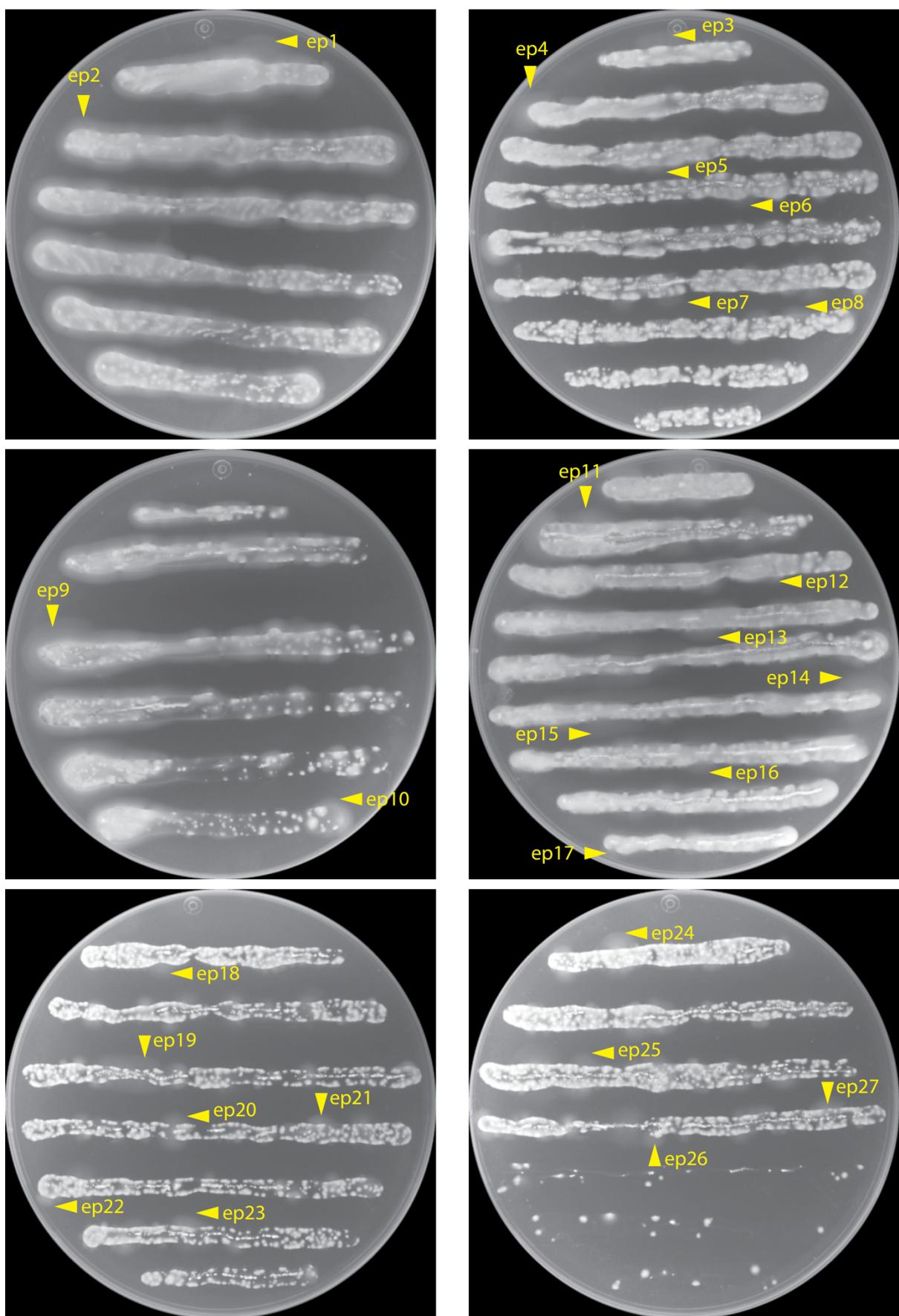
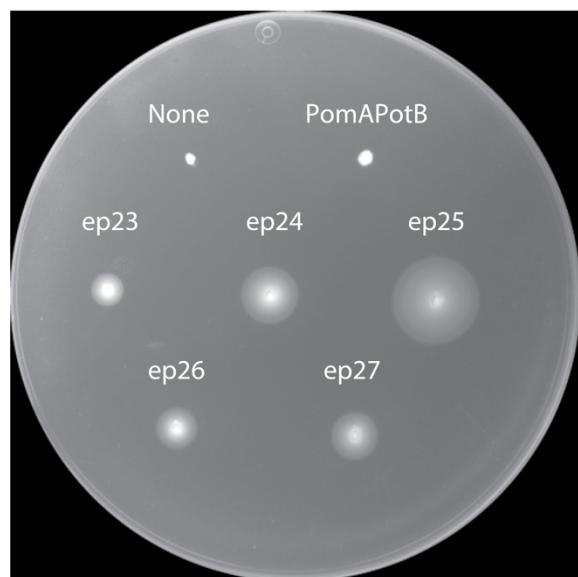
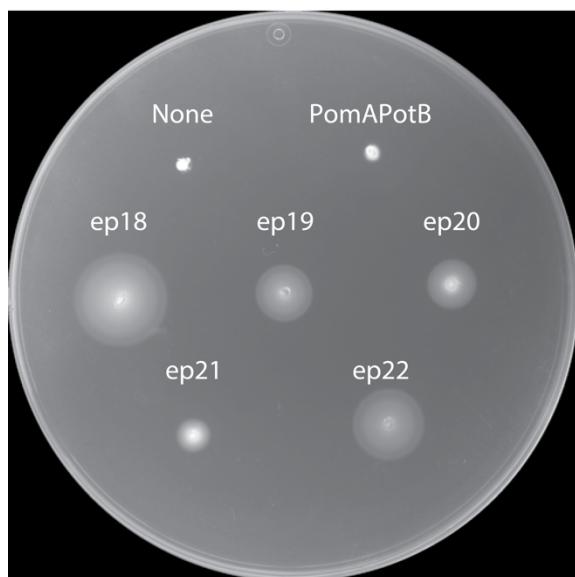
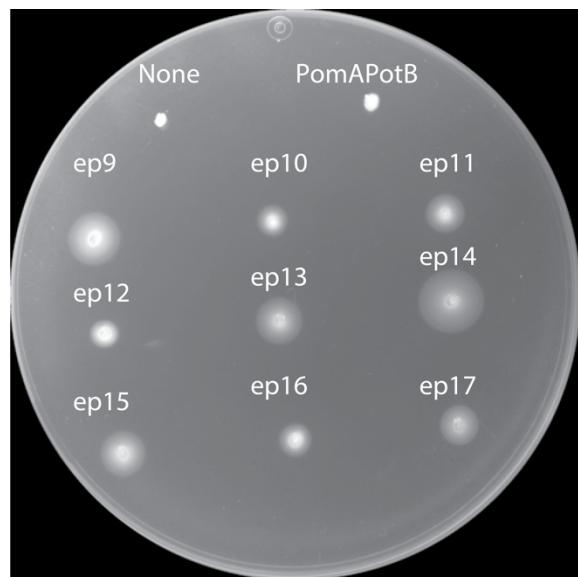
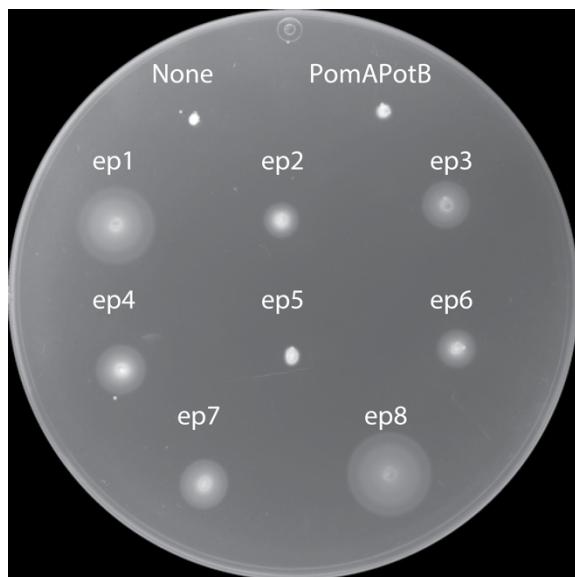


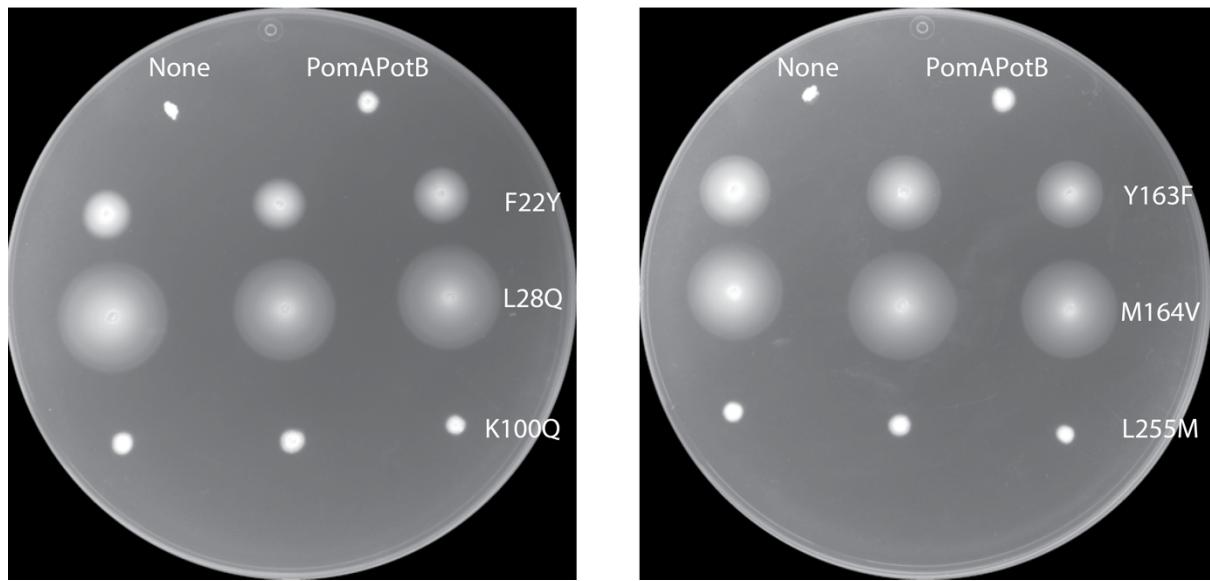
## Supplementary Material



**Figure S1: Streak Plates of EP-PCR Products.** Streak plates used to select the 27 strains identified in this work. Host: RP6665, 0.25% TB agar, 1 mM arabinose, 20  $\mu$ M phenamil, incubated at 30°C for 20 hours.



**Figure S2. Motility characterisation of screened outcomes from streak plates.** Motility characterisation for strains identified in streak plates shown in Fig. S1. Host: RP6665, 0.25% TB agar, 1 mM arabinose, 20  $\mu$ M phenamil, incubated at 30°C for 11 hours.



**Figure S3. Characterisation of functional and non-functional mutants in PotB-ep18 and PotB-ep9.** Test of individual contribution of the three mutations in PotB-ep18 (left) and PotB-ep9 (right) respectively. Host: RP6665, 0.25% TB agar, 1 mM arabinose, 20  $\mu$ M phenamil, incubated at 30°C for 17.5 hours.

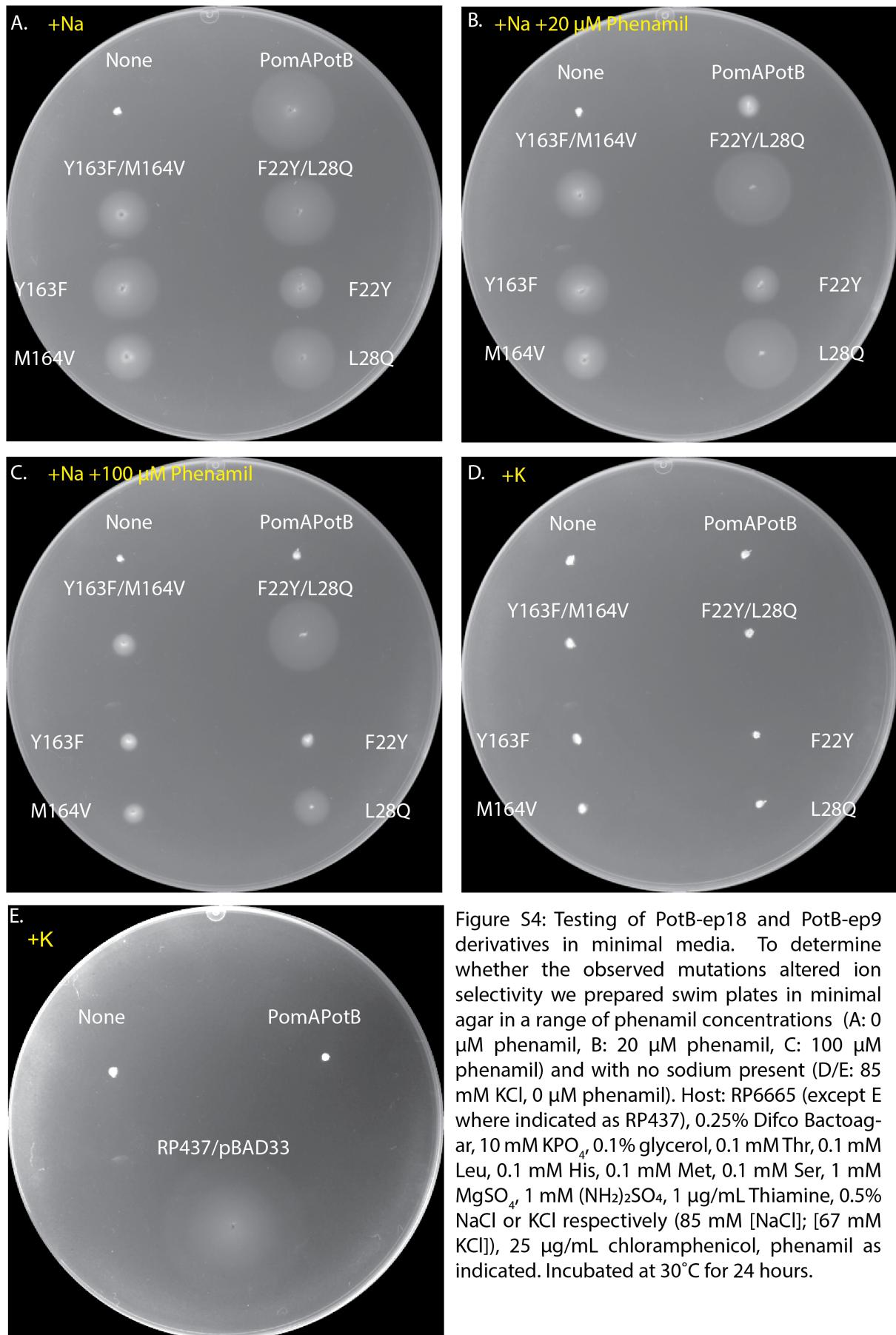
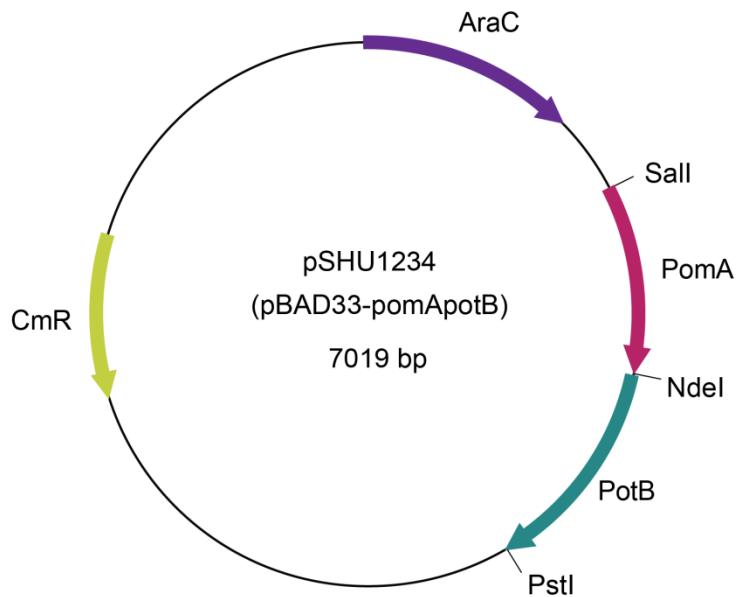
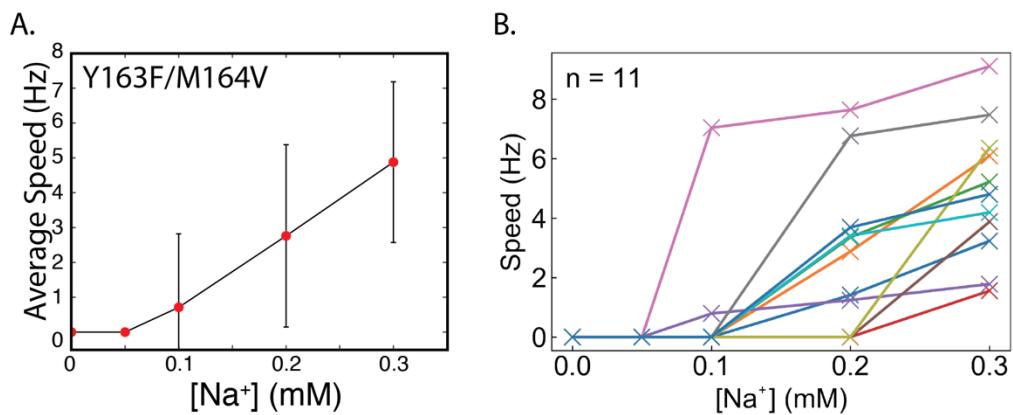


Figure S4: Testing of PotB-ep18 and PotB-ep9 derivatives in minimal media. To determine whether the observed mutations altered ion selectivity we prepared swim plates in minimal agar in a range of phenamil concentrations (A: 0 µM phenamil, B: 20 µM phenamil, C: 100 µM phenamil) and with no sodium present (D/E: 85 mM KCl, 0 µM phenamil). Host: RP6665 (except E where indicated as RP437), 0.25% Difco Bactoagar, 10 mM KPO<sub>4</sub>, 0.1% glycerol, 0.1 mM Thr, 0.1 mM Leu, 0.1 mM His, 0.1 mM Met, 0.1 mM Ser, 1 mM MgSO<sub>4</sub>, 1 mM (NH<sub>2</sub>)<sub>2</sub>SO<sub>4</sub>, 1 µg/ml Thiamine, 0.5% NaCl or KCl respectively (85 mM [NaCl]; [67 mM KCl]), 25 µg/ml chloramphenicol, phenamil as indicated. Incubated at 30°C for 24 hours.



**Figure S5. Map of plasmid pSHU1234.** Plasmid based on pBAD33 backbone for expression of PomA and PotB with introduced Ndel site between PomA and PotB to aid in targeted random mutagenesis across PotB.



**Figure S6. Repeat measurements of Y163F/M164V at low sodium.** We repeated measurements of Y163F/M164V with finer spacing of sodium concentration and tracking single cell motility during sodium concentration transitions. (A) Mean and SD motility at 0, 0.05, 0.1, 0.2, and 0.3 mM Na<sup>+</sup>. (B) Single cell motility tracked for each cell showing that all cells were motile at 0.3 mM Na<sup>+</sup>, and 9 out of 11 cells were motile at 0.2 mM Na<sup>+</sup>.

**Table S1**

EP-PCR #ID	Mutant amino acids	swarm
PotB-ep1	L15H, L35I, T60T, S73G, P74T, R154R, A207T, I265N, N269D	+++
PotB-ep2	I50T, M102V, D241G	+
PotB-ep3	L15H, L17L, F33Y, M42K, R101S, S105G, K123N, L131P, E161E, N274S, T293T	++
PotB-ep4	S38S, K100I, N180Y, L228S, H267R, E284V	++
PotB-ep5	S105G, K132N, E268G, E284K	-
PotB-ep6	S105G, L131P, K198K, P244L, H267L, S290G, V291V	+
PotB-ep7	L15H, E41G, K109I, D113N, R128S, E138E, D166N, V230V, Q273P, S290G	++
PotB-ep8	D9E, C10R, L36Q, V44I, M102L, I142I, I182I, E197D, I252F, E271D	+++
PotB-ep9	E88E, Y163F, M164V, L255M	++
PotB-ep10	T21S, F39V, I68I, L124M, K198N, K226I, D245D, A286V	+
PotB-ep11	G87G, S105S, L228F, P276P	+
PotB-ep12	N94Y, R108W, R125R, L168L, F191L, L228F, P276T, Q288L, V291V	+
PotB-ep13	F39L, F47L, Y163F, I171I, N176S, V248A, P283P, E284G, V289V	++
PotB-ep14	M30L, F32I, T63S, G77G, I252F	+++
PotB-ep15	R128R, E262G, L266L, E284V	++
PotB-ep16	D80E, I167V, P173H, E216V, L217M, Q288R, M294V	+
PotB-ep17	V44A, K100I, K123R, N180D, I182I, E197G	+
PotB-ep18	F22Y, L28Q, T56T, T60T, K100Q	+++
PotB-ep19	L36Q, G78S, I118T, E119V, L255L, Q260L, V289D, V291A	++
PotB-ep20	V34A, T56T, T63A, D66D, E72E, E97K, K109N, D159V, N180S, A247A, K282K	++

PotB-ep21	L37H, E88D, L98L, Q137L, R165R, D190V, Q273P, P295Q	+
PotB-ep22	P12P, L36Q, E88V, S105R, I118V, I145I, G196G, K198I, L217L, G220G, T236M, L239L, S240R, E281K, P283P	+++
PotB-ep23	Q92H, A172T, D223N, D245E, A286A	+
PotB-ep24	N70Y, G112S, N180Y, G196G, R229R, Q260Q, I265I, L280M, V285V	++
PotB-ep25	C8R, L28Q, M42V, F54Y, N70S, G196G, N202N, S224G, E268E, S290G, P295P	+++
PotB-ep26	C8Y, F22F, E96V, L107L, D113V, D159A, I171F, P173P, L217L, D223G, L228I, N258G, E262E, A286A, Q288L	+
PotB-ep27	I50T, L117M, R125R, A234A, K259N, I265T	++

**Table S1:** Mutation Tables for Phenamil-resistant outcomes from EP-PCR. All 27 selectable outcomes from EP-PCR are listed, with coding (red) and silent (black) mutations indicated in the second column. Comparative motility was recorded on a scale of 0-3 (-, +, ++, +++), with swim plate measurements shown in Fig. S1.

**Table S2**

	Primer ID	5>3	Purpose
QuickChange			
	1373	TTATGGATGGGACATACGCAGATTGATGTC	PotB(F22Y)-F
	1374	GACATCAAATCTCGTATGTCCCCATCCATAA	PotB(F22Y)-R
	1375	GCAGAGTTGATGTGCAGCTGATGTGTTCTT	PotB(L28Q)-F
	1376	AAGAAACACATCAGCTGCGACATCAAATCTGC	PotB(L28Q)-R
	1377	ATCGAAGAGCTGAAACAACGCATGGAGCAAAG	PotB(K100Q)-F
	1378	CTTGCTCCATGCGTTGTTCAGCTTCGAT	PotB(K100Q)-R
	1379	GCCGATGTCGAACCCTTATGCGCGACATTCT	PotB(Y163F)-F
	1380	AGAACATGTCGCGCATAAAGGGTTGACATCGGC	PotB(Y163F)-R
	1381	GATGTCGAACCCTATGTGCGCGACATTCTGCG	PotB(M164V)-F
	1382	CGCAGAACATGTCGCGCACATAGGGTTGACATC	PotB(M164V)-R
	1383	CGTCGCATCAGCCTGATGGTACTGAACAAACA	PotB(L255M)-F
	1384	TGTTTGTTCAGTACCATCAGGCTGATGCGACG	PotB(L255M)-R
	1385	GATGTCGAACCCTTGTGCGCGACATTCTGCG	PotB(Y163F_M164V)-F
	1386	CGCAGAACATGTCGCGCACAAAGGGTTGACATC	PotB(Y163F_M164V)-R
Sequencing			
	0363	GCGTCACACTTGCTATGCC	pBAD1212-f
	0364	TGGGACCACCGCGCTACTGC	pBADHind-r
	0111	GACATCGCGCTTACGGATGAAC	pomA382-403
EP-PCR			
	1176	TAACTTGGAGAATTCATATGGATGATGAAGAT	Nde-potB-F
	0104	GAAC TGCA GTC ACCT CGG TT CGG	motAmotB-TR (Pst)

**Table S2: List of primers used in this work.** Primers for QuickChange (point mutations to establish functional and non-functional mutations), Sequencing, and EP-PCR indicated respectively.

Strain	Description	Reference
RP437	Wild type for motility and chemotaxis:  F-, thr-1, araC14, leuB6(Am), fhuA31, lacY1, tsx-78, λ-, eda-50, hisG4(Oc), rfbC1, rpsL136(strR), xylA5, mtl-1, metF159(Am), thiE1	(Parkinson and Houts, 1982)
RP6665	Δ(motA-motB):  motA-motB)Δm12-13 his-4 metF(Am)159 (lac) Δu169 rpsL136 thi-1 ara-14 mtl-1 xyl-5 tonA31 tsx-78	(Block et al., 1989)

**Table S3:** List of strains used in this work.

**Non-phlyogenetic Logistic Regression (glm)**

PotB Position	r0_slope_Estimate	r0_slope_St.d. Error	r0_slope_z_value	r0_slope_Pr(> z )
F22	2.947	1.300	2.266	0.023
L28	-18.817	3765.847	-0.005	0.996

**Phlyogenetic Logistic Regression**

(phyloglm with method = logistic\_IG10)

PotB Position	r2_slope_Estimate	r2_slope_St.dErr	r2_slope_z.value	r2_slope_p.value
F22	3.195	1.338	2.387	0.017
L28	1.990	1.316	1.512	0.130

**Table S4: Correlation Analysis of Mutations at each respective site.** As per methods, we calculated a non-phylogenetic and phylogenetic general logistic model using packages glm and phyloglm in R respectively. Where the p-value of the slope is < 0.05, this indicates a significant correlation between the residue identity (ie F22 vs Y22) and PomB/MotB classification respectively.

Strain	Ion Source
MotB_Escherichia_coli_strain_K12_MOTB_ECOLI	H+
MotB_Salmonella_typhimurium_strain_LT2_MOTB_SALTY	H+
MotB_Pseudomonas_aeruginosa_strain_ATCC_15692_Q9HUL2_PSEAE	H+
MotB_Bacillus_subtilis_strain_168_MOTB_BACSU	H+
MotB_Streptococcus_pneumoniae_A0A0T8PK69_STREE	H+
MotB_Bacillus_licheniformis_strain_ATCC_14580_Q65KJ0_BACLD	H+
MotB_Streptococcus_pneumoniae_A0A0E8TCW6_STREE	H+
MotB_Helicobacter_pylori_strain_ATCC_700392_MOTB_HELPY	H+
MotS_Oceanobacillus_iheyensis_A0A2P1WLE1_9BACI	Na+
MotS_Bacillus_alcalophilus_G9I2I5_BACAO	Na+
MotS_Bacillus_subtilis_subsp_natto_BEST195_BAI864791	Na+
MotS_Bacillus_licheniformis_A0A1Q9FXY5_BACLI	Na+
MotD_Pseudomonas_aeruginosa_strain_ATCC_15692_G3XD90_PSEAE	H+
MotB_Desulfovibrio_magneticus_strain_ATCC_700980_C4XPD2_DESMR	H+
PomB_Vibrio_uginolyticus_O06874_VIBAL	Na+
PomB_Vibrio_cholerae_serotype_O1_strain_ATCC_39315_Q9KTK9_VIBCH	Na+
MotB_Aliivibrio_fischeri_KLU777421	Na+
PomB_Shewanella_oneidensis_MR_1_NP_7171461	Na+
MotB_Aquifex_aeolicus_strain_VF5_O67121_AQUAE	Na+

**Table S5: Classification of ion type used to calculate estimator.** After filtering (as per Methods) we were left with 19 strains where the ion type was identified from the literature. *Bacillus* (Fujinami et al., 2009); *E. coli* (Sowa and Berry, 2008), *Salmonella* (Minamino and Imada, 2015); *Pseudomonas* (Doyle et al., 2004); *Aquifex*, *Shewanella* (Takekawa et al., 2015); *Vibrio* (Atsumi et al., 1992); *Streptococcus* (Manson et al., 1977); *Helicobacter* (Nakamura et al., 1998).

## **Supplementary Note**

List of specific homologues from set of 948 Mot B homologues across *E. coli* K12's 308 residues that contained the mutations L28Q, Y163F, and M164V respectively. Both F22 and Y22 were present in many cases and as such is not listed.

### **L28Q**

MotB *Myxococcus fulvus* A0A0F7BJE1 MYXFU

### **Y163F**

MotB *Yersinia pestis* biovar *Orientalis* str IP275 A0A0M1V4N2 YERPE

### **M164V**

*Betaproteobacteria bacterium* RBG 16 66 20 A0A1F3YFW8 9PROT

MotB *Caballeronia terrestris* A0A158KEN9 9BURK

*Paraburkholderia monticola* A0A149PEJ5 9BURK

MotB *Spirochaetae bacterium* HGW Spirochaetae 1 A0A2N1TQI3 9SPIR

*Spirochaetes bacterium* RBG 16 49 21 A0A1G3QXZ5 9SPIR

*Desulfuromonadales bacterium* C00003093 A0A1E7IF89 9DELT

*Oceanospirillum maris* UPI000403DD69

MotB *Oceanospirillales* A0A1H5Y5D3 9GAMM

TssL *Alteromonadaceae bacterium* A0A2D8J8H6 9ALTE

*Oceanospirillum maris* UPI000403DD69

MotB *Oceanospirillales* A0A1H5Y5D3 9GAMM

MotD *Gallionellales bacterium* GWA2 60 18 A0A1G0CPT2 9PROT

MotD unclassified *Nitrosomonadales* A0A1G0DLC6 9PROT

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