Supporting Information

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SI Experimental Procedures

Construction of *Streptomyces coelicolor* Kinase Mutants. STK mutants of *S. coelicolor* M600 were generated by replacing the entire coding sequence of individual genes (*SCO1468*, *SCO2110*, *SCO2244*, *SCO3102*, *SCO3821*, *SCO3848*, *SCO4507*, *SCO7240*), or pairs of adjacent genes (*SCO3820* and *SCO3821*, *SCO4487* and *SCO4488*), or five adjacent genes (*SCO4775*–*SCO4779*) with an apramycin-resistance cassette (*apr*) deriving from pIJ773, using the PCR-targeting method of Gust et al. (1). The double and triple mutants corresponding to the three PASTA domain-containing STK genes (*SCO2110*, *SCO3821*, and *SCO3848*) were built up by converting *apr*-marked mutations into in-frame deletions as described by Gust et al. (1), and then reusing the *apr* cassette to replace the next gene. All STK mutant strains were verified by PCR and by Southern blot hybridization.

Construction of Plasmids. Phusion DNA polymerase (Finnzymes) was used in PCR for construction of plasmids, and the inserts of constructed plasmids were confirmed by DNA sequencing. Oligonucleotide primers are listed in Table S2.

For complementation of the *afsK* mutant, *afsK* and the entire 217-bp intergenic region upstream of *afsK*, including the mapped transcription start site (2), was amplified by using the primers KF549, which introduced an SpeI restriction site, and KF547, allowing the amplified fragment to be digested and ligated into the EcoRV-SpeI-cut pMS82. The resulting plasmid, pKF256, was introduced into *S. coelicolor* strains by conjugation and integrated into the chromosome at the ΦBT1 attachment site.

To fuse AfsK to a fluorescent protein, the *afsK* gene, including the promoter region, was amplified using the primers KF547 and KF548, which introduced BamHI and NdeI restriction sites, and replaced the stop codon with four glycine codons. This PCR product was ligated into BamHI-NdeI-cut pKF210 resulting in an in-frame fusion of *afsK* with *mCherry* connected by a tetraglycine linker. The resulting plasmid, pKF255, was introduced into *S. coelicolor* strains by conjugation and integrated into the chromosome at the ΦC31 attachment site.

To create an *afsK* allele that would encode a constitutively active AfsK, site-directed mutagenesis was performed by using primers KF658 and KF659 and pIJ10551 as the template. Briefly, the primers led to amplification of the entire plasmid as a linear fragment incorporating the desired mutations (T165D and T168D), which were built into primers KF658 and KF659, respectively. The primers were phosphorylated before the PCR, and the PCR product was purified and religated. To create an inducible construct, the *afsK*(T165D,T168D) allele was cut out from the resulting plasmid, and subcloned into NdeI-EcoRI-cut pIJ6902, placing the *afsK*(T165D,T168D) allele directly downstream of the thiostrepton-inducible promoter *tipAp* (3). The resulting plasmid, pKF275, was introduced into *S. coelicolor* strains by conjugation and integrated into the chromosome at the ΦC31 attachment site.

Analysis of DivIVA Phosphorylation by Immunoprecipitation and Pro-Q Diamond Staining. The appropriate *S. coelicolor* strains were grown in yeast extract-malt extract medium for 15 to 22 h. For expression of *FLAG-divIVA* from the thiostrepton-inducible promoter *tipAp*, strains were grown in the presence of 0.1 μg/mL of thiostrepton. Hyphae were harvested by centrifugation, washed twice in 10.3% (wt/vol) sucrose, and resuspended in appropriate buffer. Cell extracts were prepared in immunoprecipitation (IP) buffer (100 mM Tris-HCl, pH 8, 5% (vol/vol) glycerol, 50 mM sodium pyrophosphate, 1 mM sodium molybdate, 25 mM sodium fluoride, 25 mM glycerophosphate, 15 mM EGTA, 5 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 μM leupeptin, 1 nM calyculin A, 1 mM sodium orthovanadate). Cell lysates were prepared by sonication or by bead beating. After lysis, Triton X-100 was added to a final concentration of 1% (vol/vol) and cell lysates were cleared by centrifugation (16,000 × *g* for 30 min at 4 °C) and, when appropriate, subsequent ultracentrifugation (100,000 × *g* for 1 h at 4 °C). Protein concentrations were determined by using a Bio-Rad DC Kit.

The cleared cell lysates were used for IP essentially as described by Wang et al. (4). Briefly, pre-equilibrated anti-FLAG M2 affinity beads (Sigma-Aldrich) were mixed with equal amounts of total cell extracts and incubated from 1 h to overnight at 4 °C with gentle shaking. After three washes with IP buffer containing 1 M NaCl and then two with IP buffer containing 1 mM PMSF, bound proteins were eluted by boiling for 3 min in 2× elution buffer (125 mM Tris-HCl, pH 8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.004% bromophenol blue). When appropriate, samples were dephosphorylated for 10 min at 30 °C by using lambda protein phosphatase (Sigma-Aldrich).

Eluted proteins were separated by SDS-PAGE, and phosphorylated proteins were detected by using Pro-Q Diamond phosphoprotein gel stain (Molecular Probes). Gels were fixed twice in 50% (vol/vol) methanol/10% (vol/vol) acetic acid for 30 min and then washed three times in ultrapure water for 10 min. Gels were stained for 60 to 90 min in the dark, then destained three times in 20% (vol/vol) acetonitrile/50 mM sodium acetate, pH 4.5, for 30 min before washing in ultrapure water. Phosphorylated species were visualized by using a Typhoon 9410 Scanner (GE Healthcare) or FLA-7000 system (Fujifilm) in fluorescence mode. Subsequently, the gels were also stained with Coomassie brilliant blue.

MS. Cell extraction and IP were performed as described earlier, except that Tris-buffered saline solution (25 mM Tris-HCl, pH 8, 150 mM NaCl) supplemented with complete EDTA-free protease inhibitor mixture (Roche) was used as the buffer, and bound proteins were eluted from the M2 beads by competition with 150 ng/mL 3× FLAG peptide (Sigma-Aldrich) in Tris-buffered saline solution containing 1% (vol/vol) Triton X-100 for 1 h at 4 °C with gentle rotation. The immunoprecipitated DivIVA was digested for 10 min at 37 °C in a vortex shaker by using magnetic trypsin beads (Clontech). Without desalting or other concentration steps, the resulting digest was mixed 1:1 with a saturated matrix solution of sinapinic acid (Fluka) in 30% (vol/vol) acetonitrile, 0.1% TFA, and 1 μL was spotted onto a polished stainless steel MALDI target and air dried. A portion of digest was also dephosphorylated for 1 h at 37 °C by using glycerol-free calf intestinal alkaline phosphatase (NEB) and analyzed similarly. Cocrystallized spots of matrix and sample were washed briefly (<5 s) on the MALDI target when necessary by using 10 mM ammonium phosphate, 0.1% TFA before analysis. Myoglobin was used for calibration.

MALDI-TOF MS was carried out on an UltraFlex MALDI-TOF/TOF mass spectrometer (Bruker) in linear positive ionization mode by using a 337-nm pulsed nitrogen laser with a 50-Hz repetition rate. The source voltage (IS1) was set to 25 kV, with IS2 at 23.4 kV, pulsed ion extraction delay at 80 ns, and deflection of ions <1,000. Linear detector voltage was 1.65 kV, and 800 shots were collected per spectrum.

Analysis of Hyphal Branching Patterns. As described in more detail in elsewhere (5), it is important when measuring tip-to-branch

distances to account for biases that might artificially skew the data. To do this, we introduced a protocol that ensures that all measured hyphae have effectively the same length of 80 μm. Hyphae shorter than 80 μm were discarded, and those longer than 80 μm were trimmed so that only the 80 μm nearest the tip remained. As still images do not normally capture the exact instant at which a new branch emerges, it is necessary to infer the tip-to-branch distance at the moment of branching (failure to do so will result in biased tip-to-branch distances). Measurements from time-lapse microscopy have shown that an established tip extends at an approximately constant velocity (i.e., V_{max}) of 8 ± 4 μm/h. In contrast, newly developing branches initially extend at a v_0 of 4 ± 2 μm/h and then gradually increase in speed until they reach V_{max} after a time (T) of approximately 90 min. By using these values, we can infer, for each measured branch, a distribution for the tip-to-branch distance at the moment of branching. We do this by allowing each of V_0, V_{max}, and T to fluctuate independently according to Gaussian distributions (which are truncated to ensure $0 < V_0 < V_{max}$ and $T > 0$). For each measured branch, we randomly chose many sets (V_0, V_{max}, T), each one leading to a tip-to-branch distance (unphysical negative distances are discarded), which in turn leads to a tip-to-branch distribution for that single branch measurement. Finally, the complete measured tip-to-branch distribution is obtained by summing the normalized distributions of all individual branch measurements.

1. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* 100:1541–1546.
2. Lee PC, Umeyama T, Horinouchi S (2002) *afsS* is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2). *Mol Microbiol* 43:1413–1430.
3. Huang J, et al. (2005) Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. *Mol Microbiol* 58:1276–1287.
4. Wang SB, et al. (2009) Domains involved in the *in vivo* function and oligomerization of apical growth determinant DivIVA in *Streptomyces coelicolor*. *FEMS Microbiol Lett* 297:101–109.
5. Richards DM, Hempel AM, Flärdh K, Buttner MJ, Howard M (2012) Mechanistic basis of branch-site selection in filamentous bacteria. *PLOS Comput Biol* 8:e1002423.

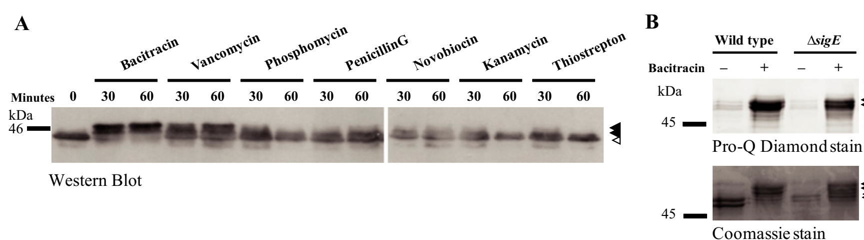

Fig. S1. (*A*) The phosphorylation state of DivIVA indicated by mobility shift was analyzed by Western blotting upon treatment with different antibiotics. Growing cultures of *S. coelicolor* WT strain were incubated for 30 min with 50 μg/mL bacitracin, 50 μg/mL vancomycin, 600 μg/mL phosphomycin, 200 μg/mL penicillin G, 25 μg/mL novobiocin, 150 μg/mL kanamycin, and 10 μg/mL thiostrepton before harvest and cell extract preparation. Closed arrowheads indicate phosphorylated and open arrowheads indicate normal DivIVA. (*B*) The phosphorylation state of DivIVA, before and after bacitracin treatment, was analyzed in a *sigE* mutant lacking RNA polymerase sigma factor σ^E. Growing hyphae of WT and mutant strain expressing *FLAG-divIVA* were incubated with 50 μg/mL bacitracin for 30 min before harvest, preparation of cell extracts, and IP. Closed arrowheads indicate phosphorylated and open arrowheads indicate normal DivIVA.

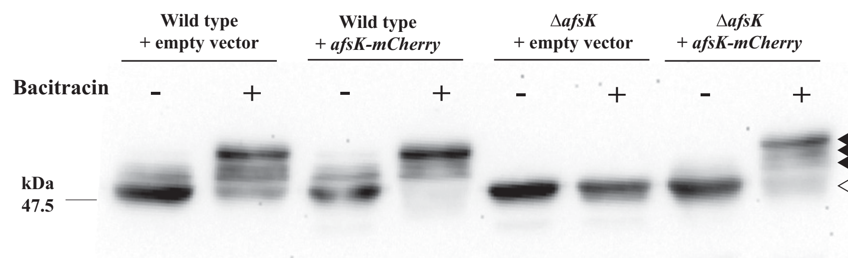

Fig. S2. Complementation test shows functionality of AfsK-mCherry. The ability of the AfsK-mCherry hybrid protein, encoded from plasmid pKF255, to restore phosphorylation of DivIVA to an *afsK* mutant strain was analyzed by Western blotting. Phosphorylation status of DivIVA is indicated by mobility shift, with unphosphorylated species indicated by an open arrowhead, whereas the more slowly migrating phosphorylated DivIVA species are indicated by closed arrowheads. Growing cultures of *S. coelicolor* were incubated for 30 min with or without 50 μg/mL bacitracin: WT strain carrying empty vector (strain M600 pKF210), WT strain expressing AfsK-mCherry (strain K326), *afsK* mutant carrying empty vector (strain M1101 pKF210), and *afsK* mutant expressing AfsK-mCherry (strain K327).

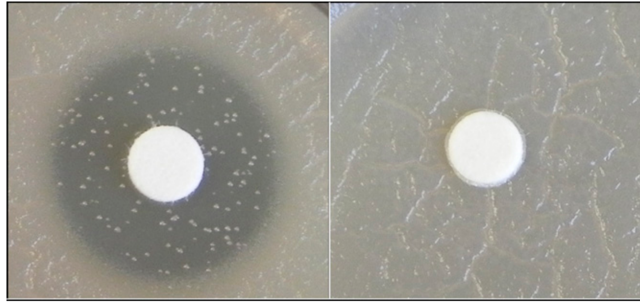

Fig. S3. Inhibition of growth caused by induced expression of the *afsK*(T165D, T168D) allele. Spores of two bacterial strains were spread evenly on TSB agar plates containing apramycin to maintain selection for the integrated plasmids. The strains were derivatives of *S. coelicolor* strain M600 carrying plasmids integrated at the ϕC31 *attB* site with the thiostrepton-inducible promoter driving expression of constitutively active AfsK (stain K335) or a control strain with empty vector (strain K336). A sterile paper disk was soaked with 15 μL of 0.1 mg/mL thiostrepton dissolved in DMSO. Plates were incubated at 30 °C for 2 d and then photographed. The clearing zone around the disk (*Left*) demonstrates that induced expression of the constitutively active AfsK kinase inhibits growth.

Table S1. Bacterial strains and plasmids

Strain/plasmid	Relevant genotype	Source
S. coelicolor A3 (1)		
M145	Prototrophic, SCP1^{-}SCP2^{-}	(2)
M600	Prototrophic, SCP1^{-}SCP2^{-}	(2)
M1101	M600 *ΔafsK::apr*	Present study
M1103	M600 *ΔSCO1468::apr*	Present study
M1104	M600 *ΔSCO2244::apr*	Present study
M1105	M600 ΔSCO*3102::apr*	Present study
M1106	M600 *ΔSCO3820-3821::apr*	Present study
M1107	M600 *ΔSCO4487-4488::apr*	Present study
M1108	M600 *ΔSCO4507::apr*	Present study
M1109	M600 *ΔSCO4775-4779::apr*	Present study
M1111	M600 *ΔSCO7240::apr*	Present study
J3376	M600 *ΔSCO2110* (inframe)	Present study
J3377	M600 *ΔSCO3821* (inframe)	Present study
J3378	M600 *ΔSCO3848* (inframe)	Present study
J3379	M600 *ΔSCO2110* (inframe) *ΔSCO3821* (inframe)	Present study
J3381	M600 *ΔSCO3821* (inframe) *ΔSCO3848* (inframe)	Present study
J3382	M600 *ΔSCO2110* (inframe) *ΔSCO3848* (inframe)	Present study
J3385	M600 *ΔSCO3821* (inframe) *ΔSCO3848* (inframe) *ΔSCO2110::apr*	Present study
K128	M600 $attB_{pSAM2}$::pKF67[*tipAp-FLAG-divIVA*]	Present study
K120	M145 $attB_{pSAM2}$::pKF67[*tipAp-FLAG-divIVA*]	(1)
K324	M600 *ΔafsK::apr* $attB_{\varphi BT1}$::pKF252[*divIVA-egfp*]	Present study
K325	M600 $attB_{\varphi BT1}$::pKF252[*divIVA-egfp*]	Present study
K326	M600 $attB_{\varphi C31}$::pKF255[*afsK-mCherry*]	Present study
K327	M600 *ΔafsK::apr* $attB_{\varphi C31}$::pKF255[*afsK-mCherry*]	Present study
K330	M600 $attB_{\varphi BT1}$::pKF252[*divIVA-egfp*] $attB_{\varphi C31}$::pKF255[*afsK-mCherry*]	Present study
K335	M600 $attB_{\varphi C31}$::pKF275[*tipAp-afsK*(T165D, T168D)]	Present study
K336	M600 $attB_{\varphi C31}$::pIJ6902[*tipAp*]	Present study
K338	M600 $attB_{\varphi BT1}$::pKF252[*divIVA-egfp*] $attB_{\varphi C31}$::pKF275[*tipAp-afsK*(T165D, T168D)]	Present study
K339	M600 $attB_{\varphi BT1}$::pKF252[*divIVA-egfp*] $attB_{\varphi C31}$::pIJ6902 [*tipAp*]	Present study
E. coli		
DH5α	Cloning strain	Laboratory stock
ET12567/pUZ8002	*dam-13::*Tn*9 dcm-6 hsdM*, carries RK2 derivative with defective *oriT* for plasmid mobilization, Kan^{r}	(2)
pGEX(M)_AfsK	*afsK* (1-331 kinase domain) amplified with VM712 and VM739, digested and cloned with BamHI and HindIII into pGEX(M)	Present study
pGEX(M)_DivIVA	*divIVA* amplified with VM748 and VM749, digested and cloned with BamHI and HindIII into pGEX(M)	Present study
pIJ773	Source of the *FRT-aac(3)IV-oriT-FRT* cassette, here referred to as *apr*	(3)
pIJ6902	Mobilizable vector that integrates at φC31 *attB* in *S. coelicolor*, carries thiostrepton-inducible promoter *tipAp*, Thio^{r} Apra^{r}	(4)
pIJ10551	*afsK* amplified with phosphorylated primers afsK fwd and afsK rev, which introduced NdeI and HindIII restriction sites, cloned in SmaI site of pUC19	Present study
pKF210	Mobilizable vector that integrates at φC31 *attB* in *S. coelicolor*, carries promoterless *mCherry* gene, Thio^{r} Apra^{r}	K.F.
pKF59	Plasmid carrying *divIVA-egfp* fusion	(5)
pKF252	*divIVA-egfp*, excised from pKF59 with XbaI and NsiI and cloned into AvrII-NsiI-cut pMS82	Present study
pKF255	*afsK* amplified with KF547 and KF548, digested and cloned with BamHI and NdeI into pKF210 to create an *afsK-mCherry* fusion	Present study
pKF256	*afsK* cloned in pMS82	Present study
pKF275	*afsK* (T165D, T168D) allele cloned into pIJ6902 under control of *tipAp*	Present study
pMS82	Mobilizable vector that integrates at φBT1 *attB* in *S. coelicolor*, Hyg^{r}	(6)
pSET152	Mobilizable vector that integrates at φC31 *attB* in *S. coelicolor*, Apra^{r}	(7)

1. Wang SB, et al. (2009) Domains involved in the *in vivo* function and oligomerization of apical growth determinant DivIVA in *Streptomyces coelicolor*. *FEMS Microbiol Lett* 297:101–109.
2. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics* (John Innes Foundation, Norwich, UK).
3. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* 100:1541–1546.
4. Huang J, et al. (2005) Cross-regulation among disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. *Mol Microbiol* 58:1276–1287.
5. Flärdh K (2003) Essential role of DivIVA in polar growth and morphogenesis in *Streptomyces coelicolor* A3(2). *Mol Microbiol* 49:1523–1536.
6. Gregory MA, Till R, Smith MC (2003) Integration site for *Streptomyces* phage phiBT1 and development of site-specific integrating vectors. *J Bacteriol* 185:5320–5323.
7. Bierman M, et al. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* 116:43–49.

Table S2. Oligonucleotide primers used in this study

Primer	Sequence*	Introduced restriction site
afsK		
Forward	AAAAACATatggtggatcagctgacg	NdeI
Reverse	TTTTTAAGCTTtcacgtcgtacgggc	HindIII
KF547	CTGGTTAACCGGATCCcccggcggccacggcgccgac	BamHI
KF548	GGAATTCCATAT**GCCGCCGCCGCCG**cgtcgtacgggcggtccccgt†	NdeI
KF549	TGGTTAACCACTAGttcacgtcgtacgggcggtccccgt	SpeI
KF658	CTGGACATGACGAACGTCGCCG‡	—
KF659	ACGGTCGTTCGAGACGCCGG‡	—
VM712	TATGGATCCgtggtggatcagctgacgcagcac	BamHI
VM739	TATAAGCTTtcagcggccgccggccgtggtggc	HindIII
VM748	TATGGATCCatgccgttgacccccgaggac	BamHI
VM749	TATAAGCTTtcagttgtcgtcctcgtcgat	HindIII

*Sequence matching the template is shown in lowercase, whereas added or changed nucleotides in the 5′-ends of oligonucleotides that did not correspond to the template are shown in capitals. Added restriction sites are underlined.

†In bold is a sequence added to incorporate a four-glycine linker region in the recombinant AfsK-mCherry.

‡The specific point mutations that were introduced are underlined in the primer sequences. They gave rise to the *afsK* mutations T168D and T165D, respectively.

Movie S1. Time-lapse imaging of *S. coelicolor afsK*$^{+}$ strain K325. Hyphae were grown on agarose pads, and images were captured every 6 min as described previously (1). Fluorescence signals from DivIVA-EGFP are shown overlaid on the phase-contrast images. (Scale bar, 12 µm.)

Movie S1

1. Hempel AM, Wang SB, Letek M, Gil JA, Flärdh K (2008) Assemblies of DivIVA mark sites for hyphal branching and can establish new zones of cell wall growth in *Streptomyces coelicolor*. *J Bacteriol* 190:7579–7583.

Movie S2. Time-lapse imaging of *S. coelicolor afsK* mutant strain K324. Hyphae were grown on agarose pads, and images were captured every 6 min as described previously (1). Fluorescence signals from DivIVA-EGFP are shown overlaid on the phase-contrast images. (Scale bar, 12 μm.)

Movie S2

1. Hempel AM, Wang SB, Letek M, Gil JA, Flärdh K (2008) Assemblies of DivIVA mark sites for hyphal branching and can establish new zones of cell wall growth in *Streptomyces coelicolor*. *J Bacteriol* 190:7579–7583.