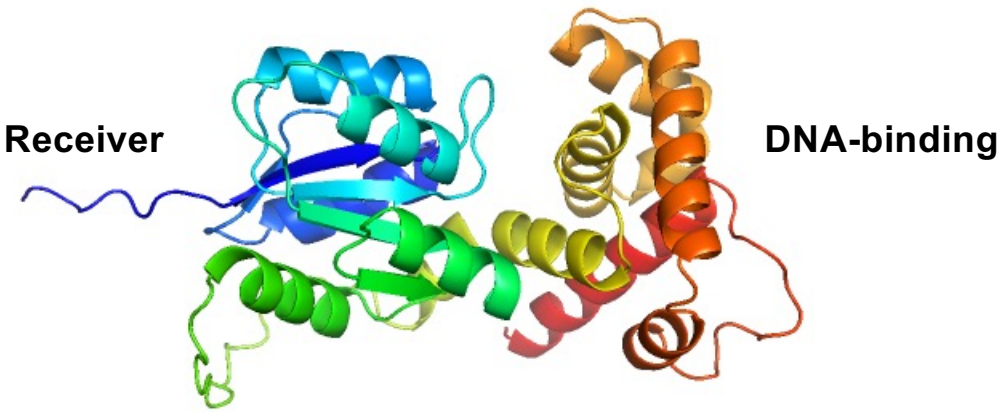


Figure S1. Predicted Spo0A structure and domain architecture. AlphaFold predicted model of *C. difficile* Spo0A (UniProt accession P52938). Spo0A consists of an N-terminal receiver domain used for protein-protein interaction and a C-terminal helix-turn-helix domain used for DNA-binding. The AlphaFold-generated predicted Spo0A PDB (AF-P52938-F1-model_v2) was color-edited using PyMOL (The PyMOL Molecular Graphics System, Version 2.4.0 Schrödinger, LLC).

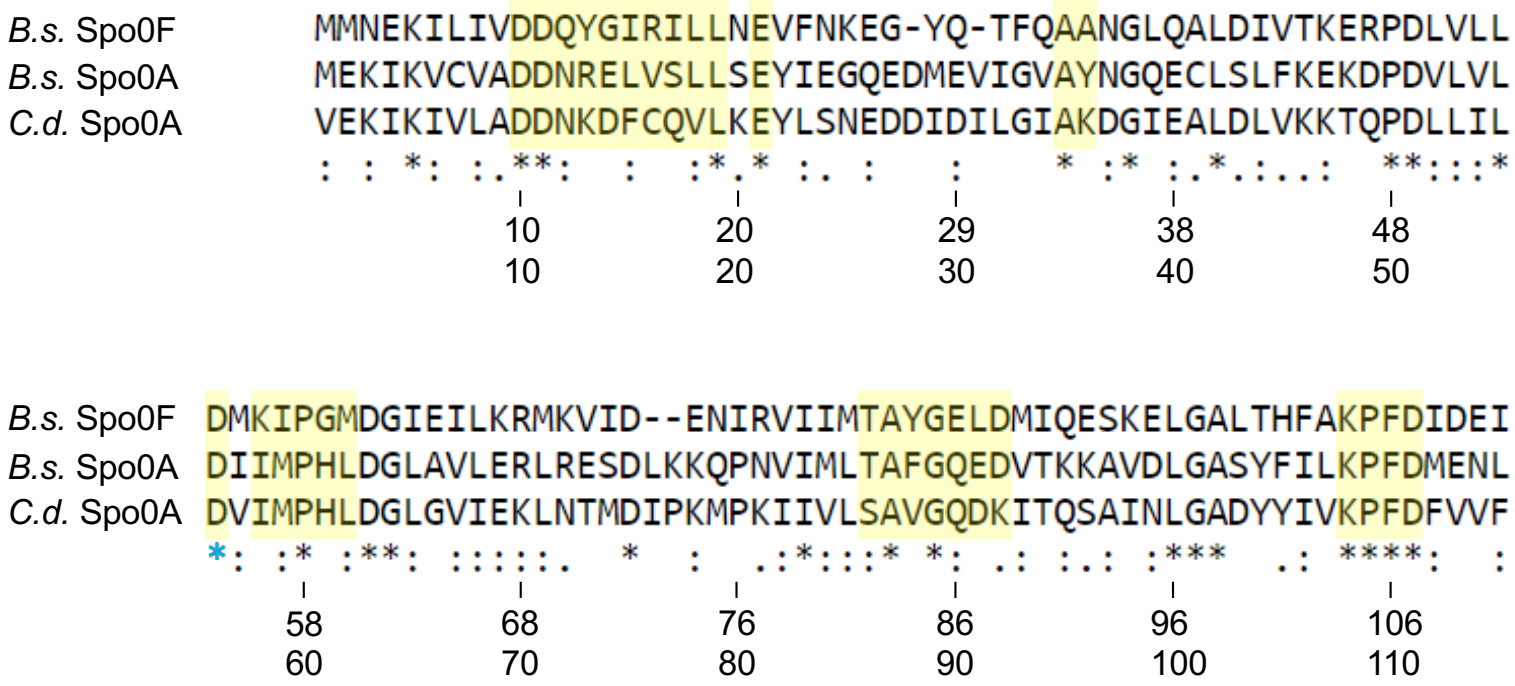
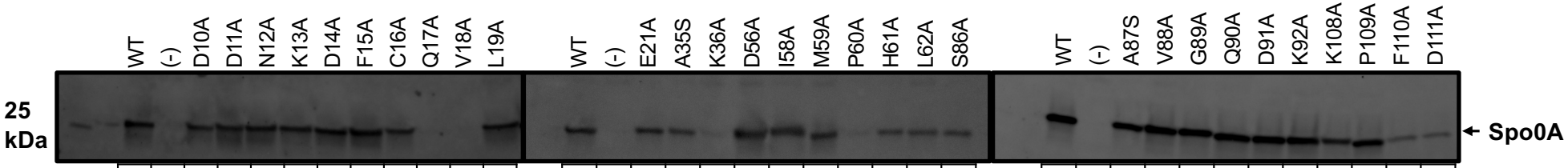


Figure S2. Alignment of receiver domain residues of *B. subtilis* Spo0A and Spo0F, and *C. difficile* Spo0A. Spo0A receiver domain of *C. difficile* aligned to *B. subtilis* Spo0F and receiver domain of Spo0A using Clustal Omega (*B.s.* = *B. subtilis*; *C.d.* = *C. difficile*). Conserved residues chosen for mutation in *C. difficile* are highlighted in yellow. Amino acid sequences of Spo0F (BSU_37130) and of the Spo0A receiver domains for *B. subtilis str. 168* (BSU_24220, top), and *C. difficile* 630 (CD630_12140, bottom). The blue star (*) is the conserved site of phosphorylation.



Fold change	1.00	**0.19**	0.98	0.85	1.00	0.99	1.01	0.99	0.97	0.53	0.62	0.59
STDEV	0.00	**0.27**	0.07	0.10	0.03	0.05	0.03	0.05	0.09	0.48	0.34	0.33

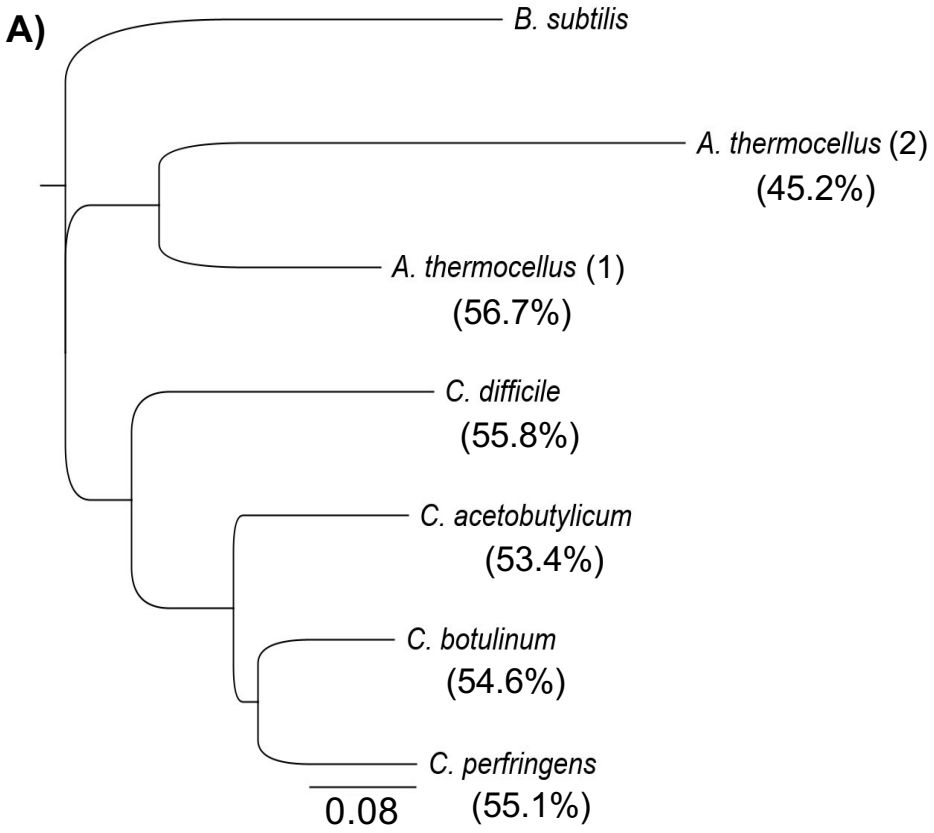
Fold change	1.00	**0.05**	0.79	0.72	0.53	0.70	0.82	0.77	0.69	1.00	0.71	0.72
STDEV	0.00	**0.03**	0.46	0.31	0.45	0.47	0.29	0.23	0.27	0.09	0.18	0.34

Fold change	1.00	**0.03**	0.98	0.97	1.30	1.23	1.21	1.18	1.17	1.09	1.05	0.97
STDEV	0.00	**0.01**	0.58	0.42	0.25	0.29	0.29	0.24	0.29	0.42	0.30	0.44

Figure S3. Stability of Spo0A mutant alleles. SDS-PAGE western blot analysis of the 30 Spo0A point mutants to assess their stability using anti-Spo0A antibody. Strains harvested after 12 h growth on 70:30 sporulation medium and 2.5 µg of total protein was loaded for each sample. A wildtype (WT) positive control, *spo0A*::*erm* pSpo0A (MC848) and the *spo0A*::*erm* pMC123 (MC855) negative control strain (-) are included with each western blot. Each blot is representative of three independent experiments. The means and standard deviation of densitometric quantification normalized to WT on each membrane are shown, and bold values indicate P ≤ .05 as determined by a one-way ANOVA with Dunnett's multiple comparisons test.

Spo0A site-directed mutation	Angstroms error estimate	Confidence in model	Sporulation frequency (%)
Wildtype	-	0.85	12.1±1.0
D10A	0.75 (0.71)	0.86	0.0002±0.0001
D11A	0.75 (0.74)	0.83	0.00055±0.0004
D14A	0.82 (0.81)	0.84	49.7±6.5
Q17A	0.78 (0.78)	0.86	<LOD
V18A	0.82 (0.77)	0.83	<LOD
D56A	0.71 (0.72)	0.85	0.008±0.001
P60A	0.74 (0.77)	0.86	<LOD
A87S	1.00 (1.22)	0.83	0.01±0.01
Q90A	1.16 (1.62)	0.84	59.3±10.7
K92A	1.32 (1.36)	0.85	33±2.7
P109A	0.99 (0.94)	0.87	76.9±9.3

Figure S4. Predicted wild type and site-directed mutant Spo0A structures. The predicted effect on protein structure of a Spo0A site-directed mutant based on comparison of angstroms error estimate and confidence in the predicted model of site-directed mutants against corresponding wildtype residue values. Wild type residue angstroms error estimates are shown in parentheses adjacent to the corresponding Spo0A site-directed mutation. Predictions made using RoseTTAFold (Robetta).



B) Spo0A % identity

	***A.t.* 2**	***B.s.***	***A.t.* 1**	***C.d.***	***C.p.***	***C.b.***	***C.a.***
***A.t.* 2**	-	45.2	54.9	47.6	49.8	47.8	48.2
B.s.	45.2	-	56.7	55.4	55.1	54.6	53.4
***A.t.* 1**	54.9	56.7	-	56.7	61.2	62.5	59.1
C.d.	47.8	55.8	57.1	-	60.2	64.6	61.3
C.p.	49.8	55.1	61.2	59.8	-	78.6	75.1
C.b.	47.8	54.6	62.5	64.1	78.6	-	76.5
C.a.	48.2	53.4	59.1	60.9	75.1	76.5	-

Figure S5 Spo0A divergence in Firmicutes. A) Dendrogram of full-length Spo0A protein coding regions rooted to the outgroup *B. subtilis* Spo0A. Percentage identity of each species' Spo0A protein sequence is shown relative to *B. subtilis*. Spo0A alignment and dendrogram tree constructed using MUSCLE Alignment plugin and Geneious Tree Builder in Geneious Prime 2020.2.2. **B)** Heatmap of the comparisons of percent identities of Spo0A from each species in **(A)**. *B.s.* = *B. subtilis*, *A.t.*1 = *A. thermocellus* Spo0A 1, *A.t.* 2 = *A. thermocellum* Spo0A 2, *C.d.* = *C. difficile*, *C.p.* = *C. perfringens*, *C.b.* = *C. botulinum*, and *C.a.* = *C. acetobutylicum*.

Supplementary Material

Table S1. Luminescence outputs from split-luciferase assay.

Strain	**Average LU/OD_{600}**
Positive control (bitLuc^{opt})	9422.6 ± 1035.5
Negative control (SmBit-LgBit)	1051.6 ± 328.0
Spo0A-SmBit	1820.5 ± 692.9
Spo0A-LgBit	845.7 ± 188.2
Spo0A-SmBit-Spo0A-LgBit	934244.3 ± 47268.6
Spo0A D10A-SmBit	2517.3 ± 456.7
Spo0A D10A-LgBit	1307.8 ± 64.2
Spo0A D10A-SmBit-Spo0A D10A-LgBit	341759.3 ± 145113
Spo0A D11A-SmBit	969.4 ± 195.5
Spo0A D11A-LgBit	1378.3 ± 44.6
Spo0A D11A-SmBit-Spo0A D11A-LgBit	399696.3 ± 145900
Spo0A D56A-SmBit	998.9 ± 141.4
Spo0A D56A-LgBit	1203.4 ± 370.1
Spo0A D56A-SmBit-Spo0A D56A-LgBit	242346.6 ± 89320.3
Spo0A I58A-SmBit	2200.3 ± 788.8
Spo0A I58A-LgBit	1609.2 ± 199.9
Spo0A I58A-SmBit-Spo0A I58A-LgBit	442895.4 ± 269303
Spo0A K108A-SmBit	2648.6 ± 196.3
Spo0A K108A-LgBit	1592.4 ± 54.6
Spo0A K108A-SmBit-Spo0A K108A-LgBit	542192.7 ± 443215

Table S2. Cloning and vector construction details

pMC566: A 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was cloned into pMC123 using BamHI/EcoRI sites.

pMC567: A single amino acid mutation (D56A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing PCR reaction with two fragments generated by using primer set oMC1251/oMC1252 and cloned into pMC123 using BamHI/EcoRI sites.

pMC656: A single amino acid mutation (N12A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing PCR reaction with two fragments generated by using primer set oMC1513/oMC1514 and cloned into pMC123 using BamHI/EcoRI sites.

pMC657: A single amino acid mutation (K13A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing PCR reaction with two fragments generated by using primer set oMC1515/oMC1516 and cloned into pMC123 using BamHI/EcoRI sites.

pMC663: A single amino acid mutation (I58A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing reaction with two fragments generated by using primer set oMC1519/oMC1520 and cloned into pMC123 using BamHI/EcoRI sites.

pMC674: A 1.3 kb *spo0A* PCR product with C-terminal 3xFLAG amplified with primers oMC1249/oMC1547 was cloned into pMC123 using BamHI/EcoRI sites.

pMC684: A single amino acid mutation (V18A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing PCR reaction with two fragments generated by using primer set oMC1517/oMC1518 and cloned into pMC123 using BamHI/EcoRI sites.

pMC685: A single amino acid mutation (H61A) within a 1.2 kb *spo0A* PCR product amplified with primers oMC1249/oMC1250 was made in a SOEing PCR reaction with two fragments generated by using primer set oMC1583/oMC1584 and cloned into pMC123 using BamHI/EcoRI sites.

pMC697: A 1.2 kb *spo0A* C16A allele (TGT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC698: A 1.2 kb *spo0A* E21A allele (GAG -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC699: A 1.2 kb *spo0A* A35S allele (GCT -> TCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC700: A 1.2 kb *spo0A* P60A allele (CCA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC701: A 1.2 kb *spo0A* A87S allele (GCA -> TCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC702: A 1.2 kb *spo0A* V88A allele (GTA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC703: A 1.2 kb *spo0A* G89A allele (GGT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC704: A 1.2 kb *spo0A* K108A allele (AAG -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC742: A 1.2 kb *spo0A* D91A allele (GAT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC768: A 1.2 kb *spo0A* M59A allele (ATG -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC769: A 1.2 kb *spo0A* L62A allele (CTA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC770: A 1.2 kb *spo0A* K92A allele (AAG -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC771: A 1.2 kb *spo0A* P109A allele (CCA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC922: Two PCR fragments were generated with oMC2437/2439 (950 bp) + oMC2441/2442 (600 bp) to create *spo0A*-SmBit-LgBit and Gibson assembled as BamHI/SacI into pAP118.

pMC924: Three PCR fragments were generated with oMC2443/2356 (150 bp) + oMC2354/2447 (950 bp) + oMC2445/2442 (550 bp) to create SmBit-*spo0A*-LgBit and Gibson assembled as BamHI/SacI into pAP118.

pMC930: Two PCR fragments were combined in SOEing PCR reaction from oMC2443/2449 and oMC2442/2448 (600 bp) and Gibson assembled into pAF256 to create SmBit-LgBit fusion.

pMC932: Two PCR fragments were combined in SOEing PCR reaction from oMC2437/2439 and oMC2441/2356 (1050 bp) and cloned into the SacI/PvuI sites of pAF257 to create *spo0A*-SmBit.

pMC944: Two PCR fragments were combined in SOEing PCR reaction from oMC2354/2447 and oMC2445/2442 (1850 bp) and cloned into the BamHI/PvuI sites of pMC932 to create *spo0A*-SmBit-*spo0A*-LgBit.

pMC965: A 1.2 kb *spo0A* D11A allele (GAC -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC966: A 1.2 kb *spo0A* D14A allele (GAT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC967: A 1.2 kb *spo0A* F15A allele (TTT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC968: A 1.2 kb *spo0A* Q17A allele (CAG -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC969: A 1.2 kb *spo0A* L19A allele (TTA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC970: A 1.2 kb *spo0A* D111A allele (GAT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC971: *spo0A* D56A-3XFLAG (SOEing product from oMC1249/1252 and oMC1251/1547) was made and cloned into pMC123 using BamHI/EcoRI.

pMC975: A 1.2 kb *spo0A* K36A allele (AAG -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC976: A 1.2 kb *spo0A* Q90A allele (CAA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC986: A 1.2 kb *spo0A* F110A allele (TTT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC1055: A 1.2 kb *spo0A* S86A allele (TCA -> GCA) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC1088: A 1.2 kb *spo0A* D10A allele (GAT -> GCT) synthesized by Genscript was cloned into pMC123 using BamHI/EcoRI sites.

pMC1097: An 850 bp *spo0A* D10A PCR fragment was amplified from pMC1088 using oMC2437/oMC2439 and was Gibson assembled into pAF256.

pMC1098: An 850 bp *spo0A* D11A PCR fragment was amplified from pMC965 using oMC2437/oMC2439 and was Gibson assembled into pAF256.

pMC1099: An 850 bp *spo0A* D56A PCR fragment was amplified from pMC567 using oMC2437/oMC2439 and was Gibson assembled into pAF256.

pMC2000: An 850 bp *spo0A* I58A PCR fragment was amplified from pMC663 using oMC2437/oMC2439 and was Gibson assembled into pAF256.

pMC2001: An 850 bp *spo0A* K108A PCR fragment was amplified from pMC704 using oMC2437/oMC2439 and was Gibson assembled into pAF256.

pMC2002: An 850 bp *spo0A* D10A PCR fragment was amplified from pMC1088 using oMC2354/oMC2447 and was Gibson assembled into pAF257.

pMC2003: An 850 bp *spo0A* D11A PCR fragment was amplified from pMC965 using oMC2354/oMC2447 and was Gibson assembled into pAF257.

pMC2004: An 850 bp *spo0A* D56A PCR fragment was amplified from pMC567 using oMC2354/oMC2447 and was Gibson assembled into pAF257.

pMC2005: An 850 bp *spo0A* I58A PCR fragment was amplified from pMC663 using oMC2354/oMC2447 and was Gibson assembled into pAF257.

pMC2006: An 850 bp *spo0A* K108A PCR fragment was amplified from pMC704 using oMC2354/oMC2447 and was Gibson assembled into pAF257.

pMC2007: An 850 bp *spo0A* D10A PCR fragment was amplified from pMC1088 using oMC2437/oMC2439 and was Gibson assembled into pAP118. An 850 bp *spo0A* D10A PCR fragment was then amplified from pMC1088 using oMC2354/oMC2447 and cloned into pAP118 using NotI and PvuI sites.

pMC2008: An 850 bp *spo0A* D11A PCR fragment was amplified from pMC965 using oMC2437/oMC2439 and was Gibson assembled into pAP118. An 850 bp *spo0A* D11A PCR fragment was then amplified from pMC965 using oMC2354/oMC2447 and cloned into pAP118 using NotI and PvuI sites.

pMC2009: An 850 bp *spo0A* D56A PCR fragment was amplified from pMC567 using oMC2437/oMC2439 and was Gibson assembled into pAP118. An 850 bp *spo0A* D56A PCR fragment was then amplified from pMC567 using oMC2354/oMC2447 and cloned into pAP118 using NotI and PvuI sites.

pMC2010: An 850 bp *spo0A* I58A PCR fragment was amplified from pMC663 using oMC2437/oMC2439 and was Gibson assembled into pAP118. An 850 bp *spo0A* I58A PCR fragment was then amplified from pMC663 using oMC2354/oMC2447 and cloned into pAP118 using NotI and PvuI sites.

pMC2011: An 850 bp *spo0A* K108A PCR fragment was amplified from pMC704 using oMC2437/oMC2439 and was Gibson assembled into pAP118. An 850 bp *spo0A* K108A PCR fragment was then amplified from pMC704 using oMC2354/oMC2447 and cloned into pAP118 using NotI and PvuI sites.