TABLE S1: *Bacillus subtilis* strains used in this study, related to STAR Methods

Strains	Genotype	Source
bBB042	*spoIIIE-gfp (spec)*	(Burton et al., 2007)
bBB069	*spoIIIE36-gfp (kan)*	(Besprozvannaya et al., 2014)
bDR2413 (168)	wild-type	(Zeigler et al., 2008)
bCR1557	*pbpG::kan*	This study
bCR1558	*pbpF::tet*	This study
bCR1592	*spoIIIM::erm*	This study
bCR1600	*pbpG::kan, spoIIIM::erm*	This study
bCR1602	*pbpF::loxP tet, spoIIIM::erm*	This study
bAT001	*pbpG::kan, pbpF::tet*	This study
bAT007	*ycgO::PspoIIIM-spoIIIM (spec), spoIIIM::erm*	This study
bAT010	*ycgO::PspoIIIM-spoIIIM (spec), pbpG::kan, spoIIIM::erm*	This study
bAT023	*ycgO::PspoIIIM-opt$_{RBS}$-gfp-spoIIIM(spec), spoIIIM::erm*	This study
bAT024	*ycgO::PspoIIIM-opt$_{RBS}$-gfp-spoIIIM(spec), spoIIIM::erm, pbpG::kan*	This study
bAT087	*amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT088	*amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::erm*	This study
bAT089	*amyE::PspoIIQ-cfp(Bs) (cat), pbpG::kan*	This study
bAT090	*amyE::PspoIIQ-cfp(Bs) (cat), pbpF::tet*	This study
bAT091	*amyE::PspoIIQ-cfp(Bs) (cat), pbpG::kan, spoIIIM::erm*	This study
bAT092	*amyE::PspoIIQ-cfp(Bs) (cat), pbpF::tet, spoIIIM::erm*	This study
bAT121	*ycgO::spoIIIM-his6 (spec), spoIIIM::erm*	This study
bAT122	*ycgO::spoIIIM-his6 (spec), pbpG::kan, spoIIIM::erm*	This study
bAT440	*spoIIIE::neo, spoIIIM::erm, ycgO::PspoIIIM-opt$_{RBS}$-gfp-spoIIIM-(spec)*	This study
bAT353	*ycgO::PspoIID-opt$_{RBS}$-gfp-spoIIIM(spec)*	This study
bAT442	*spoIIIE::neo, ycgO::PspoIID-opt$_{RBS}$-gfp-spoIIIM(spec)*	This study
bAT455	*amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT456	*amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT457	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT458	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72*	This study
bAT459	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT460	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT469	*spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT470	*spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72*	This study
bAT471	*spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT472	*spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT475	*spoIIIE::neo, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT476	*spoIIQ::erm, spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT478	*spoIIQ::erm, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT479	*spoIIQ::erm, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72*	This study
bAT480	*spoIIQ::erm, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT481	*spoIIQ::erm, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT490	*spoIIQ::erm, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT491	*spoIIQ::erm, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72*	This study
bAT492	*spoIIQ::erm, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT493	*spoIIQ::erm, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT497	*spoIIIE::neo, spoIIQ::erm, spoIIP::tet, spoIID::spec, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT498	*ycgO::PspoIIIE-spoIIIE*D584A(phleo), spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT499	*ycgO::PspoIIIE-spoIIIE*D584A(phleo), spoIIIE::neo, spoIIIM::lox72, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT500	*ycgO::PspoIIIE-spoIIIE*D584A(phleo), spoIIIE::neo, pbpG::lox72, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT501	*ycgO::PspoIIIE-spoIIIE*D584A(phleo), spoIIIE::neo, spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-cfp(Bs) (cat)*	This study

bAT538	*amyE::PspoIIQ-opt$_{RBS}$-cfp (kan), sigE::erm, yrvN::Phyperspank-opt$_{RBS}$-spoIIM-lacI (spec), ykoW::Phyperspank-opt$_{RBS}$-spoIIP-lacI (phleo), pelB::Phyperspank-opt$_{RBS}$-spoIID-lacI (cat)*	This study
bAT539	*amyE::PspoIIQ-opt$_{RBS}$-cfp (kan), spoIIQ::tet, sigE::erm, yrvN::Phyperspank-opt$_{RBS}$-spoIIM-lacI (spec), ykoW::Phyperspank-opt$_{RBS}$-spoIIP-lacI (phleo), pelB::Phyperspank-opt$_{RBS}$-spoIID-lacI (cat)*	This study
bAT540	*amyE::PspoIIQ-opt$_{RBS}$-cfp (kan), spoIIQ::tet, sigE::erm, yrvN::Phyperspank-opt$_{RBS}$-spoIIM-lacI (spec), ykoW::Phyperspank-opt$_{RBS}$-spoIIP-lacI (phleo), pelB::Phyperspank-opt$_{RBS}$-spoIID-lacI (cat), pbpG::lox72*	This study
bAT552	*pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan*	This study
bAT553	*pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, spoIIIM::lox72*	This study
bAT554	*pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72*	This study
bAT555	*pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72, spoIIIM::lox72*	This study
bAT557	*amyE::PspoIIQ-opt$_{RBS}$-cfp (kan), spoIIQ::tet, spoIIIE::lox72, sigE::erm, yrvN::Phyperspank-opt$_{RBS}$-spoIIM-lacI (spec), ykoW::Phyperspank-opt$_{RBS}$-spoIIP-lacI (phleo), pelB::Phyperspank-opt$_{RBS}$-spoIID-lacI (cat)*	This study
bAT558	*spoIIQ::tet, pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan*	This study
bAT559	*spoIIQ::tet, pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, spoIIIM::lox72*	This study
bAT560	*spoIIQ::tet, pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72*	This study
bAT561	*spoIIQ::tet, pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, spoIIIM::lox72, pbpG::lox72*	This study
bAT604	*spoIIB::erm, amyE::PspoIIQ-cfp(Bs) (cat)*	This study
bAT605	*spoIIB::erm, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72*	This study
bAT606	*spoIIB::erm, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT607	*spoIIB::erm, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72, spoIIIM::lox72*	This study
bAT608	*spoIIB::erm, amyE::PspoIIQ-cfp(Bs) (cat),spoIIIE::neo*	This study
bAT627	*spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yycR::lacO48 (cat)*	This study
bAT628	*pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yycR::lacO48 (cat)*	This study
bAT629	*spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yycR::lacO48 (cat)*	This study
bAT630	*spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT631	*pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT632	*spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT633	*spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yhdG::lacO48 (erm)*	This study
bAT634	*pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yhdG::lacO48 (erm)*	This study
bAT635	*spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yhdG::lacO48 (erm)*	This study
bAT643	*spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yrvN::lacO48 (phleo)*	This study
bAT644	*pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yrvN::lacO48 (phleo)*	This study
bAT645	*spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yrvN::lacO48 (phleo)*	This study
bAT646	*amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT647	*amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yhdG::lacO48 (erm)*	This study
bAT648	*amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yycR::lacO48 (cat)*	This study
bAT649	*amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), yrvN::lacO48 (phleo)*	This study
bAT652	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), ycgO::spoIIIE36 (spec)*	This study
bAT653	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, ycgO::spoIIIE36 (spec)*	This study
bAT654	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72, ycgO::spoIIIE36 (spec)*	This study
bAT655	*spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72, ycgO::spoIIIE36 (spec)*	This study
bAT668	*spoIIB::erm, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT669	*spoIIB::erm, spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT670	*spoIIB::erm, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT671	*spoIIB::erm, spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT0679	*ycgO::PspoIIIE-spoIIIE*D584A(phleo), spoIIIE::neo, amyE::PspoIIQ-cfp(Bs) (cat), spoIIQ::tet*	This study
bAT697	*spoIID::cat, spoIIP::tet, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT698	*spoIID::cat, spoIIP::tet, spoIIIM::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study

bAT699	*spoIID::cat, spoIIP::tet, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT700	*spoIID::cat, spoIIP::tet, spoIIIM::lox72, pbpG::lox72, amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pelB::lacO48 (kan)*	This study
bAT743	*pelB::spoIIIE36 (tet), spoIIIE::neo, spoIIIM::erm, ycgO::PspoIIIM-opt$_{RBS}$-gfp-spoIIIM-(spec)*	This study
bAT779	*spoIIP::tet, spoIID::spec, pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan*	This study
bAT810	*yycR(−7°)::tetO48 (cat), ycgO::PftsW tetR-cfp (spec) terminators PftsW lacI-mypet, pelB(+174°)::lacO48 (kan), spoIIIM::lox72, pbpG::lox72*	This study
bAT811	*spoIIQ::tet, ycgO::spoIIIM-his6 (spec), spoIIIM::erm*	This study
bAT834	*spoIIIE::kan, ycgO::spoIIIM-his6 (spec), spoIIIM::erm*	This study
bAT837	*pelB::spoIIIE36 (tet), spoIIIE::neo, ycgO::PspoIID-opt$_{RBS}$-gfp-spoIIIM(spec)*	This study
bAT844	*pelB::spoIIIE36 (tet), spoIIIE::kan, ycgO::spoIIIM-his6 (spec), spoIIIM::erm*	This study
bAT855	*ycgO::PpbpG-his6-pbpG (erm), amyE::PspoIIQ-cfp(Bs) (cat), pbpG::Kan*	This study
bAT856	*ycgO::PpbpG-his6-pbpG (erm), amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::kan*	This study
bAT857	*ycgO::PpbpG-his6-pbpG (erm), amyE::PspoIIQ-cfp(Bs) (cat), pbpG::Kan, pbpF::loxP tet*	This study
bAT858	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), amyE::PspoIIQ-cfp(Bs) (cat), pbpG::Kan*	This study
bAT859	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::kan*	This study
bAT860	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), amyE::PspoIIQ-cfp(Bs) (cat), pbpG::Kan, pbpF::loxP tet*	This study
bAT861	*ycgO::PpbpG-his6-pbpG (erm), spoIIQ::tet, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT862	*ycgO::PpbpG-his6-pbpG (erm), spoIIQ::tet, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT863	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), spoIIQ::tet, amyE::PspoIIQ-cfp(Bs) (cat), pbpG::lox72*	This study
bAT864	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), spoIIQ::tet, amyE::PspoIIQ-cfp(Bs) (cat), spoIIIM::lox72, pbpG::lox72*	This study
bAT865	*ycgO::PpbpG-his6-pbpG (erm), yycR::lacO48 (cat), amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pbpG::lox72*	This study
bAT866	*ycgO::PpbpG-his6-pbpG (erm), yycR::lacO48 (cat), amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), spoIIIM::lox72, pbpG::lox72*	This study
bAT867	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), yycR::lacO48 (cat), amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), pbpG::lox72*	This study
bAT868	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), yycR::lacO48 (cat), amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec), spoIIIM::lox72, pbpG::lox72*	This study
bAT869	*ycgO::PpbpG-his6-pbpG (erm), pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72*	This study
bAT870	*ycgO::PpbpG-his6-pbpG (erm), pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72, spoIIIM::lox72*	This study
bAT871	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72*	This study
bAT872	*ycgO::PpbpG-his6-pbpG (E94A, S365A) (erm), pelB::PspoIIIE-spoIIIE-GFP (cat), spoIIIE::kan, pbpG::lox72, spoIIIM::lox72*	This study
bAT873	*ycgO::PpbpG-his6-pbpG (erm), spoIIIE::kan, pbpG::lox72*	This study
bHC035	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (tet), pbpG::kan, spoIIIM::erm*	This study
bHC036	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (tet), pbpG::lox72*	This study
bHC040	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (E94A, S365A) (tet), pbpG::lox72*	This study
bHC046	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (E94A, S365A) (tet), pbpG::kan, spoIIIM::erm*	This study
bHC050	*spoIIQ::cat, ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (tet), pbpG::kan, spoIIIM::erm*	This study
bHC051	*spoIIQ::cat, ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (tet), pbpG::lox72*	This study
bHC052	*spoIIQ::cat, ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (E94A, S365A) (tet), pbpG::lox72*	This study
bHC053	*spoIIQ::cat, ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (E94A, S365A) (tet), pbpG::kan, spoIIIM::erm*	This study
bWX1200	*spoIIIE36, yycR(−7°)::tetO48 (cat), pelB(+174°)::lacO48 (kan), ycgO::PftsW tetR-cfp(spec) terminators PftsW lacI-mypet*	(Wang et al., 2014)

TABLE S2: Plasmids used in this study, related to STAR Methods

Plasmid	Description	Source
pAT001	*ycgO::spoIIIM (spec)*	This study
pAT003	*ycgO::PspoIIIM-gfp-spoIIIM (spec)*	This study
pAT024	*ycgO::PspoIID-opt$_{RBS}$-gfp-spoIIIM (spec)*	This study
pAT032	*ycgO::spoIIIM-His6 (spec)*	This study
pAT057	*pelB::spoIIIE-GFP (cat)*	This study
pAT090	*ycgO::spoIIIE36 (tet)*	This study
pCR204	*amyE::PspoIIQ-spoIVF$_{RBS}$-lacI-gfp (spec)*	This study
pHC3	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (tet)*	This study
pHC23	*ycgO::PspoIIQ-opt$_{RBS}$-pbpG-His6 (E94A, S365A) (tet)*	This study
pHC28	*P$_{lac}$::T18-spoIIIE (amp)*	This study
pHC29	*P$_{lac}$::T18-spoIIIM (amp)*	This study
pHC30	*P$_{lac}$::T18-pbpG (amp)*	This study
pHC32	*P$_{lac}$::T25-spoIIIE (kan)*	This study
pHC33	*P$_{lac}$::T25-spoIIIM (kan)*	This study
pHC34	*P$_{lac}$::T25-pbpG (kan)*	This study
pHC535	*P$_{lac}$::T25-slmA (kan)*	(Cho et al., 2011)
pHC538	*P$_{lac}$::T18-slmA (amp)*	(Cho et al., 2011)
pJL013a	*pelB::spoIIIE36 (tet)*	This study

TABLE S3: Oligonucleotide primers used in this study, related to STAR Methods

Primers	Sequence*
oAT001	cgcGAATTCctgtataaaaaccgagcattcgc
oAT002	cgcGGATCCttaagggtagaccgggaattt
oAT005	cgcAAGCTTacataaggaggaactactatgagtaaaggagaagaacttttc
oAT006	cgcCTCGAGgccgcttgagcctccagatgatcctttgtatagttcatccatgccatg
oAT007	cgcCTCGAGatgaagcgtctcaccttagtatg
oAT096	aaagcgacagtggcgattgcagaccagaatttctacgat
oAT097	atcgtagaaattctggtctgcaatcgccactgtcgcttt
oAT137	cgcAAGCTTccaaaaaaagcatgttgccgaact
oAT139	gacgcaatgtcatgttcatacg
oAT147	gcgaagcgccagcctgctgcgacgatcaagccgttgctg
oAT148	cagcaacggcttgatcgtcgcagcaggctggcgcttcgc
oAT160	cgcGGATCCtcaatggtgatggtgatgatgtcgccccagcca
oCR353	cgcGGATCCttatgtaatcccagcagctttacatactcatgaaggaccatgt
oCR416	ggcAAGCTTacaaaggatgatggcaatgaaaccagtaacgttatacgatg
oCR417	gcgCTCGAGcagctgcattaatgaatcggcca
oCR418	gcgCTCGAGggatcatctggaggctcaagcggcatgggaggcacaagcatgagta
oCR659	ccgGGATCCttaatggtgatggtggtgatgagggtagaccgggaatttata
oCR694	cgcGAATTCgtcggacaggcaatcaataaactg
oCR695	gcgCTCGAGaagagagctcatcatatttctcttttg
oCR696	gcgCTCGAGgttcaggcatgagtaaaggagaagaacttttcac
oCR697	cgcGGATCCttatttgtatagttcatccatgccatgtg
oCR704	gcgCTCGAGttaagggtagaccgggaatttatac
oCR716	tcgacTCTAGAgtctggttcaggcatgagtgtggcaaagaaaaaacga
oCR717	acccggGGATCCttaagaagagagctcatcatatttctc
oCR718	tcgacTCTAGAgtctggttcaggcatgaagcgtctcaccttagtatgc
oCR719	ccggGGATCCttaagggtagaccgggaatttatacgc
oCR720	tcgacTCTAGAgtctggttcaggcgtggatgcaatgacaaataaacgg
oCR721	ccggGGATCCtcaatgatgtcgccccagccattt
oHC055	gtggatgcaatgcatcatcaccatcaccatacaaataaacggctgagact
oHC056	ccgtttatttgtatggtgatggtgatgatgcattgcatccacaacgggtt

*capital letters indicate restriction sites

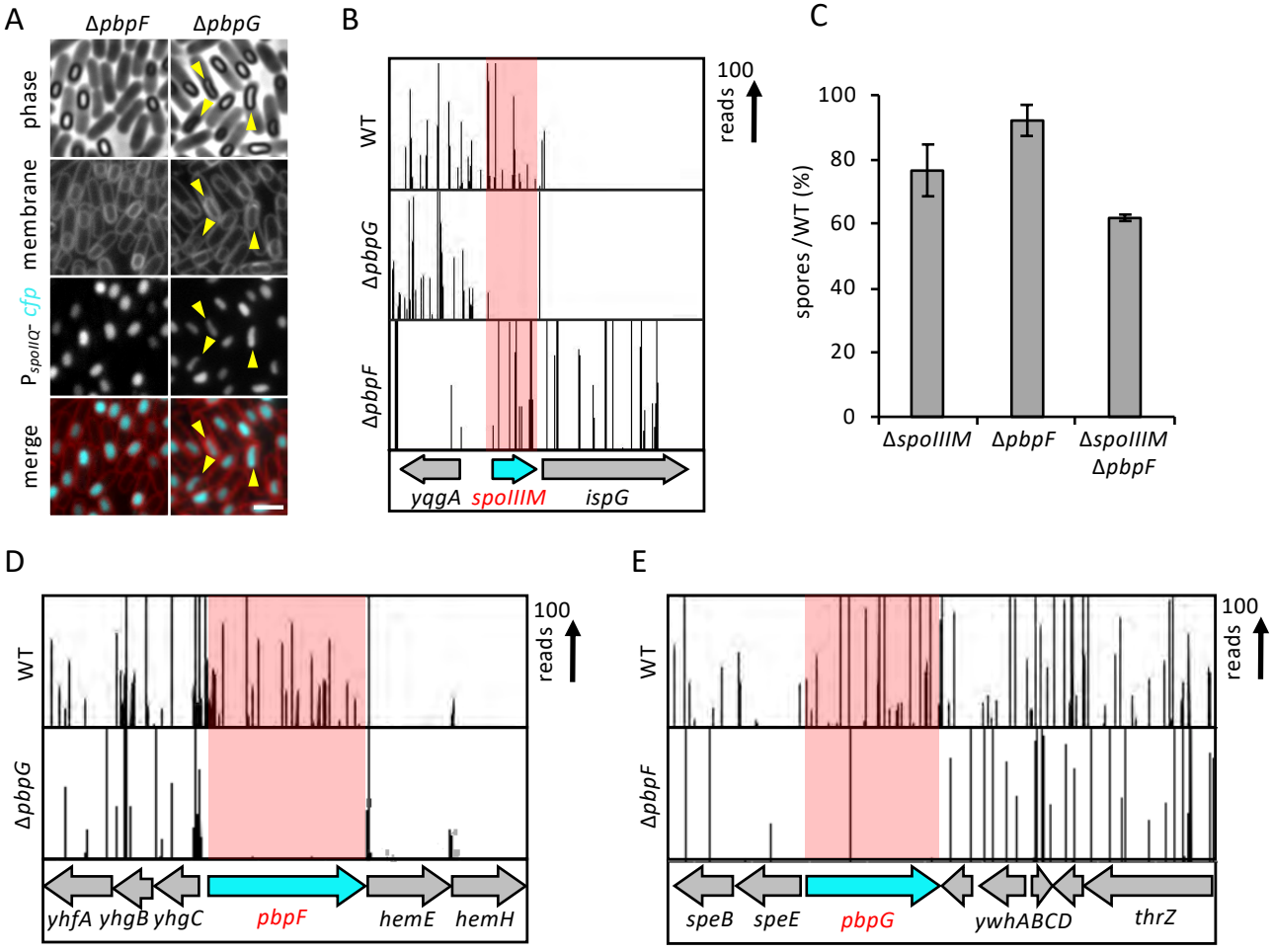


Figure S1: Morphological defect of Δ*pbpG* and Δ*pbpF* and Tn-seq profiles in the Δ*pbpG* and Δ*pbpF*, related to Figure 1. **(A)** Representative images of spore morphology in Δ*pbpF* and Δ*pbpG* strains at T3.5. Yellow arrowheads indicate jellybean-shaped forespores in the Δ*pbpG*. Scale bar, 2 µM. **(B)** Tn-seq profiles in WT, Δ*pbpG* and Δ*pbpF* after 24 hours of growth and sporulation in exhaustion medium. The height of each line reflects the number of sequencing reads at this position. Red box highlights the *spoIIIM* (*yqfZ*) locus, which is depleted for transposon insertions in the Δ*pbpG* library compared to the WT and Δ*pbpF*. **(C)** Sporulation efficiency (%, average ± SD, n=3) relative to WT of Δ*pbpF*, Δ*spoIIIM* and Δ*pbpF* Δ*spoIIIM* strains in exhaustion medium. **(D)** Tn-seq profiles in WT and Δ*pbpG* after 24 hours of growth and sporulation in exhaustion medium. The height of each line reflects the number of sequencing reads at this position. Red box highlights the *pbpF* locus, which is depleted for transposon insertions in the Δ*pbpG* library compared to WT. **(E)** Tn-seq profiles in WT and Δ*pbpF* after 24 hours of growth and sporulation in exhaustion medium. The height of each line reflects the number of sequencing reads at this position. Red box highlights the *pbpG* locus, which is depleted for transposon insertions in the Δ*pbpF* library compared to WT.

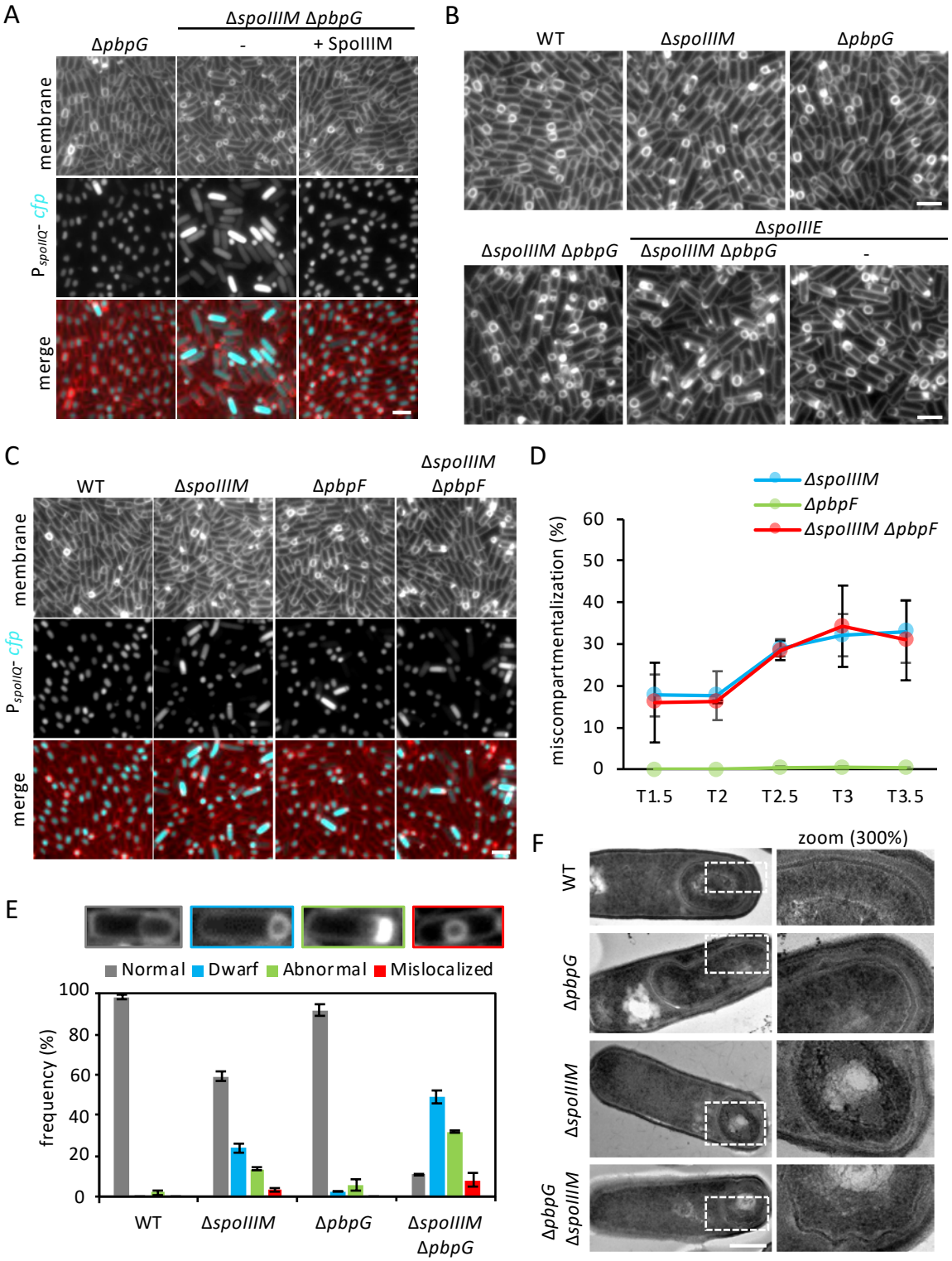


Figure S2: Forespore morphological defects in the absence of SpoIIIM and PbpG and demonstration that the miscompartmentalization phenotype is not dependent on PbpF, related to Figure 1. (A) Representative images of miscompartmentalization in Δ*pbpG*, Δ*spoIIIM* Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* complemented with SpoIIIM at T3.5. Scale bar, 2 μM. **(B)** Representative images showing morphological defects compared to wild-type (WT) in Δ*spoIIIM*, Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*spoIIIE* Δ*spoIIIM* Δ*pbpG* and Δ*spoIIIE* strains imaged at T3. Cell membranes were visualized with TMA-DPH fluorescent dye. Scale bar, 2 μM. **(C)** Representative images of miscompartmentalization in WT, Δ*spoIIIM*, Δ*pbpF* and Δ*spoIIIM* Δ*pbpF* strains at T3.5. Scale bar,2 μM. **(D)** Average frequency (± SD of 3 biological replicates) of miscompartmentalized cells during a sporulation time-course in Δ*spoIIIM* (blue), Δ*pbpF* (green) and Δ*spoIIIM* Δ*pbpF* (red) strains (*n*>950 per time-course, per strain). **(E)** Average frequency (± SD of 3 biological replicates) of normal (grey), dwarf (blue), abnormal (green) and mislocalized (red) forespores in WT, Δ*spoIIIM*, Δ*pbpG*, Δ*spoIIIM* Δ*pbpG* strains at T3 (n>200 per strain, per replicate). **(F)** Transmission electron microscopy images of wild-type WT, Δ*spoIIIM*, Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* strains, with respective zoomed-in areas indicated by white dashed boxes and shown on the right. Scale bar, 500 nm.

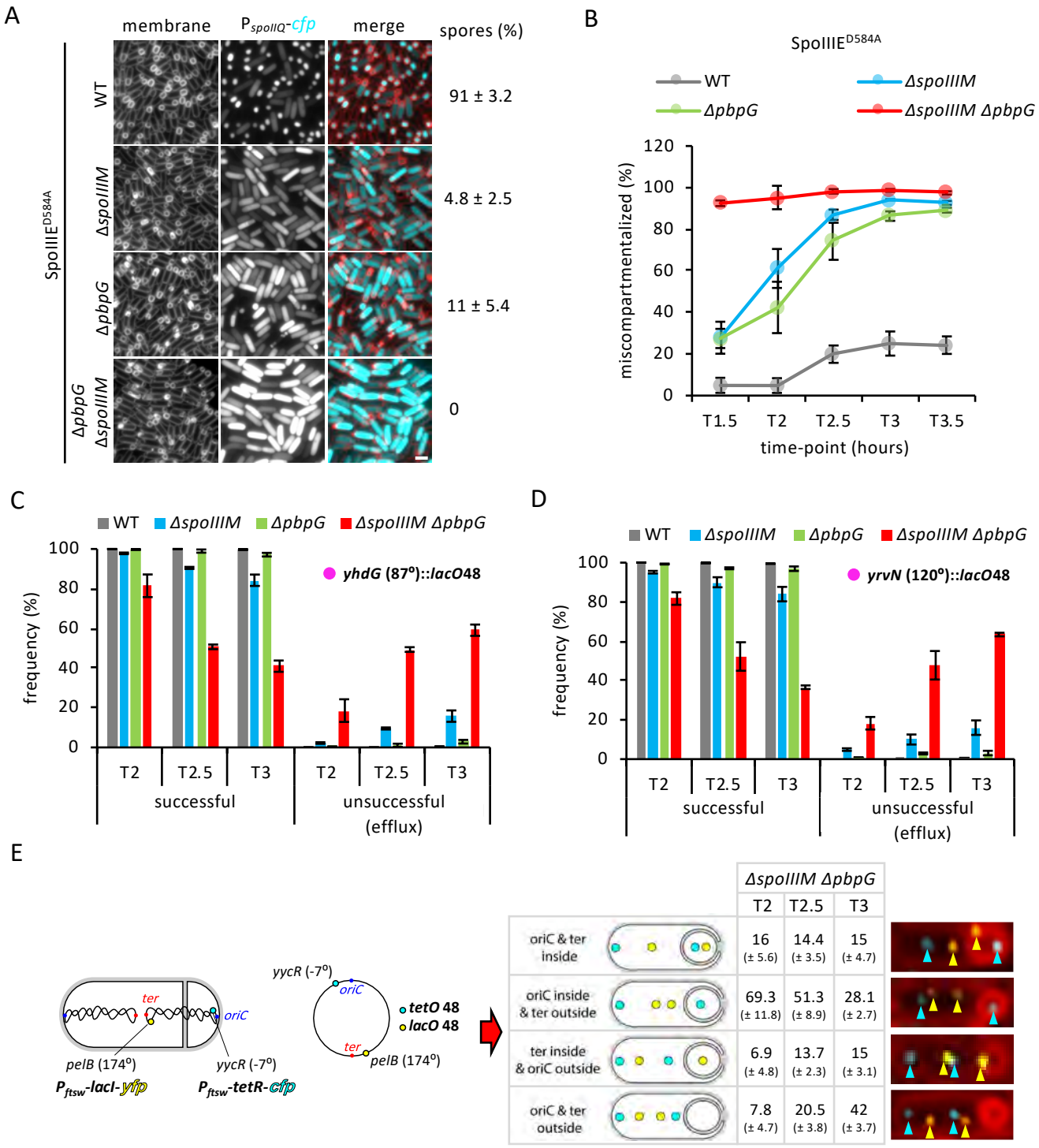
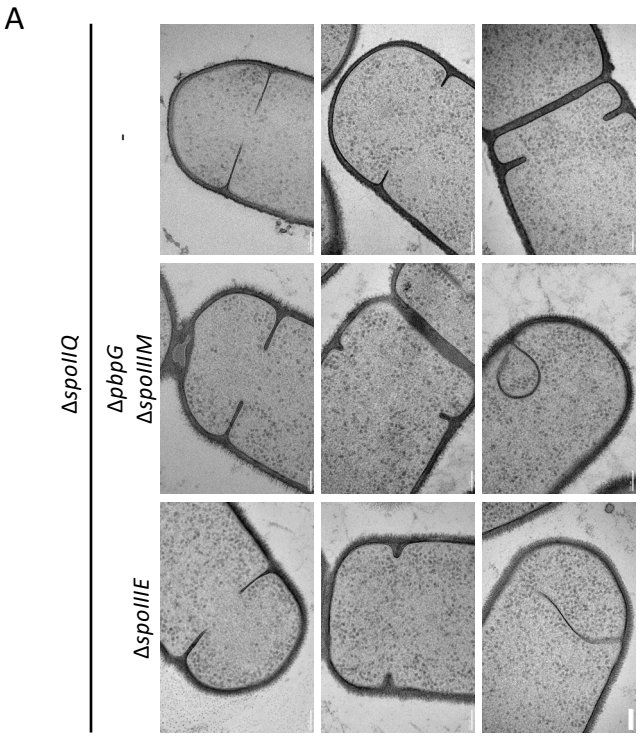
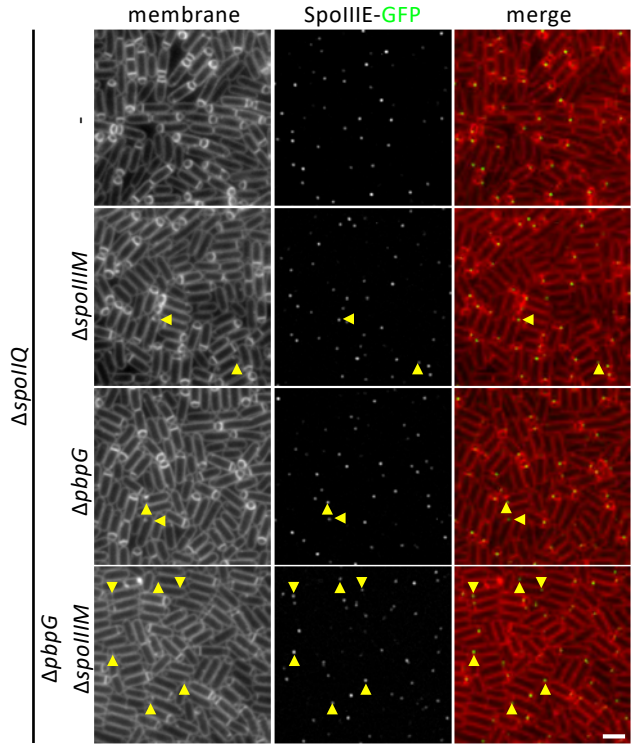
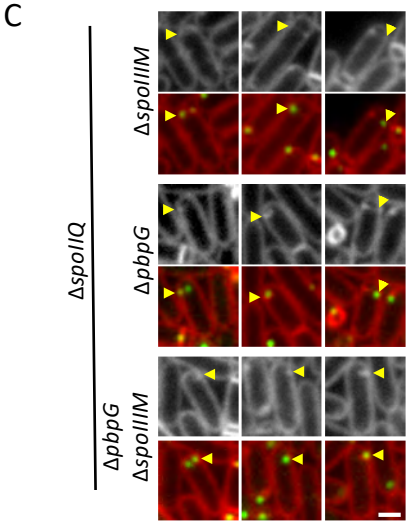


Figure S3: Evidence that SpoIIIE, SpoIIIM and PbpG are together required for compartmentalization, chromosome translocation of other chromosomal loci and evidence supporting passive chromosome efflux in the absence of SpoIIIM and PbpG, related to Figure 3. **(A)** Representative images of miscompartmentalization in the SpoIIIE^{D584A} mutant in otherwise WT, Δ*spoIIIM*, Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* strains at T3.5. Scale bar, 2 µM. **(B)** Average frequency (± SD of 3 biological replicates) of miscompartmentalized cells during a sporulation time-course in the SpoIIIE^{D584A} mutant in otherwise WT (grey), Δ*spoIIIM* (blue), Δ*pbpG* (green) and Δ*spoIIIM* Δ*pbpG* (red) strains (*n*>800 per time-course, per strain, per replicate). **(C)** Average frequency (± SD of 3 biological replicates) of cells with a LacI-GFP focus in the forespore (successful translocation) or with no or two LacI-GFP foci in the mother cell (unsuccessful translocation, efflux) during a sporulation time-course, with *lacO48* integrated at the *yhdG* locus (87°), in WT (grey), Δ*spoIIIM* (blue), Δ*pbpG* (green) and Δ*spoIIIM* Δ*pbpG* (red) strains (*n*>600 per time-course, per strain) and **(D)** at the *yrvN* locus (120°), in WT (grey), Δ*spoIIIM* (blue), Δ*pbpG* (green) and Δ*spoIIIM* Δ*pbpG* (red) strains (*n*>650 per time-course, per strain). **(E)** Diagram explaining experimental rationale of the dual TetR-CFP / *tetO* and LacI-YFP / *lacO* system for visualizing chromosome translocation and efflux TetR-CFP binds to *tetR48* sites (cyan dots) inserted at the *yycR* (-7°) locus as a proxy for the chromosomal origin (*oriC*, blue). LacI-YFP binds to *lacO48* sites (yellow dots) inserted at the *pelB* (174°) locus as a proxy for the chromosomal terminus (*ter*, red). Right: Frequency (± SD of 3 biological replicates) of Δ*spoIIIM* Δ*pbpG* cells containing both *oriC* and *ter* inside the forespore; *oriC* inside and *ter* outside the forespore; *ter* inside and *oriC* outside the forespore; both *oriC* and *ter* outside the forespore, during a sporulation time-course (*n*>30 per time-point, per replicate). The red arrow highlights cells where the origin region was effluxed into the mother but the terminus region remained within the forespore, an event that is inconsistent with active reverse translocation.





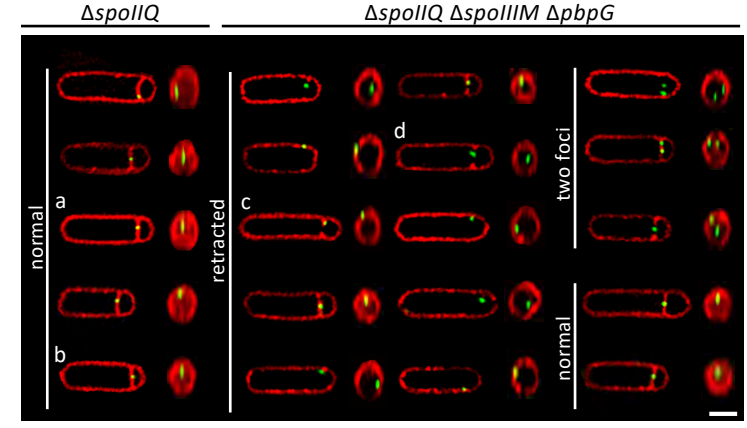


D

Time-point (hours)

SpoIIIE-GFP localization patterns

		T2				T2.5				T3			
		ΔQ	ΔQ ΔM	ΔQ ΔG	ΔQ ΔM ΔG	ΔQ	ΔQ ΔM	ΔQ ΔG	ΔQ ΔM ΔG	ΔQ	ΔQ ΔM	ΔQ ΔG	ΔQ ΔM ΔG
vegetative		97.0 (± 1)	82.6 (± 12)	79.8 (± 12)	41.1 (± 6)	97.9 (± 1)	56.9 (± 4)	50.4 (± 8)	8.9 (± 3)	98.2 (± 1)	42.0 (± 7)	32.7 (± 5)	3.1 (± 2)
retraction		0.0 (± 0)	12.6 (± 11)	15.4 (± 11)	**44.7 (± 4)**	0.0 (± 0)	37.5 (± 3)	47.3 (± 9)	**80.8 (± 6)**	0.0 (± 0)	54.3 (± 7)	64.2 (± 6)	**87.3 (± 3)**
		0.0 (± 0)	1.7 (± 2)	0.8 (± 1)	**9.7 (± 3)**	0.0 (± 0)	2.6 (± 1)	0.9 (± 0.2)	**8.9 (± 2)**	0.8 (± 1)	2.8 (± 2)	2.4 (± 1)	**8.3 (± 2)**
vegetative		2.2 (± 1)	1.8 (± 1)	2.3 (± 0.3)	2.8 (± 1)	1.6 (± 0.4)	1.1 (± 0.5)	0.9 (± 1)	1.1 (± 2)	1.0 (± 0.3)	0.5 (± 0.2)	0.7 (± 1)	1.2 (± 1)
		0.8 (± 0.5)	1.3 (± 0.2)	1.7 (± 0.4)	1.7 (± 1.3)	0.5 (± 0.4)	1.8 (± 0.3)	0.5 (± 0.2)	0.3 (± 0.2)	0.0 (± 0)	0.4 (±1)	0.0 (± 0)	0.1 (± 0.2)



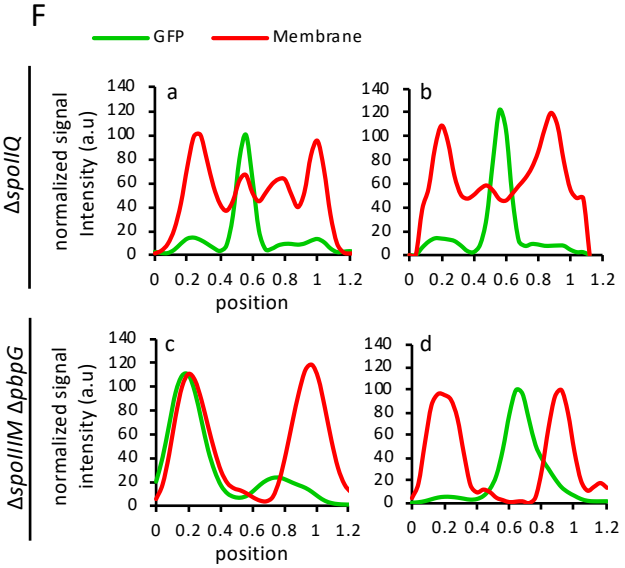


Figure S4: Septa retraction observed by transmission electron microscopy and localization of GFP-SpoIIIE in retracted septa, related to Figure 5. **(A)** Representative images of asymmetric septa in the Δ*spoIIQ* alone or combined with the Δ*spoIIIM* Δ*pbpG* and Δ*spoIIIE* at 3 hours after the onset of sporulation (T3). The septa in the Δ*spoIIIM* Δ*pbpG* and Δ*spoIIIE* are thought to represent retracted septa since based on the data in Fig. 5B & E, almost all cells have retracted septa. In the Δ*spoIIQ* because there is no septa retraction, the images shown are thought to represent forming septa, which have a similar appearance as some retracting septa. Scale bar, 100 nm. **(B)** Representative images of SpoIIIE-GFP localization in the Δ*spoIIQ* alone, or combined with Δ*spoIIIM*, Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* at 2 hours after the onset of sporulation (T2). Yellow arrowheads point to retracting septa. Scale bar, 2 μM. **(C)** Zoomed-in, representative examples of SpoIIIE-GFP localization as foci in retracted septa in the Δ*spoIIQ* combined with Δ*spoIIIM*, Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* at 3 hours after the onset of sporulation (T3). Yellow arrowheads point to retracting septa. Scale bar,1 μM **(D)** Average frequency (± SD of 3 biological replicates) of SpoIIIE-GFP localization patterns at 2 (T2), 2.5 (T2.5) and 3 hours (T3) after the onset of sporulation in the Δ*spoIIQ* alone, or combined with Δ*spoIIIM*, Δ*pbpG* and Δ*spoIIIM* Δ*pbpG* (n>150, per strain, per time-point, per replicate). **(E)** Representative images of SpoIIIE-GFP localization the Δ*spoIIQ* alone or combined with the Δ*spoIIIM* Δ*pbpG* at 2 hours after the onset of sporulation (T2), visualized using 3D-Structured Illumination Microscopy. Examples of normal septa with one SpoIIIE-GFP focus and retracted septa with one or two foci SpoIIIE-GFP foci are shown. Images are SpoIIIE-GFP (green) merged with membrane (red). Scale bar, 1 uM. **(F)** Normalized SpoIIIE-GFP and membrane signal intensity along the asymmetric septum in cells labelled in (E) with lowercase letter for Δ*spoIIQ* (a & b) and Δ*spoIIQ* Δ*spoIIIM* Δ*pbpG* (c & d).

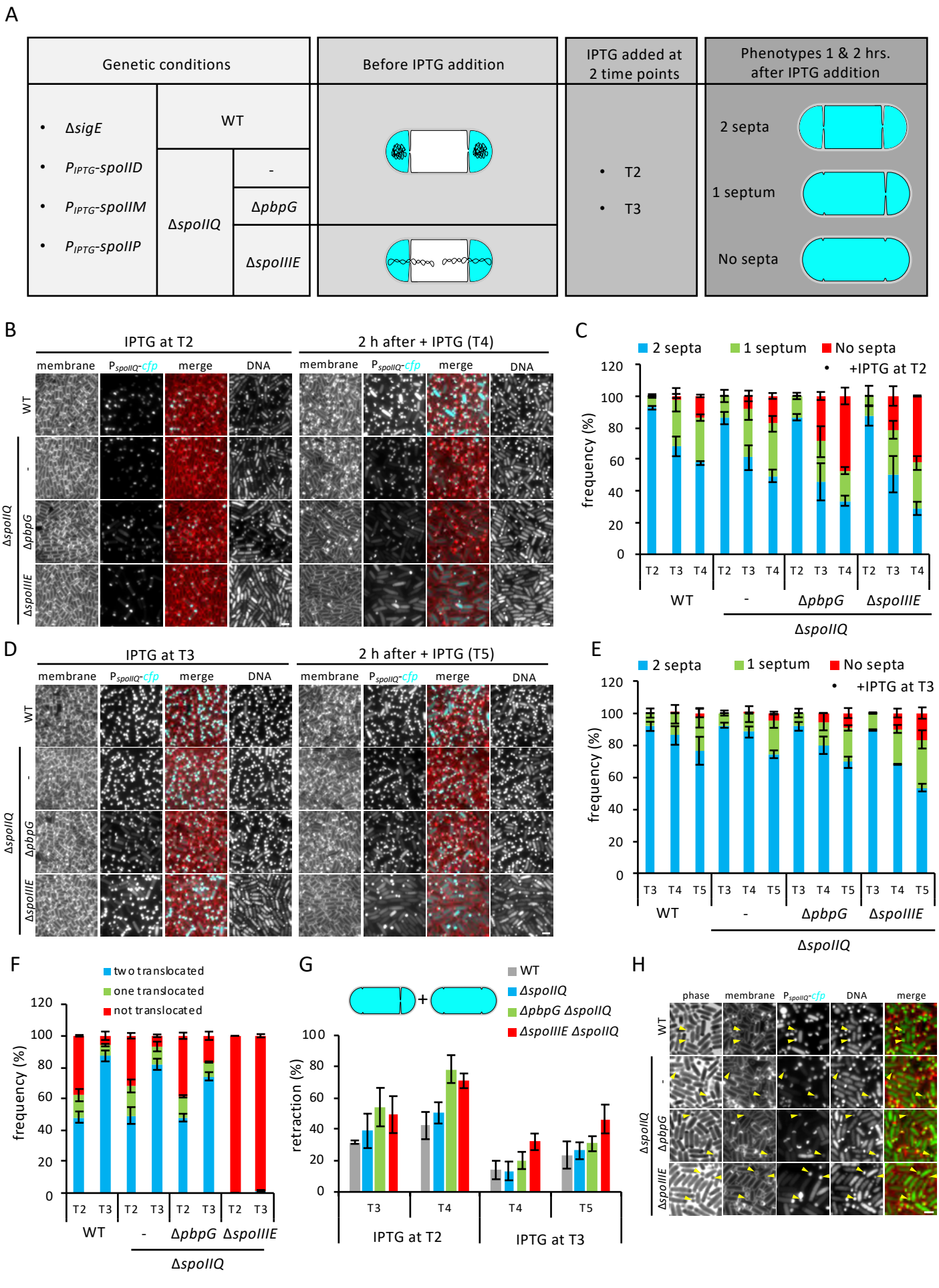

A
Genetic conditions
Before IPTG addition
IPTG added at 2 time points
Phenotypes 1 & 2 hrs. after IPTG addition
ΔsigE
PIPTG-spoIID
PIPTG-spoIIM
PIPTG-spoIIP
WT
ΔspoIIQ
-
ΔpbpG
ΔspoIIIE
T2
T3
2 septa
1 septum
No septa
B
IPTG at T2
2 h after + IPTG (T4)
membrane
PspoIIQ-cfp
merge
DNA
WT
ΔspoIIQ
C
2 septa
1 septum
No septa
+IPTG at T2
frequency (%)
WT
ΔpbpG
ΔspoIIIE
D
IPTG at T3
2 h after + IPTG (T5)
E
+IPTG at T3
F
two translocated
one translocated
not translocated
G
WT
ΔspoIIQ
ΔpbpG ΔspoIIQ
ΔspoIIIE ΔspoIIQ
retraction (%)
IPTG at T2
IPTG at T3
H
phase
membrane
PspoIIQ-cfp
DNA
merge

Figure S5: Evidence that SpoIIIE and PbpG are required for efficient septal pore closure in coordination with chromosome translocation, related to Figure 5. In our experimental system, septal PG hydrolysis is delayed relative to chromosome translocation. To achieve this, we took advantage of a previously-characterized strain that lacks the mother cell transcription factor σ^E and produces SpoIID, SpoIIP and SpoIIM under IPTG control (Rodrigues et al., 2013) . If no IPTG is added, septal PG hydrolysis does not occur and because the cells lack σE, they form two flat asymmetric septa generating two forespore compartments, each with a trapped chromosome capable of activating σ^F (monitored by the forespore-expressed CFP reporter, P_{spoIIQ}-*cfp*). If we add IPTG, we can induce the PG hydrolases that remodel the septal PG. Upon IPTG addition, if the septal pore has not yet closed, CFP will leak into the mother cell and the septal membrane will retract. Note that this strain is effectively a *spoIIIM* null, since *spoIIIM* depends on σE for its expression. **(A)** Schematic showing experimental overview. All strains are Δ*sigE* with IPTG-inducible expression of Δ*spoIID*, Δ*spoIIM* and Δ*spoIIP*. Strains tested include an otherwise wild-type (WT) strain, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE*. Before IPTG addition, cells have two polar septa, with CFP fluorescence (cyan) localized to both forespores. DNA is represented by black squiggles. Chromosomes are translocated to both forespores in WT, Δ*spoIIQ* and Δ*spoIIQ* Δ*pbpG* strains; no DNA translocation occurs in the Δ*spoIIQ* Δ*spoIIE* strain. Expression of Δ*spoIID*, Δ*spoIIM* and Δ*spoIIP* was induced with the addition of IPTG at 2 h (T2) or 3 h (T3) after the onset of starvation. Phenotypes were scored 1 h and 2 h after IPTG addition and were classified as cells having 2 septa, a single 1 septum or no septa. CFP fluorescence leaks from the forespores as septa retract. **(B)** Representative images of cells expressing CFP from a forespore-specific promoter (P_{spoIIQ}) in WT, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE* strains in a Δ*sigE* background at T2 (time of IPTG-induced expression of Δ*spoIID*, Δ*spoIIM* and Δ*spoIIP*) and T4 (2h after IPTG addition). DNA is stained with DAPI. Scale bar, 2 μM. **(C)** Average frequency (± SD of 3 biological replicates) of cells with two septa, one septum or no septa for strains shown in (B) at T2, T3 and T4, with addition of IPTG at T2 (*n*>450 per time-point, per strain, per replicate). **(D)** Representative images of cells expressing CFP from a forespore-specific promoter (P_{spoIIQ}) in WT, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE* strains in a Δ*sigE* background at T3 (time of IPTG-induced expression of Δ*spoIID*, Δ*spoIIM* and Δ*spoIIP*) and T5 (2h after=IPTG addition). DNA is stained with DAPI. Scale bar, 2 μM. **(E)** Average frequency (± SD of 3 biological replicates) of cells with two septa, one septum or no septa for strains shown in (D) at T3, T4 and T5, with addition of IPTG at T3 (*n*>600 per time-point, per strain, per replicate). **(F)** Average frequency (± SD of 3 biological replicates) of cells with two translocated, one translocated or no translocated chromosomes in WT, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE* strains during a sporulation time-course (n>600 per time-point, per strain, per replicate). **(G)** Average frequency (± SD of 3 biological replicates) of cells with septal retraction in otherwise WT, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE* strains and imaged 1 h and 2 h after IPTG addition at T2 and T3 to induce expression of *spoIID*, *spoIIM* and *spoIIP* in Δ*sigE* cells (n>600 per time-point, per strain, per replicate). **(H)** Detached spores containing DNA, in otherwise WT, Δ*spoIIQ*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIE* strains. The detached forespores (yellow arrowheads) are indicative of cytokinesis at the asymmetric septum. The DNA is stained with DAPI. Scale bar, 2 uM.

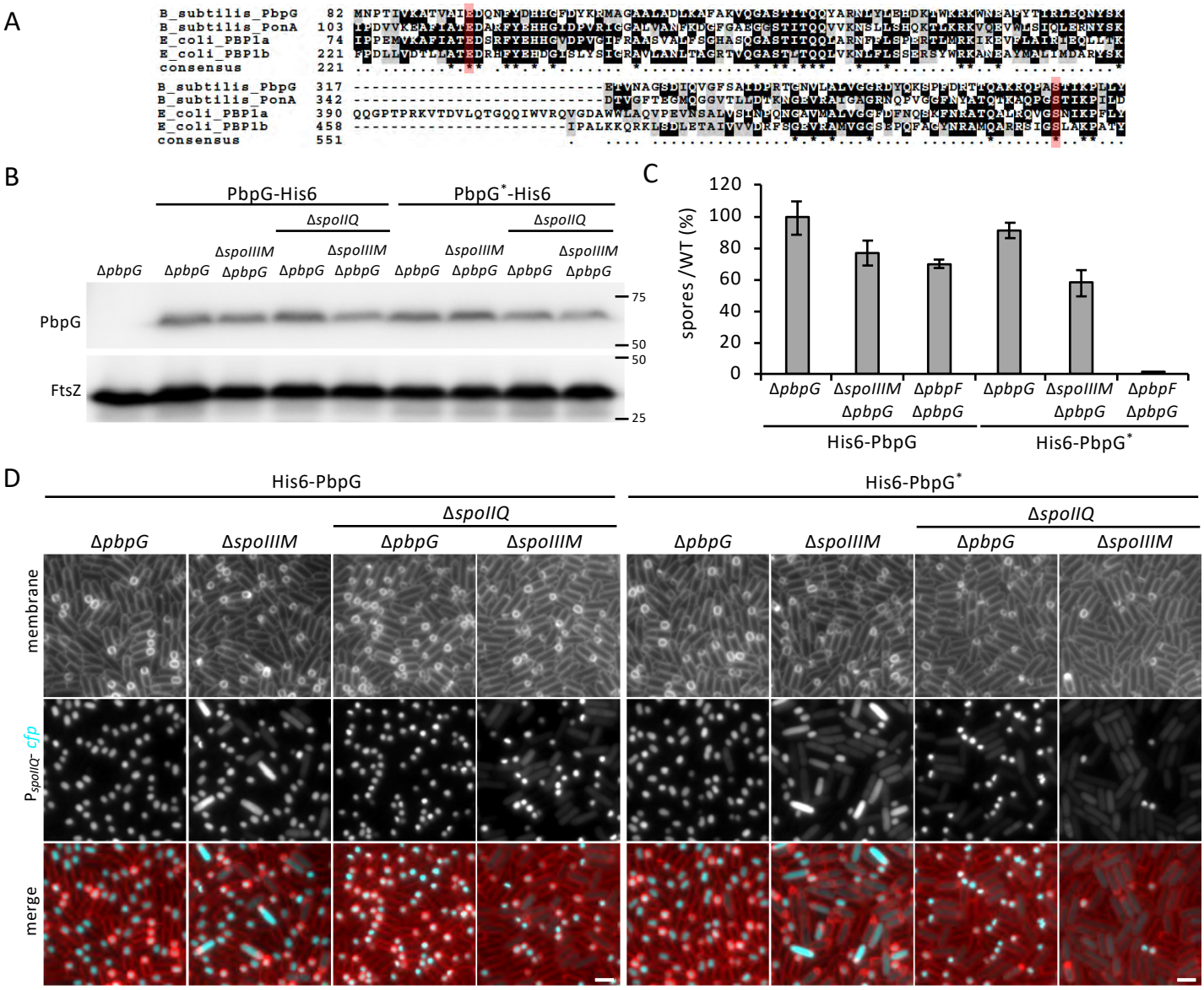


Figure S6: Validation of the PbpG catalytic mutant, related to Figure 6. **(A)** Identification of the PbpG catalytic residues using multiple sequence alignment with other Class A PBPs: PonA from *Bacillus subtilis* (Uniprot accession number: **P39793**), PBP1A and PBP1B from *Escherichia coli* (Uniprot accession numbers: P02918 and P02919, respectively). Red shaded residues are the predicted catalytic site residues involved in transglycosylation (E94) and transpeptidation (S365). **(B)** Immunoblot analysis demonstrating levels of PbpG-His6, using His6 antibodies, as a sole source in Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*spoIIQ* Δ*pbpG*, Δ*spoIIQ* Δ*spoIIIM* Δ*pbpG* and PbpG catalytic mutant (PbpG*) in Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*spoIIQ* Δ*pbpG* and Δ*spoIIQ* Δ*spoIIIM* Δ*pbpG*. Cells were collected 2 hours after the onset of sporulation (T2). The immunoblot shows that catalytic site mutations do not affect the levels of PbpG. FtsZ is used a loading control. Numbers on the right indicate molecular weight in kDa. **(C)** Sporulation efficiency of strains harboring the His6-PbpG construct as the sole source of PbpG relative to WT. Strains used were Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*pbpG* Δ*pbpF* and PbpG catalytic mutant (PbpG*) in Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*pbpG* Δ*pbpF*. His6-PbpG complements the sporulation defect of the Δ*spoIIIM* Δ*pbpG* and Δ*pbpG* Δ*pbpF*. Consistent with the idea that catalytic activity of PbpG is required for efficient sporulation, the His6-PbpG* does not complement the sporulation defect of the Δ*spoIIIM* Δ*pbpG* and Δ*pbpG* Δ*pbpF*. **(D)** Representative images of miscompartmentalization and septal retraction phenotypes in strains harboring His6-PbpG and His6-PbpG* as the sole source of PbpG. For His6-PbpG, the strains used were Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*spoIIQ* Δ*pbpG*, Δ*spoIIQ* Δ*spoIIIM* Δ*pbpG*. For His6-PbpG*, the strains used were Δ*pbpG*, Δ*spoIIIM* Δ*pbpG*, Δ*spoIIQ* Δ*pbpG*, Δ*spoIIQ* Δ*spoIIIM* Δ*pbpG*. Scale bar, 2 uM.

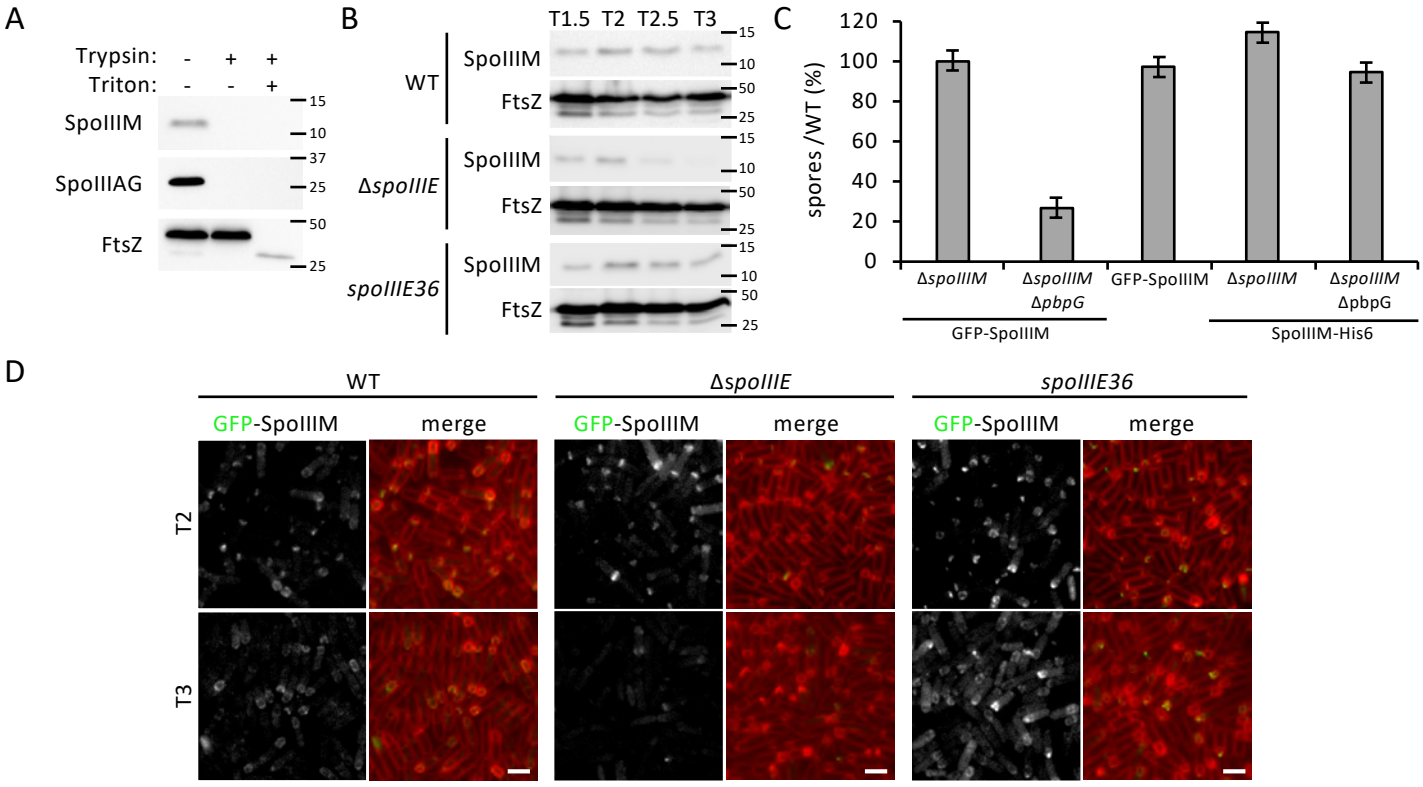


Figure S7: Validation of GFP-SpoIIIM fluorescent fusion and evidence of SpoIIIM membrane topology, related to Figure 6. (A) SpoIIIM is surface exposed and thus accessible to trypsin digestion. Immunoblot analysis using anti-His antibodies of protoplasted sporulating cells containing SpoIIIM-His6 as a sole source of SpoIIIM in strain Δ*spoIIQ* Δ*spoIIIM*, treated with Trypsin in the presence and absence of TritonX-100. Consistent with the idea that SpoIIIM is membraned-anchored, it remained cell-associated after the generation of protoplasts. As controls, the immunoblot was probed for a membrane protein with an extracellular domain (SpoIIIAG) and a cytoplasmic protein (FtsZ). **(B)** Immunoblot using anti-His antibodies of SpoIIIM-His6 in WT, Δ*spoIIIE* and SpoIIIE36 strains, showing that *spoIIIE* is required for SpoIIIM-His6 stability. FtsZ is used as a loading control. Numbers on the right indicate molecular weight in kDa. **(C)** Sporulation efficiency (%, average ± SD, n=3) relative to WT of Δ*spoIIIM* where $P_{spoIIIM}$-GFP-SpoIIIM is complemented in Δ*spoIIIM* and partially complemented in Δ*spoIIIM* Δ*pbpG* and P_{spoIID}-GFP-SpoIIIM in merodiploid background. The histogram also shows the sporulation efficiency (%, average ± SD, n=3) of the functional SpoIIIM-His6 in Δ*spoIIIM* and Δ*spoIIIM* Δ*pbpG*. **(D)** Representative images of GFP-SpoIIIM localization in WT, Δ*spoIIIE* and *spoIIIE36* strains at T2 and T3. Scale bar, 2 uM.