SwrAA activates poly- γ -glutamate synthesis in addition to swarming in *Bacillus subtilis*

Cecilia Osera, Giuseppe Amati, Cinzia Calvio and Alessandro Galizzi

Correspondence

Cinzia Calvio

cinzia.calvio@unipv.it

Dipartimento di Genetica e Microbiologia, Università degli Studi di Pavia, via Ferrata 1, 27100 Pavia, Italy

Poly- γ -glutamic acid (γ -PGA) is an extracellular polymer produced by various strains of *Bacillus*. It was first described as the component of the capsule in *Bacillus anthracis*, where it plays a relevant role in virulence. γ -PGA is also a distinctive component of 'natto', a traditional Japanese food consisting of soybean fermented by *Bacillus subtilis* (natto). Domesticated *B. subtilis* strains do not synthesize γ -PGA although they possess the functional biosynthetic *pgs* operon. In the present work we explore the correlation between the genetic determinants, *swrAA* and *degU*, which allow a derivative of the domestic strain JH642 to display a mucoid colony morphology on LB agar plates due to the production of γ -PGA. Full activation of the *pgs* operon requires the co-presence of SwrAA and the phosphorylated form of DegU (DegU~P). The presence of either DegU~P or SwrAA alone has only marginal effects on *pgs* operon transcription and γ -PGA production. Although SwrAA was identified as necessary for swarming and full swimming motility together with DegU, we show that motility is not involved in γ -PGA production. Activation of γ -PGA synthesis is therefore a motility-independent phenotype in which SwrAA and DegU~P display a cooperative effect.

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INTRODUCTION

Poly- γ -glutamic acid (γ -PGA) is an anionic polymer of increasing industrial interest composed of thousands of glutamic acid residues (1200–12000) linked by γ -glutamyl bonds (Shih & Van, 2001; Candela & Fouet, 2006). In *Bacillus subtilis* its synthesis is carried out by the *pgs* operon containing the genes *pgsB*, *pgsC*, *pgsAA* and *pgsE* (formerly *ywsC*, *ywtA,B,C*). In contrast to wild strains, most laboratory strains of *B. subtilis* do not produce γ -PGA because the *pgs* operon is not transcribed (Urushibata *et al.*, 2002). In a search for the genetic determinants that control the formation of the exopolymeric matrix in which *B. subtilis* biofilms are encased, Stanley & Lazazzera (2005) identified *degQ* and *swrAA* as the genes responsible for γ -PGA production, allowing a non-producer laboratory strain to be converted into a mucoid γ -PGA producer by simply introducing the *degQ_H* (*sacQ36*, Yang *et al.*, 1986) and *swrAA⁺* alleles (Kearns *et al.*, 2004; Calvio *et al.*, 2005). The *degQ_H* mutation is a single base substitution in the *degQ* promoter enhancing its expression (Yang *et al.*, 1986). DegQ facilitates phosphate transfer from the DegS kinase to its cognate response regulator DegU (Kobayashi, 2007) and strains carrying the *degQ_H* mutation have the same phenotype as *degU(Hy)* and *degS(Hy)* mutant strains (Amory *et al.*, 1987; Msadek *et al.*, 1990), with the latter mutations reducing the dephosphorylation rate of DegU

(Tanaka *et al.*, 1991; Dahl *et al.*, 1992) and resulting in an overall high level of phosphorylated DegU (DegU~P). SwrAA is a small protein (117 amino acids) without any recognizable structural motif from which a molecular role can be inferred. Laboratory strains carry a single nucleotide insertion in the coding region of *swrAA* leading to premature product truncation (*swrAA⁻*) (Kearns *et al.*, 2004). In a laboratory strain, complementation experiments with a wild-type (wt) copy of the gene resulted in a significant improvement in both swimming capacity and ability to swarm on semi-solid surfaces treated with surfactin (Calvio *et al.*, 2005, 2008), biasing cells into a σ^D -ON state (Kearns & Losick, 2005). The *swrAA⁻* motility phenotype could be suppressed by a *fla/che* promoter up-mutation, and microarray experiments indicated that SwrAA enhances expression of the *fla/che* operon, acting on the σ^A -dependent promoter *P_{fla/che(A)}* (Kearns & Losick, 2005). Several lines of evidence point to a cooperation between SwrAA and DegU~P in motility (Kobayashi, 2007; Verhamme *et al.*, 2007; Calvio *et al.*, 2008) and here we demonstrate not only that the two proteins must cooperate to drive γ -PGA production, but also that such an effect is achieved independently of the activation of the *fla/che* promoter.

METHODS

Bacterial strains and growth conditions. *B. subtilis* and *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. LB

Abbreviations: γ -PGA, poly- γ -glutamic acid; DegU~P, phosphorylated DegU; wt, wild-type.

broth contained 1% tryptone (Difco), 1% NaCl and 0.5% yeast extract (Difco). LB plates were prepared by the addition of 1.5% (w/v) agar. Where appropriate, antibiotics were added at the following concentrations: 60 μ g spectinomycin ml⁻¹, 1 μ g erythromycin ml⁻¹, 2 μ g kanamycin ml⁻¹, 5 μ g phleomycin ml⁻¹.

Strain construction. Bacterial strains and primers used in this study are listed in Tables 1 and 2, respectively. Our *swrAA*⁺ PB5249 strain is a spontaneous derivative of JH642 (Calvio *et al.*, 2005), while PB5370 is a marker-less strain derived from PB5249 carrying the *swrAA*⁻ gene (Calvio *et al.*, 2008).

To construct strains with *degU32*(Hy) or *degS200*(Hy) mutations, the *degS*–*degU* region was amplified with primers *loxdegS1* and *loxdegU1* from PB5213 and PB5246 chromosomal DNA (Amati *et al.*, 2004) as templates. The products were cloned into the *XhoI* and *HindIII* sites of plasmid pLoxSpec (a generous gift from E. Ferrari, Genencor, Palo Alto, CA, USA), thus generating pLoxdegS1 U(Hy) or pLoxdegS200-U1. The region upstream of *degS*–*degU* was amplified from PB5249 chromosomal DNA by using primers *loxdegS2* and *loxdegS3* and the PCR product was cloned into the corresponding site of pLoxdegS1 U(Hy) or pLoxdegS200-U1 after *NorI* digestion. The resulting recombinant plasmids pLoxSpec/degSU(Hy) and pLoxSpec/degS200 contain the spectinomycin resistance gene flanked by the region upstream of the *degS*–*degU* locus and by the *degS*–*degU* locus itself carrying either the *degU32*(Hy) or *degS200*(Hy) mutations. The recombinant plasmids, linearized with *NcoI* and *AhdI*, were transformed into strain PB5249 or PB5370. Since the resistance marker is distant from both mutations, spectinomycin-resistant transformants did or did not incorporate the desired mutations depending on the position of the recombination event. The presence of the *degU32*(Hy) and *degS200*(Hy) mutations was checked by

digestion with *Eco571* and *EcoRI*, respectively. Strains PB5381 (*degU*/S⁺ *swrAA*⁺), PB5382 (*degU*/S⁺ *swrAA*⁻), PB5383 [*degU32*(Hy) *swrAA*⁺], PB5384 [*degU32*(Hy) *swrAA*⁻], PB5390 [*degS200*(Hy) *swrAA*⁺] and PB5391 [*degS200*(Hy) *swrAA*⁻] were thus constructed.

For the construction of the *pgsB*–*lacZ* transcriptional fusions, PB5249 and PB5370 were transformed with chromosomal DNA extracted from the *pgsB*::pMUTIN4 strain BFS1235 (Schumann *et al.*, 2001), selecting for erythromycin resistance. The resulting derivatives, in which the *pgs* locus is inactivated by pMUTIN4 insertion, were in turn transformed using PB5381–84 chromosomal DNAs as donor and selected for spectinomycin resistance to give rise to strains PB5416–19. The incorporation of the *degU32*(Hy) mutation was checked by digestion with *Eco571*.

For the construction of strain PB5422, PB5249 was co-transformed with PB5383 and PB5129 DNAs. Transformants were selected for resistance to kanamycin and spectinomycin and checked for the conservation of the *swrAA*⁺ marker. For the construction of *degU32*(Hy) strains carrying the mutant *swrA* promoters, strains PB5393, PB5394 and PB5395 (Calvio *et al.*, 2008) were transformed with pLoxSpec/degSU(Hy), producing strains PB5438, PB5439 and PB5440, respectively.

To introduce the *dhsA6* mutation (Amati *et al.*, 2004) into PB5249 and PB5370, the shuttle vector pMAD (Arnaud *et al.*, 2004) was used. A PCR fragment was amplified from PB5319 (Amati *et al.*, 2004) using primers *codYF* and *nRflgBN*, digested with *EcoRI* and *NcoI* and inserted between the corresponding sites of pMAD. The resulting plasmid pMADdhs was used to transform PB5370 following the procedure described by Arnaud *et al.* (2004). The incorporation of the *dhsA6* mutation was checked by sequencing. The resulting strain was transformed with pLoxSpec/degSU(Hy),

Table 1. Strains used in this study

Strain	Relevant genotypes and phenotypes	Source or reference
<i>E. coli</i>		
DH5 α	<i>supE44 lacU169 [ϕ80lacZΔM15] hsdR17λrλ⁻mλ⁺ recA1 endA1 gyrA96 thi-1 relA1</i>	Lab strain
<i>B. subtilis</i>		
BFS1235	<i>trpC2 pgsB::pMUTIN4</i> (Em ^r)	Schumann <i>et al.</i> (2001)
PB5129	<i>trpC2 ΔflaA::kan</i> (Km ^r)	Barilla <i>et al.</i> (1994)
PB5213	<i>trpC2 degU32</i> (Hy)	Amati <i>et al.</i> (2004)
PB5246	<i>trpC2 leuA8 degS200</i> (Hy)	Amati <i>et al.</i> (2004)
PB5249	<i>trpC2 pheA1 swrAA</i> ⁺	Calvio <i>et al.</i> (2005)
PB5370	<i>trpC2 pheA1 swrAA</i> ⁻	Calvio <i>et al.</i> (2008)
PB5381	<i>trpC2 pheA1 swrAA</i> ⁺ (Sm ^r)	This study
PB5382	<i>trpC2 pheA1 swrAA</i> ⁻ (Sm ^r)	This study
PB5383	<i>trpC2 pheA1 swrAA</i> ⁺ <i>degU32</i> (Hy) (Sm ^r)	This study
PB5384	<i>trpC2 pheA1 swrAA</i> ⁻ <i>degU32</i> (Hy) (Sm ^r)	This study
PB5390	<i>trpC2 pheA1 swrAA</i> ⁺ <i>degS200</i> (Hy) (Sm ^r)	This study
PB5391	<i>trpC2 pheA1 swrAA</i> ⁻ <i>degS200</i> (Hy) (Sm ^r)	This study
PB5416	<i>trpC2 pheA1 swrAA</i> ⁺ <i>pgsB</i> ::pMUTIN4 (Em ^r) (Sm ^r)	This study
PB5417	<i>trpC2 pheA1 swrAA</i> ⁻ <i>pgsB</i> ::pMUTIN4 (Em ^r) (Sm ^r)	This study
PB5418	<i>trpC2 pheA1 swrAA</i> ⁺ <i>degU32</i> (Hy) <i>pgsB</i> ::pMUTIN4 (Ery ^r) (Sm ^r)	This study
PB5419	<i>trpC2 pheA1 swrAA</i> ⁻ <i>degU32</i> (Hy) <i>pgsB</i> ::pMUTIN4 (Ery ^r) (Sm ^r)	This study
PB5422	<i>trpC2 pheA1 swrAA</i> ⁺ <i>degU32</i> (Hy) Δ <i>flaA</i> :: <i>kan</i> (Km ^r) (Sm ^r)	This study
PB5429	<i>trpC2 pheA1 swrAA</i> ⁺ <i>degU32</i> (Hy) <i>pks</i> ::P _{<i>swrA</i>} WT- <i>lacZ</i> Δ <i>sigD</i> (Sm ^r) (Km ^r) (Em ^r)	Calvio <i>et al.</i> (2008)
PB5438	<i>trpC2 pheA1 P_{swrA}DA⁻-swrAA</i> ⁺ <i>degU32</i> (Hy) (Km ^r) (Sm ^r)	This study
PB5439	<i>trpC2 pheA1 P_{swrA}D⁻-swrAA</i> ⁺ <i>degU32</i> (Hy) (Km ^r) (Sm ^r)	This study
PB5440	<i>trpC2 pheA1 P_{swrA}DA⁻-swrAA</i> ⁺ <i>degU32</i> (Hy) (Km ^r) (Sm ^r)	This study
PB5447	<i>trpC2 pheA1 swrAA</i> ⁻ <i>dhsA6 degU32</i> (Hy) (Sm ^r)	This study

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (restriction sites are underlined)
BL468	5'-CGAAGACGTTGTCCGATATTTAAAG-3'
BL469	5'-CAACAGTCTCAGTCAAAATATCAGC-3'
BL517	5'-CTGTAAACCCAGATTATCAAATC-3'
BL518	5'-CTGCGCGGCAGTTCATGATGAT-3'
loxdegS1	5'-CCGCTCGAGATAAAAGACTGCCTATACAAATTCG-3'
loxdegS2	5'-ATAAGAATGCGGCCGCTTAGCAGGAGGGTCAGCTA-3'
loxdegS3	5'-ATAAGAATGCGGCCGCTTCCGTTCTACAACACCTA-3'
loxdegU1	5'-CCCAAGCTTGTCTGCAAGCATCTGCAGCACTTCAC-3'
codYF	5'-CGGAATTCTAGTGAATATACCTGCTTTCCC-3'
nRflgBN	5'-GCCCATGGTTATAGTTTCGGTGTATCTATATTG-3'

producing PB5447. Recombinant plasmids and chromosomal mutations were sequenced.

β -Galactosidase assay. To assay β -galactosidase activity, overnight cultures grown in Antibiotic Medium 3 (Difco) containing 0.5% glucose were diluted in fresh LB medium to an OD₆₀₀ of 0.2. Cells were grown at 37 °C with shaking and samples (1 ml) were taken at 30 min intervals for OD₆₀₀ readings. Cell pellets were resuspended in 1 ml Z buffer (Miller, 1972) containing 100 µg lysozyme and 10 µg DNase I and lysed by 20 min incubation at 37 °C. β -Galactosidase activity was calculated as described by Miller (1972) according to the formula $(A_{420} \times 1.5) / (OD_{600} \times \text{sample volume in ml} \times \text{reaction time in min} \times 0.00486)$. Experiments were repeated at least three times and a representative result is shown.

RNA isolation and RT-PCR. Total RNA was isolated from cells grown in LB at 37 °C to OD₆₀₀ 1.2 as previously described (Amati *et al.*, 2004). RNA template (500 ng) was reverse transcribed using random primers and ThermoScript Reverse Transcriptase (Invitrogen), following the manufacturer's instructions. The resulting cDNA was used as template in PCRs with the following primer pairs: BL517 and BL518 for *pgsB*, and BL468 and BL469 for *veg* (Gilman & Chamberlin, 1983). PCR products were analysed by 1% agarose gel electrophoresis.

Motility and γ -PGA production assays. Swimming and swarming assays were performed as already described (Calvio *et al.*, 2008). Motility was scored as positive when cells colonized the entire 8.5 cm plate in 13 h at 30 °C. γ -PGA production was considered positive only when colonies displayed a mucoid phenotype on LB plates after 16 h incubation at 37 °C.

γ -PGA isolation. Cells were grown at 37 °C with shaking in LB. A 5 ml sample of the culture supernatant was collected at t_1 (1 h after the transition phase) to isolate γ -PGA as described by Stanley & Lazazzera (2005), omitting the dialysis step. Roughly 2% (v/v) of the recovered γ -PGA samples were loaded on 6% acrylamide gels stained with 0.5% methylene blue in 3% acetic acid for 30 min and destained in H₂O.

RESULTS AND DISCUSSION

Genetic requirements for γ -PGA production

γ -PGA production could be induced in a domestic *B. subtilis* strain (JH642) by transformation with the *degQ_H* and *swrAA*⁺ alleles (Stanley & Lazazzera, 2005). Since the

degQ_H and the *degU32(Hy)* mutants have similar phenotypes as regards protease production, motility and transformation efficiency (Amory *et al.*, 1987), and taking into account that DegQ enhances DegU phosphorylation (Kobayashi, 2007), we sought to determine if a laboratory strain carrying the *degU32(Hy)* and the *swrAA*⁺ alleles could also activate the *pgs* operon. As expected, the *degU32(Hy)* as well as the *degS200(Hy)* mutations were independently able to convert *swrAA*⁺ laboratory strains into γ -PGA producers (Fig. 1, lanes 1 to 3) with a translucent and mucoid colony morphology. γ -PGA production was dependent on a functional *pgs* operon, since its disruption eliminated mucoid morphology on LB plates (data not shown). Thus, the *degQ_H* allele can be substituted by an increase in the intracellular levels of DegU~P caused by mutations in either DegU or DegS. As shown for *degQ_H* (Stanley & Lazazzera, 2005), also the *degU32(Hy)* and *degS200(Hy)* mutations activated γ -PGA production exclusively in *swrAA*⁺ strains, as isogenic *swrAA*[−] strains were non-mucoid and γ -PGA recovery was

Fig. 1. γ -PGA production in strains with different genetic backgrounds: electrophoretic separation of γ -PGA purified from strains with different combinations of the *swrAA* and *degS/U* alleles. The isogenic strains carry the *swrAA* gene either in the wt form (+) or with a truncating mutation (−) and the *degS/U* alleles either in the wt form (SU^{wt}) or with the *degU32(Hy)* (U^{hy}) or the *degS200(Hy)* (S^{hy}) mutations, as indicated. The strains are: PB5381 in lane 1, PB5383 in lane 2, PB5390 in lane 3, PB5384 in lane 4 and PB5391 in lane 5. γ -PGA was purified from the culture supernatant as described in Methods.

highly compromised (Fig. 1, compare lanes 2 and 4 or 3 and 5).

Cooperativity of DegU~P and SvrAA

To assess the relative contribution of SvrAA and DegU~P to the synthesis of γ -PGA, the expression level of the *pgs* operon was measured by RT-PCR in four isogenic laboratory strains differing in the status of the *svrAA* (wt or mutant) and *degU* (wt or Hy) alleles. The expression of *veg*, a gene constitutively expressed throughout the cell cycle, was used as an internal control (Gilman & Chamberlin, 1983). In a *degU*⁺ *svrAA*[−] genetic background *pgs* transcripts were not detected and only trace amounts were found in a *degU*⁺ *svrAA*⁺ strain (Fig. 2a, lanes 2 and 1, respectively). The *pgs* operon showed limited expression in a *degU32*(Hy) *svrAA*[−] background; full activation required the presence of both *degU32*(Hy) and *svrAA*⁺ alleles (Fig. 2a, lanes 4 and 3, respectively).

By using a *pgsB-lacZ* translational fusion, transcription of the *pgs* operon could be measured by β -galactosidase assay in the different genetic backgrounds (Fig. 2b). There was no production of β -galactosidase in *degU*⁺ strains, irrespective of the *svrAA* allele present; in a *degU32*(Hy) *svrAA*[−] background the *pgs-lacZ* fusion gave only 58 Miller units, while a fivefold higher activity (309 Miller units) was achieved in a *degU32*(Hy) *svrAA*⁺ background, in line with the results obtained by RT-PCR. On the whole, the data shown in Fig. 2 demonstrated that the effect of SvrAA and DegU~P is cooperative rather than additive. DegU~P alone activated *pgs* transcription only to a minimal level in the absence of SvrAA (see also lanes 4 and 5 in Fig. 1) while the presence of the sole *svrAA*⁺ allele (in a *degU*⁺ background) had no effect on the *pgs* operon activation (see also lane 1 in Fig. 1). Moreover, over-expression of SvrAA from an inducible promoter in a *degU*⁺ strain did not activate the *pgs* operon (data not shown). Thus, the contribution of SvrAA is effective only in the presence of high levels of DegU~P.

Taken together these data indicate that the activation of the *pgs* operon is dependent on the co-presence of SvrAA and a high level of DegU~P and that the two proteins act synergistically. Cooperativity between SvrAA and DegU has already been observed in motility: SvrAA allows full swimming and swarming exclusively when DegU is present and does not confer any motility advantage if the two-component system DegS/U is deleted (Calvio *et al.*, 2008).

Control of *svrAA* expression and γ -PGA production

It had been previously shown that *svrAA* transcription is driven by two promoters, a putative σ^A -dependent promoter $P_{svrAA(A)}$ activated by DegU~P, and a σ^D -dependent promoter $P_{svrAA(D)}$ (Calvio *et al.*, 2008). Since the *degU32*(Hy) mutation prevents SigD production by blocking the $P_{fla/chl(A)}$ (Amati *et al.*, 2004), in a *degU32*(Hy) genetic

Fig. 2. Expression of the *pgs* operon in different genetic backgrounds. The expression of the *pgs* operon in four isogenic strains differing in the status of the *svrAA* and *degU* genes was assessed by RT-PCR (a) or by translational fusions to *lacZ* (b). (a) RT-PCR on total RNA extracted at t_1 (1 h after the transition phase) from strains grown in LB medium. The '+' and '−' indicate the wt or mutant *svrAA* allele, respectively, while the status of *degU* is indicated as 'wt' or 'hy' in the case of the *degU32*(Hy) mutation. Transcripts corresponding to *pgsB* and *veg*, as indicated on the right, were amplified with specific primers (see Methods for details). The strains used are: PB5381 (lane 1), PB5382 (lane 2), PB5383 (lane 3) and PB5384 (lane 4). (b) Relative levels of β -galactosidase (on the left axis) obtained from *pgsB-lacZ* fusions inserted in the different genetic backgrounds. The dashed line refers to the typical growth curve displayed by these strains, with the OD₆₀₀ values given on the right axis; t_0 indicates the transition between the exponential and stationary phases of growth. The strains used are: PB5416 (■, *degU*⁺ *svrAA*⁺), PB5417 (▲, *degU*⁺ *svrAA*[−]), PB5418 (□, *degU*^{Hy} *svrAA*⁺) and PB5419 (△, *degU*^{Hy} *svrAA*[−]). Solid symbols are grouped at the baseline.

background only the $P_{svrAA(A)}$ is active (Calvio *et al.*, 2008). Indeed, mutations in the *svrAA* promoters had different outcomes on γ -PGA production, with deletion of $P_{svrAA(D)}$ (Fig. 3, P_{D-} in lane 3) having a slightly negative effect, but mutation of $P_{svrAA(A)}$ critically compromising it (Fig. 3, P_{A-} in lane 4). As expected, an even stronger reduction of γ -PGA was obtained when both promoters were mutated (Fig. 3, P_{DA-} in lane 5). However, it can be excluded that the role of DegU~P is limited to the induction of *svrAA* expression, since SvrAA overexpression in a *degU*⁺ strain had no effect on γ -PGA production, as reported above.

Fig. 3. γ -PGA production in P_{swrAA} mutants. Electrophoretic separation of γ -PGA purified from *degU32(Hy)* strains carrying the mutant (lane 1, PB5384) or the functional (lanes 2–5) *swrAA* allele. The functional *swrAA* allele was expressed from the wt promoter (lane 2, PB5383) or from promoters carrying mutations in the consensus for the binding of SigD (lane 3, PB5439), SigA (lane 4, PB5438) or both (lane 5, PB5440). A schematic representation of the status of the *swrAA* promoter region present in each strain is given above with the same lane numbering. The symbol ' Δ ' indicates a deletion.

γ -PGA activation by SwrAA does not require the expression of the *fla/che* operon

The molecular role played by SwrAA is still unclear, although in transcriptional profiling assays SwrAA was found to enhance transcription of the motility and chemotaxis operon *fla/che* acting on $P_{fla/che(A)}$ (Kearns & Losick, 2005). If this was the exclusive role played by SwrAA, then a gene(s) belonging to the *fla/che* operon including *sigD*, or a σ^D -dependent gene, had to be responsible for γ -PGA production. As the *fla/che* operon is switched off in a *degU32(Hy)* background (Amati *et al.*, 2004; Calvio *et al.*, 2008) this hypothesis is unlikely, unless the candidate gene(s) can escape *degU32(Hy)* repression. This occurrence was ruled out by the following two sets of experiments: either perturbing the *fla/che* operon in *degU32(Hy) swrAA⁺* strains or, conversely, maintaining the *fla/che* transcription in a *degU32(Hy) swrAA⁻* background. Results from these experiments are summarized in Table 3. In the first instance, a *sigD* null mutation (Calvio *et al.*, 2008) or a 21 kb deletion in the *fla/che* operon (Δ *flaA*, Barilla *et al.*, 1994) did not affect γ -PGA production in a *degU32(Hy) swrAA⁺* background. In the second instance, the effect of the *dhsA6* mutation (Amati *et al.*, 2004), a $P_{fla/che(A)}$ up-mutation, was analysed in a *degU32(Hy) swrAA⁻* strain. This single base substitution in $P_{fla/che(A)}$ allowed swarming and full swimming motilities in the absence of SwrAA but did not confer on this strain the ability to produce γ -PGA. Not even overexpression of SigD from an inducible promoter was sufficient for γ -PGA production in a *degU32(Hy) swrAA⁻* strain. Indeed, SigD

Table 3. γ -PGA production and motility in different *degU32(Hy)* strains

Strain	Relevant genotype			Phenotype*	
	<i>swrAA</i>	<i>fla/che</i>	<i>sigD</i>	Motility	γ -PGA
PB5383	wt	wt	wt	N	Y
PB5384	–	wt	wt	N	N
PB5429	wt	wt	Δ †	N	Y
PB5422	wt	Δ †	wt	N	Y
PB5384 pSigD	–	wt	(+ + +)‡	N	N
PB5447	–	<i>dhsA6</i> §	wt	Y	N

*The motility and γ -PGA phenotypes are referred to the ability to swarm and fully swim on appropriate media (Calvio *et al.*, 2008) and to display a mucoid colony morphology, as described in Methods. 'Y' represents full capacity and 'N' indicates the lack of the phenotype.

†The Δ symbol indicates that the locus was replaced by a resistance marker.

‡SigD was present on a multicopy plasmid (Barilla *et al.*, 1994) and was overexpressed from an IPTG-inducible promoter.

§ $P_{fla/che(A)}$ carries an up-mutation that allows it to escape the *degU32(Hy)* repression (Amati *et al.*, 2004) and to become independent of *swrAA* for full activation.

overexpression represses transcription of the γ -PGA biosynthetic genes *pgsB,C,AA* while it activates *ywtD* (Kearns & Losick, 2005), the gene coding for PgdS, an enzyme involved in γ -PGA degradation (Suzuki & Tahara, 2003). This is additional evidence that SwrAA must activate γ -PGA synthesis through a pathway different from the one acting on the *fla/che* operon.

Concluding remarks

This study has shown that the role played by SwrAA in *pgs* operon expression is independent of its action on $P_{fla/che(A)}$ and therefore the role of DegU32(Hy) and SwrAA in γ -PGA production is independent of their effect on motility.

Activation of DegU and of a second transcriptional regulator such as Spo0A has been recently found to be required for the expression of *aprE*, *yvcA* and *yvaB* in *B. subtilis* (Veening *et al.*, 2008a; Verhamme *et al.*, 2009) and such a dual regulation has been described as a logic AND gate (Veening *et al.*, 2008b). Although it would be tempting to add the *pgs* operon to the list of genes regulated by AND gate systems, the lack of phenotypes produced by SwrAA alone (in the absence of DegU~P) rules out the existence of two independent regulators, as required by this logic circuitry. The data presented here and elsewhere (Calvio *et al.*, 2008) support a model in which SwrAA interacts with DegU~P, thereby enhancing/modifying its properties, although currently there is no evidence supporting a physical contact between the two proteins.

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