

1 **Title**

2 Cytoplasmic contractile injection systems mediate cell death in *Streptomyces*

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15

16 **Abstract**

17 Contractile injection systems (CISs) are bacteriophage tail-like structures that mediate bacterial
18 cell-cell interactions. While CISs are highly abundant across diverse bacterial phyla,
19 representative gene clusters in Gram-positive organisms remain poorly studied.

20 Here we characterize a CIS in the Gram-positive multicellular model organism *Streptomyces*
21 *coelicolor* and show, that in contrast to most other CISs, *S. coelicolor* CIS (CIS^{Sc}) mediate cell
22 death in response to stress and impact cellular development. CIS^{Sc} are expressed in the
23 cytoplasm of vegetative hyphae and not released into the medium. Our cryo-electron
24 microscopy structure enabled the engineering of non-contractile and fluorescently tagged CIS^{Sc}
25 assemblies. Cryo-electron tomography showed that CIS^{Sc} contraction is linked to reduced
26 cellular integrity. Fluorescence light microscopy furthermore revealed that CIS^{Sc} contraction
27 mediates cell death upon encountering different types of stress. Finally, the absence of
28 functional CIS^{Sc} had an impact on hyphal differentiation and secondary metabolite production.

29 Our results provide new functional insights into CISs in Gram-positive organisms and a
30 framework for studying novel intracellular roles, including regulated cell death and life cycle
31 progression in multicellular bacteria.

32 Introduction

33 Bacteria exist in highly competitive environments that require them to interact with a range of
34 organisms. To respond to potential stressors, bacteria have evolved complex strategies to
35 mediate potential antagonistic interactions¹. One such response is the deployment of cell-
36 puncturing nanodevices called bacterial contractile injection systems (CIS), which are large
37 macromolecular protein machines that can translocate cytotoxic effectors into the extracellular
38 space or directly into target cells^{2,3,4,5}. In general, CIS are composed of a contractile sheath that
39 encloses an inner tube loaded with effectors, which is fitted with a baseplate complex that
40 facilitates attachment to the membrane. A conformational change in the baseplate complex
41 triggers the contraction of the outer sheath, which leads to the propulsion of the inner tube into
42 the target.

43 Phylogenetic analyses have indicated that these CISs are conserved across diverse microbial
44 phyla including Gram-negative and Gram-positive bacteria, as well as archaea^{6,7}. CIS are
45 commonly classified as Type VI secretion systems (T6SS) or extracellular CIS (eCIS), based
46 on their modes of action. Anchored at the host's cytoplasmic membrane, T6SSs function by a
47 cell-cell contact-dependent mechanism, wherein the T6SS injects effectors directly into a
48 neighboring cell^{8,9,10,11,12}. By contrast, eCIS are assembled in the bacterial cytoplasm of the
49 donor cell and are subsequently released into the extracellular space, where they can bind to
50 the surface of a target cell, contract and puncture the cell envelope¹³. Recently, a third mode of
51 action was described in multicellular *Cyanobacteria*¹⁴. This system is also assembled in the
52 bacterial cytoplasm and it then attaches to the thylakoid membrane where it potentially induces
53 lysis of the cell upon stress, resulting in the formation of “ghost cells” which may in turn
54 proceed to interact with other organisms¹⁴.

55 Of the hundreds of putative CIS gene clusters detected in sequenced bacteria, all well
56 characterized examples have come from two closely related clades and have been exclusively
57 examined in Gram-negative bacteria. Characterized CIS representatives include
58 “metamorphosis-associated contractile structures” (MACs) from *Pseudoalteromonas*
59 *luteoviolacea*¹⁵, the “T6SS subtype *iv*” (T6SS^{*iv*}) in *Candidatus Amoebophilus asiaticus*¹⁶,
60 “antifeeding prophages” (AFPs) from *Serratia*¹⁷, “Photorhabdus Virulence Cassettes” (PVCs)
61 from *P. asymbiotica*¹⁸, and two newly characterized CIS from the marine bacteria
62 *Algoriphagus machipongonensis*¹⁹ and *Cyanobacteria*¹⁴.

63 Strikingly, 94 of 116 sequenced Gram-positive actinomycetes of the genus *Streptomyces* were
64 shown to encode a potential CIS gene cluster^{6,7}. A previous report suggested that CIS from *S.*
65 *lividans* were involved in microbial competition, however, the mechanism remains unknown²⁰.
66 *Streptomyces species* are abundant soil bacteria and renowned for their complex developmental
67 life cycle and their ability to produce an array of clinically relevant secondary metabolites²¹.
68 The *Streptomyces* life cycle begins with the germination of a spore and the generation of germ
69 tubes which grow by apical tip extension and hyphal branching to form a dense vegetative
70 mycelium. Upon nutrient depletion, non-branching aerial hyphae are erected, which eventually
71 synchronously divide into chains of uni-nucleoid spores²². Notably, the production of these
72 important molecules is tightly coordinated with the developmental life cycle²¹.

73 Here, we provide evidence that CISs from the model organism *Streptomyces coelicolor* (CIS^{Sc})
74 function intracellularly and belong to a new class of contractile injection systems that exist as
75 free-floating, fully assembled particles in the cytoplasm and mediate cell death in response to
76 stress conditions. Additionally, we found that the absence of CISs affects the coordinated
77 cellular development and secondary metabolite production of *S. coelicolor*, indicating a wider
78 role of CIS from *Streptomyces* in the multicellular biology of these important bacteria.

79

80 **Results and Discussion**

81 ***Streptomyces* express cytoplasmic CIS during vegetative growth**

82 Previous bioinformatic studies revealed that the majority of sequenced *Streptomyces* genomes
83 harbor a highly conserved cluster of eCIS genes related to the poorly studied CIS IId subtype^{6,7}.
84 This was further confirmed by our phylogenetic analyses using sheath protein sequences from
85 known producers of CIS and from two representative *Streptomyces* species, namely *S.*
86 *coelicolor* and *S. venezuelae* (Fig. 1a).

87 Closer inspection of the *Streptomyces* CIS gene clusters from *S. coelicolor* (*sco4244-Sco4260*)
88 and *S. venezuelae* (*vnz_28875-vnz_28935*) suggested that both species encode 10 and 11 core
89 structural components of the phage-tail-like systems, respectively^{6,7} (Fig. 1b/c). Based on this
90 sequence similarity, we renamed the genes from *Streptomyces* to *cis1-16*. Both CIS gene
91 clusters encode two inner tube homologs (*cis1a* and *cis1b*) as well as additional proteins of
92 unknown function. Cis10, a PAAR-repeat containing protein, is only present in *S. venezuelae*.
93 Genes encoding a tail fiber protein (Afp13), which mediates eCIS binding to target cells, and
94 a tail measure protein (Afp14), involved in regulating the length of eCIS particles¹⁷, are absent
95 in both CIS gene clusters.

96 To test whether *S. coelicolor* and *S. venezuelae* produced CIS, we purified sheath particles from
97 crude cell lysates, followed by negative stain electron microscopy (EM) imaging. We observed
98 typical contracted sheath-like particles in crude extracts from wild-type (WT) *S. coelicolor* and
99 *S. venezuelae*, while no such assemblies were seen in strains carrying a deletion in *cis2* (Δ CIS,
100 Fig. 1d). Subsequent mass spectrometry analysis of the purified particles detected peptides
101 from Cis1a (inner tube) and Cis2 (sheath) (Extended Data Table 1), confirming that the CIS
102 gene clusters from *Streptomyces* encode CIS-like complexes. We noticed that *S. coelicolor*
103 produced approximately 50 times more sheath particles compared to *S. venezuelae* (Extended
104 Data Fig. 1a/b). Therefore, we focused on the characterization of CIS from *S. coelicolor* (CIS^{Sc})
105 in subsequent experiments.

106 To test if CIS^{Sc} displayed a mode of action similar to canonical eCIS, we investigated whether
107 CIS^{Sc} were released from cells into the extracellular space. Using automated Western blotting,
108 we analyzed the culture supernatant and whole cell extracts from WT and Δ CIS *S. coelicolor*
109 cells that were grown for 48 h in liquid medium. Interestingly, we detected the two key CIS^{Sc}
110 components Cis1a (inner tube) and Cis2 (sheath) only in whole cell lysates but not in the

111 supernatant of cultures of the WT or the complemented Δ CIS mutant (Fig. 1e). SDS-PAGE
112 analysis of concentrated culture supernatants further confirmed that all tested samples
113 contained protein (Extended Data Fig. 1c). These findings suggest that the entire CIS^{Sc}
114 assembly is retained in the cytoplasm, unlike typical T6SS (inner tube protein translocated into
115 the medium) and unlike eCIS (full assemblies released into the medium)^{8,19}. Next, to visualize
116 the localization of CIS^{Sc} *in situ*, we imaged hyphae of *S. coelicolor* and *S. venezuelae* by cryo-
117 electron tomography (cryoET). While intact *S. coelicolor* hyphae could be imaged directly, *S.*
118 *venezuelae* was too thick and had to be thinned by cryo-focused ion beam (FIB) milling prior
119 to imaging. We predominantly found extended CIS that appeared to be free-floating in the
120 cytoplasm, a behavior that is inconsistent with a T6SS mode of action (Fig. 1f/g). Taken
121 together, these results indicate that CIS from *Streptomyces* may play a role in intracellular
122 processes, which would be distinct from the previously described functions for T6SS and eCIS.

123

124 **Structure, engineering and subcellular localization of CIS^{Sc}**

125 To obtain insights into the structural details of the CIS^{Sc} contractile sheath-tube module, we
126 performed single particle cryoEM (helical reconstruction) of purified sheath particles from WT
127 *S. coelicolor*, which had a homogeneous length of ~140 nm (Fig. 2a-c). The resulting map of
128 the contracted sheath reached a resolution of 3.6 Å (Extended Data Fig. 2a/b). Contracted
129 sheath proteins adopt a right-handed helical array with an inner diameter of 115 Å and an outer
130 diameter of 233 Å (Fig. 2b). Similar to the recently described sheath structures observed in
131 AlgoCIS¹⁹ and tCIS¹⁴, the CIS^{Sc} sheath is comprised of only one protein (Cis2). Cis2 monomers
132 consist of three domains and are well conserved in *S. coelicolor* and *S. venezuelae*, sharing
133 ~65% sequence identity (Extended Data Fig. 2c). From the resulting map, it was possible to
134 build *de novo* domains 1 and 2, which contribute to the sheath wall (Fig. 2c). The additional
135 domain 3, which is located on the sheath surface, seems to be highly flexible. The overall
136 contracted structure of Cis2 is similar to sheaths of previously characterized systems^{23,24,18}.

137 In order to be able to purify the extended form of the CIS^{Sc} sheath tube module from *S.*
138 *coelicolor* cell lysates, we set out to engineer non-contractile CIS^{Sc} based on the information
139 from the contracted Cis2 structure and based on similar previous approaches in *V. cholerae*²⁵
140 and enteroaggregative *E. coli*²⁶ (Extended Data Fig. 3a). Different sets of two (IE), three (IEG)
141 and five (IEGVG) amino acid residues were inserted into the N-terminal linker of Cis2 after
142 position G25, resulting in the mutants CIS-N2, CIS-N3, and CIS-N5, respectively. For the CIS-

143 N2 and CIS-N3 mutants, less than 30% and 50% were found in extended form, respectively
144 (Extended Data Fig. 3b/c). For the CIS-N5 non-contractile mutant, more than 95% of the
145 complexes were seen in the extended conformation (Extended Data Fig. 3d). *In vitro*, the length
146 of the CIS-N5 non-contractile mutant was homogeneous at ~230 nm (Fig. 2d). Moreover, mass
147 spectrometry analyses confirmed the presence of most CIS^{Sc} components, indicating the
148 stability of the complex (Fig. 1b, Extended Data Table 1).

149 Next, we optimized the purification of CIS-N5 and performed cryoEM. Helical reconstruction
150 was used to generate an EM map, which we then used to build *de novo* the sheath-tube (Cis2-
151 Cis1a) module in extended conformation at 3.9 Å resolution (Extended Data Fig. 3e/f). Domain
152 3 of the extended sheath (Cis2) was again too flexible to be resolved. The tube (Cis1a) structure
153 and fold are highly similar to the tube structures already described for other CISs (Fig. 2f,
154 Extended Data Fig. 3g). The comparison of the sheath (domains 1/2) in the extended vs. the
155 contracted states revealed an increase in diameter and shortening of the length upon
156 contraction, similar to other CISs (Fig. 2b/e)^{14,19, 24,18,27}.

157 Guided by the high-resolution structure of the sheath module (Fig. 2a-f), we engineered a
158 fluorescently tagged CIS^{Sc} by inserting YPet at position I274 in the Cis2 monomer.
159 Subsequently, we used this Cis2-YPet sandwich fusion to complement the *S. coelicolor* $\Delta cis2$
160 mutant *in trans* (Extended Data Fig. 4a). Using negative stain EM and cryoET, we confirmed
161 that YPet-tagged CIS^{Sc} were able to assemble into extended particles and to contract,
162 suggesting that these fluorescently labeled CIS^{Sc} particles were functional (Extended Data Fig.
163 4b/c). This enabled us to visualize the subcellular localization of CIS^{Sc} in vegetatively growing
164 hyphae using time-lapse fluorescence light microscopy (fLM). Multiple CIS^{Sc}-YPet foci were
165 found inside the hyphae but not in extracellular space. The foci were largely static or displayed
166 short-range movements within the hyphae (Fig. 2g/h and Extended Data Movie 1). CIS^{Sc}-YPet
167 foci were stable over the course of the experiment and did not reveal significant changes in the
168 shape or intensity of the fluorescence. While this indicates the absence of firing events during
169 the experiment, the resolution in fLM and the relatively short length of the CIS^{Sc} may hamper
170 the detection of firing events (in contrast to the much longer T6SSs^{8,28}).

171 Taken together, our structural data allowed us to engineer non-contractile and fluorescently
172 tagged CIS^{Sc}, which revealed the presence of scattered CIS^{Sc} in *S. coelicolor* hyphae.

173

174

175 **CIS^{Sc} contraction state correlates with the integrity of the cell membrane**

176 Our initial cryoET data of *S. coelicolor* cells indicated that contracted CIS^{Sc} were frequently
177 found in hyphae that displayed a damaged cell membrane. To explore this correlation further,
178 we first acquired low magnification two-dimensional (2D) cryoEM images. Based on the
179 contrast of individual hyphae in these 2D images (Fig. 3a), we classified the hyphae into three
180 distinct groups: (1) ‘intact hyphae’ (dark appearance in 2D) with mostly intact cytoplasmic
181 membrane and occasional vesicular membranous assemblies that are reminiscent of “cross-
182 membranes”²⁹ (Fig. 3b); (2) ‘partially lysed hyphae’ with a mostly disrupted/vesiculated
183 cytoplasmic membrane (reduced contrast in 2D), indicative of cytoplasmic leakage (Fig. 3c);
184 and (3) membrane-less ‘ghost cells’ (lysed hyphae; hardly visible in 2D) that only consisted of
185 the peptidoglycan cell wall (Fig. 3e). Representative hyphae of each group (n=90) were imaged
186 by cryoET (270 tomograms in total, n=3 experiments) and the conformational state and *in situ*
187 localization of the CIS^{Sc} was determined (Fig 3b-g). In addition, we performed 3D volume
188 segmentation of selected full tomograms.

189 As observed before for intact hyphae (Fig. 1f), individual CIS^{Sc} particles were always found in
190 the extended conformation and localized in the cytoplasm (Fig. 3b). By contrast, in partially
191 lysed hyphae (Fig. 3c), the ratio of extended to contracted CIS^{Sc} was 2:1. CIS^{Sc} particles often
192 appeared to cluster in the vicinity of membranous structures (Fig. 3d). Notably, we found that
193 in some cases, the extended CIS^{Sc} aligned perpendicular to membrane patches or vesicles with
194 the baseplate complex facing the membrane, indicating that CIS^{Sc} may interact with the
195 cytoplasmic membrane (Fig. 3c/d). In contrast, ghost cells only displayed CIS^{Sc} particles in the
196 contracted state and which were often clustered (Fig. 3f).

197 Collectively, these results indicated that the conformational state of CIS^{Sc} correlates with the
198 integrity of the cell and that CIS^{Sc} may play an intracellular role as a consequence of cellular
199 stress and either directly or indirectly lead to cell death. Hence, we hypothesized that such
200 stress conditions could result in the recruitment of CIS^{Sc} to the membrane and trigger firing.

201

202 **CIS^{Sc} contraction mediates cell death under stress conditions**

203 To test this hypothesis, we explored whether upon encountering stress, the presence of CIS^{Sc}
204 and their contraction could mediate cell death. To generate a marker for cell viability, we
205 inserted *sfgfp* under the control of a constitutive promoter *in trans* in *S. coelicolor* WT, in the
206 Δ CIS null mutant, and in the non-contractile mutant (CIS-N5). In order to label intact and

207 partially lysed hyphae, cells were incubated with the fluorescent membrane dye FM5-95. We
208 first used correlated cryo-light and electron microscopy (CLEM) to confirm that the detected
209 cytoplasmic and membrane fluorescence correlated with the physiological state of the hyphae
210 (Extended Data Fig. 5). To assess the level of cell death in the imaged strains, we used fLM
211 and quantified the ratio of sfGFP signal (indicator of viable hyphae) to FM5-95 signal
212 (indicator of intact and lysed hyphae) in the different strains. Cells were grown for 48 h in
213 liquid, a time-point at which CIS^{Sc} can be detected in hyphae (Fig. 1d/e).

214 During non-stress conditions, the WT, the Δ CIS and the CIS-N5 mutant strains displayed a
215 similar sfGFP/FM5-95 ratio, indicating that none of the strains showed a significant difference
216 in viability (Fig. 4a/c). In parallel, we challenged the same *S. coelicolor* strains with a sub-
217 lethal concentration of the bacteriocin nisin (1 μ g/ml) for 90 min, which causes the formation
218 of membrane pores and eventually will lead to the disruption of cell envelope integrity³⁰. In
219 the WT, we found that ~50% of the analyzed hyphae displayed signs of cell death (Fig. 4b/d).
220 Strikingly, in the CIS-deficient strain and the non-contractile CIS^{Sc} mutant there was no
221 dramatic induction of cell death upon nisin treatment (Fig. 4b/d).

222 To investigate whether other stress factors could induce cell death, we repeated the experiments
223 and challenged *S. coelicolor* with the membrane depolarising agent CCCP (carbonyl cyanide
224 3-chlorophenylhydrazone) and with UV stress, to induce DNA damage (Extended Data Fig.
225 6a/b). In line with our previous results, the treatment of vegetative hyphae with CCCP or with
226 UV radiation both led to an increase in cell death by 25% in the WT but not in hyphae of the
227 Δ CIS or the CIS-N5 mutant strain (Fig. 4e/f). In parallel, we also purified CIS^{Sc} from crude
228 cell extracts obtained from non-stressed and stressed samples that were used for fLM imaging.
229 By negative-stain EM imaging, we confirmed the presence of CIS^{Sc} particles in hyphae of the
230 WT and in the CIS-N5 mutant strain, and the absence of sheath particles in the Δ CIS mutant
231 (Extended Data Fig. 7). The abundance of CIS^{Sc} in non-stressed and stressed samples was
232 comparable, which was also confirmed by the detection of Cis1a/2 proteins by Western blotting
233 analysis of non-stressed vs. nisin-treated hyphae (Extended Data Fig. 8a/b).

234

235 **CIS^{Sc} contribute to the multicellular development of *Streptomyces***

236 Earlier studies indicated that the expression of the *S. coelicolor* CIS gene cluster is coordinated
237 with the *Streptomyces* life cycle³¹. To follow the expression of CIS^{Sc} during the developmental

238 life cycle, we constructed a fluorescent reporter strain in which expression of *ypet* was driven
239 by the *cis2* promoter ($P_{cis2-ypet}$). Since *S. coelicolor* only completes its spore-to-spore life cycle
240 when grown on solid medium, glass coverslips were inserted at a 45-degree angle into agar
241 plates inoculated with spores. Coverslips with attached *S. coelicolor* hyphae were removed and
242 imaged every 24 h for four days by fLM. Fluorescent signal indicated that the *cis2* promoter
243 was primarily active in vegetative hyphae at the 48-h time point (Fig. 5a). In parallel, we
244 determined CIS^{Sc} protein levels in surface-grown WT *S. coelicolor* over the life cycle.
245 Consistent with our fluorescence reporter experiment, Cis1a/2 levels were highest in vegetative
246 mycelium that was harvested after 30 h and 48 h of incubation (Extended Data Fig. 9a). These
247 results are also in agreement with published transcriptomics data from *S. venezuelae*, showing
248 the specific induction of the CIS^{Sv} gene cluster during vegetative growth (Extended Data Fig.
249 S9b)³².

250 Since a previous study on *S. lividans* reported a putative role of CISs in inter-species
251 interactions²⁰, we performed a series of growth competition assays but did not observe any
252 obvious differences in fitness between the WT and CIS^{Sc}-mutants (Supplementary Table 2).
253 We therefore then tested whether the expression of functional CIS^{Sc} had an effect on the timely
254 progression of the *S. coelicolor* life cycle, using WT, Δ CIS, CIS-N5 and a complemented
255 strain. First, we detected sporulating hyphae and spores by imaging surface imprints of plate
256 grown (R2YE agar) colonies at different time points. All strains consistently completed their
257 life cycle and synthesized spores (Fig. 5b). Importantly, in contrast to the WT and the
258 complemented strain, both Δ CIS and CIS-N5 mutants sporulated markedly earlier (72 h vs. 96
259 h for the WT and the complemented mutant). These results were further corroborated by
260 quantifying the number of spores produced by the individual strains under the same
261 experimental conditions (Fig. 5c).

262 In addition to the accelerated cellular development in CIS^{Sc} mutants, we also noticed by the
263 appearance of the cultures from same strains grown in liquid R2YE, that production of the two
264 characteristic pigmented secondary metabolites in *S. coelicolor*, actinorhodin (blue)³³ and
265 undecylprodigiosin (red)³⁴, was significantly reduced, compared to the WT and the
266 complemented Δ CIS mutant (Extended Data Fig. 9c). This was further confirmed by a
267 quantification of the total amount of actinorhodin (intracellular and secreted) produced over a
268 period of 72 h. Both Δ CIS and CIS-N5 mutants produced approximately 70 % less actinorhodin
269 compared to the WT and the Δ CIS complementation strain (Δ CIS/CIS⁺) (Extended Data Fig.

270 9d). Moreover, in contrast to the observed delay in sporulation, the actinorhodin production in
271 the CIS^{Sc} mutants was not just delayed, but it never reached WT levels until the end of the
272 experiment.

273 Altogether, we showed that deleting or expressing non-functional CIS^{Sc} results in significant
274 changes in the *S. coelicolor* life cycle progression, which also affects secondary metabolite
275 production.

276

277 **Conclusions**

278 Here we show that CIS particles from *Streptomyces* are functionally distinct from related eCIS
279 and T6SS. Our data from fLM imaging, cryoET imaging and Western blotting all indicate
280 consistently that CIS^{Sc} were assembled free floating in the cytoplasm, however, under our
281 experimental conditions, they were not found to be released into the medium, nor were they
282 seen attached to the cytoplasmic membrane. This argues against a mode of action as a typical
283 eCIS. In addition the *Streptomyces* CIS gene cluster does not contain a typical tail fiber-like
284 protein for binding of a potential target cell. This also speaks against a typical T6SS mode of
285 action, since it is difficult to imagine how a CIS^{Sc} acting as a T6SS would fire through the thick
286 peptidoglycan cell wall in the Gram-positive host organism. Therefore, our data points to an
287 intracellular function, which is supported by further observations that are discussed below.

288 CryoET imaging revealed a significant fraction of partially or fully lysed cells in a vegetative
289 culture. Interestingly, the degree of cell lysis strongly correlated with the presence of contracted
290 CIS^{Sc} assemblies. fLM imaging, on the other hand, showed that under different types of stress
291 conditions, cell death was induced in a WT strain but significantly less in mutants that did not
292 express CIS^{Sc} or that expressed non-contractile CIS^{Sc}. Importantly, cell viability was not
293 compromised by these stress conditions in CIS-deficient or non-contractile mutant strains.
294 CIS^{Sc} contraction is therefore required for inducing cell death once a culture encounters stress.
295 We speculate that cell lysis could be achieved by membrane- or cell wall-targeting effectors
296 that are loaded into the CIS^{Sc} and released upon contraction. This could either happen upon
297 CIS^{Sc} binding to the cytoplasmic membrane followed by contraction, or by contraction of a free
298 floating CIS^{Sc} releasing effectors into the cytoplasm, which in both examples might trigger cell
299 death. A similar mode of action was recently proposed for thylakoid-anchored CISs in
300 multicellular cyanobacteria¹⁴.

301 In addition to mediating death of the host cell in response to stress, we showed that CIS^{Sc}
302 contraction also plays a role in the timely progression of the *Streptomyces* life cycle, evidenced
303 by the earlier onset of sporulation in CIS^{Sc} mutants. Cell death has been proposed as a distinct
304 process in the developmental programme of *Streptomyces*³⁵. However, the underlying
305 molecular mechanism has remained unclear. We speculate that contracting CIS^{Sc} could induce
306 hyphal cell death, which impacts the *Streptomyces* multicellular development. Notably,
307 increased cell death has been reported to occur at the center of colonies^{36,37}. These regions are
308 thought to be limited in nutrient and/or oxygen supply, which in turn may be perceived as stress
309 and trigger CIS^{Sc}-mediated cell death.

310 In addition, the morphological differentiation of *Streptomyces* colonies is tightly coordinated
311 with the production of secondary metabolites, which are often secreted into the environment
312 where they can provide a competitive advantage²¹. We showed that CIS^{Sc} mutants were not
313 only significantly affected in the timing of the onset of sporulation, but also in the production
314 of the secondary metabolite actinorhodin. We speculate that the delay of sporulation in the WT
315 (and the complemented strain) may be advantageous to allow the coordinated production and
316 release of key secondary metabolites such as toxins, proteases or signaling molecules. The lack
317 of functional CIS^{Sc} in both mutant strains could lead to improper timing of cell cycle
318 progression, resulting in early sporulation, which may in turn lead to lower amounts of
319 actinorhodin production.

320 In conclusion, our data provide new functional insights into CISs in a Gram-positive model
321 organism and a framework for studying new intracellular roles of CIS, including regulated cell
322 death and life cycle progression.

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- 472
- 473

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484

485 **Author contributions**

486 B.C., S.S. and M.P. conceived the project. B.C. conducted cryoFIB milling and cryoET; B.C.
487 optimized the sample preparation, collected and processed the cryoEM data, reconstructed the
488 cryoEM map, built and refined the structural models, performed correlative cryo-light and
489 electron microscopy, determined sporulation efficiency and actinorhodin production; B.C. and
490 S.S. conducted fluorescent light microscopy; S.S. generated *Streptomyces* strains; J.W.S. and
491 S.S. performed automated Western blot analyses; B.C., J.W.S, S.S. and M.P. wrote the
492 manuscript.

493

494 **Declaration of interest**

495 The authors declare no competing interests.

496

497 **METHODS**

498 **Bacterial strains, plasmids, and oligonucleotides.**

499 Bacterial strains, plasmids, and oligonucleotides can be found in Supplementary Tables 3-4. *E.*
500 *coli* strains were cultured in LB, SOB, or DNA medium. *E. coli* cloning strains TOP10 and
501 DH5 α were used to propagate plasmids and cosmids. *E. coli* strain BW25113/pIJ790 was used
502 for recombineering cosmids³⁸. For interspecies conjugation, plasmids were transformed into *E.*
503 *coli* ET12567/pUZ8002. Where necessary, media was supplemented with antibiotics at the
504 following concentrations: 100 μ g/ml carbenecillin, 50 μ g/ml apramycin, 50 μ g/ml kanamycin,
505 50 μ g/ml hygromycin.

506

507 *Streptomyces coelicolor* and *Streptomyces venezuelae* strains were cultivated in LB, MYM,
508 TSB, TSB-YEME, or R2YE liquid medium at 30 °C in baffled flasks or flasks with springs, at
509 250 rpm or grown on LB, MYM, SFM, R2YE medium solidified with 1.5% (w/v) Difco
510 agar³⁹. Where necessary, media was supplemented with antibiotics at the following
511 concentrations: 25 μ g/ml apramycin, 5 μ g/ml kanamycin, 25 μ g/ml hygromycin, 12.5-25
512 μ g/ml nalidix acid.

513

514 **Generation of *Streptomyces* mutant strains**

515 The λ RED homologous recombination system was used to isolate gene replacement mutations
516 using PCR-directed mutagenesis (ReDirect) of the *S. coelicolor* cosmid StD-49 and the *S.*
517 *venezuelae* cosmid P11-F14, containing the CIS gene cluster^{40,38}. Genes encoding the sheath
518 (*sco4253*, *vnz_28920*) or the whole CIS-sheath operon (*sco4253-SCO4251*, *vnz_28920-28910*)
519 were replaced with the *aac3(IV)-oriT* resistance cassette from pIJ773. Mutagenized cosmids
520 (pSS480, pSS481, pSS489, pSS490) were transformed and subsequently conjugated from *E.*
521 *coli* ET12567/pUZ8002 to wild-type *S. coelicolor* or *S. venezuelae*. Exconjugants that had
522 successfully undergone double-homologous recombination were identified by screening for
523 apramycin-resistance and kanamycin sensitivity. Deletion of the respective CIS mutant genotypes
524 were subsequently verified by PCR.

525

526 **Phylogenetic analysis**

527 The phylogenetic analysis of the different contractile injection systems (from eCIS, T6SS,
528 phage and CIS from *Streptomyces*) were examined using the putative sheath proteins.

529 Alignment and generation of the phylogenetic tree was performed as previously reported^{16,19}.
530 First, the amino acid sequences from 16 sheath proteins were aligned by the MUSCLE online
531 tool^{41,42}. Standard parameters were applied for multiple sequence alignment. Then, MEGAX
532 program⁴³ was used to reconstruct phylogenetic trees using the Maximum Likelihood (ML)
533 method and bootstrap values (1000 resamples) were applied to assess the robustness of the tree.

534

535 **Sheath preparation of CIS from *Streptomyces* for negative-stain EM** 536 **and mass spectrometry**

537 *S. venezuelae* was cultivated either in 30 mL LB or MYM liquid medium for 14 hours and of
538 *S. coelicolor* strains were grown in 30 ml TSB, TSB-YEME or R2YE liquid medium for 48
539 hours, respectively. *Streptomyces* cultures were pelleted by centrifugation (7000×g, 10 min, 4
540 °C), resuspended in 5 ml lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5×CellLytic B
541 (Sigma-Aldrich), 1 % Triton X-100, 200 µg/ml lysozyme, 50 µg/ml DNase I, pH 7.4), and
542 incubated for 1 hour at 37°C. Cell debris was removed by centrifugation (15000×g, 15 min, 4
543 °C) and cleared lysates were subjected to ultra-centrifugation (150000×g, 1 h, 4 °C). Pellets
544 were resuspended in 150 µl resuspension buffer (150 mM NaCl, 50 mM Tris-HCl,
545 supplemented with protease inhibitor cocktail (Roche), pH 7.4). Proteins in the CIS preparation
546 were subjected to negative stain EM imaging⁴⁴ and mass spectrometry at the Functional
547 Genomics Center Zürich.

548

549 **Negative stain electron microscopy**

550 4 µl of purified sheath particles were adsorbed to glow-discharged, carbon-coated copper grids
551 (Electron Microscopy Sciences) for 60 s, washed twice with milli-Q water and stained with
552 2 % phosphotungstic acid for 45 s. The grids were imaged at room temperature using a Thermo
553 Fisher Scientific Morgagni transmission electron microscope (TEM) operated at 80 kV.

554

555 **Mass spectrometry analysis**

556 To confirm the presence of predicted CIS components from *Streptomyces*, isolated sheath
557 particles were subjected to liquid chromatography–mass spectrometry analysis (LC–MS/MS).
558 First, the samples were digested with 5 µl of trypsin (100 ng/µl in 10 mM HCl) and
559 microwaved for 30 min at 60 °C. The samples were then dried, dissolved in 20 µl ddH₂O with

560 0.1% formic acid, diluted in 1:10 and transferred to autosampler vials for liquid
561 chromatography with tandem mass spectrometry analysis. A total of 1 μ l was injected on a
562 nanoAcquity UPLC coupled to a Q-Exactive mass spectrometer (ThermoFisher). Database
563 searches were performed by using the Mascot swissprot and tremble_streptomycetes search
564 program. For search results, stringent settings have been applied in Scaffold (1% protein false
565 discovery rate, a minimum of two peptides per protein, 0.1% peptide false discovery rate). The
566 results were visualized by Scaffold software (Proteome Software Inc., Version 4.11.1).

567

568 **Automated Western blot analysis**

569 Automated Western blot analysis (WES) of liquid grown *Streptomyces* strains was essentially
570 performed as described previously⁴⁵. Cell pellets were resuspended in 0.4 ml of sonication
571 buffer (20 mM Tris pH 8.0, 5 mM EDTA, 1x EDTA-free protease inhibitors [Sigma Aldrich])
572 and subjected to sonication at 4.5-micron amplitude for 7 cycles of 15 seconds on/15 seconds
573 off. Samples were centrifuged at 14,000 RPM for 15 minutes at 4°C. The supernatants were
574 removed and subjected to a Bradford Assay (Biorad). Equivalent total protein concentrations
575 (0.2 mg/ml) were assayed using the automated Western blotting machine WES (ProteinSimple,
576 San Jose, CA) according to the manufacturer's guidelines. For the detection of Cis1a and Cis2
577 protein, antibodies for α -Cis1a (GenScript) and α -Cis2 (GenScript) were used at a
578 concentration of 1:200. For detection of WhiA 0.5 μ g of total protein and anti-WhiA
579 (Polyclonal, Cambridge Research Biochemicals) at 1:100 dilution was used⁴⁶.

580 For the detection of Cis1a and Cis2 in culture supernatants, *S. coelicolor* WT, SS387 and SS395
581 were grown in duplicate in TSB medium for 48 h. Cultures were pelleted and 20 ml supernatant
582 obtained from each culture were concentrated to approximately 1 ml using Amicon Ultra-15,
583 10K spin column (Millipore). Total protein samples were further processed as described above.
584 In parallel, an aliquot of each sample was loaded onto a 12 % Teo-Tricine/SDS precast protein
585 gel (Expedian) to demonstrate the presence of proteins in the culture supernatants. SDS-gels
586 were stained with InstantBlue (Sigma-Aldrich) and scanned.

587 For the automated Western blot analysis of surface-grown *S. coelicolor* samples from R2YE
588 plates, mycelium was scraped of sterile cellophane discs that had been placed on top of solid
589 R2YE medium. Mycelia were removed at the described time points and washed with 1X PBS.
590 The supernatant was discarded and the pellet frozen. Pellets were treated and WES ran as
591 above. All virtual Western blots were generated using the Compass software for simple western
592 (Version 6.0.0). Data of protein abundance was plotted using GraphPad Prism (Version 9.3.1).

593 For WES analyses of Cis1a and Cis2 abundance following nisin stress *S. coelicolor* WT.
594 cultures were grown in TSB medium at 30 °C for 48 hours, after which they were split and
595 normalized to the same optical density. To one culture replicate, nisin was added to a final
596 concentration of 1 µg/ml and to the other, the diluent (0.05% Acetic acid) was added in equal
597 volume. After which 2 ml aliquots were removed from each sample and pelleted at 13,000
598 RPM. Pellets were treated as above but were additionally probed with an α-WhiA antibody at
599 1:100 concentration. The band intensities for Cis1a and Cis2 were normalized against the band
600 intensity of WhiA and plotted in GraphPad Prism (Version 9.3.1) with the standard deviation.

601

602 **Fluorescence light microscopy and image analysis**

603 For imaging protein localization and fluorescent promoter reporter fusion in *S. coelicolor*, a
604 Zeiss Axio Observer Z.1 inverted epifluorescence microscope fitted with a sCMOS camera
605 (Hamamatsu Orca FLASH 4), a Zeiss Colibri 7LED light source, a Hamamatsu Orca Flash
606 4.0v3 sCMOS camera, and a temperature-controlled incubation chamber was used. Images
607 were acquired using a Zeiss Alpha Plan-Apo 100x/1.46 Oil DIC M27 objective with a YFP
608 excitation/emission bandwidths of 489–512 nm/520–550 nm. Still images and time-lapse
609 images series were collected using Zen Blue (Zeiss) and analyzed using Fiji⁴⁷.

610 To monitor the activity of the fluorescent sheath promoter fusion in *S. coelicolor*, spores of
611 strain SS484 were spotted onto solid R2YE medium and grown alongside a microscopic
612 coverslips that had been inserted into the agar at an approximately 45 ° angle. Plates were
613 incubated at 30 °C for up to 4 days. At the indicated time points, glass coverslips with attached
614 hyphae were removed and mounted onto slides affixed with 1 % agar pads and imaged.

615 For time-lapse imaging of *S. coelicolor* expressing a fluorescently labelled sheath protein
616 (SS389), cells were first grown in TSB-YEME for 40 h and a 2 µl sample of the culture was
617 immobilized on a 1 % agarose pad prepared with filtered culture medium and using a Gene
618 Frame (Thermo Scientific). Experiments were performed at 30 °C and growing hyphae were
619 imaged every 5 min. Image collection and analysis was performed using Zen Blue (Zeiss) and
620 Fiji, respectively⁴⁷.

621

622 **Plunge freezing of *Streptomyces* hyphae**

623 For cryo-electron tomography (cryoET), *Streptomyces* cells were mixed with 10 nm Protein A
624 conjugated colloidal gold particles (1:10 v/v, Cytodiagnosics) and 4 µl of the mixture was
625 applied to a glow-discharged holey-carbon copper EM grid (R2/1 or R2/2, Quantifoil). The

626 grid was automatically blotted from the backside for 4-6 s in a Mark IV Vitrobot by using a
627 Teflon sheet on the front pad, and plunge-frozen in a liquid ethane-propane mixture (37%/63%)
628 cooled by a liquid nitrogen bath.

629 For single particle cryoEM (SPA), the *S. coelicolor* CIS particles (from WT CIS and non-
630 contractile CIS), collected after sheath preparation, were vitrified using a Vitrobot Mark IV
631 (Thermo Fisher Scientific). 4 μ l of samples were applied on glow-discharged 200 mesh
632 Quantifoil Gold grids (R 2/2). Grids were blotted for 5 s and plunged into liquid ethane-propane
633 mix (37 %/63 %). Frozen grids were stored in liquid nitrogen until loaded onto the microscope.

634

635 **Cryo-focused ion beam milling**

636 A standard protocol was used to perform cryo-focused ion beam milling (CryoFIB milling) on
637 *S. venezuelae*⁴⁸. Plunge-frozen grids were clipped into cryoFIB-autoloader grids (Thermo
638 Fisher Scientific), then transferred into a liquid nitrogen bath of a loading station (Leica
639 Microsystems) and mounted into a 40 ° pre-tilted SEM grid holder (Leica Microsystems). The
640 holder was transferred with a VCT100 cryo-transfer system (Leica Microsystems) into a Helios
641 NanoLab600i dual beam FIB/scanning electron microscope (SEM, Thermo Fisher Scientific).
642 Grids were coated with platinum precursor gas for 6 s and checked with SEM at 3-5 kV (80
643 pA) to evaluate grid quality and identify targets. Lamella were milled in multiple steps using
644 the focused gallium ion beam (43 nA to 24 pA) until a thickness ~250 nm was achieved. The
645 holder was returned to the loading station using the VCT100 transfer system. Unloaded grids
646 were stored in liquid nitrogen prior to cryoET imaging.

647

648 **Cryo-electron tomography**

649 Intact or cryoFIB-milled *Streptomyces* cells were imaged by cryoET⁴⁹. Images were recorded
650 on Titan Krios 300 kV microscopes (Thermo Fisher Scientific) equipped with a Quantum LS
651 imaging filter operated at a 20 eV slit width and with K2 or K3 Summit direct electron detectors
652 (Gatan). Tilt series were collected using a bidirectional tilt-scheme from -60 to +60 ° in 2 °
653 increments. Total dose was 130-150 e⁻/Å² and defocus was kept at -8 μ m. Tilt series were
654 acquired using SerialEM⁵⁰, drift-corrected using alignframes, reconstructed and segmented
655 using IMOD program suite⁵¹. To enhance contrast, tomograms were deconvolved with a
656 Wiener-like filter⁵².

657

658 **SPA data collection and image processing**

659 CryoEM datasets of *S. coelicolor* contracted sheath and extended sheath-tube module were
660 collected as movie stacks using the SerialEM program on Titan Krios EM operating at 300 kV
661 and equipped with an energy filter and a K2 Summit camera. The movie frames of each
662 collected stack were aligned and summed up into one single micrograph with dose weighting
663 at the binning factor of 2 using MotionCor2. The CTF parameter of the micrographs were
664 estimated using Gctf. Pixel size at specimen level was 1.4 Å and target defocus ranged from
665 1.5 µm to 3.5 µm. Each stack contains 50 frames, and the accumulated electron dose rate was
666 ~60 e⁻/Å².

667 The image processing of contracted sheath and extended sheath-tube from *S. coelicolor* was
668 performed as previously reported¹⁹. The particles were picked manually using Relion 3.0⁵⁴.
669 The particle extraction was performed in “Extract helical segments” mode to extract helical
670 segments. The structural determination of the contracted sheath and the extended sheath-tube
671 module was performed using helical reconstruction in Relion 3.0⁵⁵.

672 For the contracted sheath, the final 3.6 Å resolution structure of contracted sheath was obtained
673 from 4,838 particles applied with 6-fold symmetry and helical parameters (rise = 17.22 Å, twist
674 = 26.58 °) (Extended Data Fig. 2a).

675 For the extended sheath-tube module, the final 3.9 Å resolution structure of the extended
676 sheath-tube module was determined from 18,822 particles calculated with 6-fold symmetry
677 and helical parameters (rise = 38.50 Å, twist = 23.10 °) (Extended Data Fig. 3e).

678 The resolutions of relative reconstruction maps were estimated based on the gold-standard
679 Fourier Shell Correlation (FSC) = 0.143 criteria⁵⁶. The local resolution estimations of
680 individual maps were performed using the local resolution module in Relion 3.0 and examined
681 using UCSF Chimera⁵⁷ (Extended Data Fig. 2b and Extended Data Fig. 3f).

682

683 **Structure modeling**

684 Proteins were built *de novo* using COOT⁵⁸. Models were iteratively refined using RosettaCM⁵⁹
685 and real-space refinement implemented in PHENIX⁶⁰. Sheath protein could only be partially
686 modeled and in some cases side chains were not assigned. Final model validation was done

687 using MolProbity⁶⁰ and correlation between models and the corresponding maps were
688 estimated using mtriage⁶⁰.

689 All visualizations were done using PyMOL, UCSF Chimera⁵⁷ or ChimeraX⁶¹.

690

691 **Correlative cryo-light and electron microscopy**

692 For correlative cryo-light and electron microscopy, frozen grids containing *S. coelicolor* WT
693 were transferred to CMS196V3 Linkam cryo-stage and imaged using a 100x numerical
694 aperture 0.74 objective on a LSM900 Airyscan 2 Zeiss microscope driven by ZEN Blue
695 software (Version 3.5). Fluorescence images of areas of interest were manually correlated with
696 the corresponding TEM square montage using SerialEM^{50,62}.

697

698 **Fluorescence-based cell viability assay**

699 To express sfGFP constitutively in *Streptomyces* strains, the coding sequence for sfGFP was
700 introduced downstream of the constitutive promoter *ermE** on an integrating plasmid vector
701 (pIJ10257). The plasmid was introduced by conjugation to *S. coelicolor* strains (WT, Δ CIS and
702 CIS-N5). These strains were inoculated into 30 ml of TSB liquid culture and incubated at 30
703 °C with shaking at 250 rpm in baffled flasks for 48 h. Where appropriate, nisin and CCCP (or
704 0.002% DMSO) were added to a final concentration of 1 μ g/ml and 10 μ M, respectively.
705 Cultures were incubated for a further 90 min. For UV exposure, 10 ml of the *S. coelicolor*
706 cultures were transferred into a petri dish and treated with Sankyo Denki Germicidal 68 T5
707 UV-C lamps for 10 mins in a Herolab UV DNA crosslinker CL-1. Then, 1 ml aliquots were
708 centrifuged for 5 min at 13,000 rpm, washed twice with PBS, and resuspended in 1 ml of PBS
709 with 5 μ g/ml FM5-95 membrane stain. The cell suspension and membrane stain were mixed
710 by vortexing and kept in the dark at room temperature for 10 min. The suspension was then
711 centrifuged for 5 min at 13,000 rpm, washed twice with PBS, and resuspended in 50 μ l of PBS.
712 10 μ l of samples were immobilized on 1% agar pads and imaged on the Thunder imager 3D
713 cell culture microscope at room temperature. First, tile scan images were acquired on the Las
714 X Navigator plug-in of Leica Application Suite X (LasX) software (Version 3.7.4.23463), and
715 100 targets were picked manually. Then z-stack images with HC PL APO 100x objective were
716 acquired at an excitation of 475 nm and 555 nm under GFP (green) and TRX (red) filters
717 respectively. Images were processed using LasX software to apply thunder processing and
718 maximum projection, FIJI to create segmentation and quantify the live (sfGFP)/total cells

719 (FM5-95) area ratio⁴⁷ and statistical analysis was performed on GraphPad Prism 9 (Version
720 9.3.1).

721

722 **Cover glass impression of *Streptomyces* spore chains**

723 Spore titers of relevant strains were determined by standard techniques. 10⁷ CFU of
724 *S. coelicolor* strains (WT, SS387, SS393 and SS395) were spread onto R2YE agar plates and
725 grown at 30 °C. Sterile glass cover slips were gently applied to the top surface of each bacterial
726 lawn after 48 h, 72 h and 96 h post inoculation. Cover slips were then mounted onto glass
727 microscope slides and imaged using a 40x objective on a Leica Thunder Imager 3D Cell
728 Culture. Images were processed using FIJI⁴⁷.

729

730 **Actinorhodin production assay**

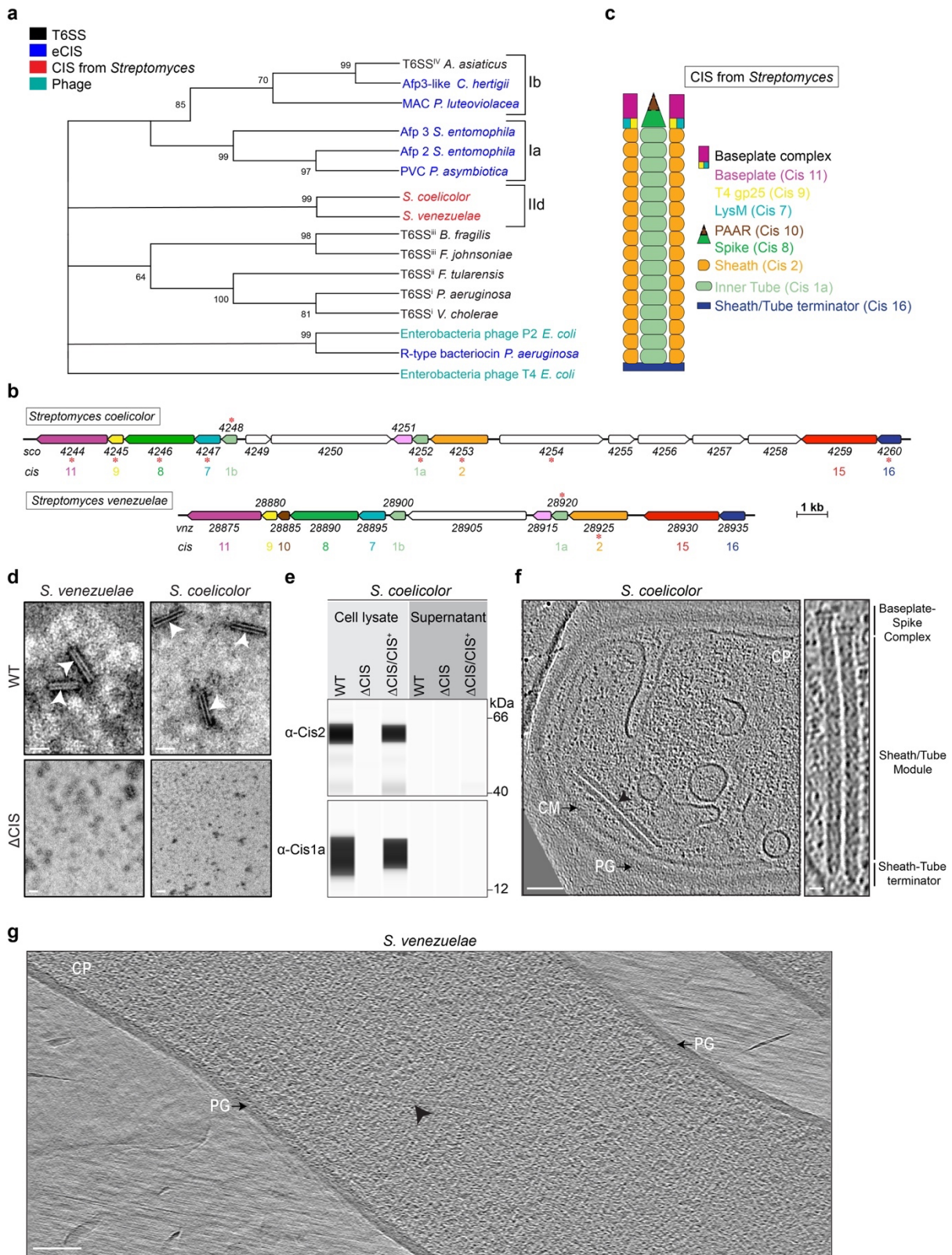
731 *S. coelicolor* strains (WT, SS387, SS393 and SS395) were inoculated into 30 ml R2YE liquid
732 media at a final concentration of 1.5 x 10⁶ CFU/ml. Cultures were grown in baffled flasks at
733 30 °C overnight. Cultures were standardized to an OD₄₅₀ of 0.5 and inoculated in 30 ml of fresh
734 R2YE liquid medium. For visual comparison of pigment production, images of the growing
735 culture were taken between t = 0 and t = 72 h (as indicated in Extended Data Fig. 9c). For
736 quantification of total actinorhodin production, 480 µl of samples were collected at the same
737 time points where images were taken. 120 µl of 5M KOH was added, samples were vortexed
738 and centrifuged at 5000 x g for 5 min. The weight of each tube was recorded. A Synergy 2
739 plate reader (Biotek) was used then to measure the absorbance of the supernatant at 640 nm.
740 The absorbance was normalized by weight of the wet pellet.

741

742 **Data availability**

743 Representative reconstructed tomograms (EMD-XXXXX, EMD-XXXXX, EMD-XXXXX,
744 EMD-XXXXX, EMD-XXXXX, EMD-XXXXX and EMD-XXXXX) and SPA cryoEM maps
745 (EMD-XXXXX and EMD-XXXXX) have been deposited in the Electron Microscopy Data
746 Bank. Atomic models (PDB: XXXX and PDB: XXXX) have been deposited in the Protein
747 Data Bank. All other data are available from the authors upon reasonable request.

748 **Figures**



749

750

751

752

753 **Figure 1: Different *Streptomyces* species express cytoplasmic CIS assemblies.**

754 **a.** Phylogenetic analysis of representative sheath protein sequences shows that homologs from
755 *Streptomyces* form a monophyletic clade. Numbers indicate bootstrap values, color code
756 denotes different modes of action. Subclades Ia, Ib and IId are based on the dbeCIS database⁶.

757 **b.** Representative gene clusters from *Streptomyces* encode conserved CIS components. The
758 schematic shows the gene arrangement of the CIS gene clusters from *S. coelicolor* A3(2)
759 (CIS^{Sc}) and *S. venezuelae* NRRL B-65442 (CIS^{Sv}) with gene locus tags. Color code indicates
760 conserved gene products. CIS components were numbered based on similarities to previously
761 studied CIS (AFP)^{14,63}. Asterisks indicate gene products that were detected by mass
762 spectrometry after CISs purification (Supplementary Table 1).

763 **c.** The schematic illustrates a putative CIS assembly from *Streptomyces*. Color-code is based
764 on the predicted gene function shown in (b).

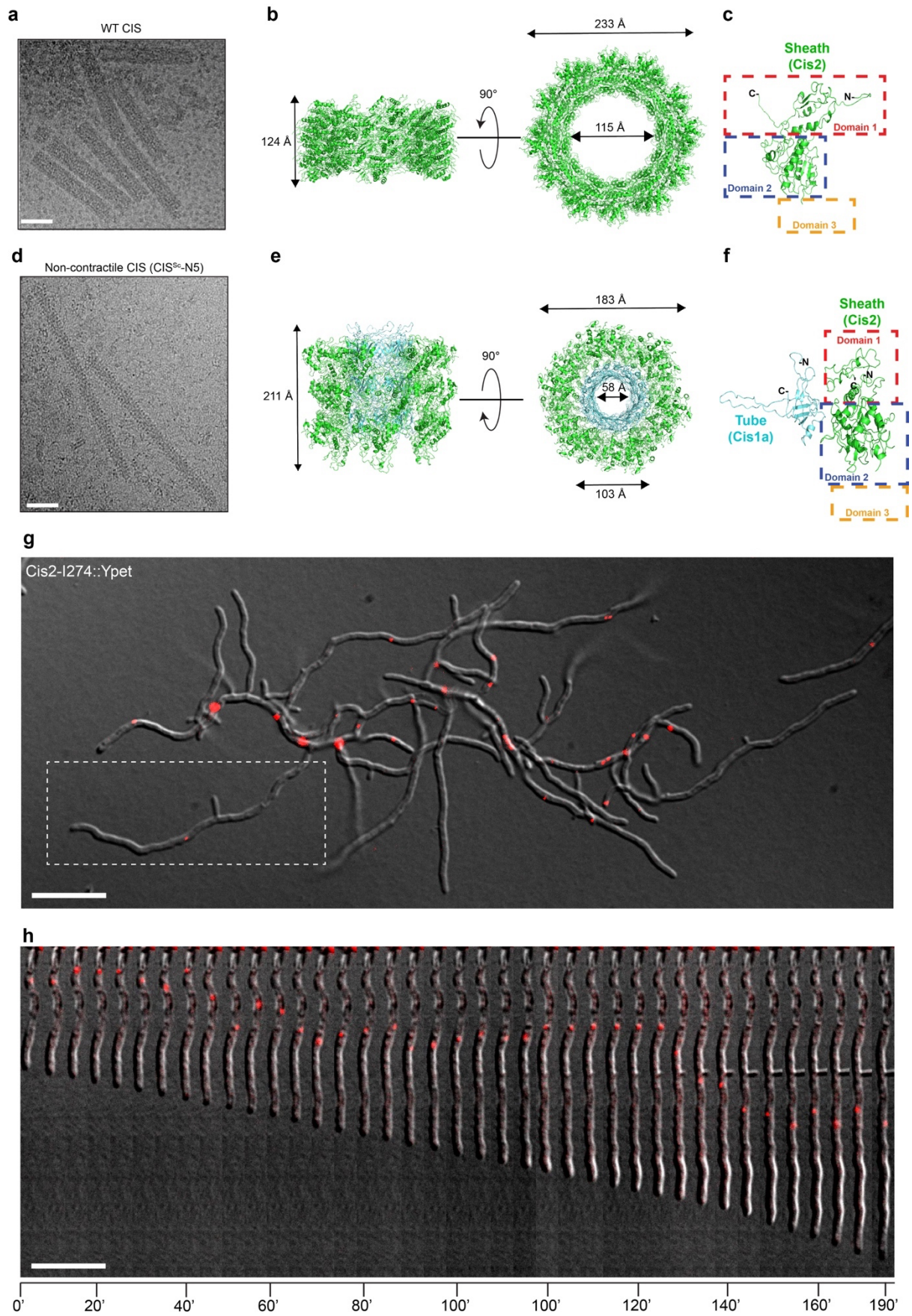
765 **d.** The gene *cis2* is required for CIS assembly. Shown are negative-stain EM images of crude
766 sheath preparations from WT and Δ CIS mutant strains of *S. coelicolor* and *S. venezuelae*. White
767 arrowheads indicate contracted sheath-like structures. Shown are representative micrographs
768 of three independent experiments. Bars, 80 nm.

769 **e.** CIS^{Sc} proteins are detected in the cell lysate but not secreted into the supernatant. Shown is
770 the automated Western blot analysis of cultures of *S. coelicolor* WT, Δ CIS mutant, and a
771 complementation (Δ CIS/CIS⁺). The presence of the sheath protein (Cis2) and the inner tube
772 protein (Cis1a) in whole cell lysates and concentrated culture supernatants was probed using
773 polyclonal antibodies against Cis1a/2. Experiments were performed in biological replicates.
774 For the control SDS-PAGE gel see Extended Data Fig. 1.

775 **f.** Shown is a cryo-electron tomogram of a WT *S. coelicolor* hypha, revealing two cytoplasmic
776 extended CIS^{Sc} assemblies (arrowhead). PG, peptidoglycan; CM, cytoplasmic membrane; CP,
777 cytoplasm. Putative structural components are indicated on the right. Bars, 75 nm and 12.5 nm
778 (magnified inset).

779 **g.** Shown is a cryo-electron tomogram of a cryoFIB milled WT *S. venezuelae* hypha, revealing
780 one cytoplasmic extended CIS^{Sv} assembly (arrowhead). PG, peptidoglycan; CP, cytoplasm.
781 Bar, 140 nm.

782



783

784

785 **Figure 2: Structure and subcellular localization of CIS^{Sc}.**

786 **a.** Shown is a representative cryo-electron micrograph of a sheath preparation from WT *S.*
787 *coelicolor* that was recorded for structure determination. All sheath structures were seen in the
788 contracted state. Bar, 40 nm.

789 **b.** Shown is a section of the CIS^{Sc} sheath cryoEM structure in the contracted conformation.

790 **c.** Shown is a ribbon representation of the Cis2 monomer in its contracted state. Dashed
791 rectangles highlight the positions of domains 1 (red), 2 (blue) and 3 (orange, not resolved
792 because of high flexibility).

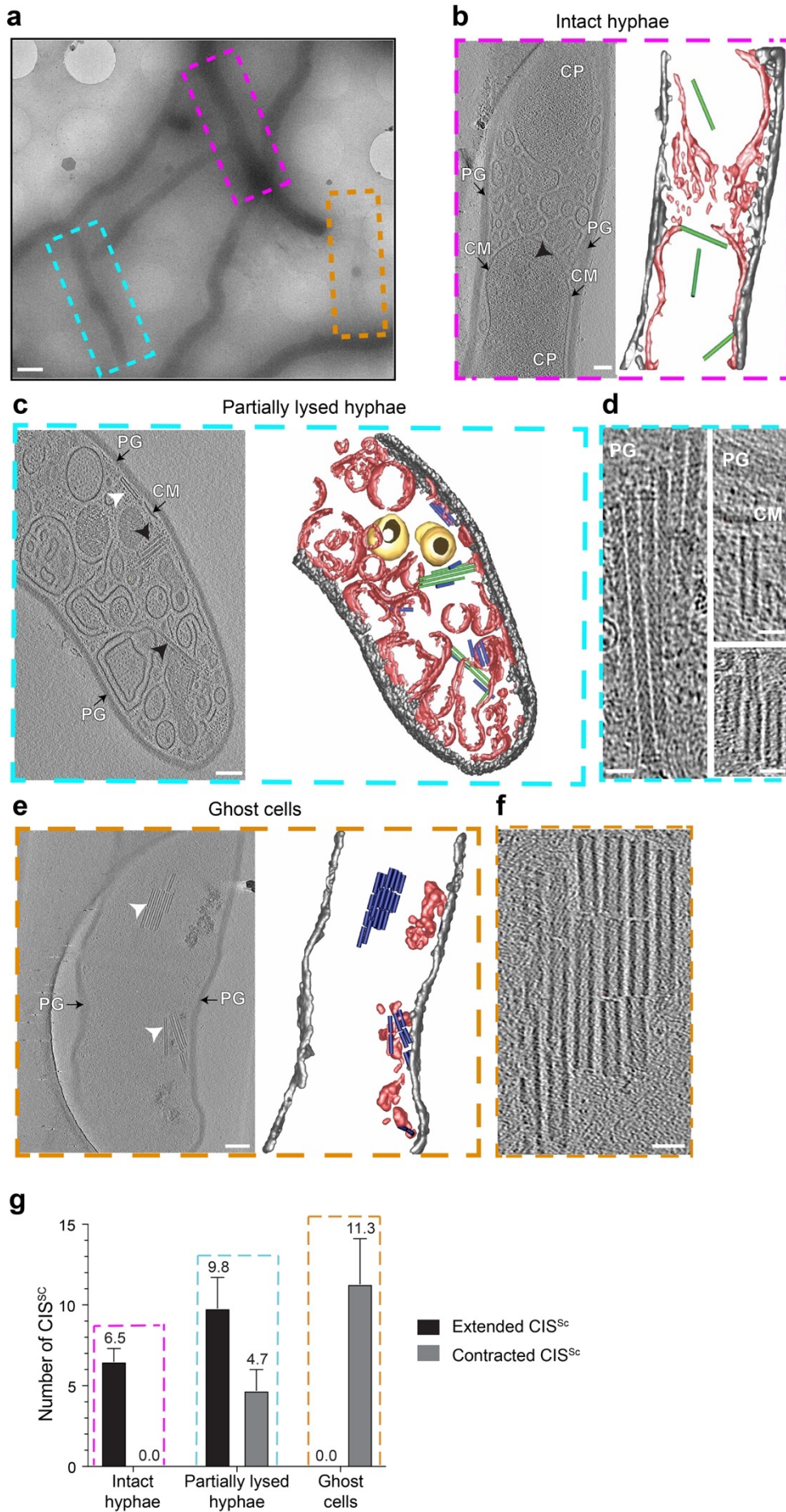
793 **d.** Shown is a representative cryo-electron micrograph of a sheath preparation from *S.*
794 *coelicolor* expressing a non-contractile mutant of Cis2 (CIS-N5). More than 95% of all
795 structures were seen in the extended state. Bar, 40 nm.

796 **e.** Shown is ribbon representation of a section of the *S. coelicolor* Cis2 (sheath)-Cis1a (inner
797 tube) cryoEM structure in the extended conformation that was solved using the non-contractile
798 mutant.

799 **f.** Shown is a ribbon representation of the Cis2 monomer (non-contractile mutant) in its
800 extended state. Dashed rectangles highlight the positions of domains 1 (red), 2 (blue) and 3
801 (orange, not resolved because of high flexibility).

802 **g.** Insights from the cryoEM structures enabled us to tag Cis2 with a fluorescent tag (YPet) for
803 subsequent time-lapse imaging to determine the localization of assembled CIS^{Sc}. Shown is a
804 still image from Supplementary Movie 1, showing scattered fluorescent foci inside vegetative
805 hyphae. Cells were first grown in TSB-YEME for 40 h and then spotted onto an agarose pad
806 prepared from culture medium and subsequently imaged by time-lapse fLM. White rectangle
807 highlights hypha shown in (h). Bar, 10 μ m.

808 **h.** Fluorescently tagged CIS^{Sc} remained largely static or showed short-range movements over
809 time. Shown is an image montage of a representative growing *S. coelicolor* hypha from
810 Supplementary Movie 1. Images were acquired every 5 min. Bar, 10 μ m.

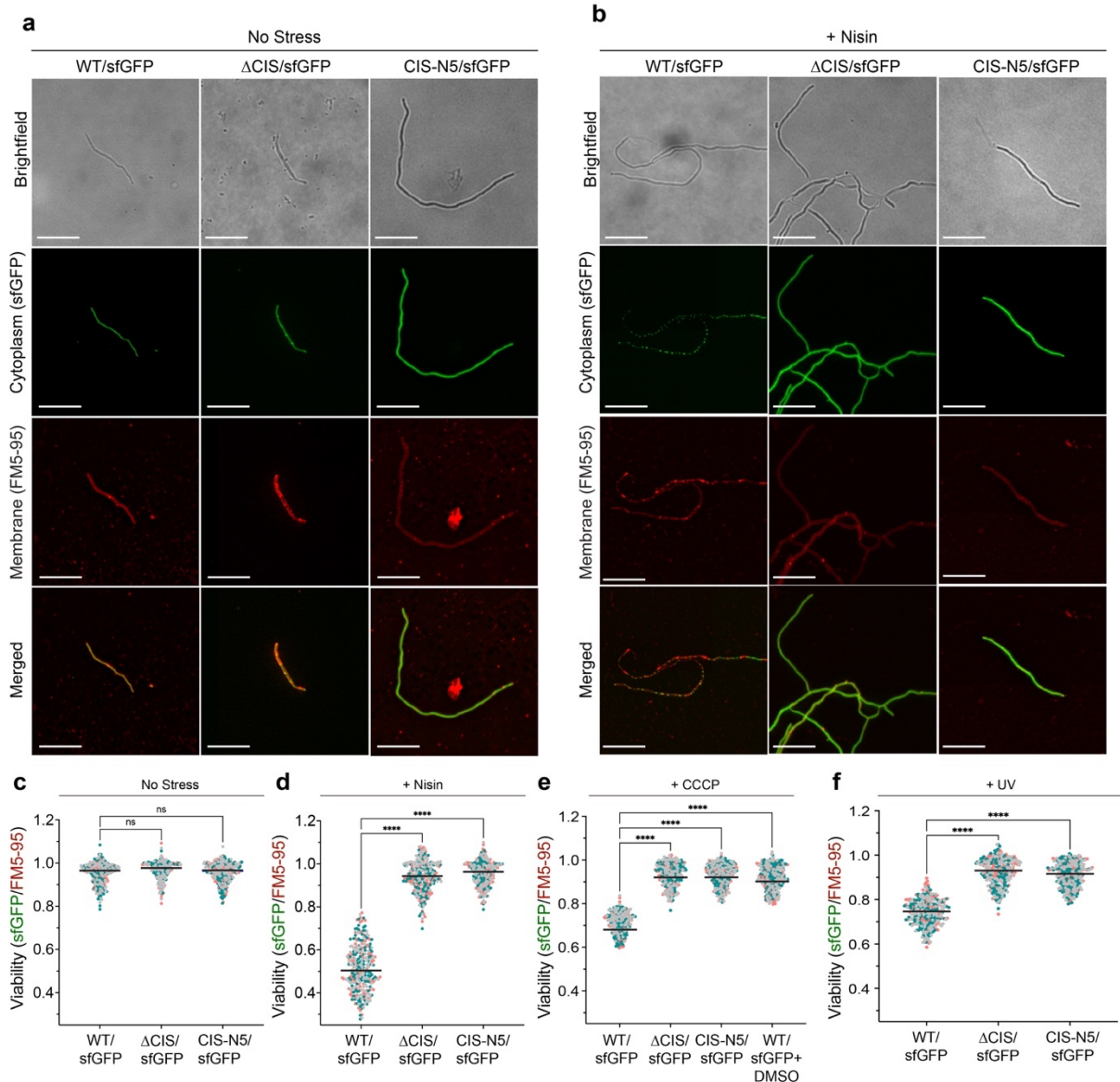


812 **Figure 3: Sheath contraction is linked to reduced cellular integrity**

813 **a.** Shown is a representative low magnification 2D cryoEM image of WT *S. coelicolor* hyphae
814 during vegetative growth. Hyphae were divided into three classes based on their density in such
815 images and based on their structure in cryo-tomograms: (1) ‘intact hyphae’ (purple box), (2)
816 ‘partially lysed hyphae’ (cyan box), and (3) ‘ghost cells’ (orange box). Bar, 1 μm .

817 **b-f.** Shown are representative cryo-tomographic slices and 3D renderings of hyphae of the
818 three classes (corresponding to the regions boxed in a). ‘Intact hyphae’ (b) had a mostly intact
819 cytoplasmic membranes and occasional vesicular membranous assemblies that are reminiscent
820 of “cross-membranes”²⁹. ‘Partially lysed hyphae’ (c) showed a mostly disrupted/vesiculated
821 cytoplasmic membrane. ‘Ghost cells’ (e) contained only remnants of membranes and a mostly
822 intact peptidoglycan cell wall. Note the frequent occurrence of CIS^{Sc} assemblies in extended
823 (black arrowheads/green) and contracted (white arrowheads/blue) conformations. Magnified
824 views of clusters of CIS^{Sc} seen in cryo-tomograms are shown in d/f. PG/grey, peptidoglycan;
825 CM/red, cytoplasmic membrane/membranes; CP, cytoplasm; yellow, storage granules. Bars,
826 75 nm in b/c/e and 25 nm in d/f.

827 **g.** Sheath contraction correlates with cellular integrity, showing the presence of only extended
828 CIS^{Sc} in the class ‘intact hyphae’, and the presence of only contracted CIS^{Sc} in ‘ghost cells’.
829 Shown is a quantification of extended and contracted CIS^{Sc} per tomogram of WT *S. coelicolor*
830 hyphae. Results are based on three independent experiments, with n=30 tomograms for each
831 class of cells.



832

833 **Figure 4: *S. coelicolor* with functional CIS^{Sc} show increased cell death upon stress.**

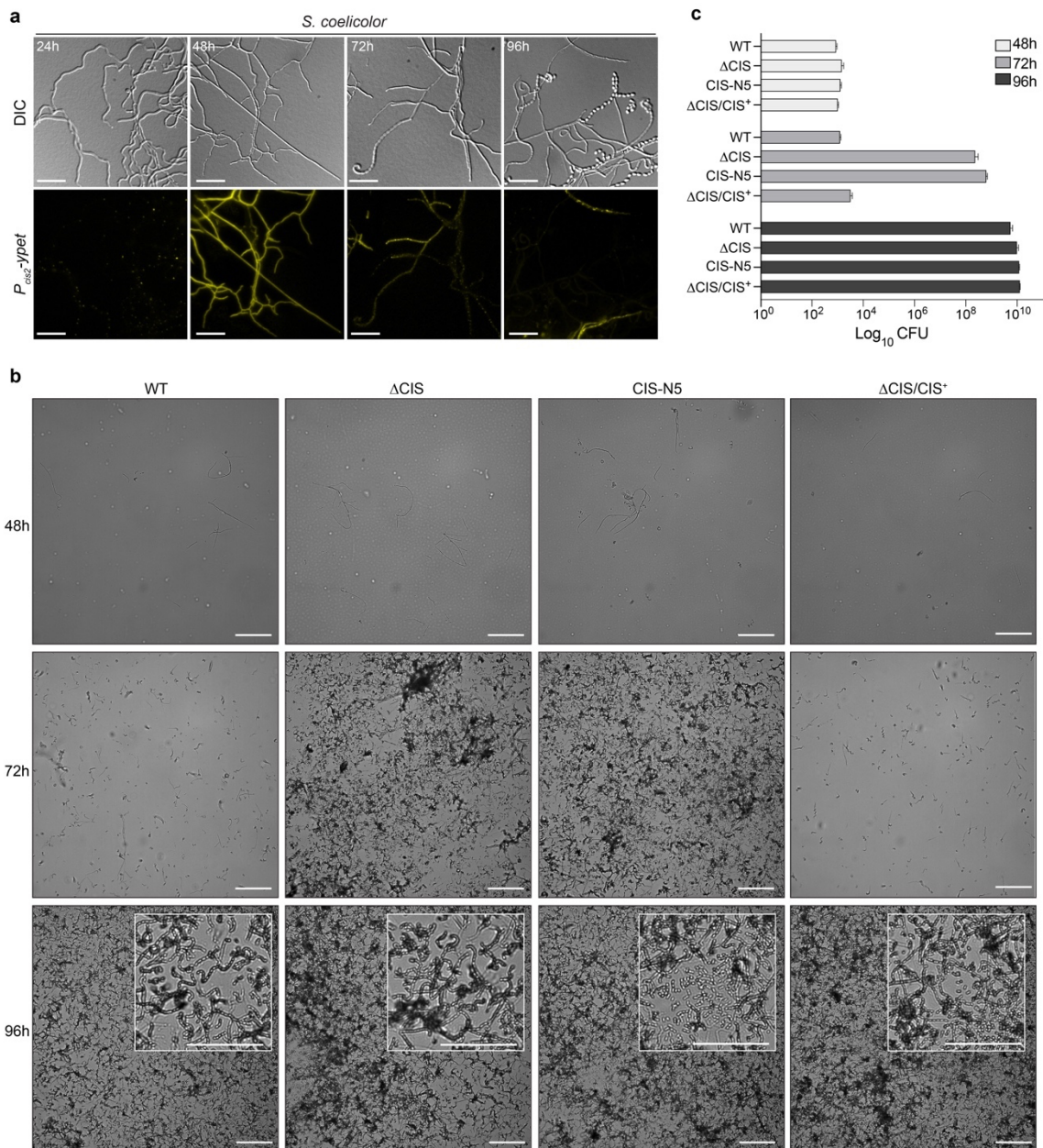
834 **a/b.** fLM (shown are representative images) was used to determine the ratio between live cells
 835 (cytoplasmic sfGFP) and total cells (membrane dye FM5-95) after growth in the absence of
 836 stress (a) or in the presence of nisin stress (b). *S. coelicolor* WT/sfGFP, Δ CIS/sfGFP and CIS-
 837 N5/sfGFP were grown in TSB for 48 h and were then treated with 1 μ g/ml nisin for 90 min.
 838 Bars, 10 μ m.

839 **c/d.** The quantification of the experiments in a/b showed no significant differences between the
 840 WT strain and both CIS^{Sc} mutants under conditions without stress. In contrast, nisin-stressed
 841 WT cells showed a significantly higher rate of cell death compared to both nisin-stressed
 842 mutants. Superplots show the area ratio of live to total hyphae. Black line indicates the mean
 843 ratio derived from biological triplicate experiments (n=100 images for each experiment). ns

844 (not significant) and **** (p < 0.0001) were determined using a one-way ANOVA and Tukey's
845 post-test.

846 **e/f.** To test the induction of cell death under other stress conditions, the same strains were
847 treated with the protonophore CCCP (10 μM, or 0.002 % DMSO as mock control) (e) or UV
848 light (f) for 10 min. Similar to nisin stress, we detected a significant difference in cell death
849 induction between WT and both CIS^{Sc} mutants. See Extended Data Fig. 6a/b for representative
850 fLM images.

851



852

853 **Figure 5: Functional CIS^{Sc} are involved in *Streptomyces* multicellular development.**

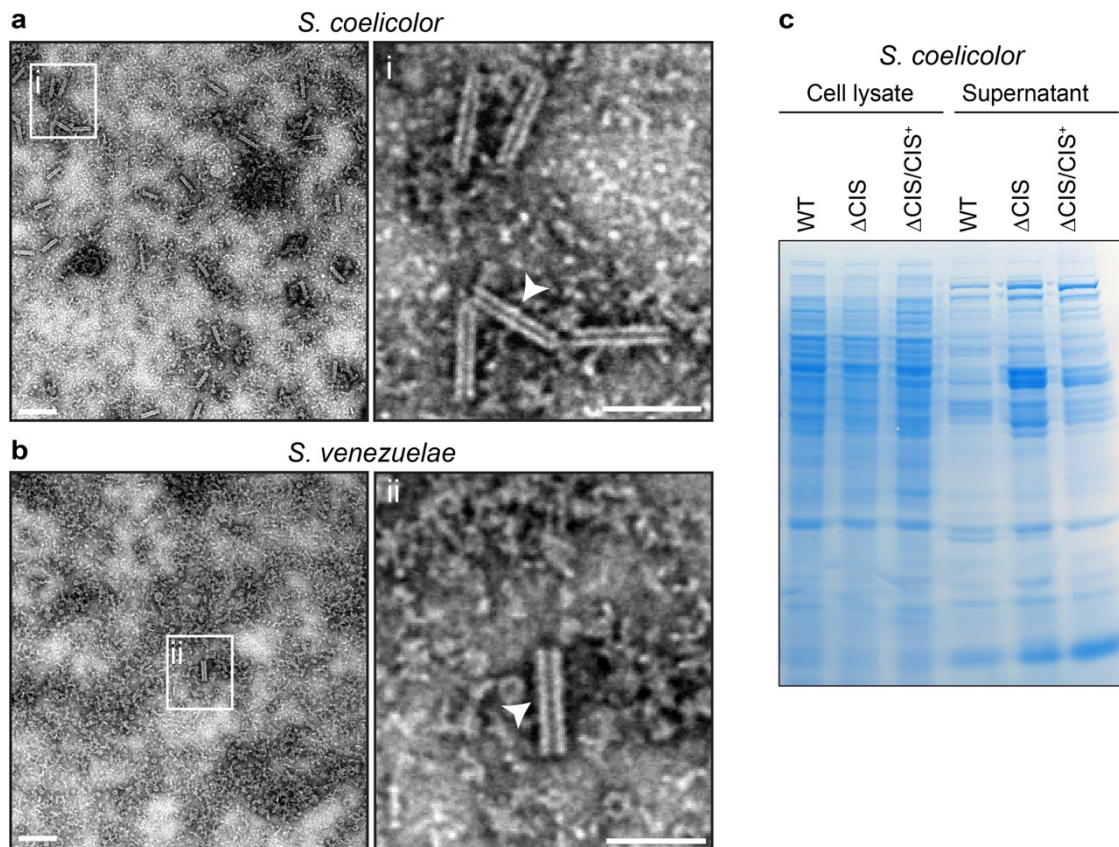
854 **a.** Microscopic analysis of *S. coelicolor* WT cells expressing a fluorescent promoter fusion to
855 the sheath promoter *p_{cis2-ypet}* *in trans*, showing that the sheath operon of the CIS^{Sc} cluster is
856 predominantly expressed during vegetative growth (48 h). Shown are representative
857 micrographs of surface-grown *S. coelicolor* hyphae that attached to a microscopic cover glass
858 inserted into the inoculated agar surface at a 45-degree angle. Plates were incubated over 96 h
859 at 30 °C and imaged at the indicated time-points. Experiments were performed in biological
860 triplicates. Bars, 10 μ m.

861 **b.** Representative brightfield images of surface imprints of plate-grown colonies of *S.*
862 *coelicolor* WT, the CIS^{Sc} mutant strains Δ CIS, CIS-N5, and the complemented mutant

863 Δ CIS/CIS⁺. Images were taken at the indicated timepoints. Only hyphae undergoing
864 sporulation or spores will attach to the hydrophobic cover glass surface. Insets show magnified
865 regions of the colony surface containing spores and spore chains. Note that strains with
866 functional CIS sporulate later. Bars, 50 μ m.

867 **c.** Shown is a quantification of spore production (colony forming unit, CFU) in the same strains
868 as above, revealing at 72 h much higher CFUs (spores) in both CIS mutants. Strains were grown
869 on R2YE agar and spores were harvested after 48 h, 72 h and 96 h of incubation. Data shows
870 mean values and standard deviation obtained from biological triplicate experiments.

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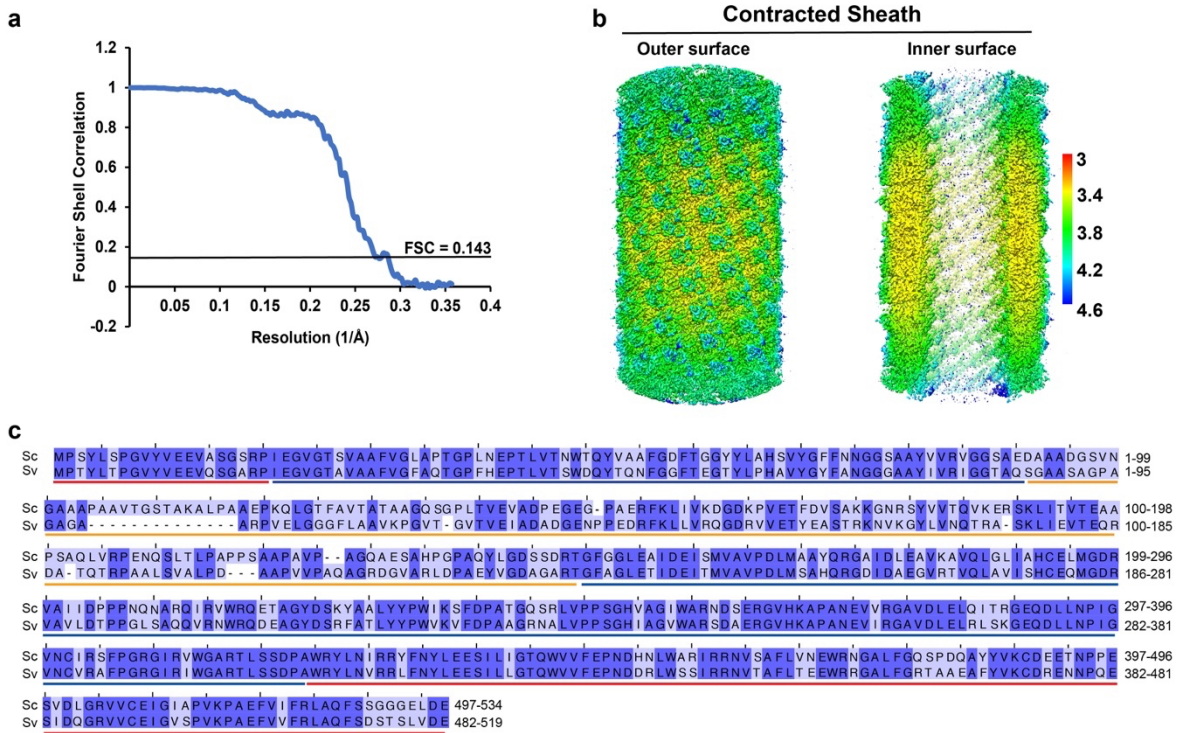


872

873 **Extended Data Figure 1: EM and SDS-PAGE analyses of *Streptomyces* supernatant and**
874 **lysate**

875 **a/b.** Representative negative-stain electron micrographs of crude sheath preparations from WT
876 *S. coelicolor* and *S. venezuelae*. Under the conditions used, the majority of isolated CIS from
877 *Streptomyces* was contracted (insets i/ii). Bars, 80 nm.

878 **c.** Control SDS-PAGE stained with Coomassie-blue showing the presence of protein in
879 concentrated culture supernatants that were used for the detection of Cis1a/2 by automated
880 Western blot analysis in Figure 1e. Samples were obtained from WT *S. coelicolor*, the Δ CIS
881 mutant and the complemented mutant Δ CIS/CIS⁺. Loaded were 10 μ g of protein per sample.



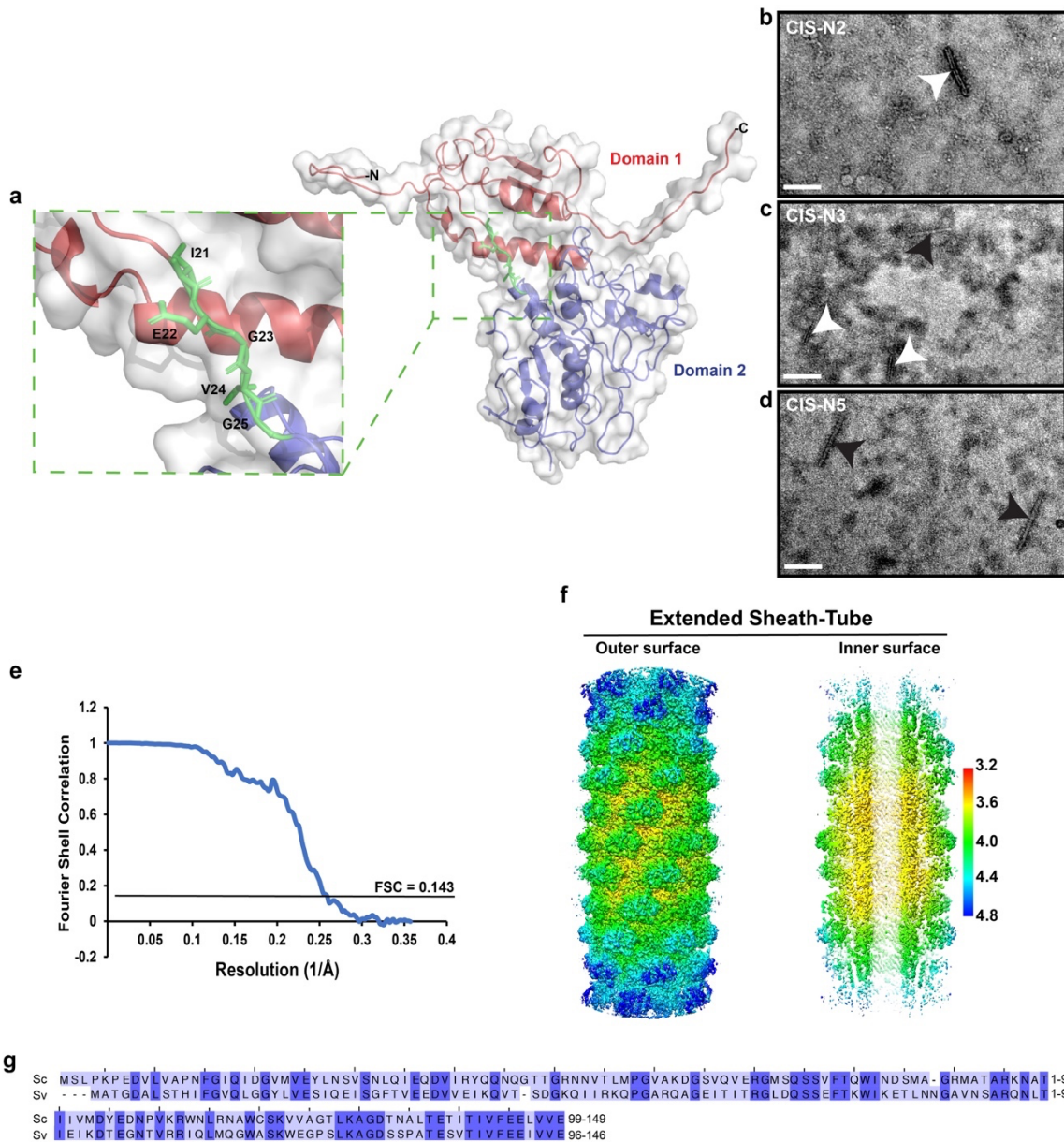
882

883

884 **Extended Data Figure 2: Structure and sequence analysis of *Streptomyces* contracted**
 885 **sheath Cis2.**

886 **a/b.** Gold-standard Fourier shell correlation (FSC) curve (a) and local resolution maps (b) of
 887 the contracted sheath (Cis2) structures from *S. coelicolor*.

888 **c.** Protein sequence alignment showing the high sequence conservation between Cis2 proteins
 889 from *S. coelicolor* (Sc) and *S. venezuelae* (Sv). Colors indicate level of sequence similarity
 890 (light blue, similar; dark blue, identical). Positions of domain 1 (red), domain 2 (blue) and
 891 domain 3 (orange) are indicated.



892

893

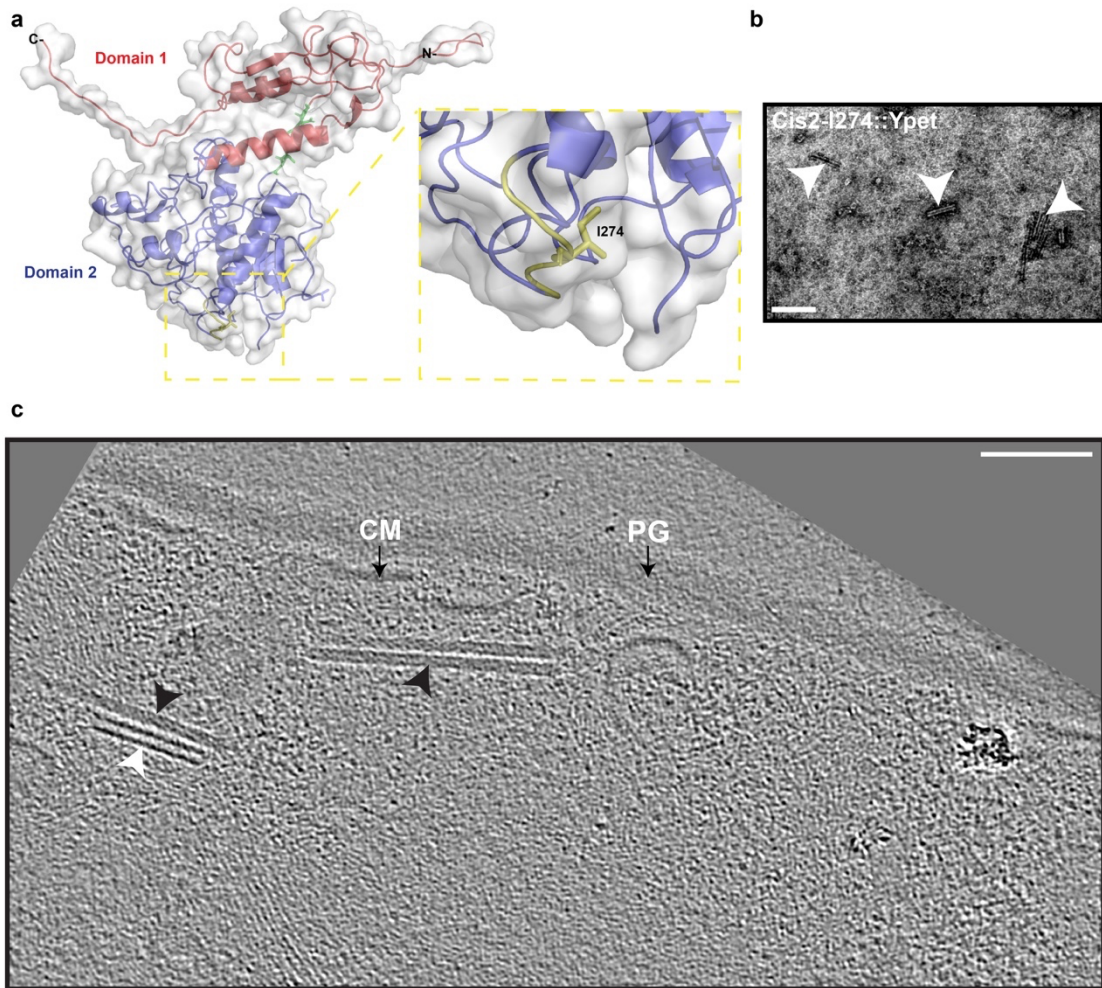
894 **Extended Data Figure 3: Engineering and structure of a non-contractile CIS^{Sc} mutant.**

895 **a.** Surface (grey) and ribbon (colored) representation of the contracted sheath structure from *S.*
896 *coelicolor*. Shown in the enlarged inset is the WT linker comprising residues I21 to G25
897 (green). In order to engineer non-contractile CIS^{Sc} mutants, additional residues were inserted
898 after position G25 (IE for CIS-N2, IEG for CIS-N3, IEGVG for CIS-N5).

899 **b-d.** Negative-stain electron micrographs of CIS particles from *S. coelicolor* strains expressing
900 the CIS^{Sc} mutant versions CIS-N2, CIS-N3 and CIS-N5. Sheath mutants carrying the CIS-N5
901 allele showed the highest fraction of extended structures. Arrowheads indicate contracted
902 (white) and extended (black) CIS^{Sc} particles. Bar, 140 nm.

903 **e-f.** Gold-standard Fourier shell correlation (FSC) curve (e) and local resolution maps (f) of the
904 extended *S. coelicolor* CIS^{Sc}-N5 sheath-tube module.

905 **g.** Protein sequence alignment showing the high conservation of Cis1a proteins from *S.*
906 *coelicolor* (Sc) and *S. venezuelae* (Sv). Colors indicate level of sequence similarity (light blue,
907 similar; dark blue, identical).



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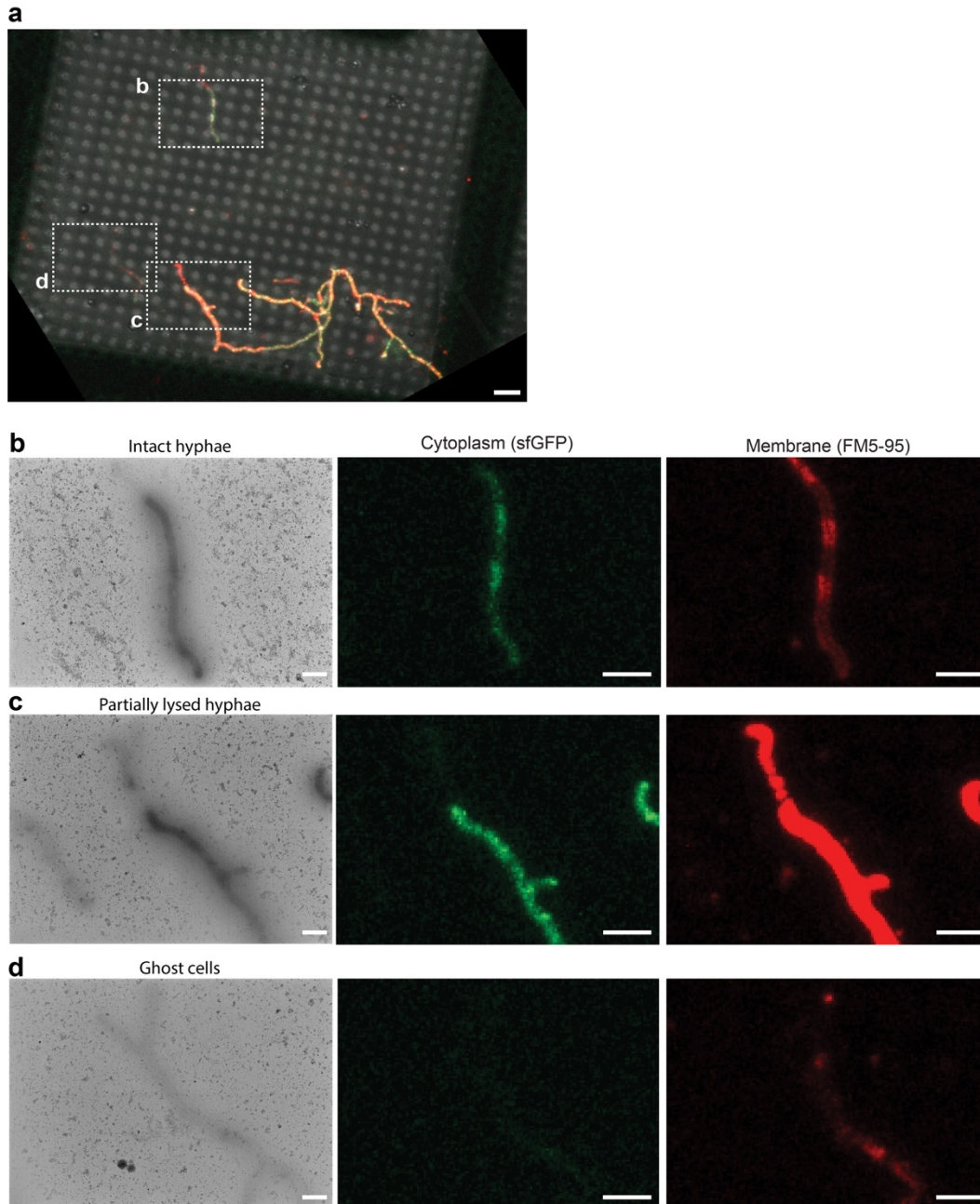
909

910 **Extended Data Figure 4: Generation of a functional Cis2-YPet sandwich fusion.**

911 **a.** Surface and ribbon diagram of the contracted sheath structure from *S. coelicolor* indicating
912 the insertion site of Ypet at residue I274 to generate a fluorescent sheath-Ypet sandwich fusion
913 (Cis2::I274-Ypet), which was used to complement a *S. coelicolor* Δ Cis2 mutant.

914 **b.** Negative-stain electron micrograph of purified CIS particles from *S. coelicolor*
915 Δ *cis2/cis2::I274-ypet*⁺ showing contracted CIS^{Sc} assemblies (white arrowheads). Bar, 140 nm.

916 **c.** Representative cryoET slice of Δ *cis2/cis2::I274-ypet*⁺ hyphae containing a contracted (white
917 arrowhead) and extended (black arrowhead) CIS^{Sc} particles in the cytoplasm. PG,
918 peptidoglycan; CM, cytoplasmic membrane. Bar, 75 nm.



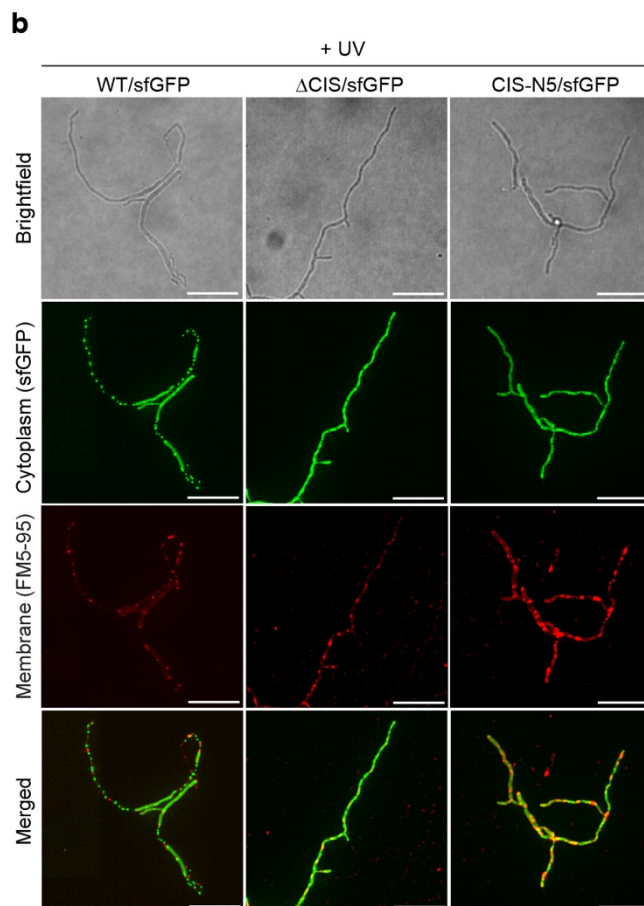
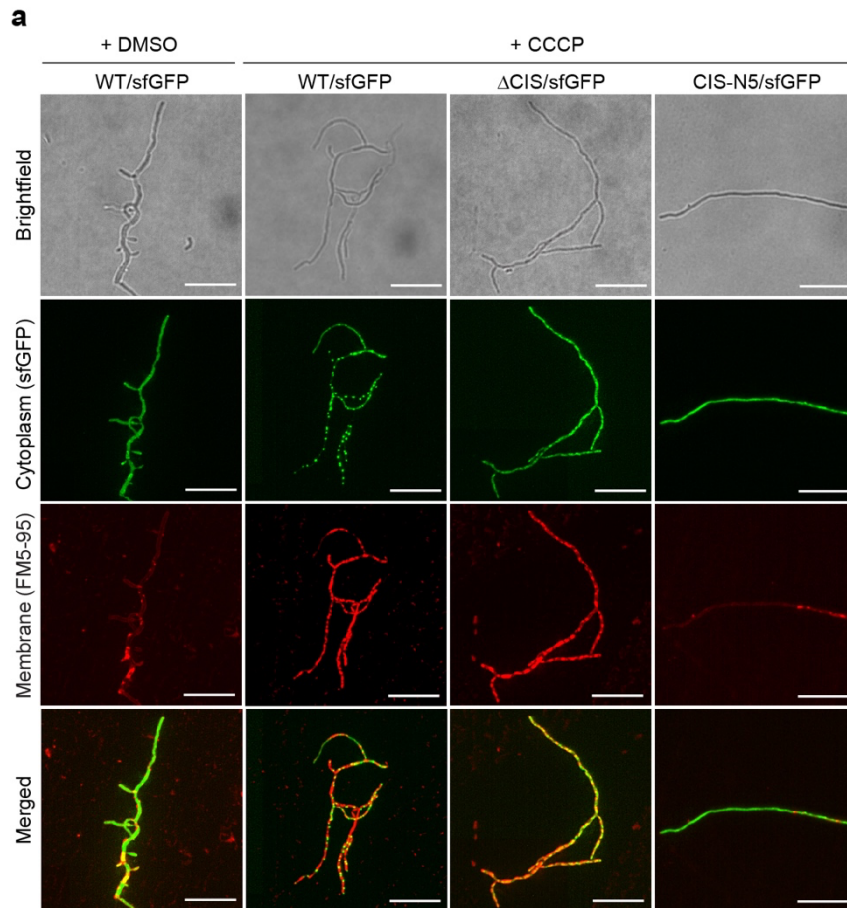
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921 **Extended Data Figure 5: Validation of hyphal membrane integrity using correlative cryo-**
922 **fLM and cryoEM (Cryo-CLEM).**

923 **a.** CryoFLM overview image of vegetative hyphae of WT *S. coelicolor* expressing cytoplasmic
924 sfGFP from a constitutive promoter and stained with the membrane dye FM5-95. The
925 membrane staining pattern and sfGFP fluorescence signal were used to identify the classes
926 ‘intact hyphae’, ‘partially lysed hyphae’ and ‘ghost cells’. The boxed areas were further
927 analyzed in (b-d). Bar, 6 μ m.

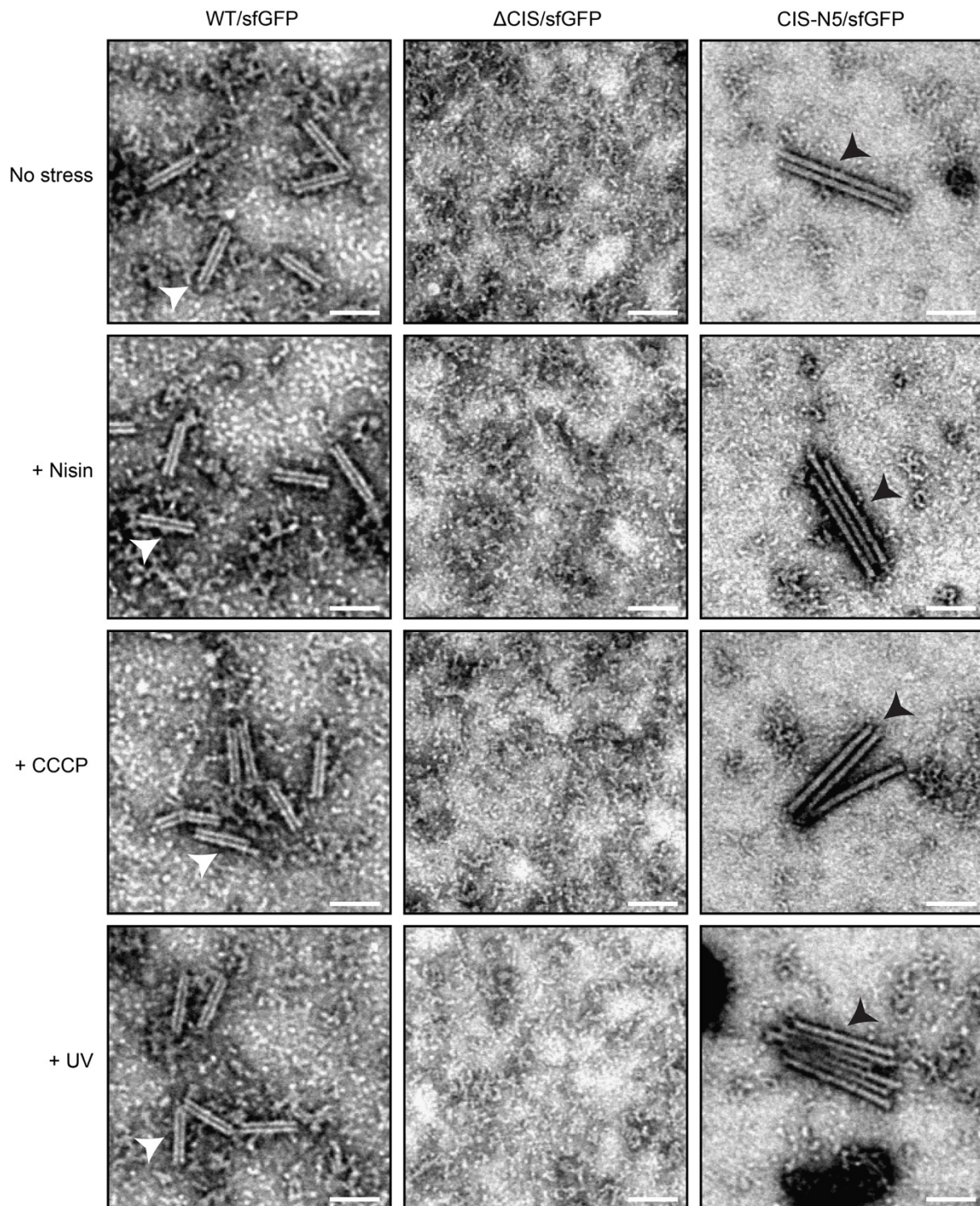
928 **b-d.** Shown are cryoEM 2D projection images (left) and the corresponding cryo-fLM images
929 of examples of ‘intact hyphae’ (b), ‘partially lysed hyphae’ (c) and ‘ghost cells’ (d). Bars, 2
930 μm .
931



933 **Extended Data Figure 6: Functional CIS^{Sc} production promotes stress-induced cell death.**
934 **a/b.** fLM (shown are representative images) was used to determine the ratio between live cells
935 (cytoplasmic sfGFP) and total cells (membrane dye FM5-95) after growth in the presence of
936 10 μ M CCCP (or 0.002 % DMSO as mock control) (a), or after exposure to UV light (b). *S.*
937 *coelicolor* WT/sfGFP, Δ CIS/sfGFP and CIS-N5/sfGFP were grown in TSB for 48 h and treated
938 with CCCP or DMSO for 90 min or were exposed to UV light for 10 min. Bars, 10 μ m. The
939 quantification for both experiments is shown in Fig. 4 e/f.

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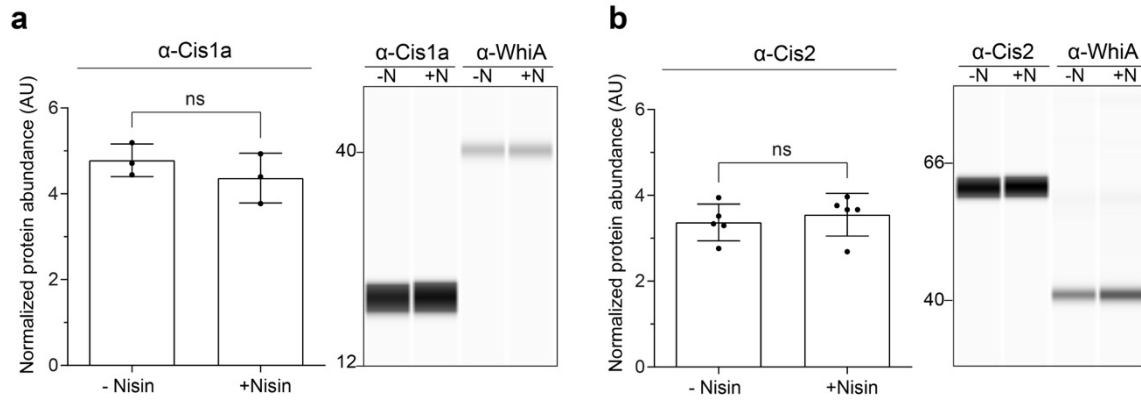
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943 **Extended Data Figure 7: Cell envelope and UV stress do not affect the overall appearance**
944 **of purified CIS^{Sc} particles.**

945 Negative-stain electron micrographs of CIS particles purified from *S. coelicolor* WT/sfGFP,
946 ΔCIS/sfGFP and CIS-N5/sfGFP exposed to no stress, 1 μg/ml nisin, 10 μM CCCP and UV
947 treatment. Arrowheads indicate contracted (white) and extended (black) CIS^{Sc}. Bars, 90 nm.



948

949

Extended Data Figure 8: Nisin-stress does not lead to increased CIS^{Sc} production.

950

a/b. Quantification (left) and automated Western blot (right) analysis, showing the abundance

951

of Cis1a (a) and Cis2 (b) in WT *S. coelicolor* cell lysates in the presence of nisin (+N) and

952

absence of nisin (-N). Cells were grown in TSB for 48 h, followed by treatment with 1 μg/ml

953

nisin for 90 min. Equal amounts of total protein were subjected to automated Western blot

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analysis and probed with α-Cis1a, α-Cis2 and α-WhiA polyclonal antibodies. Analysis was

955

performed in biological triplicate experiments. Cis1a/2 protein levels were normalized to WhiA

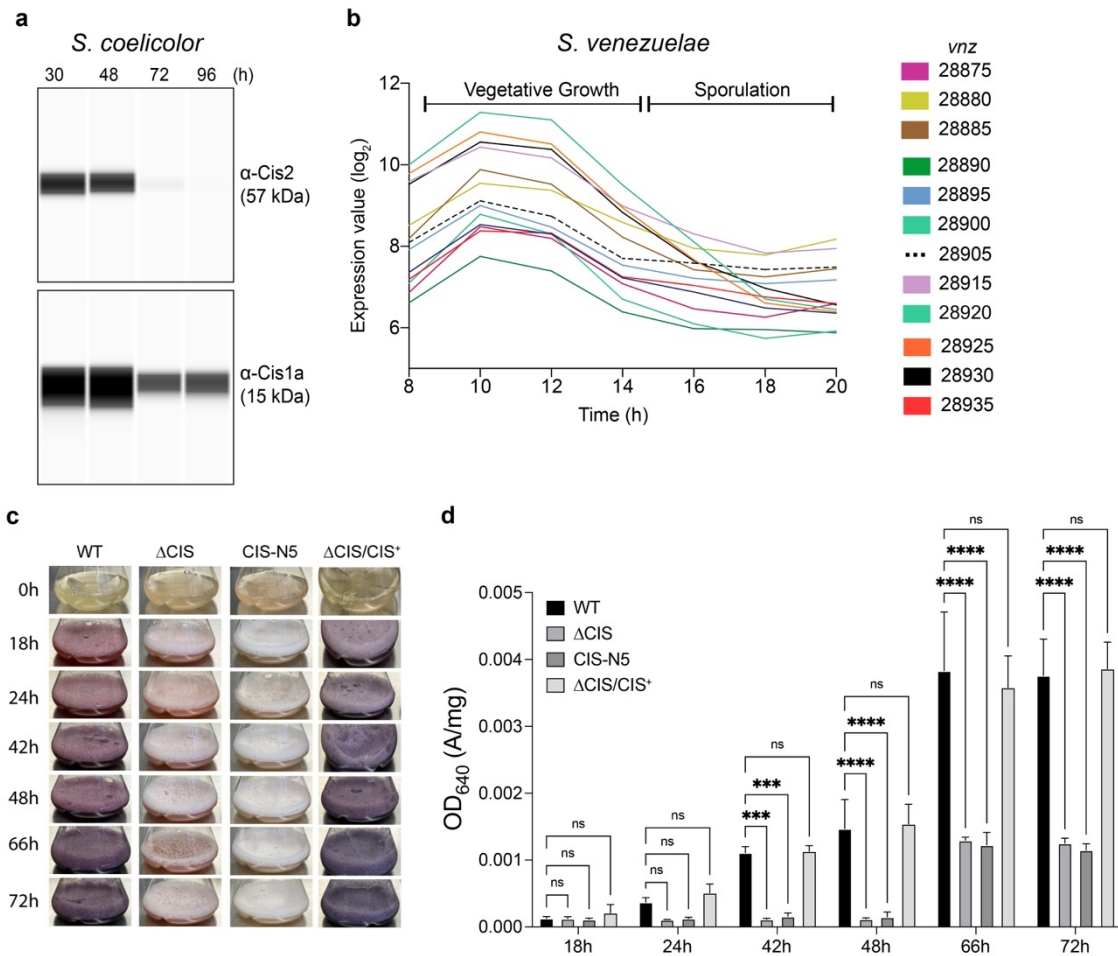
956

levels. Shown are the mean values and standard error. ns (not significant) and *p*-value was

957

determined using a two-tailed *t*-test.

958



959

960

961 **Extended Data Figure 9: *Streptomyces* CIS proteins are expressed during vegetative**
 962 **growth and impact secondary metabolite production.**

963 **a.** Automated Western blot showing the expression of Cis1a (inner tube) and Cis2 (sheath) in
 964 hyphae of WT *S. coelicolor* over a time-course of 96 h. *S. coelicolor* was grown on cellophane
 965 discs on top of R2YE agar. Automated Western blot analysis was performed in biological
 966 duplicate experiments. Equal amounts of protein lysate were loaded and Cis1a and Cis2 were
 967 detected using polyclonal α -Cis1a and α -Cis2 antibodies.

968 **b.** Transcription profile of the *S. venezuelae* CIS gene cluster over the entire life cycle³².

969 **c.** Comparison of the coloration pattern of *S. coelicolor* WT, the CIS^{Sc} mutant strains Δ CIS,
 970 CIS-N5 and the complemented mutant Δ CIS/CIS⁺ in R2YE liquid media. Coloration is
 971 indicative of actinorhodin (purple) and undecylprodigiosin (red) production³⁴. Note the
 972 difference in coloration between WT/complementation mutant as compared to both CIS^{Sc}
 973 mutants. Images of each culture flask were taken at the indicated time points.

974 **d.** Quantification of total actinorhodin production of the samples shown in (c). The optical
 975 density OD₆₄₀ is an indicator for actinorhodin production⁶⁴. OD₆₄₀ of the culture supernatants

976 was measured and normalized to pellet weight. Note the significant differences that were
977 detected between WT/complementation mutant as compared to both CIS mutants at later time
978 points. Bar plots and error bars represent three biological replicates. p-values (**p < 0.001
979 and ****p < 0.0001) were calculated using one-way ANOVA and Tukey's post-test. ns, not
980 significant.
981

982 **Supplementary Movie 1.** Time-lapse movie related to Figure 2g/h showing the spatiotemporal
983 localization of fluorescently tagged CIS particles in growing *S. coelicolor* hyphae. Images were
984 acquired every 5 min. Bar, 10 μ m.

985

986 **Supplementary Table 1.** List of peptides detected by mass spectrometry in samples of purified
 987 CIS from WT *S. coelicolor* (ScoWT) and *S. venezuelae* (SvenWT), the corresponding Δ Cis2
 988 mutants and the *S. coelicolor* non-contractile CIS^{Sc} mutant CIS-N5. Experiments were
 989 performed in biological replicates.

Protein ID	CIS ID	<i>S. coelicolor</i> WT	<i>S. coelicolor</i> Δ cis2	<i>S. coelicolor</i> CIS-N5	<i>S. venezuelae</i> WT	<i>S. venezuelae</i> Δ cis2
Sco4244/Vnz_28875	Cis11	-	-	29% coverage / 10 total unique peptide	-	-
Sco4245/Vnz_28880	Cis9	-	-	21% coverage / 2 total unique peptide	-	-
Vnz_28885	Cis10	-	-	-	-	-
Sco4246/Vnz_28890	Cis8	-	-	46% coverage / 18 total unique peptide	-	-
Sco4247/Vnz_28895	Cis7	-	-	51% coverage / 8 total unique peptide	-	-
Sco4248/Vnz_28900	Cis5	-	-	39% coverage / 4 total unique peptide	-	-
Sco4251/Vnz_28915	-	-	-	-	-	-
Sco4252/Vnz_28920	Cis1	27% coverage / 4 total unique peptide	-	32% coverage / 5 total unique peptide	17% coverage / 3 total unique peptide	-
Sco4253/Vnz_28925	Cis2	48% coverage / 26 total unique peptide	-	51% coverage / 28 total unique peptide	38% coverage / 17 total unique peptide	-
Sco4254	-	-	-	10% coverage / 6 total unique peptide	-	-
Sco4259/Vnz_28930	Cis15	-	-	-	-	-
Sco4260/Vnz_28935	Cis16	-	-	24% coverage / 4 total unique peptide	-	-

990

991

992 **Supplementary Table 2.** Experimental approaches to study effects of CIS^{Sc} on interspecies
 993 competition
 994

Target organisms	Functional assay	Procedures
<i>Saccharomyces cerevisiae</i> <i>Escherichia coli</i> <i>Bacillus subtilis</i> <i>Micrococcus luteus</i> <i>S. venezuelae</i>	Killing assay on plate or in liquid	Co-incubation with <i>S. coelicolor</i> wild-type, Δ CIS ^{Sc} and CIS-N5 mutant strains
		Co-incubation with purified CIS ^{Sc} particles
<i>Lactococcus lactis</i> (Nisin producer)	Killing assay on plate	Co-incubation with nisin-treated <i>S. coelicolor</i> wild-type, Δ CIS ^{Sc} and CIS-N5 mutant strains
Wax moth larvae	Injection into larvae gut	Injection of purified CIS ^{Sc} particles from <i>S. coelicolor</i> wild-type, Δ CIS ^{Sc} and CIS-N5 mutant strains

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997 **Supplementary Table 3:** Bacterial strains, plasmids and cosmids used in this study.

Strain	Description	Construction	Source
<i>Escherichia coli</i> strains			
TOP10	<i>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (Str^R) endA1 nupG</i>	Cloning	Invitrogen
ET12567/pUZ8002	<i>F⁻ dam13::Tn9 dcm6 hsdM hsdR recF143::Tn10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL hisG4 tsx-78 mtl-1 glnV44</i>	ET12567 with helper plasmid pUZ8002	¹
BW25113/pIJ790	<i>Δ(araD-araB)567 ΔlacZ4787(::rrnB-4) lacIp-4000(lacIQ), λ-rpoS369(Am) rph-1 Δ(rhaD-rhaB)568 hsdR514</i>	BW25113 containing λ RED recombination plasmid pIJ790	²
<i>Streptomyces</i> strains			
<i>S. venezuelae</i> NRRL B-65442	Wild Type (Sv-WT)		³
<i>S. coelicolor</i> M145	Wild Type (Sc-WT) SCP1 ⁻ SCP2 ⁻ derivative from <i>S. coelicolor</i> A3(2)		⁴
SS381	<i>Sv-WT Δvnz_28920::apr</i>	chromosomal <i>vnz_28920</i> (<i>cis2</i>) locus deleted using pSS489	This study
SS383	<i>Sc-WT Δsco4253::apr</i>	chromosomal <i>sco4253</i> (<i>cis2</i>) locus deleted using pSS480	This study

SS387	<i>Sc-WT Δsco4253-4251::apr</i>	chromosomal <i>sco4253-4251</i> locus deleted using pSS480	This study
SS389	<i>Sc-WT Δsco4253::apr attB ΦBT1 Sco4253-I274-ypet_Sco4252-51, hyg^R</i>	pSS501 integrated at ϕ BT1 attachment site of SS383	This study
SS392	<i>Sc-WT Δsco4253-51::apr attB ΦBT1 sco4253-N3-sco4252-51, hyg^R</i>	pSS503 integrated at ϕ BT1 attachment site of SS387	This study
SS393	<i>Sc-WT Δsco4253-51::apr attB ΦBT1 sco4253-N5-sco4252-51, hyg^R</i>	pSS504 integrated at ϕ BT1 attachment site of SS387	This study
SS394	<i>Sc-WT Δsco4253-51::apr attB ΦBT1 sco4253-N2-sco4252-51, hyg^R</i>	pSS505 integrated at ϕ BT1 attachment site of SS387	This study
SS395	<i>Sc-WT Δsco4253-51::apr attB ΦBT1 sco4253-51, hyg^R</i>	pSS500 integrated at ϕ BT1 attachment site of SS387	This study
SS430	<i>Sc-WT ΦBT1 P_{ermE*}-sfgfp, hyg^R</i>	pSS150 integrated at ϕ BT1 attachment site of Sc-WT	This study
SS431	<i>Sc-WT Δsco4253::apr attB ΦBT1 P_{ermE*}-sfgfp, hyg^R</i>	pSS150 integrated at ϕ BT1 attachment site of SS383	This study
SS459	<i>Sc-WT Δsco4253-51::apr attB ΦBT1 sco4253-N5-sco4252-51, P_{ermE*}-sfgfp, hyg^R</i>	pSS610 integrated at ϕ BT1 attachment site of SS387	This study
SS484	<i>Sc-WT ΦBT1 P_{cis2}-ypet, hyg^R</i>	pSS619 integrated at ϕ BT1 attachment site of Sc-WT	This study
Plasmids			

pIJ773	pBluescript KS (+) containing the apramycin resistance gene <i>apr</i> and <i>oriT</i> of plasmid RP4, flanked by FRT sites (Apr^R). Used as template for the amplification of the <i>apr-oriT</i> cassette for 'REDIRECT' PCR targeting, Apr^R _		5
pIJ10257	Cloning vector for the conjugal transfer of DNA (under control of the <i>ermE</i> * constitutive promoter). Integrates at the $\Phi BT1$ attachment site, Hyg^R		6
pIJ10770	Cloning vector for the conjugal transfer of DNA from <i>E. coli</i> to <i>Streptomyces</i> spp. Integrates at the $\Phi BT1$ attachment site. Hyg^R		4
pIJ10772	Modified pIJ10770, carries <i>mcherry</i> for construction of C-terminal fluorescent gene fusion. Integrates at the $\Phi BT1$ attachment site, Hyg^R		4
pUC19	<i>E. coli</i> multicopy cloning vector, $Carb^R$		7
pIJ12738	Derivative of pGM1190, an intermediate copy number, conjugative plasmid containing the temperature-	Used as intermediated cloning vector	8

	sensitive replication origin of pSG5, Apr ^R		
pIJ10773	Modified pIJ10770, carries <i>ypet</i> for construction of C-terminal fluorescent gene fusion. Integrates at the Φ BT1 attachment site, Hyg ^R	Codon-optimised <i>ypet</i> was PCR amplified with primer 34/4b followed by restriction digestion with XhoI/KpnI and ligation into pIJ10770 cut with XhoI/KpnI	This study
pSS150	pIJ10257 carrying <i>P_{ermE*}-sfgfp</i> , Hyg ^R	Codon-optimised <i>sfgfp</i> was PCR amplified with primer 268/269 followed by restriction digestion with NdeI/XhoI and ligation into pIJ10257 cut with NdeI/XhoI	This study
pSS480	Mutated cosmid StD-49 for REDIRECT containing Δ <i>sco4253::apr</i> , Km ^R , Carb ^R , Apr ^R	The <i>sco4253</i> coding sequence on the cosmid vector StD-49 was replaced by an oriT-containing apramycin resistance cassette, which was amplified from pIJ773 using primer 1037/1038.	This study
pSS481	Mutated cosmid StD-49 for REDIRECT containing Δ <i>sco4253-4251::apr</i> , Km ^R , Carb ^R , Apr ^R	The <i>sco4253-51</i> coding sequence on the cosmid vector StD-49 was replaced by an oriT-containing apramycin resistance cassette, which was amplified from	This study

		pIJ773 using primer 1037/1039.	
pSS489	Mutated cosmid P11-F14 for REDIRECT containing Δ <i>vnz28920::apr</i> , Km ^R , Carb ^R , Apr ^R	The <i>vnz28920</i> coding sequence on the cosmid vector P11-F14 was replaced by an oriT-containing apramycin resistance cassette, which was amplified from pIJ773 using primer 1048/1049.	This study
pSS494	pIJ12738 carrying <i>sco4253::2IIE</i> (CIS ^{Sc} -N2), Apr ^R	Insertion of "IE" at amino acid position 21 in Sco4253. Plasmid was generated via Gibson Assembly from PCR fragments generated using genomic DNA and primer 1057/1058 and 1059/1060 and pIJ12738 cut with HindIII	This study
pSS495	pIJ12738 carrying <i>sco4253::2IEG</i> (CIS ^{Sc} -N3), Apr ^R	Insertion of "IEG" at amino acid position 21 in Sco4253 Plasmid was generated via Gibson Assembly from PCR fragments generated using genomic DNA primer 1061/1057 and 1059/1060 and pIJ12738 cut with HindIII	This study

pSS496	pIJ12738 carrying <i>sco4253::2IEGVG</i> (CIS ^{Sc} -N5), Carb ^R	Insertion of " IEGVG " at amino acid position 21 in <i>Sco4253</i> . Plasmid was generated via Gibson Assembly from PCR fragments generated using genomic DNA primer 1057/1062 and 1063/1060 and pIJ12738 cut with HindIII	This study
pSS497	pUC19 carrying <i>sco4253-51</i> , Carb ^R	Amplification of <i>sco4253-4251</i> from genomic DNA with primer 1091/1092 followed by Gibson Assembly into pUC19 cut with HindIII/EcoRI	This study
pSS498	pSS497 carrying <i>sco4253::ypet(I274)-Sco4252-51</i> , Carb ^R	Insertion of <i>ypet</i> with linker after AA I274 in <i>sco4253</i> . pSS497 was amplified with primer 1075/1078, <i>ypet with 7AA linker</i> was amplified with primer 1076/1077, both fragments were combined using Gibson Assembly	This study
pSS500	pIJ10770 carrying <i>sco4253-51</i> , Hyg ^R	<i>Sco4253-4251</i> was PCR amplified with primer 1042/1101, digested with HindIII/NdeI and ligated into pIJ10770 cut with HindIII/NdeI	This study

pSS501	<i>pIJ10770</i> carrying <i>sco4253::ypet(I274)-sco4252-51</i> , Hyg ^R	<i>sco4253::ypet(I274)-Sco4252-51</i> was PCR amplified with primers 1042/1101 from pSS498, digested with HindIII/NdeI and ligated into pIJ10770 cut with HindIII/NdeI	This study
pSS503	<i>pIJ10770</i> carrying <i>CIS^{Sc}-N3 (sco4253::2IEG-sco4252-51)</i> , Hyg ^R	Fragment 1: <i>sco4253-N3-4251</i> from pSS495 was PCR amplified with primer 1042/1043 and digested with HindIII/NruI; Fragment 2: <i>sco4251-53</i> was PCR amplified from pSS494 with primer 1042/1102 and digested with NruI/AvrII; Triple ligation of both fragments with pIJ10770 cut with HindIII/AvrII	This study
pSS504	<i>pIJ10770</i> carrying <i>CIS^{Sc}-N5 (sco4253::2IEGVG-sco4252-51)</i> , Hyg ^R	fragment 1: <i>sco4253-N5-4251</i> from pSS496 was PCR amplified with primer 1042/1043 and digested with HindIII/NruI; Fragment 2: <i>sco4251-53</i> was PCR amplified from pSS494 with primer 1042/1102 and digested with NruI/AvrII; Triple	This study

		ligation of both fragments with pIJ10770 cut with HindIII/AvrII	
pSS505	<i>pIJ10770</i> carrying <i>CIS^{Sc}-N2</i> (<i>sco4253::21IE-sco4252-51</i>), Hyg ^R	Fragment 1: <i>sco4253-N2-4251</i> from pSS494 was PCR amplified with primer 1042/1043 and cut with HindIII/NruI; Fragment 2: <i>sco4251-53</i> was PCR amplified from pSS494 with primer 1042/1102 and cut with NruI/AvrII; Triple ligation of both fragments into pIJ10770 cut with HindIII/AvrII	This study
pSS610	pSS504 with <i>P_{ermE*}-sfgfp</i> , Hyg ^R	<i>P_{ermE*}-sfgfp</i> fragment was isolated from pSS150 by restriction digestion with Bsu361/AvrII and ligated between the Bsu361/AvrII site of pSS504	This study
pSS619	pIJ10773 with <i>P_{str2}-ypet</i> , Hyg ^R	<i>sco4253</i> promoter region (<i>P_{str2}</i>) was PCR amplified from genomic DNA with primer 1403/1404 and cloned into pIJ10773 cut with NdeI/XhoI using Gibson Assembly	This study
Cosmids			
StD-49	Cosmid vector containing coding sequence for <i>S</i> .		http://strepdb .

	<i>coelicolor</i> CIS gene cluster, Km ^R , Carb ^R		streptomyces.org.uk
P11-F14	Cosmid vector containing coding sequence for <i>S.</i> <i>venezuelae</i> CIS gene cluster, Km ^R , Carb ^R		http://strepdb. streptomyces.org.uk

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999 **Supplementary Table 3 References**

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1024 **Supplementary Table 4: Oligonucleotides used in this study.**

Name	Sequence (5' → 3')
4b	GGGGTACCTCACTTGTACAGCTCGTTCATG
34	ATACTCGAGATGGTCTCCAAGGGCGAGG
268	TTAATTAACATATGTCCCGCCTGAAGCCGCGCTGCACCTCCCTGGAGATGTCCAAGGGCGAGGAGCTGTTC
269	ATATACTCGAGCTCCGGGCCCAGCTCCGGGCCCAGCTTGTACAGCTCGTCCATGCCGTG
1037	GAGCAGAGCATGCCGTCTACCTGTGCGCCCGGCGTCTACATTCCGGGGATCCGTCGACC
1038	GCGATCCGCCTACTCGTCCAGTTCGCCGCCGCGCTGGATGTAGGCTGGAGCTGCTTC
1039	TCGCGTACGTCACGATTCCCCAGGCGGCTCCCGCCGAGTGTAGGCTGGAGCTGCTTC
1042	ATTAAAGCTTCCCTCCTGACACGCCGTCACC
1043	AATTAATTCATATGCTCGTCCAGTTCGCCGCCGC
1048	GGAGCGAGCATGCCGACGTACCTACCCCGGGCGTGTACATTCCGGGGATCCGTCGACC
1049	ACGTACGCAGTTCACGCCGATCGGGTTGAGCAGGTCCTGTGTAGGCTGGAGCTGCTTC
1050	CGGCGTCCGTCAGCCGCGCTGGAAGAACTCCACCTCCGCTGTAGGCTGGAGCTGCTTC
1057	CACGACGTTGTAACGACGGCCAGTGCCAAGTGTGTCGTGCGCCGTCCCGTGGTC
1058	GGCCGCCACCGACGTGCCACTCCCTCGATCTCGATCGGGCGCGAGCCGCTGGCCA
1059	ATCGAGGGAGTGGGCACGTC
1060	GCGGATCCTCTAGAGTCGACCTGCAGCCCAAGTTCTTTCGAACACGATGGTGATGG
1061	GGCCGCCACCGACGTGCCACTCCCTCGATGCCCTCGATCGGGCGCGAGCCGCTGGCCA
1062	GCCCACTCCCTCGATGCCGACGCCCTCGATCGGGCGCGAGCCGCTGGCCA
1063	ATCGAGGGCGTCGGCATCGAGGGAGTGGGCACGTC
1075	CCGAGCCTTCGAGGATCGCGCCGCGCTGGTAGG
1076	CGCGGCGCGATCCTCGAAGGCTCGGGGCAGGGG
1077	GGCCTCCAGGTCGCTCCCCTGGCCGGAGCCCG
1078	CGGCCAGGGGAGCGACCTGGAGGCCGTCAAAGC
1091	TGACCATGATTACGCCAAGCTTCCCTCCTGACACGCCGTCAC
1092	AAACGACGGCCAGTGAATTCGCGATCTCCTCGTGCAGCC
1101	AATTAATTCATATGCGCGATCTCCTCGTGCAGCC
1403	TGATAAGTTTATCAAGCTTAGATTCTCATATGGTTCAAGCGGTCCGACACG
1404	GTGAACAGCTCCTCGCCCTTGAGACCATCTCGAGCTCTCCTCGGGGTACGAGACAG

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