Supplementary Material

Just-in-Time Control of Spo0A Synthesis in *Bacillus subtilis* by multiple regulatory mechanisms.

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Table S1. *B. subtilis* strains used in this study.

Name	Genotype		Construction or Reference
PY79	prototrophic		(31)
RL3170	*Δspo0H::cat*		lab collection
RL4259	*Δspo0B::Km*		lab collection
786	*Δ$P_s$spo0A*		this study, PY79 cured from psk5
875	*ΔP_v O_{1-3}spo0A*		this study, PY79 cured from pAC306
883	*ΔO_{1-3}spo0A*		this study, PY79 cured from pAC313
894	*Δ$P_v$spo0A*		this study, PY79 cured from pAC328
ABS988	*amyE::$P_{s(wt)}$-0A-lacZ- spc*		this study, pAC407 -> PY79
ABS1085	*amyE::$P_{s(wt)}$-0A-lacZ- spc::cat*		this study, pDAG32 -> ABS988
ABS955	*amyE::$P_{s(ΔO1-3)}$-0A-lacZ- spc*		this study, pAC370 -> PY79
ABS1112	*amyE::$P_{s(wt)}$-0A-lacZ- spc::cat*	*Δspo0B::km*	this study, RL4259 -> ABS1085
ABS1149	*amyE::$P_{s(ΔO1-3)}$-0A-lacZ- spc*	*Δspo0B::km*	this study, RL4259 -> ABS955
ABS999	*amyE::$P_{s(ΔA-rich)}$-0A-lacZ- spc*		this study, pAC413 -> PY79
ABS956	*amyE::$P_{s(ΔO1-3 Arich)}$-0A-lacZ- spc*		this study, pAC374 -> PY79
ABS1048	*amyE::$P_{s(m1)}$-0A-lacZ- spc*		this study, pAC463 -> PY79
ABS1055	*amyE::$P_{s(m2)}$-0A-lacZ- spc*		this study, pAC458 -> PY79
ABS1205	*amyE::$P_{s(m3)}$-0A-lacZ- spc*		this study, pAC507 -> PY79
ABS1172	*amyE::$P_{s(m2m3)}$-0A-lacZ- spc*		this study, pAC497 -> PY79
ABS1042	*amyE::$P_{s(m4)}$-0A-lacZ- spc*		this study, pAC453 -> PY79
ABS1173	*amyE::$P_{s(m1m2m3)}$-0A-lacZ- spc*		this study, pAC498 -> PY79
ABS1034	*amyE::$P_{s(ins5)}$-0A-lacZ- spc*		this study, pAC434 -> PY79
ABS1035	*amyE::$P_{s(ins11)}$-0A-lacZ- spc*		this study, pAC436 -> PY79
ABS1223	*amyE::$P_{s(wt-VG)}$-0A-lacZ- spc*		this study, pAC527 -> PY79
ABS1224	*amyE::$P_{s(m2-VG)}$-0A-lacZ- spc*		this study, pAC528 -> PY79
ABS1226	*amyE::$P_{s(m3-VG)}$-0A-lacZ- spc*		this study, pAC531 -> PY79
ABS1227	*amyE::$P_{s(m2m3-VG)}$-0A-lacZ- spc*		this study, pAC533 -> PY79
ABS1229	*amyE::$P_{v(wt)}$-lacZ- spc*		this study, pAC557 -> PY79
ABS1230	*amyE::$P_{v(m2)}$-lacZ- spc*		this study, pAC559 -> PY79
ABS1231	*amyE::$P_{v(m3)}$-lacZ- spc*		this study, pAC538 -> PY79
ABS1234	*amyE::$P_{v(m1)}$-lacZ- spc*		this study, pAC563 -> PY79
ABS1045	*amyE::$P_{vs(wt)}$-lacZ- spc*		this study, pAC454 -> PY79
ABS1041	*amyE::$P_{vs(mi)}$-lacZ- spc*		this study, pAC450 -> PY79
ABS997	*amyE::$P_{vs(wt)}$-0A-lacZ- spc*		this study, pAC408 -> PY79
ABS962	*amyE::$P_{vs(mi)}$-0A-lacZ- spc*		this study, pAC391 -> PY79
ABS1050	*amyE::$P_{vs(wt)}$-lacZ- spc*	*Δspo0H::cat*	this study, RL3170 -> ABS1045
ABS1049	*amyE::$P_{vs(mi)}$-lacZ- spc*	*Δspo0H::cat*	this study, RL3170 -> ABS1041
ABS1021	*amyE::$P_{vs(wt)}$-0A-lacZ- spc*	*Δspo0H::cat*	this study, RL3170 -> ABS997
ABS1019	*amyE::$P_{vs(mi)}$-0A-lacZ- spc*	*Δspo0H::cat*	this study, RL3170 -> ABS962
ABS957	*amyE::$P_{s(mi)}$-0A-lacZ- spc*		this study, pAC378 -> PY79

Table S2. Plasmids used in this study.

Plasmids	Description	Reference or construction
pSK5	plasmid for marker-less deletion of P_s	(27)
pMAD	plasmid for in frame marker-less deletion	(1)
pDG1728	plasmid for integrating *lacZ* fusion at *amyE*	*
pET28a	plasmid for expression of recombinant protein in E. coli with a 6xhis tag	Novagen
pAC571	pET28a derivative, carrying full length *spo0A* orf under IPTG inducible promoter	AC883-885 (NcoI-XhoI)
	pMAD derivative carrying *spoIVB-spo0A* intergenic fragment:	
pAC306	deleted for P_v and O_{1-3}	AC739-740 (NcoI-EcoRI) + AC744-742 (EcoRI-BamHI)
pAC313	deleted for O_{1-3}	AC739-743 (NcoI-EcoRI) + AC744-742 (EcoRI-BamHI)
pAC328	deleted for P_v	AC739-740 (NcoI-EcoRI) + AC741-742 (EcoRI-BamHI)
	pDG1728 derivatives carrying Ps (but not Pv) in translational fusion with *lacZ*:	
pAC407	wild type	AC796-781 (EcoRI-SalI)
pAC378	containing 8 mutations between O1 and O2	AC783-781 (EcoRI-SalI)
pAC370	deleted of O1-O3	AC786-781 (EcoRI-SalI)
pAC413	deleted of the A-rich area	AC796-788+AC789-781 (EcoRI-SalI)
pAC374	deleted of O1-O3 and the A-rich area	AC787-781 (EcoRI-SalI)
pAC463	mutated on O1	AC816-781 (EcoRI-SalI)
pAC458	mutated on O2	AC814-781 (EcoRI-SalI)
pAC507	mutated on O3	AC820+821-781 (EcoRI-SalI)
pAC497	mutated on O2 and O3	AC814 on pAC507 (EcoRI-SalI)
pAC453	mutated on O4	AC796-812+AC813-781 (EcoRI-SalI)
pAC498	mutated on O1, O2 and O3	AC811 on PAC497 (EcoRI-SalI)
pAC434	with a 5 bp insertion	AC796-809+AC808-781 (EcoRI-SalI)
pAC436	with a 11 bp insertion	AC796-811+AC810-781 (EcoRI-SalI)
pAC527	containing P_{spoVG} SigH binding site	QC AC852-853 on pAC407
pAC528	containing P_{spoVG} SigH binding site, and mutated on O2	QC AC852-853 on pAC458
pAC531	containing P_{spoVG} SigH binding site, and mutated on O3	QC AC852-853 on pAC507
pAC533	containing P_{spoVG} SigH binding site, and mutated on O2 and O3	QC AC852-853 on pAC497
	pDG1728 derivatives carrying Pv (but not Ps) in transcriptional fusion with *lacZ*:	
pAC557	wild type	AC792-881 (EcoRI-BamHI)
pAC559	mutated on O2	AC792-857+AC856-881 (EcoRI-BamHI)
pAC538	mutated on O3	AC792-859+AC858-881 (EcoRI-BamHI)
pAC563	mutated on O1	AC792-855+AC854-881 (EcoRI-BamHI)
	pDG1728 derivative carrying complete P_{spo0A} :	
pAC454	in transcriptional fusion with lacZ, and wild type	AC792-880 (EcoRI-BamHI)
pAC450	in transcriptional fusion with lacZ, and containing 8 mutations between O1 and O2	AC792-793+AC794-880 (EcoRI-BamHI)
pAC408	in translational fusion with lacZ, and wild type	AC792-781 (EcoRI-SalI)
pAC391	in translational fusion with lacZ, and containing 8 mutations between O1 and O2	AC792-793+AC794-781 (EcoRI-SalI)

* Guerout-Fleury, A. M., N. Frandsen, and P. Stragier. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene 180:57-61.

Table S3. Oligonucleotides used in this study.

name	sequence
AC-470	ttgccagtcacgttacgttattagttatag
AC-739	cctccatggcgcaaaacgatcggggatatt
AC-740	ttcgaattcgtgaagagggatcaaaataaaaaa
AC-741	aaagaattctttttcgacaaattcacgtttccttg
AC-742	gctggatccaatactgctttgcgatgatggcgcac
AC-743	gaagaattcaaatcttcttttgtatattttaccg
AC-744	ggggaattcaaacagagaaaaacatagaataacaaag
AC-766	gcaacacaaactttaattttctccac
AC-781	ttcgtcgacttctatatattcacttaacaggc
AC-783	aaatatacagaattcgatttttcgacaaattgagctatggaagtttgtca
AC-786	acagaattcgaaaaacagagaaaaacatagaataac
AC-787	actgaattcggtgattatgatttttttagaggg
AC-788	cataatcacctttcgactaaaaatgaaatttgac
AC-789	tagtcgaaaggtgattatgatttttttagaggg
AC-792	caggaattcatatttatggaaaagaaaaagcaagctgac
AC-793	ttccatagctcaatttgtcgaaaaatcttcttttgtat
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AC-796	acagaattcgatttttcgacaaattcacgtttccttg
AC-808	ttggtgattatcttatgatttttttagagggtatatag
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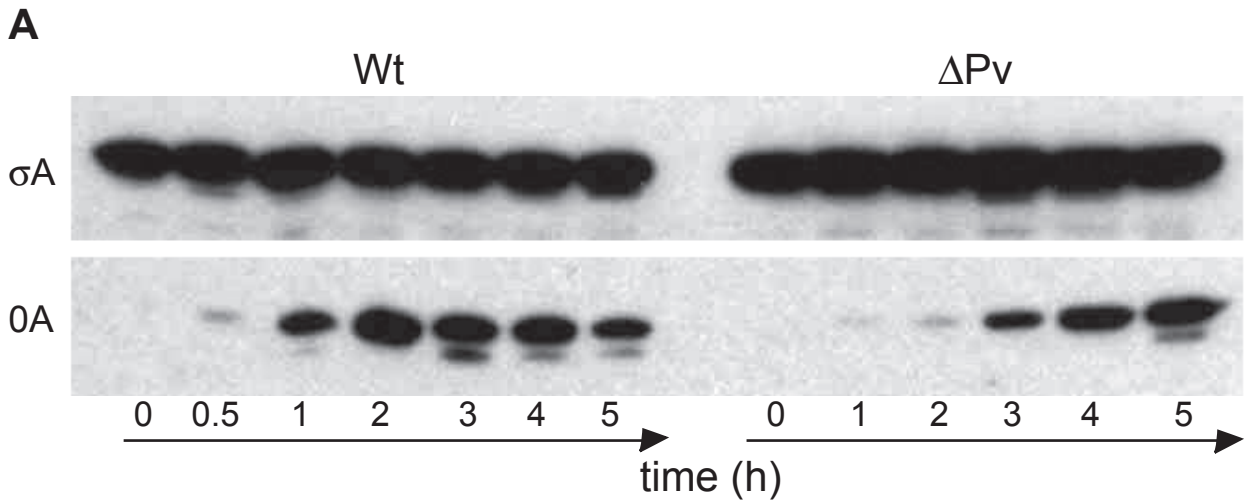
AC-810	ttggtgattatctagaggctatgatttttttagagggtatatag
AC-811	aaatcatagcctctagataatcaccaatattagtggcatatcttt
AC-812	gctacatgtttacattctccgtgaccgctatataccct
AC-813	agggtatatagcggtcacggagaatgtaaacatgtagc
AC-814	acagaattcgatttttcgacaaattcacgtttccttgtttattaaatttcattt
AC-816	acagaattcgatttttatataaattcacgtttccttg
AC-820	cacgtttccttgtttgtcaaatttcatttttatttaaaaaac
AC-821	acagaattcgatttttcgacaaattcacgtttccttgtttgtcaaatttc
AC-852	cagaaaaaatcgtggaattgaaacatgtagcaagggtgaatcctgtt
AC-853	gatttttttctgaaatcctgctaaaaaatcataatcaccaatattagtggc
AC-854	gatttttatataaattcacgtttccttgtt
AC-855	gaatttatataaaaatcttcttttgtatattttac
AC-856	ccttgtttattaaatttcatttttagtcgaaaaac
AC-857	gaaatttaataaacaaggaaacgtgaatttgtcg
AC-858	catttttatttttaaaaacagagaaaaacatagaataac
AC-859	tttttctctgtttttaaaataaaaatgaaattt
AC-880	ggaggatccatgtagttaacaggattcacccttgctacat
AC-881	ggaggatcctctaaaaaaatcataatcaccaat
AC-883	cggccAtggagaaaattaaagtttgtgttgctgat
AC-885	ctcctcgagttaagaagccttatgctctaacctcagc

Fig Sup 1


A
Wt
ΔPv
σA
0A
0 0.5 1 2 3 4 5
0 1 2 3 4 5
time (h)

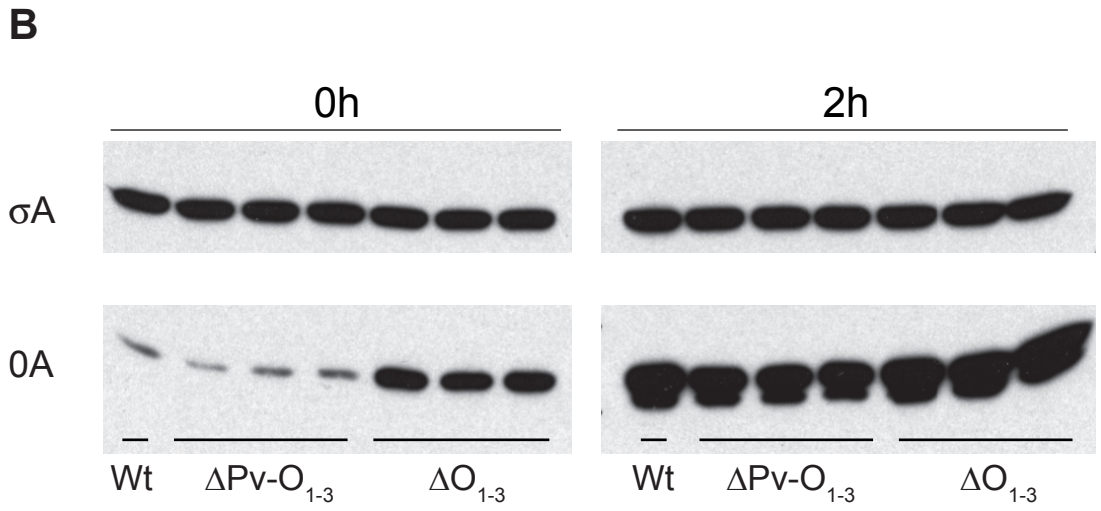

B
0h
2h
σA
0A
Wt
ΔPv-O1-3
ΔO1-3
Wt
ΔPv-O1-3
ΔO1-3

Figure sup. 1. Immunoblot analysis of 0A accumulation.

Panel A shows that accumulation of 0A is delayed and slowed in absence of P_v. Immunoblot analysis of 0A accumulation during sporulation was performed using samples taken during sporulation at the indicated times after resuspension. Equal amounts of protein, as quantified by the Bradford technique, were loaded as confirmed by the control immunoblot using anti-SigA (σ^A). Samples were taken from shaking cultures at 37°C of wild type cells (Wt; PY79) and cells mutant for P_v (ΔP_v; Abs894).

Panel B shows that O_1-O_3 contains a regulatory element controlling P_s.

Presented is an immunoblot analysis of 0A accumulation during sporulation similar to Fig 2A, except that a longer exposure time was performed in order to observe 0A at time 0. Blots were performed using samples taken just before sporulation induction by resuspension (0h) and 2 h post-induction (2h). Equal amounts of protein, as quantified by the Bradford technique, were loaded as confirmed by the control immunoblot using anti-SigA (σ^A) antibodies. Samples were taken from triplicate shaking culture at 37°C of wild type cells (Wt; PY79), cells mutant for $O_{1\text{-}3}$ ($\Delta O_{1\text{-}3}$; Abs883), and P_v plus $O_{1\text{-}3}$ ($\Delta P_v\, O_{1\text{-}3}$; Abs875).

Fig Sup 2

	time	0	+ -	90	+ -
I	**wt**	**1.0**	0.0	**14.2**	6.5
	m1	**0.8**	0.3	**16.5**	3.1
II	**m4**	**0.4**	0.1	**3.4**	0.7
	m3	**1.8**	0.3	**2.7**	1.5
III	**m2**	**5.7**	1.5	**17.7**	5.6
IV	**m2 m3**	**5.9**	2.3	**5.4**	2.6
	m1 m2 m3	**5.9**	2.6	**6.2**	5.6
	Δ123	**5.8**	2.5	**5.7**	2.2
	ΔA rich	**5.3**	2.3	**4.5**	3.7
	Δ123-A rich	**5.5**	2.8	**1.9**	0.6

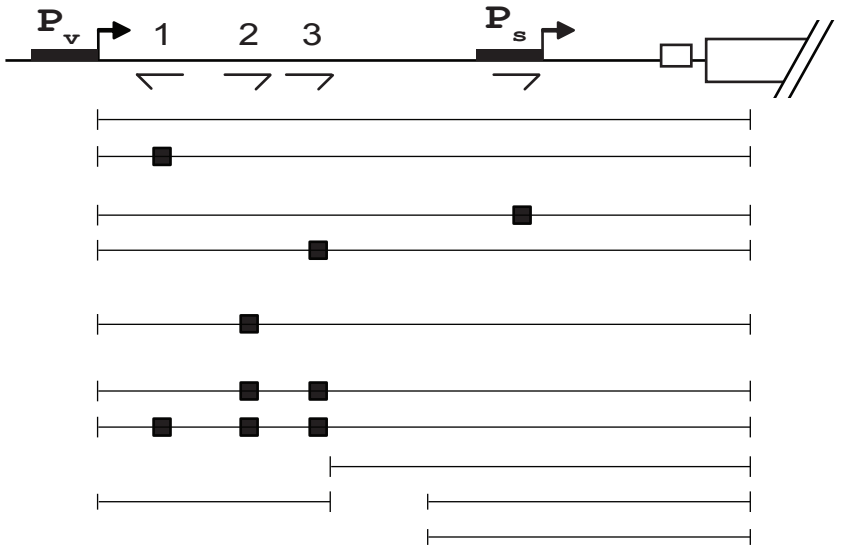


Figure sup. 2. Mutagenesis of a P_s-*lacZ* translational reporter fusion reveals four categories of mutants.

Table (left hand panel) reports β-galactosidase activity of different translational fusions with *lacZ* that are depicted in right hand panel. Two time points, representative of exponential and sporulation phase of growth, were reported: time 0 corresponds to sample harvested just before induction of sporulation by resuspension, and time 90 to cells collected 90 minutes after induction. Values are presented as a ratio as compared to the specific activity of the wild type fusion during exponential growth (thus, the ratio for the wild type fusion at time 0 was 1). Bold values are averaged ratio of specific activities, followed by the standard deviations.

Four categories of phenotypes are:

I- Wild type: expression was minimal during exponential phase and strongly induced during sporulation; wild type (wt; Abs988), mutant of O_1 (m1; Abs1048).

II- Shut down: expression is minimal in every condition tested; mutant of site 3 (m3; Abs1205) or site 4 (m4; Abs1042).

III- De-repressed: intermediate level of expression during exponential phase and full induction during sporulation; mutant of site 2 (m2; Abs1055).

IV- 0A insensitive: expression is almost constant from time 0 to time 90; double mutant for site2 and 3 (m2m3; Abs1172), triple mutant for site 1, 2 and 3 (m1m2m3; Abs1173), fragment lacking O_{1-3} (ΔO_{1-3}; Abs955), fragment lacking only A-rich area (ΔA-rich; Abs999), fragment starting downstream of the A-rich area (Δ123 A-rich; Abs956).

Fig Sup 3

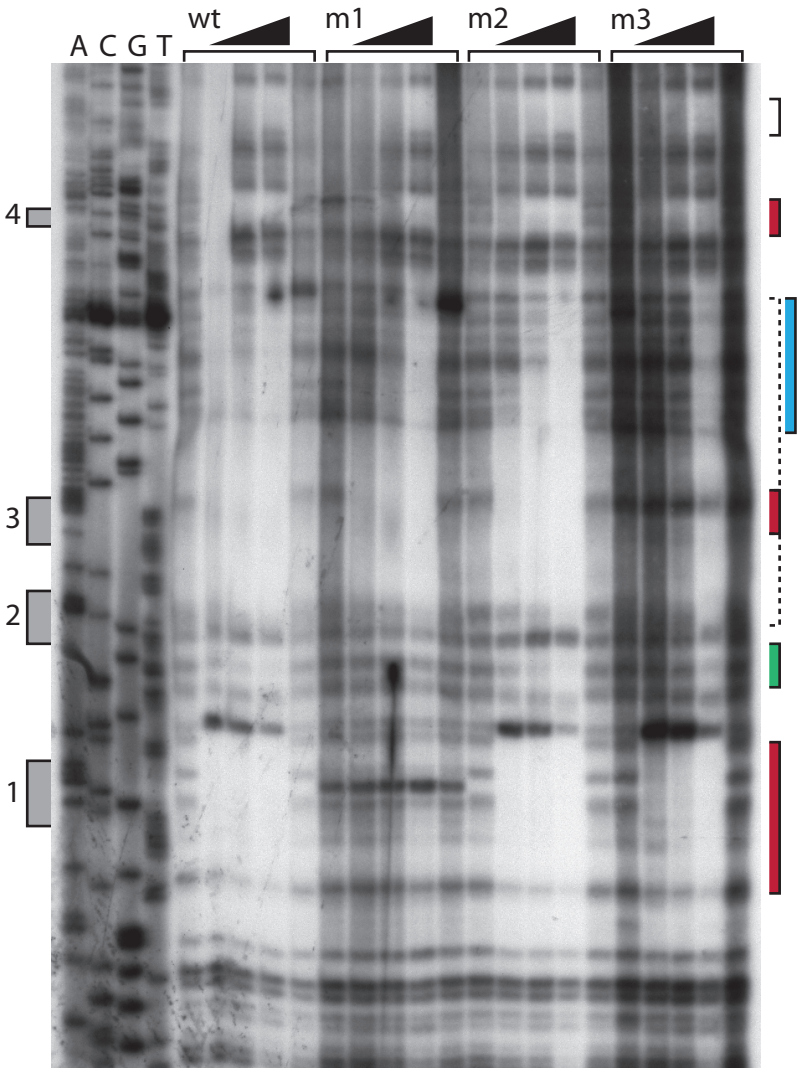


Figure sup. 3. Mutations in O_1 and O_3 impair 0A binding to corresponding sites. DNase I footprinting assays were performed using purified 0A and labeled DNA fragments as described in material and methods. 364 bp DNA fragments were amplified from plasmid pAC407 (wt), pAC463 (m1), pAC458 (m2) or pAC507 (m3) and incubated with varying amount of purified native 0A (0, 1, 3, 10, 0 μM). First four lanes correspond to dideoxy-sequencing ladders produced with the same labeled primer. Boxes on the left indicated the consensus 0A binding sequences. Bars on the right indicate areas protected from DNase I digestion and the dotted line indicates extension of the protection occurring at higher 0A concentration. Red boxes are areas matching with 0A-boxes O1, O3 and O4; the green box marks a protected area appearing only when O1 and O3 and present; the blue box marks an area protected at high 0A concentration despite the absence of protection on box O3.

Fig Sup 4

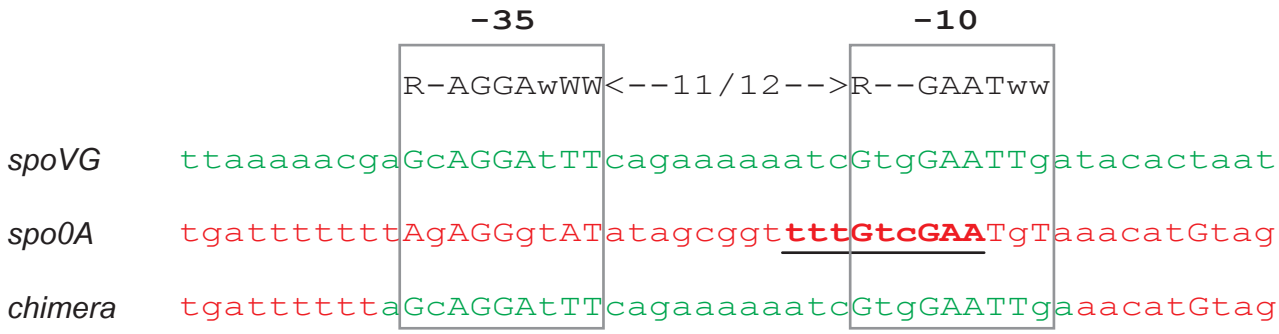

-35
-10
R-AGGAwWW<--11/12-->R--GAATww
spoVG ttaaaaacgaGcAGGAtTTcagaaaaaatcGtgGAATTgatacactaat
spo0A tgatttttttAgAGGgtATatagcggttttGtcGAATgTaaacatGtag
chimera tgatttttaGcAGGAtTTcagaaaaaatcGtgGAATTgaaacatGtag

Figure sup. 4. Construction of a chimeric promoter between P_s and P_{spoVG}. 31 bp corresponding to the *spoVG* promoter (green) and containing the -35 and -10 sequences for σ^H–RNA polymerase were introduced in place of the -35 and -10 sequences of the *spo0A* P_s promoter (red) to create a chimeric regulatory region. Aligned are sequences of *spoVG*, *spo0A* and the chimeric promoter corresponding to the region containing the -35 and -10 sequences. Capital letters indicate bases matching the σ^H–RNA polymerase consensus binding motif (Upper row - according to(3)). The *spo0A* operator O_4 is highlighted with underlined bold letters.

Fig sup 5

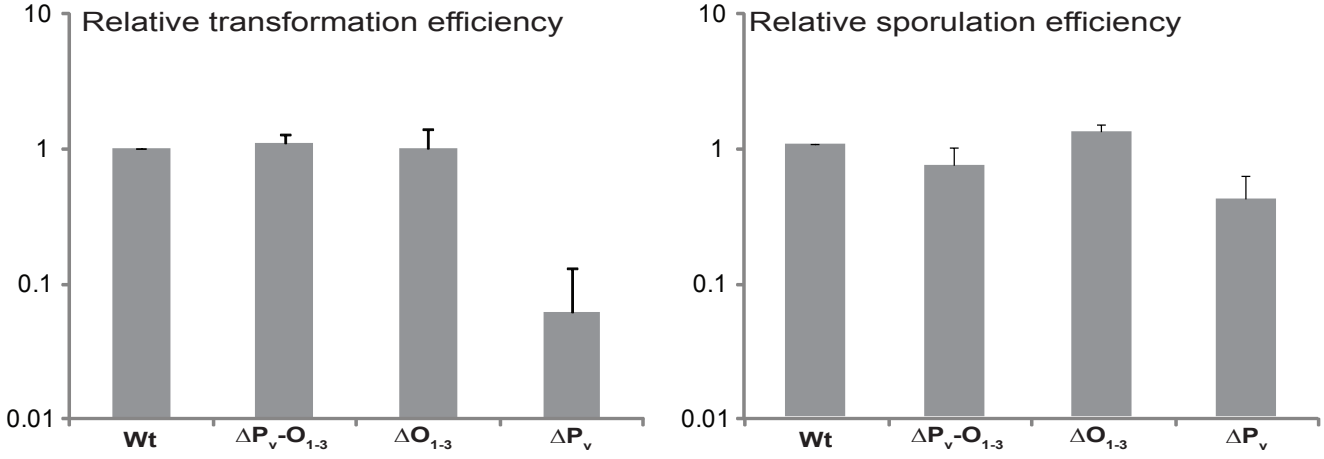

Relative transformation efficiency
10
1
0.1
0.01
Wt
ΔPv-O1-3
ΔO1-3
ΔPv
Relative sporulation efficiency
10
1
0.1
0.01
Wt
ΔPv-O1-3
ΔO1-3
ΔPv

Figure sup. 5. A mutant lacking P_v is slightly inhibited for sporulation and impaired for DNA-mediated transformation (competence).

Transformation and sporulation efficiencies were compared between wild type cells (Wt; PY79), cells mutant for $O_{1\text{-}3}$ ($\Delta O_{1\text{-}3}$; Abs883), P_v (ΔP_v; Abs894), and P_v plus $O_{1\text{-}3}$ ($\Delta P_v\ O_{1\text{-}3}$; Abs875), and expressed as the ratio to the wild type. For the competence assay (left panel), cells were transformed with 0.5 µg of DNA conferring resistance to spectinomycin and frequencies of transformants were calculated as the ratio of spectinomycin-resistant cells to the total number of cells. Values are averages of two independent experiments. For the sporulation assay, the frequency of sporulation after 30 h in sporulation conditions was calculated as the number of heat-resistant cells after 20 min at 80°C to the total number of cells. Values are averages of at least two independent experiments.