Supplemental Information for

A two-dimensional ratchet model describes assembly initiation of a specialized bacterial cell surface

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SUPPLEMENTAL FIGURES 1-4

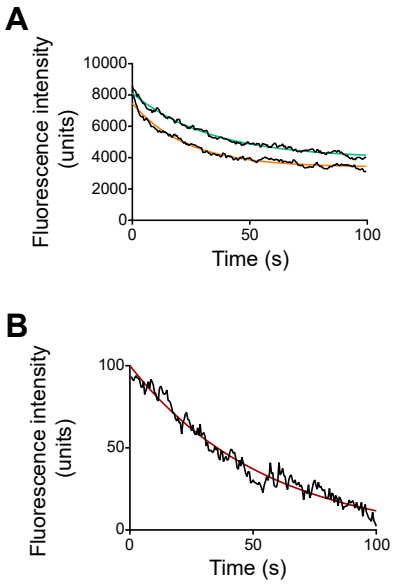

Figure S1. Photobleaching rate determined for in vitro and in vivo experimental setups. (A) Immobilized Cy3-Biotin molecules on a neutravidin-coated coverslip were visualized using a 561 nm laser with an excitation power of .250 mW. Images were acquired using HiLo every 500 ms for 200 frames at a similar angle used in the experimental setup. Fluorescence intensities of both regions of over time were fit with a one-phase exponential decay model (orange and green, respectively). Region 1 (orange) displayed a time constant of 33.34 s (R squared = .9773); region 2 (green) displayed a time constant of 23.13 s (R squared = .9833). Average photobleaching rate in vivo for Cy3 dye was therefore calculated as 28.2 s. (B) Sporulating cell producing SpoVM-HALO were labeled with 5 μM JF549 3 h after induction of sporulation, incubated for 30 minutes at room temperature, washed 3x with 1 mL PBS then resuspended in 100 μL PBS and images were acquired every 500 ms for 200 frames with a laser power of 0.400 mW (same power used to collect experimental data). A defined region (square) was chosen from saturating areas of three different cells and analyzed for photobleaching. Average time profile (black) from selected regions of three different cells were fit with nonlinear regression trendline (maroon; R squared = 0.8792).

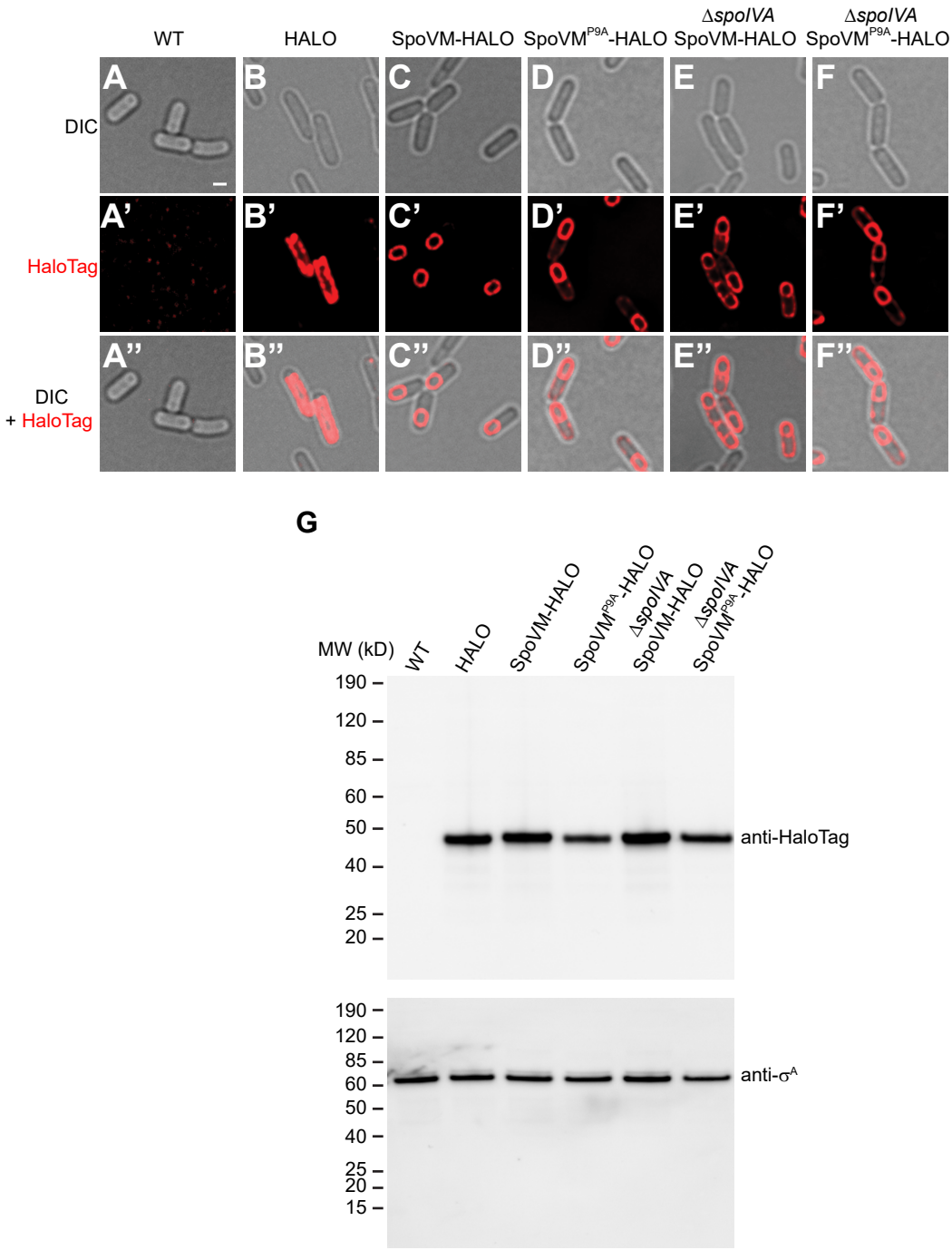

Figure S2. Subcellular localization of SpoVM-HALO in sporulating *B. subtilis*. (A-F) Fluorescence micrographs of various strains of *B. subtilis* 2.5 h after induction of sporulation. (A) wild type (PY79), (B) wild type producing free HaloTag protein (EAP24), (C) SpoVM-HALO (EAP5), (D) SpoVM^{P9A}-HALO (EAP15); or *spoIVA* deletion strain producing (E) SpoVM-HALO (EAP26) or (F) SpoVM^{P9A}-HALO (EAP18). All localization results are consistent with past observations in the lab using GFP fusions. Scale bar: 1 μm. (G) Immunoblot analysis of cell extracts of *B. subtilis* strains used in (A-F) 3 h after induction of sporulation using antisera specific to either HaloTag (top) or σ^A (loading control; bottom) to evaluate intracellular protein levels.

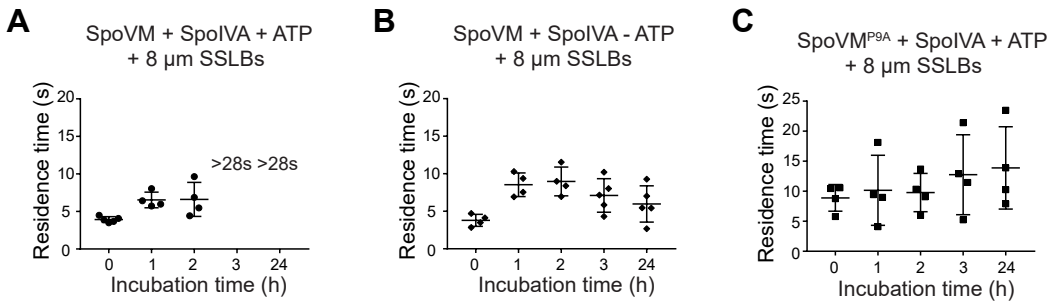

Figure S3. SpoIVA polymerization drives longer SpoVM-Cy3 residence times in vitro on 8-µm SSLBs. (A-B) Residence times of SpoVM-Cy3 measured 0 h, 1 h, 3 h, and 24 h after initiating the binding reaction on 8-µm SSLBs in the presence of (A) SpoIVA and ATP, or (B) SpoIVA and absence of ATP. (C) SpoVM^{P9A}-Cy3 residence times in the presence of SpoIVA and ATP. “>28 s” indicates that single molecule imaging conditions could not be attained and that residence times likely exceeded the timescale for photobleaching.

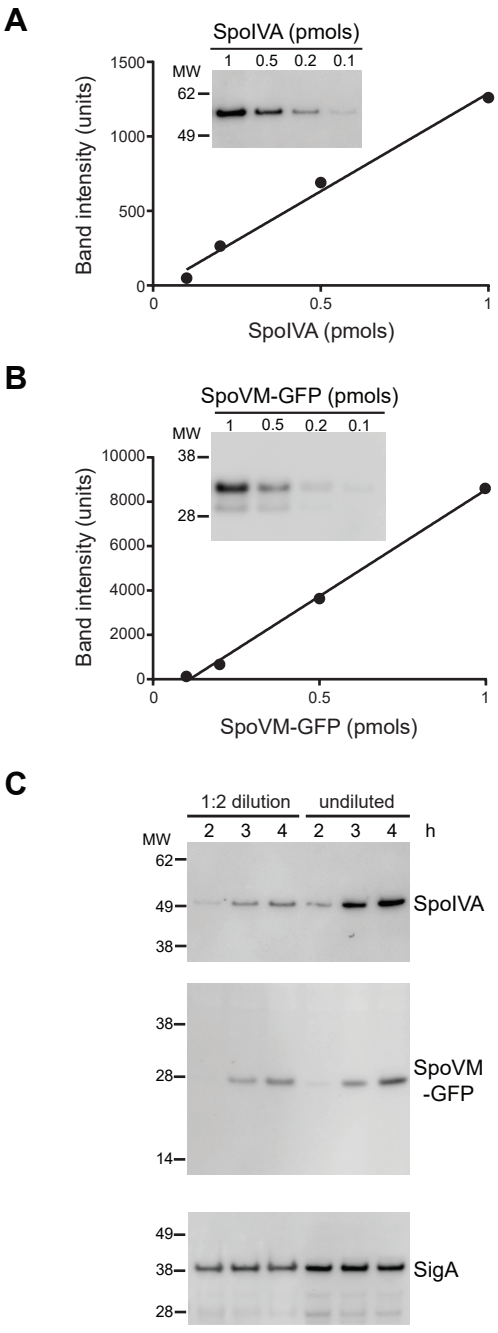

Figure S4. Intracellular concentrations of SpoVM and SpoIVA. (A) Band intensities of various amounts of recombinant, purified SpoIVA detected by immunoblotting (inset) using antisera raised against purified SpoIVA. (B) Band intensities of various amounts of recombinant, purified SpoVM-GFP detected by immunoblotting (inset) using antisera raised against purified GFP. (C) Representative immunoblots of extracts of sporulating *B. subtilis* cells producing SpoVM-GFP from a single ectopic chromosomal locus under control of the spoVM promoter, harvested at times indicated after the induction of sporulation, and probed with antisera raised against SpoIVA (top), GFP (middle), or σ^A (bottom). Extracts were loaded either 1:2 diluted or undiluted, as indicated. MW: relative migration of molecular weight markers (kD) indicated to the left.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. SpoVM-Cy3 binding to 2-μm SSLBs. Scale bar: 2 μm.

Movie S2. SpoVM-Cy3 binding to 8-μm SSLBs. Scale bar: 2 μm.

Movie S3. SpoVM-HALO localization in the absence of coat assembly. Strain EAP26; scale bar: 2 μm.

Movie S4. SpoVM-Cy3 binding to 2-μm SSLBs in the presence of SpoIVA + ATP at t = 0 h. Scale bar: 2 μm.

Movie S5. SpoVM-Cy3 binding to 2-μm SSLBs in the presence of SpoIVA + ATP at t = 3 h. Scale bar: 2 μm.

Movie S6. SpoVM-HALO localization the presence of coat assembly. Strain EAP5; scale bar: 2 μm.

Table S1: Parameters for the allosteric sigmoidal fit of adsorption of SpoVM-Cy3 and SpoVM^{P9A}-Cy3 onto differently curved membranes. B_{max}, maximal fitted binding value; h, Hill coefficient; $K_{1/2}$, concentration of protein producing half-maximal binding. Errors are SEM (n=3 independent trials, each trial containing data from >30,000 SSLBs).

Protein	SSLB diameter (μm)	B_{max} (units/μm^2)	h	$K_{1/2}$ (μM)
SpoVM-Cy3	2	87.1 (± 3.0)	3.5 (± 0.8)	0.64 (± 0.04)
SpoVM-Cy3	8	90.0 (± 8.3)	2.2 (± 0.7)	1.1 (± 0.17)
SpoVM^{P9A}-Cy3	2	98.9 (± 4.6)	1.2 (± 0.2)	0.54 (± 0.06)
SpoVM^{P9A}-Cy3	8	85.9 (± 5.6)	1.8 (± 0.5)	0.60 (± 0.09)

Table S2: Residence times of SpoVM-Cy3 and SpoVM^{P9A}-Cy3 bound to 2-μm and 8-μm SSLBs at various bulk unlabeled protein concentrations.

Protein	SSLB diameter (μM)	[SpoVM-Cy3] (μM)	[SpoVM] (μM)	Residence time of SpoVM-Cy3 (sec)	# SSLBs	# long-lived binding events
SpoVM-Cy3	2	0.003	0	5.6	14	169
				7.9	12	119
				10.7	14	130
				18.9	6	49
				8.9	7	85
				10.5	10	101
				5.7	9	61
				6.9	8	111
				12.1	6	56
				13.6	7	69
			0.01	6.9	8	59
				7.9	5	32
				5.5	4	61
				8.1	3	51
				13.8	5	76
				3.7	4	58
				10.8	5	78
			1	9.2	7	35
				4.6	11	197
				5.3	9	156
				5.0	7	121
				9.4	7	165
				7.9	6	112
				6.7	15	150
				7.5	7	35
			3	7.3	7	381
				6.2	13	494
				6.2	13	573
				6.9	12	566
				7.8	5	293
				8.0	5	303
				8.6	3	215
				5.4	4	257
			5	9.0	5	245
				7.1	8	441
				5.9	13	632
				6.2	10	562
				6.9	7	325
				8.0	7	305
				6.6	6	335
				8.9	5	246
			10	4.6	10	91

				5.3	7	127
				4.0	13	175
				4.0	12	223
				7.8	10	123
				7.5	10	182
				3.9	19	298
				4.5	9	126
SpoVM-Cy3	8	0.003	0	7.3	2	70
				3.4	1	225
				2.9	1	134
				1.4	1	112
				2.5	1	207
				4.2	1	190
				3.8	1	199
		0.010	0	2.7	1	104
				1.5	1	33
				4.0	1	95
				2.5	1	49
				3.1	1	52
				2.5	1	69
				2.1	1	156
			1	3.0	1	279
				1.1	1	69
				4.1	1	92
				2.4	1	71
				1.8	1	204
				1.6	1	213
				5.3	1	253
				2.4	1	234
				1.8	1	256
			3	4.3	1	404
				2.6	2	515
				3.6	1	284
				6.8	1	263
				2.7	1	328
				3.0	1	379
				2.6	2	440
				3.0	2	628
				2.6	3	329
				2.8	1	427
			5	1.7	1	533
				2.4	1	503
				2.8	1	317
				2.2	1	159
				1.9	1	189
				2.0	1	201
				2.5	1	226
				4.3	1	195
			10	2.7	2	400

				2.8	1	120
				3.5	1	268
				3.2	1	219
				3.2	1	186
				2.9	2	641
				2.9	1	135
				2.9	1	105
SpoVM^{P9A}-Cy3	2	0.003	0	5.1	2	53
				5.3	6	68
				6.6	3	52
				11.5	4	82
				7.2	5	111
				13.1	7	73
				11.3	9	151
				26.5	5	123
				10.9	12	175
				12.7	9	195
			0.01	4.4	5	56
				2.3	5	33
				3.2	6	31
				7.8	5	48
				16.5	3	21
				4.9	3	47
				8.6	3	90
				3.1	4	55
				20.9	6	129
			1	6.8	20	129
				6.5	6	116
				7.4	5	65
				3.6	6	83
				7.7	7	39
				5.3	7	65
				12.4	4	135
				14.6	7	80
				5.7	5	91
				4.7	8	103
			3	2.7	14	165
				2.2	12	91
				2.9	15	111
				5.6	17	108
				6.9	18	166
				3.9	16	122
				5.7	15	145
				4.6	19	277
				7.1	12	199
				5.2	11	225
			5	3.7	2	96
				6.0	4	148
				6.9	2	76

				7.8	5	147
				2.9	5	81
				4.5	4	60
				5.7	6	151
				5.9	3	103
				4.5	3	75
				7.8	10	226
			10	6.1	2	62
				7.7	7	247
				5.3	4	141
				8.5	4	155
				5.9	3	126
				5.6	3	130
				6.8	3	147
				5.9	3	74
				8.0	5	176
				12.4	2	82
SpoVM^{P9A}-Cy3	8	0.010	0	14.2	1	71
				3.2	1	195
				3.3	1	74
				3.5	1	140
				1.9	1	93
				18.3	1	84
			1	2.3	1	381
				3.0	1	356
				2.8	1	100
				6.2	1	201
				4.8	1	645
				16.8	1	83
				5.0	1	173
				4.9	1	373
				4.4	1	360
			3	2.7	1	134
				4.6	2	207
				2.9	3	190
				4.1	4	237
				6.7	1	45
				4.0	1	95
				5.0	2	110
				1.4	1	46
				4.4	1	75
				6.3	1	67
			5	4.0	1	640
				3.1	1	430
				3.4	1	705
				3.9	1	751
				2.9	1	637
				3.8	1	719
				3.3	1	684

	10	3.3	1	750
		3.6	2	1204
		2.4	1	655
		2.5	1	587
		2.9	1	673
		2.7	1	547
		4.2	1	737

Table S3: Residence times of SpoVM-HALO and SpoVM^{P9A}-HALO bound to the forespore and mother cell membranes at various timepoints during sporulation in the absence of subsequent coat assembly. JF549 (200 pM) used.

Strain/ Genotype	Time after the induction of sporulation (h)	Residence time on FS membrane (sec)	Residence time on MC membrane (sec)	# cells	# long-lived binding events on FS	# long-lived binding events on MC
EAP26 *ΔspoIVA::neo amyE::spoVM-HALO*	3	10.8	8.7	3	58	25
		14.2	22.5	2	46	30
		42.5	42.6	1	22	10
		18.6	11.7	2	15	17
		17.4	15.6	2	38	15
		7.4	45.0	2	27	14
		8.8	17.5	5	137	72
		13.6	5.4	7	118	55
		28.1	9.3	4	84	56
		8.1	6.5	4	94	79
		0.9	8.2	5	87	48
		14.0	9.5	4	83	34
	4	7.2	5.7	2	52	34
		5.2	6.0	2	21	21
		33.9	22.5	4	58	32
		27.8	7.6	3	41	22
		19.5	27.1	1	12	13
		4.6	7.7	2	34	21
		22.0	1.6	2	28	20
		18.0	7.1	3	59	40
EAP18 *ΔspoIVA::neo amyE::spoVM^{P9A}-HALO*	3	20.7	19.7	6	57	47
		22.2	9.1	2	35	39
		4.4	10.0	3	43	32
		10.5	24.4	5	86	59
		13.8	9.6	6	72	63
		14.6	15.1	6	65	71
	4	11.0	7.1	4	54	67
		15.2	8.1	4	75	76
		6.9	4.6	4	70	57
		7.9	6.2	8	145	108
		10.7	18.3	6	109	63
		19.2	10.1	5	145	101
		17.1	8.6	5	120	47
		14.8	7.6	5	114	119
		11.6	14.6	5	103	69
EAP24 *amyE::spoVM promoter HALO*	3	6.4	4.9	4	36	50
		5.2	2.0	4	40	23
		4.4	5.8	7	42	63

Table S4: Residence times of SpoVM-Cy3 in the presence of SpoIVA bound to 2-µm SSLBs at increasing incubation time periods.

[SpoIVA] (µM)	[SpoVM-Cy3] (µM)	[SpoVM] (µM)	ATP (mM)	Time of incubation (hrs.)	Residence time of SpoVM-Cy3 (sec)	# SSLBs	# long-lived binding events
2	0.003	3	4	0	4.9	22	623
					6.4	20	949
					4.8	6	291
					5.6	7	305
				1	4.2	2	126
					8.7	2	59
					5.8	11	385
					9.2	2	96
				2	5.3	2	111
					6.5	2	114
					10.5	2	85
					8.2	3	147
				3	> 28 s	N.D.	N.D.
				24	> 28 s	N.D.	N.D.
[SpoIVA] (µM)	**[SpoVM-Cy3] (µM)**	**[SpoVM] (µM)**	**ATP (mM)**	**Time of incubation (hrs.)**	**Residence time of SpoVM-Cy3 (sec)**	**# SSLBs**	**# long-lived binding events**
2	0.003	3	0	0	8.4	3	133
					10.2	2	67
					4.5	7	160
					6.8	3	137
				1	4.9	3	55
					9.7	5	196
					10.6	4	334
					7.6	4	150
				2	9.4	2	113
					9.4	6	227
					9.1	2	90
					11.0	3	99
				3	5.9	3	123
					4.7	2	84
					9.0	4	148
					5.4	5	175
				24	10.0	2	79
					6.5	3	107
					6.3	2	100
					5.0	4	153
[SpoIVA^{K30A}] (µM)	**[SpoVM-Cy3] (µM)**	**[SpoVM] (µM)**	**ATP (mM)**	**Time of incubation (hrs.)**	**Residence time of SpoVM-Cy3 (sec)**	**# SSLBs**	**# long-lived binding events**
2	0.003	3	4	0	6.7	3	77

					8.1	2	99
					4.0	6	192
				1	5.8	1	30
					6.4	7	195
					7.4	2	89
					4.4	2	102
				2	3.1	2	44
					4.4	2	53
					8.8	5	144
				3	9.2	3	88
					5.7	2	75
					5.5	3	113
					4.7	6	235
				24	3.1	1	37
					14.8	1	17
					6.3	1	71
[SpoIVA] (μM)	**[SpoVM^{P9A}-Cy3] (μM)**	**[SpoVM^{P9A}] (μM)**	**ATP (mM)**	**Time of incubation (hrs.)**	**Residence time of SpoVM-Cy3 (sec)**	**# SSLBs**	**# long-lived binding events**
2	0.003	3	4	0	7.6	4	114
					17.9	2	94
					9.4	3	145
					10.3	5	144
				1	10.1	8	142
					7.0	5	91
					6.6	5	110
					7.3	6	122
				2	6.1	9	101
					8.2	2	40
					10.4	6	77
					9.0	9	90
				3	13.0	4	52
					6.7	6	53
					17.0	1	22
					9.2	4	55
				24	11.3	3	38
					3.5	2	105
					9.4	2	54
					4.9	2	59

Table S5: Residence times of SpoVM-HALO and SpoVM^{P9A}-HALO bound to the forespore and mother cell membranes at various timepoints during sporulation in the presence of subsequent coat assembly. JF549 (200 pM) used.

Strain/ Genotype	Time after the induction of sporulation (h)	Residence time on FS membrane (sec)	Residence time on MC membrane (sec)	# cells	# long-lived binding events on FS	# long-lived binding events on MC
EAP5 *amyE::spoVM-HALO*	3	11.7	9.2	5	68	31
		11.3	2.6	4	79	50
		22.5	5.9	7	145	87
		15.7	5.9	6	126	44
		10.7	5.1	8	142	69
		22.6	8.2	11	156	39
		33.8	4.6	7	106	37
		14.9	5.7	6	103	36
	4	8.3	5.4	2	39	32
		12.9	5.4	14	335	227
		15.0	6.9	4	84	42
		10.9	11.8	4	70	45
		23.4	7.1	6	130	81
		12.7	14.9	4	90	57
		41.6	9.6	6	136	45
		15.6	12.1	8	159	47
EAP15 *amyE::spoVM^{P9A}-HALO*	3	19.2	9.7	8	92	52
		6.7	7.2	10	61	63
		15.7	6.7	9	148	102
		5.1	11.3	8	41	49
		12.2	10.3	14	107	89
		17.3	8.8	9	58	63
		22.3	27.7	14	137	155
		13.3	8.1	5	40	32
		17.3	19.0	11	53	54
	4	17.9	8.8	6	91	72
		9.5	8.5	9	117	184
		8.7	9.5	13	155	122
		8.9	6.4	7	112	117
		9.0	10.2	8	113	64
		8.1	5.9	8	105	103
	6	6.3	7.7	3	70	76
		9.5	10.0	5	131	122
		5.0	3.3	9	166	149
		5.5	5.1	4	28	31
		12.8	14.4	4	109	102
		8.7	7.0	8	280	234

EAP24	3	6.4	4.9	4	36	50
amyE::spoVM		5.2	2.0	4	40	23
promoter HALO		4.4	5.8	7	42	63

Table S6: Residence times of SpoIVA-Cy3 bound to 2-μm SSLBs at various bulk unlabeled protein concentrations.

Protein	SSLB diameter (μm)	[SpoIVA-Cy3] (nM)	[SpoIVA] (μM)	Residence time of SpoIVA-Cy3 (sec)	# SSLBs	# long-lived binding events
SpoIVA-Cy3 + 3 μM SpoVM	2	0.003	0	11.8	4	66
				6.4	9	129
				10.4	10	141
				23.5	5	66
				12.1	11	159
		0.003	0.007	9.5	5	156
				15.3	6	181
				12.5	8	258
				22.3	6	156

Table S7: SpoVM and SpoIVA intracellular concentrations via immunoblot analysis. Errors are SD.

Protein	Time after induction of sporulation (hr.)	Molecules per sporangium [SpoIVA-Cy3] (nm)
SpoVM-GFP	2	55,000 (± 23000)
	3	150,000 (± 57000)
	4	270,000 (± 150000)
SpoIVA	2	45,000 (± 31000)
	3	140,000 (± 69000)
	4	190,000 (±90000)

SUPPLEMENTAL MATERIALS AND METHODS

Strain construction and general methods

Strains CVO1195 (*amyE::spoVM-gfp cat*), CVO1395 (*amyE::spoVM^{P9A}-gfp cat*) (11), KR128 (*spoIVA::neo amyE::spoVM-gfp cat*), KR209 (*spoIVA::neo amyE::spoVM-gfp cat thrc::spoIVA spec*), and KR160 (*thrC::spoIVA-GFP)* (16) have been previously described. Plasmids pEAP9 and pEAP10 containing *spoVM-halo ampR* and *spoVM^{P9A}-halo ampR*, respectively, for insertion in the *amyE* locus in *B. subtilis* were generated by PCR amplifying *halo* from plasmid TetRpe-HALO (34) and either *spoVM* or *spoVM^{P9A}* containing abutted 5' HindIII and 3' BamHI restriction sites from KR103 and KR102 chromosomal DNA (16) and cloning into plasmid pDG1662 (35) digested using *BamH*I-HF and *Hind*III-HF. pEAP9 and pEAP10 were transformed into PY79 to generate strains EAP5 (*amyE::spoVM-halo*) and EAP15 (*amyE::spoVM^{P9A}-halo*), respectively. Strain KP73 (*spoIVA::neo2*) (21) was transformed with pEAP9 and pEAP10 to generate strains EAP18 (*spoIVA::neo2 amyE::spoVM-halo*) and EAP26 *(spoIVA::neo2 amyE::spoVM^{P9A}-halo*), respectively. Strain EAP24 was created by transforming plasmid pEAP23 (pDG1662 backbone, *halo* cloned downstream of *spoVM* promoter) into PY79. His_6-SpoIVA, His_6-SpoIVA^{K30A} (18), and SpoVM-GFP-His_6 (12) were purified as described previously. Protein amount was quantified using NanoDrop spectrophotometer. For immunoblot analysis, cells were induced to sporulate by resuspension in SM medium (36). Cells were harvested at different times after induction of sporulation, cell extract was prepared as described (37), and separated by SDS-PAGE. Samples were immunoblotted using Anti-HaloTag monoclonal antibody (Promega), or rabbit antiserum raised against purified SpoIVA, GFP, or σ^A, and Starbright Blue 700 Goat Anti-Mouse IgG (BioRad Laboratories) and imaged using a ChemiDoc gel and blot imaging system (Fig. S2).

Epifluorescence microscopy

All images were taken at room temperature using a DeltaVision Core microscope system (Applied Precision/GE Healthcare) equipped with a Photometrics CoolSnap HQ2 camera and

environmental chamber. Ten planes were acquired every 200 nm. Resulting images were deconvolved using SoftWorx software. For FRAP analysis, KR160 was induced to sporulate by resuspension in media that contained 20 μg/ml FM4-64. Cells were harvested by centrifugation 80% of the supernatant was removed, and cells were resuspended in the remaining 20% of the culture medium. 3 μl suspension were placed on a coverslip as described above and covered with an agarose pad containing 0.17 μg/ml FM4-64. Images were acquired in an environmental chamber at 30°C every 15 s for 17.5 min. Samples were photobleached with laser set at 10% power for an exposure time of 0.1 s using FITC excitation/emission filters. For competition experiment, various concentrations of SpoVM-Cy3 were incubated with 2 μm SSLBs and 8 μm SSLBs (at concentration of equal surface area) and either 0.3 μM or 0.9 μM SpoIVA + 4 mM ATP or buffer only in a total volume of 40 μL. Samples were incubated at 37°C for 3 h with shaking every 5 min for 5 min (350 RPM). 3 μl suspension were placed on a poly-L-lysine coated coverslip under a 1% (w/v) agarose pad as described above. Z-sectioning images were acquired starting at the bottom of the sample using 0.20 μm optical section spacing, 60 optical sheets, and 12 μm sample thickness. Images were taken with a 0.200 exposure time at 10% power in mCherry channel and subsequently with a 0.0250 exposure time at 32% power in POL channel. Cells were harvested 5 h after induction of sporulation and resuspended in one-tenth volume phosphate-buffered saline. FM4-64 (1 μg/ml final concentration) was added to visualize membranes as required. 3 μl suspension were placed on a poly-L-lysine coated coverslip (Mattek Corp) and a 1% (w/v) agarose pad prepared with distilled water was placed on top of the sample. When imaging HaloTag ligand JF549, cells were resuspended in PBS containing 50 nM JF549, incubated at room temperature for 30 min, washed 3 times with PBS, and first imaged without membrane dye using the mCherry channel. 50 μl MitoTracker Green FM (100 nM final concentration) was then placed on the agarose pad, incubated for ~15 min, and membranes were visualized at each previous field of view using the GFP channel.

SSLB preparation

Defined lipids in chloroform were vacuum dried overnight at room temperature then hydrated using 1 mL ultrapure water at 42°C for 1 hour, with vortexing every fifteen minutes to ensure suspension. Resuspended lipids underwent five cycles of freezing in an ethanol/dry ice bath and thawing at 42°C. Lipids were subjected to bath sonication for 1 hour to generate SUVs. At this point, the suspension became transparent and was spun down at 13,000 × g for 10 minutes to remove debris. Silica beads of 2 μm (Polysciences, Inc.) or 8 μm (Cospheric LLC) diameter were cleaned through three sequential washes of ultrapure water, methanol, 1 M NaOH, and ultrapure water and stored in 200 μL ultrapure water. Next, 400 μL SUVs, 200 μL of prewashed silica beads, and 6 μL of 100mM $CaCl_2$ were combined, vortexed vigorously, and incubated at 42°C for 1 hour with occasional vortexing. Following incubation, the suspension was centrifuged at 13,000xg for 10 minutes and washed three times with ultrapure water. The final pellet was resuspended in 1 mL buffer (50 mM Tris, 400 mM NaCl, pH 7.5) and stored at 4°C for up to one week.

Flow cytometry

Reactions were incubated at 42°C for 4 hours with alternative shaking (700 rpm) and resting every 5 minutes to keep the SSLBs suspended in solution. Each reaction volume was diluted in buffer to a total of 1 mL and immediately analyzed on a FACSCanto II (BD Biosciences, Dan Jose, CA) flow cytometer. A 488-nm laser and 530/30 bandpass filter were used to detect fluorescence at varying protein concentrations. Voltages were set to 200 V for forward scatter, 250 V for side scatter, and 400 V. Approximately 20,000 particles were evaluated at each concentration. To evaluate SpoIVA-FITC binding to SpoVM-saturated SSLBs, SSLBs were first incubated overnight with 3 μM SpoVM in buffer A, then incubated with SpoIVA-FITC in the presence of 3 μM SpoVM in buffer A using a forward scatter of 350 V, side scatter of 150 V, and B530 channel of 350 V. To determine copy number of SpoIVA on SSLBs, we determined SpoIVA-Alexa488 binding to

SpoVM-saturated SSLBs and compared the fluorescence intensity to known standards of molecules of equivalent soluble fluorochrome affixed onto beads (Bangs Laboratories).

Single molecule fluorescence microscopy

Single molecule imaging was conducted on a custom-built Olympus IX 71 microscope coupled with a 150X Oil-immersion objective lens (Olympus, N.A. = 1.45), a multi-band dichroic (405/488/561/633 BrightLine quad-band bandpass filter, Semrock, USA) and a piezo z-stage (Madcity Lab, USA). The lasers were focused into the back pupil plane of the objective to generate wide-field illumination. A xy translation stage with a mirror was placed in a plane conjugated to the back pupil plane to change the angle of the laser beam at the sample plane for generating HiLo illumination. The emission was collected by the same objective passing through a quad-band bandpass emission filter (FF01-446/523/600/677-25, Semrock, USA) in front of the electron-multiplying charge-coupled device (EMCCD) (iXon DU-888, Andor Technologies). An AOTF (Acousto-optics) and DAQ (National Instruments) were used to modulate the intensity and wavelength of multiple laser lines. The microscope, AOTF, lasers and the camera were controlled through Micro-manager (an open source software program). To image SSLBs, glass coverslips (25 mm Deckgläser) were cleaned using plasma (Plasma Etch, Inc.), coated with poly-D-lysine (0.1 mg/mL), and blocked using 3% BSA. Enclosed metal holders were used to secure the coverslip during imaging. 200 µL of SSLBs (1 mg/mL) were added to coverslip and incubated for approximately 5 minutes before washing 3x with buffer (50 mM Tris + 100 mM NaCl). 200 µL of the buffer was left on coverslip for imaging. Varying concentrations of protein were added to a tube containing oxygen scavenger buffer (165 U/ml Glucose Oxidase, 4.5 mg/mL Glucose, 2mM Trolox, 2170 U/ml Catalase) for a final volume of 200 µL. The solution was added to the coverslip and imaged using a 561 nm laser with excitation power of 0.250 mW. Data was acquired every 500 ms for 200 frames. Data from approximately 8 different regions of interest was obtained per

coverslip. Transmission images were taken using brightfield illumination following single molecule acquisitions. For experiments requiring the addition of SpoIVA, SSLBs (1 mg/mL) of defined diameter were incubated with SpoVM-Cy3 (3 nM), SpoVM (3 μM), purified His_6-SpoIVA (2 μM), ATP (4 mM), $MgCl_2$ (10 mM), and oxygen scavenger buffer in a total volume of 300 μL. Samples were imaged immediately or incubated at 37°C for the time reported, and then imaged. All imaging parameters remained the same for these experiments.

To image live cells, cultures were induced to sporulate by resuspension and 1 mL was harvested at various timepoints by centrifugation. Cell pellets were resuspended in 100 μL PBS containing 200 pM JF549 and incubated at room temperature for 30 minutes. Cells were washed three times with PBS and resuspended in 100 μL PBS. 3 μL of cells were pipetted onto a coverslip (cleaned previously using plasma) and a 1% agarose pad was placed over sample. Using a 561 nm laser and excitation power of 0.400 mW, images were acquired every 500 ms for 200 total frames. The coordinates of each region of interest were recorded. Following all data collection, 100 μL PBS containing 100 nM of Mitotracker Green FM was added to the top of the agar pad and incubated for ~15 minutes to visualize membranes. Each region of interest was revisited and imaged using X-Cite 110LED (Excelitas Technologies).

Residence time analysis

Data were analyzed using custom written MATLAB software (39) The MATLAB scripts, SLIMFAST/evalSPT, which implements the Multiple-Target-Tracing algorithm (39, 40) were used to localize and track single molecules. Briefly, single molecules are first localized with 2D Gaussian fitting subject to a log-likelihood ratio test with a ‘localization error’. A maximal expected diffusion constant was set to connect localizations between consecutive frames. We used the following algorithm parameters for SPT: localization error: 10^{-6}; detection box: 7 (px); deflation loops: 15; maximum off-time (frames): 3; maximum number of competitors: 1; maximal expected

diffusion constant (μm^2/s): 0.1. Regions of interest (around beads, forespores, or mother cells) were generated from original brightfield images. These regions were segmented and used as a mask to filter tracking data for each individual SpoVM-Cy3 molecule and the survival distribution were fitted to a two-component exponential decay suggesting two binding behaviors of SpoVM - a short-lived component binding lasting less than one frame and a long-lived component binding lasting one frame or more. Residence times were corrected for photobleaching. A graphical user interface of this software implementation is available in the supplemental appendix as supplemental software. To measure the photobleaching rate of Cy3, the coverslip was cleaned as described above and incubated with 200 μL of 1 mg/mL BSA-biotin in PBS for 5 minutes. The solution was removed and after washing 3 times in PBS, the coverslip was incubated with 200 μL 1 mg/mL neutravidin in PBS for 5 minutes and washed 3 times in PBS supplemented with 10 mM $MgCl_2$. 3 nM of Cy3-Biotin was imaged on the surface of the coverslip. Images were acquired every 200 ms using a 561-nm laser with an excitation power of 0.250 mW.

Simulation

$$5P + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_5B + 3A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_5BA_3 + 2A \xrightarrow{k_3} P_5BA_5$$

$$\frac{\mathrm{d}P_5B}{\mathrm{dt}} = [P]^5[B]k_1 - [P_5B]k_{-1} - [P_5B][A]^3k_2 + [P_5BA_3]k_{-2} \qquad \text{Eq. 1}$$

$$\frac{\mathrm{d}P_5BA_3}{\mathrm{dt}} = [P_5B][A]^3k_2 - [P_5BA_3]k_{-2} - [P_5BA_3][A]^2k_3 \qquad \text{Eq. 2}$$

$$\frac{\mathrm{d}P_5BA_5}{\mathrm{dt}} = [P_5BA_3][A]^2k_3 \qquad \text{Eq. 3}$$

$$\frac{dP}{dt} = 5[P_5B]k_{-1} - [P]^5[B]k_1 \qquad \text{Eq. 4}$$