}DA^-$*-lacZ*; Cm Sp	PB5383 → PB5403
PB5412	*trpC2 pheA1 swrAA* mutant *degU32*(Hy) *pks*::P_{swrA}*WT-lacZ*; Cm Sp	PB5383 → PB5404
PB5413	*trpC2 pheA1 swrAA* mutant *degU32*(Hy) *pks*::$P_{swrA}A^-$*-lacZ*; Cm Sp	PB5383 → PB5405
PB5414	*trpC2 pheA1 swrAA* mutant *degU32*(Hy) *pks*::$P_{swrA}D^-$*-lacZ*; Cm Sp	PB5383 → PB5406
PB5415	*trpC2 pheA1 swrAA* mutant *degU32*(Hy) *pks*::$P_{swrA}DA^-$*-lacZ*; Cm Sp	PB5383 → PB5407
PB5426	*trpC2 pheA1 swrAA* mutant *pks*::P_{swrA}*WT-lacZ*; Δ*sigD*; Cm Em	pLD11 → PB5404
PB5427	*trpC2 pheA1 swrAA*$^+$ *pks*::P_{swrA}*WT-lacZ*; Δ*sigD*; Cm Em	pLD11 → PB5400
PB5428	*trpC2 pheA1 swrAA* mutant *degU32*(Hy) *pks*::P_{swrA}*WT-lacZ*; Δ*sigD*; Cm Em Sp	PB5383 → PB5426
PB5429	*trpC2 pheA1 swrAA*$^+$ *degU32*(Hy) *pks*::P_{swrA}*WT-lacZ*; Δ*sigD*; Cm Em Sp	PB5383 → PB5425

EcoRV (for D^- mutations) or BfaI (for A^- mutations). To introduce the mutations into PB5370 (*swrAA* mutant), generating strains PB5396 to PB5399, the same procedure was followed, but plasmids were digested with ScaI, allowing recombination upstream of the nine-adenine stretch in the *swrAA* gene. The integrative vector pJM783 (27), which generates transcriptional fusions to *lacZ*, was used for studying *swrAA* promoter activity. Because of the deficiency of unique restriction sites in pJM783, pCAP^s (Roche) vector was used as an intermediate step. A DNA segment corresponding to the *swrAA* promoter was amplified from PB5249 chromosomal DNA using primers Up-PromA and A-rev and was inserted in the MluNI restriction site in the catabolite gene activator protein gene. This plasmid was first digested with SphI, filled by T4 DNA polymerase, and digested with EcoRI, to extract a fragment containing the P_{swrA}

TABLE 2. Primers used in this study

Primer	Sequence (restriction sites underlined)
A-rev	5′-ACCGCTCGAGTTGTGAACCCCCATTTTCTTTATACAGATAAGCAC-3′
DPSigA F	5′-GTTGCCTATCTTTGTTTACTTCAAAATATAAGAAG-3′
DPSigA R	5′-CTTCTTATATTTTGAAGTAAACAAAGATAGGCAAC-3′
DPSigD F	5′-GGACTGTTATTACCCATCAATATATGAGAGAGAC-3′
DPSigD R	5′-GTCTCTCTCATATATTGATGGGTAATAACAGTCC-3′
FlgB2	5′-GCTTAATATCCGCTCTGCTCAAGGCA-3′
mk120	5′-GCGCTGCAATATTGTGGTTAATTCTC-3′
mk170	5′-GTTCTTTTGGCTCGCACTGTTGTTTG-3′
mk237	5′-AGATCGCAAGACCTGCTGCGTC-3′
Pflg	5′-TGAAGCTTGGAATTGACGCCCC-3′
PKS-for	5′-GGGAATTCGCTTACACCCTGCAGGTC-3′
PKS-rev	5′-CCCGAATTCAACCAGTCTTTG-3′
upFla/Che	5′-TCTCGGGTTGAAAGTCTTTCTATG-3′
Up-PromA	5′-CCGAATTCTTTGTGCTTAAAGAGATTATGGATC-3′
yvjD3220	5′-ACGGAATTCTTATTACAAAGCGGTACAGACCGC-3′
yvjD2661	5′-CGCGAATCCGCAATGAAACCGAGAGGAATC-3′
yvjB.rev.X	5′-CGCCGCTCGAGCACAAAGAAACAGGAGATG-3′
yvjB-F	5′-GGGGTACCAATGGGGGACGGCAGCAAC-3′
yvzD1	5′-CTGGAATCCTTGAAGAGGGCAAGTATTGTG-3′
yvzD-R	5′-GCGGATCCGCGCTTTTTAACAGTTCAATTCC-3′
301	5′-CGGGATCCCAAGATCGCCCGCTTCCCGGAT-3′
302	5′-AAAACTGCAGCATTGTTATCCCCCTAATACCT-3′
303	5′-CCATCGATGAATCTGCTGGAAAAAGTGATACA-3′
304	5′-GGGGTACCCGCTTTATTTTCTATGAGTTTCTCAT-3′

TABLE 3. Plasmids used in this study

Plasmid	Description	Reference
pJM114	Integrative vector; Km	27
pCCP$_{swrA}$WT	pJM114 P_{swrA}WT-*swrAA*$^+$; Km	This study
pCCP$_{swrA}A^-$	pJM114 $P_{swrA}A^-$-*swrAA*$^+$; Km	This study
pCCP$_{swrA}D^-$	pJM114 $P_{swrA}D^-$-*swrAA*$^+$; Km	This study
pCCP$_{swrA}DA^-$	pJM114 $P_{swrA}DA^-$-*swrAA*$^+$; Km	This study
pJM783	Integrative vector, promoterless *lacZ* preceded by rbs_{spoVG}; Cm	27
pJM783P$_{swrA}$WT	pJM783 P_{swrA}WT-*lacZ*; Cm	This study
pJM783P$_{swrA}A^-$	pJM783 $P_{swrA}A^-$-*lacZ*; Cm	This study
pJM783P$_{swrA}D^-$	pJM783 $P_{swrA}D^-$-*lacZ*; Cm	This study
pJM783P$_{swrA}DA^-$	pJM783 $P_{swrA}DA^-$-*lacZ*; Cm	This study
pHCMC05	Replicative vector; P_{spac} promoter; Cm	24
pSwrAA	pHCMC05 P_{spac}-*swrAA*; Cm	This study
pDG148	Replicative vector; P_{spac} promoter; Km Bm	35
pBG9	FlgM-overexpressing plasmid	2
pYFC-11	SigD-overexpressing plasmid	5
pJM109B	Integrative vector; Em	27
pLD11	*sigD*::Em in pJM109B	This study

promoter that was cloned into the EcoRI and SmaI site of pJM783, creating pJM783A. A 700-bp fragment from the *pksA* gene was amplified from PB5249 chromosomal DNA using primers PKS-for (EcoRI site) and PKS-rev (EcoRI site) and inserted into the EcoRI site in pJM783A. One clone carrying the *pksA* fragment, in the orientation opposite P_{swrA}, was selected, creating the plasmid pJM783P_{swrA}WT. Mutations of P_{swrA} were introduced in pJM783P_{swrA}WT by site-directed mutagenesis PCR using primers DPSigD F and DPSigD R and/or DPSigA F and DPSigA R. These plasmids were used to transform strains PB5249 and PB5370, obtaining strains PB5400 to PB5407. The P_{fliDST}-*lacZ* reporter fusion inserted in *pks* was derived by transformation with DNA from a strain carrying $P_{fliD\text{-}224}$ (9).

To insert the *degU32*(Hy) mutation, strains from PB5400 to PB5407 were transformed with chromosomal DNA of PB5383, in which the *degU32*(Hy) mutation is associated with a spectinomycin resistance gene, obtaining strains PB5408 to PB5415. Construction of PB5383 will be described in detail elsewhere (G. Amati et al., unpublished data).

To place the *swrAA* coding sequence under control of the IPTG-inducible P_{spac} promoter on a replicative vector, chromosomal DNA of PB5249 was amplified with primers yvzD1 (BamHI) and yvzD-R (BamHI). After BamHI digestion, the fragment was ligated to BamHI-restricted pHCMC05 (24), thus producing pSwrAA. This plasmid and the empty pHCMC05 were used to transform PB5249 and PB5370, alone or in combination with chromosomal DNA from PB5342 or PB5343, selecting for chloramphenicol and kanamycin resistance when required. Several different clones were tested in motility assays.

To inactivate *sigD*, its flanking regions were cloned into the corresponding restriction sites of plasmid pJM109B (27), using primers 301 (BamHI) and 302 (PstI) and primers 303 (ClaI) and 304 (KpnI), respectively, giving pLD11 (constructed by L. De Riso in our laboratory). PB5404 and PB5400 were transformed with pLD11 and selected with erythromycin, giving strains PB5426 and PB5427, respectively. Both strains were subsequently transformed with chromosomal DNA of PB5383, obtaining strains PB5428 and PB5429, respectively.

Plasmids, listed in Table 3, were verified by sequencing.

Motility assays. Cells, previously grown on LB broth-1.5% agar plates with appropriate antibiotic, were seeded onto the center of an 8.5-cm plate containing freshly prepared LB broth plus 0.2% agar to evaluate swimming motility. Swarming was evaluated on 8.5-cm plates containing freshly prepared LB broth with 0.7% agar. On the surface of swarming plates, 10 μl of 2 mM surfactin was spread, and plates were dried for 10 min in a 40°C incubator before cells were spotted at the center of the plate. Swimming and swarming plates were incubated at 30°C; diameters of halos due to bacterial migration were recorded 13 or 16 h postinoculation, as indicated. For complementation experiments with pSwrAA, swimming and swarming plates contained 5 μg/ml chloramphenicol; when appropriate, 1 mM IPTG was added.

β-Galactosidase activity assay. To assay β-galactosidase activity, overnight cultures, grown in peptone-yeast extract and 0.5% glucose, were washed with physiological solution and diluted, in fresh LB medium supplemented with 100 μM of $FeCl_3$, to an optical density at 600 nm (OD_{600}) of 0.2. Samples were taken at 30-min intervals for OD_{600} readings, and β-galactosidase activity was determined. The β-galactosidase activity, based on OD_{600} readings, was calculated according to the formula $(OD_{420} \times 1.5)/(OD_{600} \times$ sample volume in ml $\times$ reaction time in min $\times$ 0.00486) and expressed as modified Miller units (MU) (19). Results shown are the means of at least three different experiments, done in duplicate.

Sporulation efficiency was calculated by plating serial dilutions of samples taken from the growth curves directly or after a 10-min incubation at 80°C as published previously (25).

Protein purification. *Bacillus subtilis* RNA polymerase core enzyme was purified from MH5636 essentially as described previously (28). The RNA polymerase fraction was dialyzed against DB (50 mM sodium phosphate, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50% glycerol), and aliquots were stored at −70°C. The protein concentration, determined by the Bradford assay, was 2.8 mg/ml. Recombinant FlgM was overexpressed from pBG9 and purified as described previously (2), omitting the size-exclusion chromatographic step. In fractions containing FlgM, glycerol was added to a final concentration of 20%. The FlgM concentration was 0.3 mg/ml. Recombinant SigD was overexpressed from pYFC-11 (5) in *E. coli* BL21(DE3) and purified as described previously (11) with the following modifications: cells were grown to mid-logarithmic phase at 37°C in 1 liter of LB medium with 100 mg ampicillin. IPTG was added to 0.5 mM, and cells were harvested after 3 h of further incubation. The cell pellet was resuspended in 20 ml TNEG (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) and lysed by sonication. Triton X-100 was added to an 0.5% (vol/vol) final concentration, and the inclusion bodies were recovered by centrifugation. The pellet was washed twice with 20 ml of TNEG containing 0.5% Triton X-100. The pellet was finally resuspended in 10 ml of TNEG containing 0.4% Sarkosyl and incubated for 30 min at 20°C before centrifugation. The supernatant was slowly diluted 10 times with TNEG at 4°C and dialyzed for 16 h against 1 liter TNEG. After centrifugation, the supernatant was applied on a Q-Sepharose column (GE Healthcare) equilibrated with TNEG, and after washing, SigD was eluted with a linear salt gradient from buffer TNEG to TNEG containing 1 M NaCl. Peak fractions were pooled, dialyzed against 1 liter of TNEG containing 50% glycerol, and stored at −80°C in aliquots. The concentration of SigD was 2 mg/ml. Purified recombinant SigA was a generous gift from A. Albertini.

FIG. 1. The promoter of the *swrA* operon. DNA sequence of the 361-nt region upstream of the *swrAA* start codon (*TTG*). Sequences corresponding to the putative σ^D- and σ^A-dependent promoters are underlined, and the promoter's identity is indicated above (33). The consensus sequence for σ^D-dependent promoters is TAAA-$(N)_{12–16}$-GCCGATAT, whereas for σ^A promoters it is TTGACA-$(N)_{13–22}$-TATAAT. An arrow above the DNA sequence specifies the estimated σ^D-directed transcription start site (position −235/−236). Nucleotides deleted to obtain the mutant promoters are in bold.

Runoff transcription assay. Runoff transcription assays were performed with promoter fragments PCR amplified from the different pCCP$_{swrA}$ plasmids or from PB5249 chromosomal DNA (for $P_{fla/che}$ and P_{hag}). For P_{swrA} Up-PromA and A-rev primers were used; primers upFla/Che and FlgB2 amplified $P_{fla/che}$, and primers Pflg and mk120, mk170, or mk237 were used to produce P_{hag}. Reactions (25-μl mixtures) were performed as follows: 3.4 pmol of *B. subtilis* RNA polymerase was preincubated for 15 min on ice with 34 pmol of σ^D or 50 pmol of σ^A and 60 pmol of FlgM when indicated. The protein complexes were then transferred in tubes containing 0.25 pmol template DNA, 1 μl RNasin (Promega), 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 mM KCl, and 1 mM dithiothreitol and incubated for 8 min at 37°C. ATP, CTP, and GTP (1 mM each, final concentration); UTP (0.12 mM); and 0.2 μl of [α-^{32}P]UTP were added. Incubation was stopped after 8 min by addition of 100 μl of stop solution (2.5 M NH_4OAc, 10 mM EDTA, 0.2 mg/ml glycogen). Following phenol-chloroform extraction and ethanol precipitation, samples were analyzed by electrophoresis on 6% acrylamide-8 M urea-polyacrylamide gels. Dried gels were exposed on film.

RESULTS

Identification of the *swrAA* promoter in vitro. Inspection of the region upstream of the *swrAA* open reading frame allowed for the identification of sequences that have high homology to the consensus sequences of σ^A (SigA)- and σ^D (SigD)-dependent promoters (Fig. 1).

In order to confirm the validity of our in silico analysis, we set up in vitro transcription assays for the promoter of the *swrA* operon (P_{swrA}) using purified *B. subtilis* RNA polymerase. The entire sequence depicted in Fig. 1, starting from the ρ-independent terminator of the preceding *ctpB* gene (*yvjB*) up to the *swrAA* start codon (excluded), was used as a template (hereafter referred to as wt) with either σ^D or σ^A RNA polymerase (E) holoenzyme. Parallel reactions were run using the same

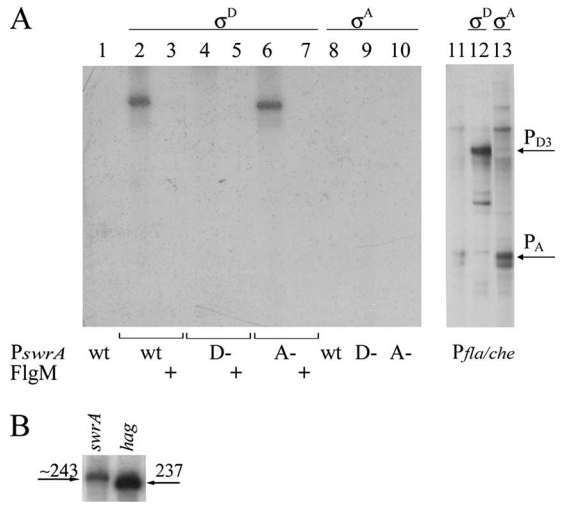

FIG. 2. In vitro *swrA* transcription is driven by the σ^D-dependent promoter. (A) The entire sequence shown in Fig. 1 and the promoter region of the *fla*/*che* operon were used as templates in runoff experiments with purified *B. subtilis* core RNA polymerase only (lanes 1 and 11) plus recombinant SigD (lanes 2 to 7 and lane 12) or SigA (lanes 8 to 10 and lane 13). The P_{swrA} template sequence was in the wild-type form (wt) or contained a deletion of the σ^D promoter (D−) or of the σ^A promoter (A−). To confirm the identity of the sigma factor, the anti-σ^D factor FlgM was added where indicated (+). The E(σ^D) product obtained from the A^- mutant *swrA* promoter (lane 6) migrates faster than the product from the wt promoter (lane 2) because of the deletion of 3 nt in the mutant template. (B) Transcription products obtained with E(σ^D) from P_{swrA}WT (left) and P_{hag}237 (right). The length of P_{swrA} transcript was estimated from the migration of P_{hag}237, P_{hag}270, and P_{hag}120 (not shown).

template in which the putative consensus sequences for either σ^D or σ^A had been deleted (D^- or A^-, respectively), as specified in Fig. 1.

As shown in Fig. 2A, both the wt and A^- templates can be efficiently transcribed by the *B. subtilis* Eσ^D, and transcription is blocked by the addition of the σ^D-specific anti-sigma factor FlgM (10, 22) (lanes 3 and 7). As expected, when the D^- template is used, transcription is completely abolished (lane 4), confirming that the *swrA* operon indeed possesses a σ^D-dependent promoter located between 267 and 243 nt upstream of the initiation codon.

No σ^A-dependent transcription could be detected, while in the same conditions the *fla*/*che* promoter (8) could be transcribed by both sigma factors (Fig. 2A, lanes 12 and 13). Thus, although the putative σ^A-dependent P_{swrA} is closer to the σ^A consensus than $P_{fla/che(A)}$ (TAGACT—17 nt–TACAAT) (8), it is not recognized by Eσ^A. From these data we concluded that the putative σ^A-dependent promoter identified in silico is not functional in vitro.

The site of *swrA* transcription initiation could not be determined by primer extension analysis despite experiments that were conducted under several conditions (with several enzymes, at different temperatures, and in different media) both in $degU^{wt}$ and in *degU32*(Hy) genetic backgrounds (see below). This could possibly be attributed to the presence of RNA secondary structures that can be predicted in the 5′ untranslated region of the *swrA* operon. To overcome this technical problem, the *hag* promoter was used as a ruler in runoff assays, since the σ^D-dependent transcription start site for *hag*, coding for flagellin in *B. subtilis*, is well characterized (20). Templates spanning different lengths but beginning from the same position in the *hag* promoter were transcribed and run in parallel with a P_{swrA} transcript, whose initiation was approximately mapped at position −235/−236 (Fig. 2B), indicated by an arrow in Fig. 1.

Assessment of promoter activity by transcriptional fusions. To verify our findings in vivo, we constructed transcriptional fusions to *lacZ* of the entire P_{swrA} region shown in Fig. 1, in the pJM783 plasmid (27). A fragment of 700 bp containing the *pksA* gene was also cloned upstream of P_{swrA}, in the opposite transcriptional orientation, to direct plasmid integration into the nonessential *pks* locus. Transcriptional fusion constructs were also generated using the promoter deletions indicated in Fig. 1 both alone and in combination. Strains integrating these constructs in *pks* will be identified as P_{swrA}WT-*lacZ*, $P_{swrA}A^-$-*lacZ*, and $P_{swrA}D^-$-*lacZ*, for the wild-type sequence and the single mutations, and as $P_{swrA}DA^-$-*lacZ* for the double mutant. Each construct was inserted in both $swrAA^+$ (PB5249) and *swrAA* mutant (PB5370) isogenic strains. The transcriptional fusions were assayed in liquid LB medium during a prolonged growth curve (shown by a dotted line in Fig. 3). This medium was chosen because it is used for standard swimming and swarming motility assays (13) and allowed a direct comparison with data obtained from motility experiments (see below). As shown in Fig. 3, in $swrAA^+$ strains β-galactosidase is produced at comparable levels by P_{swrA}WT-*lacZ* and $P_{swrA}A^-$-*lacZ*; the mutation of the *sigD* promoter, $P_{swrA}D^-$-*lacZ*, completely abolished transcription, as occurs with the double mutant $P_{swrA}DA^-$-*lacZ*. Thus, under these conditions, *swrA* is an operon exclusively transcribed by σ^D.

It has been shown that SwrAA stimulates transcription of the *fla*/*che* operon and of *sigD* contained therein, thus increasing transcription of σ^D-dependent genes (14). Therefore, according to what we observed, SwrAA should indirectly stimulate its own expression. Indeed, at $T_{0.5}$ β-galactosidase levels from P_{swrA}WT-*lacZ* are higher in $swrAA^+$ than in *swrAA* mutant strains, as can be better appreciated from insets A and B in Fig. 3. The same is true for the σ^D-dependent *fliDST* promoter P_{fliDST} (4, 9), which shows higher activity in $swrAA^+$ than in *swrAA* mutant strains (data not shown).

To reinforce these results, we determined that P_{swrA}WT activity in a Δ*sigD* background is extremely low (Fig. 3B), giving an average of 1.29 MU (range, 0.70 to 2.04 MU) between $T_{-0.5}$ and T_7 (not shown). These data confirm the results obtained by runoff experiments; altogether they indicate that the identified consensus is in fact recognized by σ^D, which is the sole promoter driving *swrA* expression in these conditions, and prove the existence of a transcriptional feedback loop between *swrA* and *fla*/*che* operons.

It is conceivable that *swrAA* (*yvzD*) has never been identified as a σ^D-dependent gene in array experiments (14, 32) because the level of *swrA* promoter activity, measured as β-galactosidase MU, is low. At $T_{0.5}$ it reaches a maximum of 16.55 MU (Fig. 3).

Extending our examination up to T_7 (7 h after the transition phase) and by using LB medium, we were able to reproducibly detect three main time intervals of σ^D activity (Fig. 3). We detected a peak of activity centered around $T_{0.5}$, which has

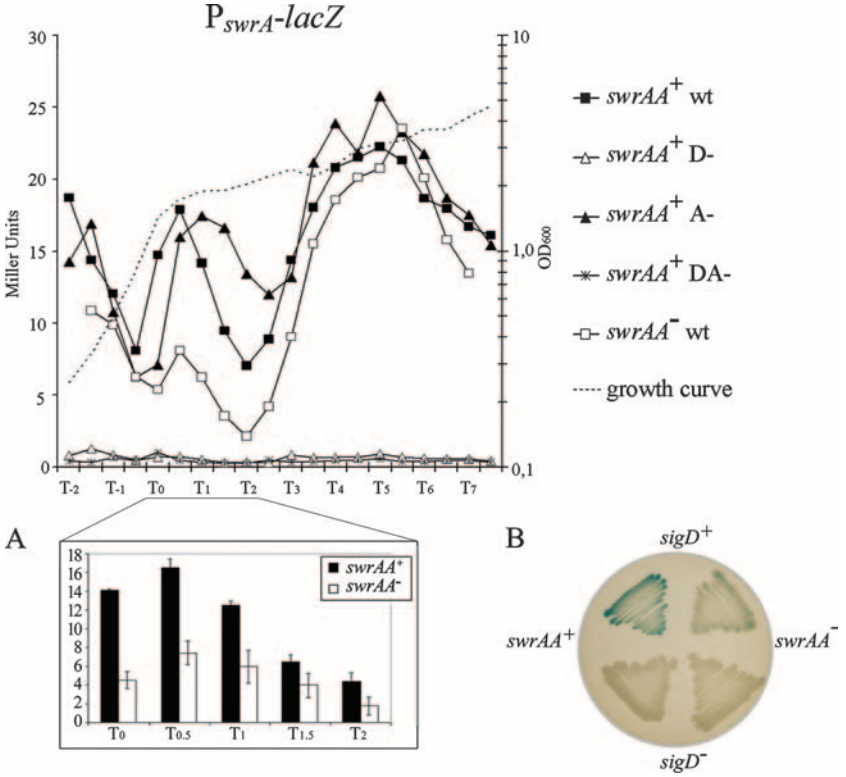

FIG. 3. P$_{swrA}$-*lacZ* is transcribed by the σ^D-dependent promoter. Transcriptional fusions in *degU*^{wt} strains were assayed during growth in liquid LB medium, and activities from the wt and mutant *swrA* promoters are compared. The *swrAA*$^+$ strains are PB5400 for the wt promoter, PB5402 for D$^-$, PB5401 for A$^-$, and PB5403 for DA$^-$. Strain PB5404 carries P$_{swrA}$WT-*lacZ* and is an *swrAA* mutant. β-Galactosidase units are shown on the left axis; time, in hours before and after the transition phase (T_0), is shown on the *x* axis. The typical growth curve for these strains is represented by a dotted line, and the corresponding OD_{600} values are on the right axis. An enlargement from T_0 to T_2 is shown in panel A: β-galactosidase units of P$_{swrA}$WT-*lacZ* in *swrAA*$^+$ and *swrAA* mutant strains are displayed. Bars refer to the means ± standard errors of the means ($\sigma_{n-1}/n^{1/2}$). The same strains shown in panel A are displayed in the upper part of the plate in panel B: PB5400 (*swrAA*$^+$ *sigD*$^+$) is on the left and PB5404 (*swrAA* mutant *sigD*$^+$) is on the right. In the lower part of the plate the strains carry a *sigD* deletion: PB5427 (*swrAA*$^+$ Δ*sigD*) is on the left and PB5426 (*swrAA* mutant Δ*sigD*) on the right. Note that transcription from P$_{swrA(A)}$ is not perceptible even on solid plates. The LB plate contains 1.5% agar and 100 μg/ml X-Gal and was incubated for 8 h at 37°C, followed by a 72-h incubation at room temperature.

already been described for σ^D-dependent promoters, and a later activation burst around T_5, which could be medium specific and which, to our knowledge, has never been described before. We verified that sporulation did not begin at T_7 (data not shown). The activity observed at T_{-2} is also unclear and could depend on medium composition (for a substantial discussion on this matter, see reference 21); indeed, the same profile can be obtained with the σ^D-dependent *fliDST* promoter (4, 9) (not shown).

Transcription of *swrA* is regulated by *degU*. Since σ^D-directed transcription is shut off in the presence of the *degU32*(Hy) mutation, due to direct repression of P$_{fla/che(A)}$ (1, 36), *swrA* transcription should also be silenced in *degU32*(Hy) strains. In fact, strains bearing the *degU32*(Hy) mutation are nonmotile (1, 23, 37). Therefore, to confirm the σ^D dependence of P$_{swrA}$, we introduced the *degU32*(Hy) allele in all strains carrying P$_{swrA}$-*lacZ* and assessed β-galactosidase activity in these conditions. Surprisingly, in contrast to what happens with the well-characterized σ^D-dependent *fliDST* promoter (data not shown), transcription of P$_{swrA}$ is not shut off. We found a consistent enhancement in β-galactosidase activity clearly visible on LB plates containing X-Gal (data not shown). When the activity was measured during a growth curve in liquid medium, this enhancement was localized around T_5, where 61.42 MU was measured (Fig. 4). Using the mutant promoters, we established that P$_{swrA}$ activity is no longer dependent on SigD. In fact, there is no difference in P$_{swrA}$WT activity measured in the single mutant *degU32*(Hy) strains (Fig. 4) or in the double mutant *degU32*(Hy) Δ*sigD* strains (data not shown), confirming that SigD is not involved. Rather, transcription depends on the putative σ^A-dependent promoter whose deletion leads to the loss of β-galactosidase expression (indicated as A$^-$ in Fig. 4). Thus, the second promoter, silent in liquid medium in a *degU*^{wt} background and not recognized by Eσ^A in vitro, becomes active in the presence of DegU~P in conditions in which σ^D-directed transcription is blocked. It can be excluded that induction of the putative σ^A-dependent P$_{swrA}$ is due to the lack of interference created by SigD binding, since in *degU*^{wt} strains such a promoter is not used even in the P$_{swrA}$D$^-$-*lacZ* mutant (Fig. 3). These data are supported by microarray and Northern blotting data obtained by Kobayashi, in which the *swrA* operon is shown to be positively regulated by *degU* and *degS* (15).

Unfortunately the binding of DegU or DegU32(Hy) proteins to P$_{swrA}$ could not be detected (not shown). Therefore, it cannot be excluded that *degU* activates P$_{swrA}$ indirectly.

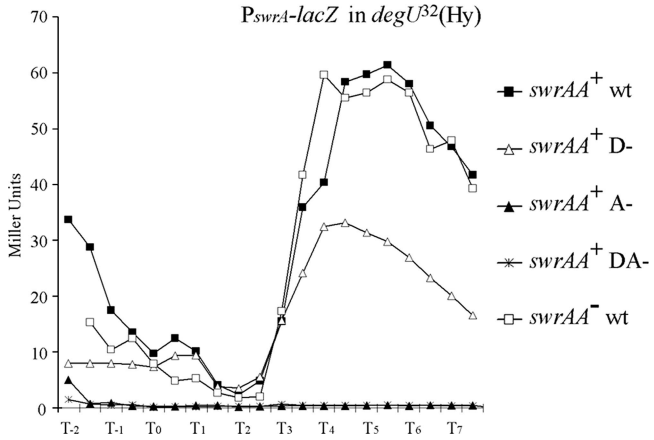

FIG. 4. P_{swrA}-*lacZ* is transcribed by the putative σ^A-dependent promoter in *degU32*(Hy) strains. The assay is the same as that shown in Fig. 3, but all strains carry a *degU32*(Hy) mutant allele. The *swrAA*$^+$ strains are PB5408 for the wt promoter, PB5410 for D$^-$, PB5409 for A$^-$, and PB5411 for DA$^-$. Strain PB5412 carries P_{swrA}WT-*lacZ* and is an *swrAA* mutant.

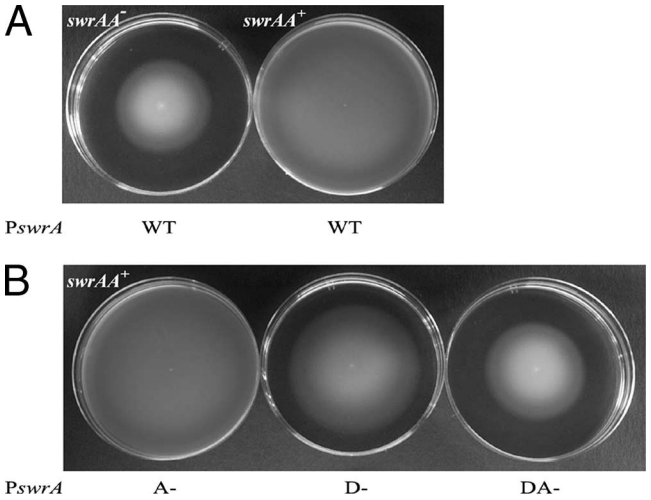

FIG. 5. Swimming depends mainly on the σ^D-dependent promoter. (A) Swimming ability is different in *swrAA*$^+$ (PB5392) and *swrAA* mutant strains (PB5396). (B) Swimming ability in *swrAA*$^+$ strains carrying mutations in the putative σ^A-dependent *swrA* promoter (A−, PB5393), in the σ^D-dependent *swrA* promoter (D−, PB5394), and in both promoters (DA−, PB5395).

Currently, there are no clear explanations for the reduced level of transcription obtained with the $P_{swrA}D^-$-*lacZ* construct, which could be due to a structural effect of the deletion on DNA conformation.

In vivo analysis of the effects of *swrA* promoter mutations in swimming and swarming. The *swrA* operon not only is crucial for swarming motility but also improves swimming as a result of a dramatic increase in the number of flagella (3, 14, 31). We hence determined the effect of mutations in P_{swrA} on motility phenotypes in *swrAA*$^+$ strains, where the gene is functional.

The WT, A$^-$, D$^-$, and DA$^-$ promoter mutations were inserted into the *swrA* locus by double crossover, driving transcription of the *swrA* operon. These strains will be identified hereafter as P_{swrA}WT, $P_{swrA}D^-$, $P_{swrA}A^-$, and $P_{swrA}DA^-$. Swimming plates were freshly prepared with LB medium supplemented with a low concentration of agar (0.2%). Strains were inoculated on the center of the surface with a toothpick and incubated at 30°C for 13 h. As shown in Fig. 5A, *swrAA*$^+$ strains swim better than *swrAA* mutant strains and this ability is unaffected in the $P_{swrA}A^-$ mutant (Fig. 5B), confirming that swimming depends mainly on a functional σ^D-dependent promoter. Unexpectedly, in the $P_{swrA}D^-$ mutant swimming is only slightly reduced (Fig. 5B), suggesting that a minor role must be played by the remaining σ^A-dependent promoter, which is, although silent in β-galactosidase assays, partially active on swimming plates in vivo. As expected, the $P_{swrA}DA^-$ mutant loses *swrAA* activity and becomes indistinguishable from an *swrAA* mutant strain.

Since our laboratory strains are derived from strain 168 and are *sfp*^{0}, it is possible to observe swarming migration on LB plates enriched with 0.7% agar upon addition of surfactin (13). Swarming motility is restricted to *swrAA*$^+$ strains (Fig. 6A). As expected the $P_{swrA}DA^-$ mutation eliminates P_{swrA} activity and thus eliminates swarming. Surprisingly, however, both the $P_{swrA}D^-$ and $P_{swrA}A^-$ single mutations have no effect on this behavior (Fig. 6). This finding strongly points to equivalent roles for the two promoters in swarming conditions. Thus, on semisolid surfaces the putative σ^A-dependent *swrAA* promoter is fully functional and can replace σ^D-directed *swrAA* transcription, indicating that either promoter alone is sufficient for swarming migration.

Kunst and Rapoport (17) analyzed the transcription of *sacB*, a *degU*-controlled gene, and reported that *sacB* expression is eightfold higher on agar plates than in liquid medium. In liquid medium a comparable level of expression could be obtained by the addition of 1 M NaCl, and the assay demonstrated that this effect was dependent on a functional DegS-DegU two-component system (17). We presume that the surface of the plates is exposed to evaporation, leading to a gradual increase in solute

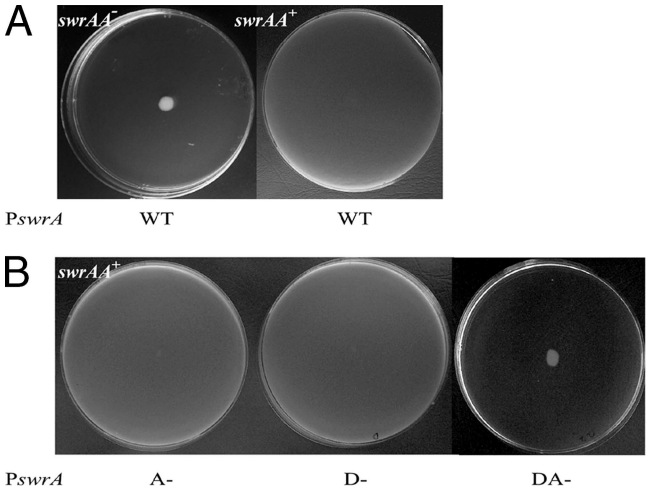

FIG. 6. Each *swrA* promoter is sufficient for swarming. (A) Swarming ability in *swrAA*$^+$ (PB5392) and *swrAA* mutant (PB5396) strains. (B) Swarming ability in *swrAA*$^+$ strains carrying mutations in the putative σ^A-dependent *swrA* promoter (A−, PB5393), in the σ^D-dependent *swrA* promoter (D−, PB5394), and in both promoters (DA−, PB5395).

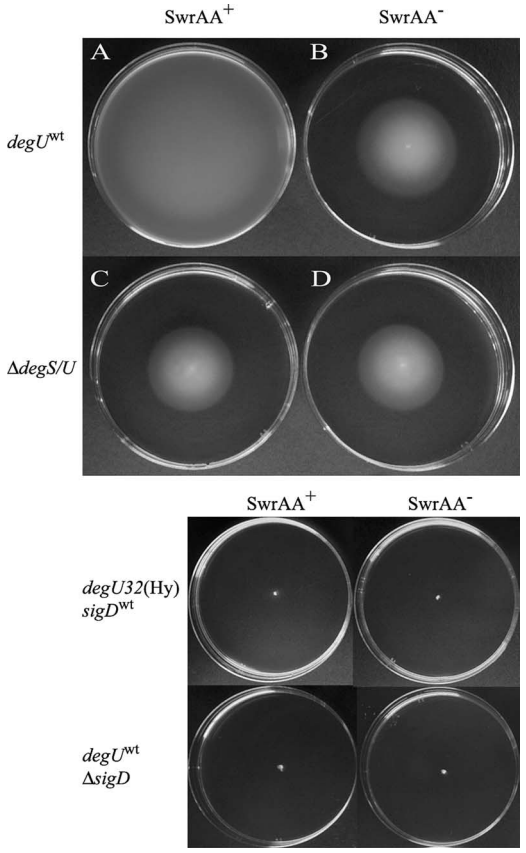

FIG. 7. Both *swrAA* and *degU* are required for full motility. Swimming in LB medium for strains *swrAA*$^+$ *degU*^{wt} (PB5249) (A), *swrAA* mutant *degU*^{wt} (PB5370) (B), *swrAA*$^+$ Δ*degS/U* (PB5343) (C), and *swrAA* mutant Δ*degS/U* (PB5342) (D). There is no difference in the swimming abilities of *swrAA* mutant and *swrAA*$^+$ Δ*degS/U* strains. Nonmotile strains are shown below as controls: they are PB5408 [*degU32*(Hy) *swrAA*$^+$ *sigD*^{wt}], PB5412 [*degU32*(Hy) *swrAA* mutant *sigD*^{wt}], PB5425 (*degU*^{wt} *swrAA*$^+$ Δ*sigD*), and PB5426 (*degU*^{wt} *swrAA* mutant Δ*sigD*) as indicated.

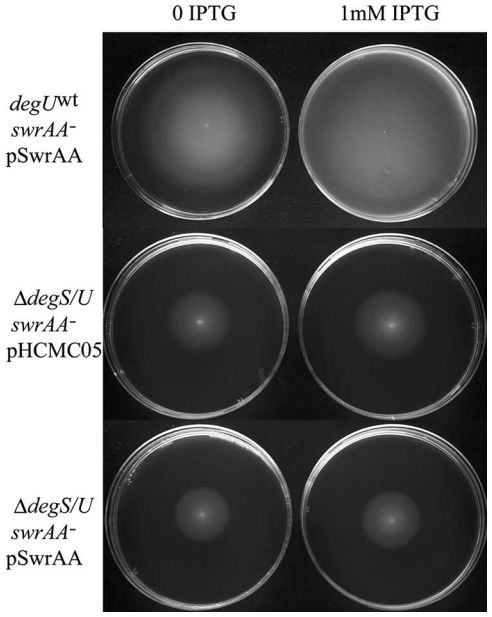

FIG. 8. SwrAA overexpression does not complement the swimming defect in a Δ*degS/U* mutant. Plasmid pSwrAA was transformed into a *degU*^{wt} *swrAA* mutant strain (PB5370) and into a Δ*degS/U swrAA* mutant strain (PB5342). As a control, the empty plasmid pHCMC05 was used. Swimming plates contained antibiotics and were supplemented with 1 mM IPTG, where indicated. Identical results were obtained with *swrAA*$^+$ strains (not shown).

concentration. Salinity changes are thought to be perceived through the DegS-DegU system (17, 29, 34), leading to gradual phosphorylation of DegU, although characteristics other than salinity could vary as well between liquid and solid media.

The low level of activity of $P_{swrA(A)}$ in *degU*^{wt} backgrounds impairs measurements of its activity by β-galactosidase assays even on solid media (e.g., in Fig. 3B). Nevertheless, the σ^A promoter becomes functionally more active in media with increasing agar concentrations (0.2% and 0.7%) as shown in Fig. 5 and 6.

DegU∼P and SwrAA work together to activate swarming motility. Swarming requires a low level of DegU∼P (15, 37), although the constitutively active *degU32*(Hy) mutant is completely nonmotile (1, 15, 23, 37). When a *degS-degU* deletion (Δ*degS/U*) was introduced in our *swrAA*$^+$ and *swrAA* mutant strains, not only was there an impairment in swarming, as already reported (15, 37), but swimming was affected as well (Fig. 7). The positive effect exerted by *swrAA* (compare A and B in Fig. 7) was completely lost, and the swimming halos of *swrAA*$^+$ and *swrAA* mutant strains became indistinguishable (compare C and D in Fig. 7). This demonstrates that also in swimming the copresence of DegU and SwrAA is required for full motility. As the putative σ^A-dependent *swrAA* promoter is stimulated by DegU∼P (Fig. 4), we asked if the requirement of DegU∼P in swarming could be related to the activation of *swrA* transcription. If this were the case, SwrAA overexpression from an IPTG-inducible promoter would complement the motility defect in strains lacking DegU. Therefore, *swrAA* was overexpressed from the P_{spac} promoter of the multicopy plasmid pHCMC05 (24) in Δ*degS/U* strains, overcoming the dependence of *swrAA* transcription on *degU*. Although this construct is able to complement an *swrAA* mutant strain, both in swimming (Fig. 8) and in swarming (not shown), SwrAA overexpression is not sufficient to complement the swimming (Fig. 8) and swarming (not shown) defect in Δ*degS/U* strains. Therefore, the DegU∼P requirement in swimming (Fig. 7 and 8) and swarming (15, 37) is not to be attributed solely to its contribution to *swrA* transcription.

DISCUSSION

We described the characteristics of the *swrA* operon transcription driven by two promoters: a σ^D-dependent promoter, used in liquid growth (Fig. 3 and 5), and a putative σ^A-dependent promoter, triggered in the presence of DegU∼P (Fig. 4) and on surface growth (Fig. 6). Currently, there is no explanation for the lack of use of the putative σ^A-dependent P_{swrA}, both in vitro and, more importantly, during liquid growth in a *degU*^{wt} background; despite the close resemblance to a σ^A-directed promoter, there is no formal proof that it is indeed recognized by $E\sigma^A$. It is an issue that requires further experimental investigation.

Our findings demonstrate that in planktonic conditions SwrAA is transcribed solely by the σ^D-dependent promoter and closes an autoregulatory loop, where it stimulates *fla/che* transcription (14), raising SigD levels and consequently flagellar gene expression and motility, besides enhancing its own transcription (Fig. 3A). The existence of a mechanism of positive autoregulation for motility genes, sufficient to set up the phenomenon of bistability (7), had already been predicted (14). However, further experiments are needed to prove that SwrAA is involved in this mechanism.

On swarming plates the levels of DegU phosphorylation are probably moderately raised due to the environmental conditions. This results in a change in *swrA* transcription: namely, the second P_{swrA} promoter becomes active (Fig. 6). It should be kept in mind that in *degU*^{wt} strains the σ^D-dependent *swrA* promoter remains functional (Fig. 6), as the level of DegU~P is not as high as in *degU32*(Hy) mutants, and this allows the maintenance of the positive feedback action.

Furthermore, our data enhance the importance of *degU*, demonstrating that beyond its requirement for swarming in undomesticated and laboratory strains of *B. subtilis* (15, 37), it plays a role also in swimming (Fig. 7), at least in *swrAA*$^+$ domestic strains. SwrAA expression does not simply counteract the negative effect of DegU~P on $P_{fla/che}$, as in this case a Δ*degS/U swrAA* mutant strain should fully swim and swarm, while the copresence of DegU~P and SwrAA is necessary to achieve complete motility. To reinforce this scenario, there is at least another phenotype dependent on the concerted action of *degU32*(Hy) and SwrAA, which is the activation of the *pgs* operon, driving the synthesis of γ-polyglutamic acid (Amati et al., unpublished). Unfortunately, preliminary pull-down experiments could not demonstrate any physical interactions between SwrAA and DegU.

The same model holds true if a third player is involved, which can be envisaged as the product of a gene regulated by SwrAA and DegU~P together. Future studies will be focused on determining if such a factor exists.

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