A contractile injection system mediates developmentally regulated intra-strain cell death in *Streptomyces coelicolor*

Maria Vladimirov¹, Ruo Zhang², Stefanie Mak¹, Justin R. Nodwell¹, and Alan R. Davidson¹²

¹Department of Biochemistry, ²Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada
Abstract

Extracellular contractile injection systems (eCIS) are encoded in diverse clades of bacterial species. Although closely related to contractile phage tails, these entities can inject toxic proteins into eukaryotic cells. The roles of eCIS in mediating cytotoxic activities has led to a view of them as defense mechanisms that are not central to normal bacterial lifecycles. Here, we provide evidence that eCIS play an entirely distinct role in Streptomyces coelicolor (Sco), where they appear to participate in the complex developmental process of this species. In particular, we have shown that Sco produces eCIS particles as a part of its normal growth cycle and that strains lacking functional eCIS particles exhibit pronounced alterations in their developmental program. Most intriguingly, eCIS-deficient mutants display significantly reduced levels of cell death and altered morphology during liquid growth. These data suggest that Sco eCIS function by inducing intra-strain lethality rather than by attacking foreign species.
Introduction

Many strains of bacteria encode extracellular contractile injection systems (eCIS)\(^1\)-\(^3\). eCIS are closely related to contractile-tailed bacteriophage (phage) tails. Like tails, they are composed of a long tube attached to a baseplate (Fig. 1f). The proteins comprising the eCIS tail-like structure are similar in sequence to phage proteins. However, genomic regions encoding eCIS are distinguished from phage tail regions by invariably encoding an AAA+ ATPase of unknown function and a tail terminator protein (Tr) that is a member of the Pvc16_N (PF14065) Pfam family\(^4\)-\(^5\). They also often encode recognizable toxin proteins\(^2\),\(^6\),\(^7\). All of the eCIS with characterized functions mediate interactions between bacteria and eukaryotic cells. *Serratia* and *Photorhabdus* species release eCIS structures, known as Anti-feeding prophages (Afp) and *Photorhabdus Virulence Cassettes* (PVC), respectively, that mediate killing of insect cells\(^8\),\(^9\). This killing occurs through the injection of insecticidal toxins encoded downstream of the eCIS operon\(^8\)-\(^10\). MACs (Metamorphosis Associated Contractile structures) are a distinct eCIS produced by the marine bacterium, *Pseudoalteromonas luteoviolacea*, that are required for the morphogenesis of the tubeworm *Hydroides elegans* in a mutualistic interaction\(^11\). The structures of several other eCIS have been examined in detail\(^12\)-\(^14\), but their functions have not been determined.

eCIS regions have clearly been disseminated through horizontal gene transfer and this has resulted in their appearing sporadically across diverse bacterial clades\(^2\),\(^3\). A striking exception to this pattern is seen in the *Streptomyces* genus where the majority of species (>70%) encode at least one eCIS. *Streptomyces* propagate through a complex developmental program. Germinated spores initially grow to form extensive vegetative hyphae by a combination of tip extension and branching\(^15\). Upon nutrient deprivation or stress, programmed developmental and morphological changes result in formation of aerial hyphae and subsequent formation of spores. This “metabolic switch” is accompanied by a large shift in gene expression and the onset of secondary metabolite production, many of which are antibiotics\(^16\),\(^17\).

Given the uniquely complex lifestyle of *Streptomyces* and the prevalence of eCIS encoding regions in this genus, we hypothesized that eCIS may play a fundamental role in the developmental program of these bacteria. To test this idea, we initiated studies on the eCIS
encoding region of the model strain *Streptomyces coelicolor* A3(2) (*Sco*), which has a single eCIS-encoding region. The goals of these experiments were to determine whether eCIS are produced as part of the normal *Sco* lifecycle and what role these little characterized entities might be playing.

**Results**

**eCIS tail-like structures can be purified from *Sco* lysates**

To determine whether wild-type (WT) *Sco* produces eCIS particles, cell lysates of *Sco* cultures grown in liquid medium for 72 hr were subjected to a standard eCIS purification protocol (see Methods). Examination of the resulting purified samples using transmission electron microscopy (TEM) revealed abundant phage tail-like particles with average lengths of 300 nm and widths of 21 nm (Fig. 1a-b, 1d). These particles closely resemble previously observed eCIS particles with a rounded cap on one end and a baseplate on the other as indicated by arrows (Fig. 1b)\(^5,18,19\). The baseplate is somewhat smaller than other eCIS and fibers, which are attached to the baseplates of some eCIS\(^5,11,20\), were not detectable. The purified samples also contained many empty sheath structures of varying length (Fig. 1a, white arrowheads).

Strikingly, TEM micrographs of the extracellular fraction (supernatant collected after cell pelleting) revealed only what appeared to be emptied out sheaths left behind after eCIS contraction. These structures were 100-150 nm long and 28-35 nm wide with a typical striation pattern characteristic of polymerized tail sheath, but they had no density in the center and were wider than mature wild-type eCIS tails (Fig. 1c,d). Tail sheath (TS) protein was also detected in the extracellular (E) fraction by Western blots (Supplementary Fig. 1a,b). The strictly cytoplasmic protein, ActR, was not detected in the extracellular fraction (Supplementary Fig. 1b), suggesting that the appearance of TS protein in this fraction was not due to generalized cell lysis. *Sco* strains bearing deletions in the genes encoding either the tail sheath (ΔTS) or the baseplate wedge proteins (ΔBP) (Fig. 1e) displayed no tail-like structures in extracellular samples or cellular lysates. Western blots confirmed the absence of these proteins from the mutant strain preparations (Supplementary Fig. 1 a,c).
The presence of a gene encoding an AAA+ ATPase is a universal feature of eCIS genomic regions, yet no function had been ascribed to this enzyme. To investigate this issue, we generated a deletion in the Sco ATPase gene (sco4259) and performed TEM experiments as described above on lysates of this mutant. The eCIS particles produced appeared identical to those produced by the WT strain (Supplementary Fig. 2a). Similar levels of extracellular empty sheaths were also observed (Supplementary Fig. 2b,c). These results imply that the ATPase is not required for the production of eCIS-like particles with normal appearance. This finding agrees with work on the Photobacterium eCIS, which also showed that normal looking eCIS particles are formed in the absence of the ATPase.

We used mass spectrometry to determine the composition the purified tail-like particles produced by Sco. As expected, most of the eCIS structural proteins (Fig. 1e) that have phage tail homologues were detected in all three purifications (TR, TS, TT, BH2, BW2) while others of these conserved structural proteins (BH1 and BW1) were detected in at least one of three separate purifications performed (Supplementary Tables 1 and 2). BW3 was the only conserved structural protein that was not detected in any purifications. Two proteins of unknown function, encoded by genes sco4242 and sco4256, were detected in all three purifications, and one other, encoded by sco4251, was detected in at least one purification. The AAA+ ATPase was not detected in purified eCIS particles. We also performed mass spectrometry on eCIS-related particles purified from the extracellular fraction. In this case we detected only TS and TT proteins, which are the likely components of the empty contracted sheaths observed by TEM. Importantly, purified preparations produced from the ΔBP mutant contained only TT and TS proteins, demonstrating that the detection of most eCIS proteins requires the proper eCIS assembly, which cannot occur in the absence of the baseplate wedge proteins. Aggregates of TS and TT protein may still be purified by high-speed centrifugation. Overall, these mass spectrometry experiments show that the tail-like particles that we have purified are indeed produced from the eCIS region of Sco and that the composition of these particles is the same as other eCIS.

**eCIS do not contribute to killing of other species by Sco**

A previous study implicated the eCIS of Streptomyces lividans as being responsible for a growth inhibitory activity against the yeast, Saccharomyces cerevisiae. Since the components of S.
E. coli eCIS are very similar in sequence to those of the Sco system, it was of interest to determine whether the Sco eCIS also displayed this activity. As shown in Fig. 2, we found that WT Sco did indeed inhibit the growth of S. cerevisiae and that this activity was not seen for the ΔTS strain. As Sco is known to produce several antibiotics that might contribute to the observed growth inhibition, we tested the ability of Sco strain M1152, in which genomic regions required for synthesis of the four major Sco antibiotics are deleted (∆act, ∆red, ∆cpk), to inhibit yeast growth. We confirmed that this strain still produces eCIS-related particles (Supplementary Fig. 3a). We found that neither strain M1152 nor a mutant version of this strain that does not produce eCIS (M1152ΔTS) inhibited yeast growth (Fig. 2). These results imply that antibiotics are required for the growth inhibition of S. cerevisiae and that the absence of eCIS may indirectly affect the inhibitory activity by perturbing antibiotic production.

Similar to our results with S. cerevisiae, we found that Sco inhibited growth of the Gram-positive bacterial species, Bacillus subtilis, and Micrococcus luteus, and that this inhibition was partially attenuated in the ΔTS strain (Fig. 2). However, strain M1152 and the ΔTS mutant lacked this activity. Finally, we found that Sco inhibited the growth of four different Streptomyces species, but the ΔTS strain displayed the same level of inhibition, implying that the eCIS was not required for the growth inhibition (Supplementary Fig. 3b). In summary, these growth inhibition experiments demonstrate that Sco does inhibit the growth of some bacterial species and S. cerevisiae, but there does not appear to be a direct role for the eCIS in this activity.

**ECIS mutants exhibit decreased hyphal death and reduced pellet size during differentiation in liquid cultures**

Given the apparent lack of killing of other species by the Sco eCIS, we postulated that eCIS might mediate an intra-strain cell killing activity during the normal developmental program. To visualize and quantify levels of cell death during Sco differentiation, we used a well-established bacterial viability assay. Samples are stained with SYTO 9, which labels both live and dead cells, and with propidium iodide (PI), which penetrates and stains only bacteria with damaged membranes, which are likely dead. In the presence of both stains, live bacteria appear green whereas dead bacteria appear red. After 48 hr of growth in liquid media, WT Sco grew mycelial pellets as has been previously observed. The average diameter of these pellets was 85 µm (Fig. 3a). The same size pellets were seen in the eCIS-deficient strains. By quantitating the...
staining by SYTO9 and PI, we calculated the ratio of live to dead cells in the WT cultures to be 0.8 (Fig. 3a). The live/dead ratios were slightly less in the mutant cultures though the difference was not significant. Differences between the WT and the eCIS-deficient strains became evident at the 54 and 74 hr of growth points. WT samples developed many large mycelial pellets ranging from 100 to 300 µm in diameter that displayed increased PI staining in the center (Fig. 3b,c). By contrast, in both eCIS-deficient mutants, the preponderance of observed hyphal pellets remained small with the average pellet sizes being half that of WT Sco. In addition, the live/dead cell ratios of the mutants were double those of WT (Fig. 3b,c). Overall, these data show that the development of WT Sco in liquid media is accompanied by formation of large mycelial pellets and considerable levels of cell death. Mutants lacking eCIS particles significantly deviate from this behavior displaying smaller cell pellets and a much lower frequency of cell death.

To address the role of the AAA+ ATPase protein in eCIS function we performed the same experiment as described above using the ∆ATPase Sco mutant strain. Although this strain produces eCIS particles with a normal appearance, the mycelial pellets produced by this strain after 54 hr of growth in liquid media resembled those produced by the eCIS-deficient mutant strains. These pellets were approximately half the size of the WT pellets, and they displayed half the frequency of cell death (Supplementary Fig. 4b). This trend continued at the 74 hr time point (Supplementary Fig. 4c). As with the other eCIS-deficient mutants, the mycelial pellets of the ∆ATPase strain resembled WT after 48 hr of growth (Supplementary Fig. 4a). These data demonstrate that the presence of tail-like particles alone does not mediate cell death and that the ATPase performs a function that is required for the biological activity of the eCIS particles.

**Sco strains lacking eCIS display altered development**

*Sco* follows a developmental program characterized by a shift from formation of vegetative hyphae to aerial hyphae, which is followed by spore formation. To determine whether the lack of eCIS might perturb this program, we compared the growth of the WT and ∆TS strains on solid media. At early stages of growth, visual examination of the agar plates did not reveal any significant differences, with all strains producing similar vegetative lawns. However, after 48 hours, eCIS mutants had visibly developed faster in terms of producing the pigmented secondary metabolite, actinorhodin (Figure 4a, top panel). At later time points, any visible apparent differences disappeared, and by 72 hours all strains had developed comparable lawns of aerial
mycelia (Figure 4a, bottom panel). In liquid media, we observed that WT and eCIS-deficient strains exhibited similar growth rates (Supplementary Fig. 5).

It should be noted that the production of actinorhodin by eCIS-deficient strains was highly variable depending on the media used. In some conditions of liquid or solid media growth, these mutants produced considerably less pigment than WT (Supplementary Fig. 6a,b,c). Since the regulation of actinorhodin production is very complicated\textsuperscript{26-30}, it is difficult to rationalize these variable effects. However, the perturbation of secondary metabolite production in the absence of eCIS is consistent with a role for it in the developmental program.

We used a microscopy approach to gain a clearer assessment of aerial hyphae formation at intermediate time points in the developmental program. We applied glass cover slips to the surface of the growing bacterial lawn. Since the surface of the cover slips is hydrophobic, only aerial hyphae, which have a hydrophobic coating, stick to them. Adhered hyphae were then detected through microscopic examination. No cells were seen on cover slips that had been applied to the surface of lawns in any of the samples up to 35 hr post-germination. At 42 hr, the WT strains had yet to produce any visible aerial hyphae, while the ΔTS and ΔBP mutant strains showed well-developed aerial hyphal strands clustered in numerous regions on the microscope slide (Fig. 4b). This trend continued at 55 hr of growth where the WT strains had developed a few dispersed hyphal strands compared to the eCIS-deficient mutants, which displayed larger, more developed clusters of hyphae (Fig. 4b).

**eCIS-encoding genes are developmentally regulated**

Since the absence of eCIS appeared to perturb the $Sco$ developmental program, we wondered if the eCIS-encoding genes were expressed in a developmentally regulated pattern. To this end, we fused four known eCIS region promoters (Fig. 5a; Supplementary Fig. 7), to plasmid-borne genes encoding luciferase activity ($luxCDABE$)$^{31}$. Introduction of these plasmids into WT $Sco$ revealed that all four eCIS promoters were active and displayed the same temporal pattern of expression. This pattern was similar to the promoter of the major sigma factor gene $hrdB$\textsuperscript{32}, which acts during vegetative growth and to the promoter of $redD$. This gene encodes the transcriptional regulator of the $red$ biosynthetic cluster, which is transcribed at early stages of the metabolic switch\textsuperscript{33,34}. These results show that the eCIS operons are expressed during the
vegetative growth phase under normal growth conditions, right before the metabolic switch that leads to aerial hyphae formation and sporulation.

Our observed transcriptional pattern for the eCIS promoters is consistent with previous observations that eCIS expression is regulated by bldA. This gene is required for proper morphological development, secondary metabolism, and sporulation. It encodes the only tRNA for the rare leucine codon, TTA, present in 2-3% of Streptomyces genes. The production of the eCIS transcriptional regulator encoded by sco4263 is dependent on BldA as it contains a TTA codon. We analyzed 56 Streptomyces eCIS regions and found that 42 contained at least one gene containing an in-frame TTA codon. Of the identified TTA-containing genes, 22 are putative eCIS regulatory genes (Supplementary Data Sheets 1 and 2). These findings indicate that eCIS regions are often regulated by bldA, which ensures that eCIS particles will accumulate in conjunction with the morphogenetic transition.

Identification of a putative toxin and fiber for Streptomyces eCIS

To address the question of whether eCIS may perform a shared function among many Streptomyces species, we sought to identify conserved features particular to Streptomyces eCIS. To this end, we searched publicly available Streptomyces genomes and identified 153 eCIS-encoding regions within 127 Streptomyces genomes (Supplementary Data Sheet 3). eCIS have been classified previously into 6 distinct subtypes based on sequence similarity and gene arrangement. The majority (> 80%) of Streptomyces species including Sco encode a type IIId eCIS, as has been previously noted; thus, we assume that any conserved eCIS function must be performed by this type.

Most eCIS regions encode proteins with domains associated with toxin activity. These proteins are thought to be assembled into the eCIS particle and injected into target cells to mediate cell killing. We were not able to identify a protein encoded frequently within Streptomyces eCIS regions that contained an identifiable toxin domain. However, a BLAST search with the Sco4256 protein, which we found to be a component of the Sco eCIS particle (Supplementary Table 1), revealed closely related proteins encoded in 90% of the 105 Streptomyces class IIId eCIS (Supplementary Data Sheet 4). Alignment of a representative group of sequences and structural analysis using HHpred suggested a three domain architecture with the second domain
predicted to be a transmembrane helical region (Fig. 6a, Supplementary Data Sheet 5). The first
domain, present across all homologues, shows high probability similarities to periplasmic
chaperones involved in pilus assembly among other structures and families (Supplementary Data
Sheet 5). Approximately one third of homologues, including the one from Sco, possess a C-
terminal third domain with similarity to various bacterial toxins and hemolytic lectins (Fig. 6b;
Supplementary Data Sheet 5). These proteins may be delivered by eCIS as toxic cargo.
Interestingly, homologues of these proteins were also identified in class IId eCIS regions in
genomes of other Actinobacterial species and several species of filamentous Cyanobacteria and
Chloroflexi (Fig. 6a; Supplementary Data Sheet 4). Proteins with highly significant similarity to
Sco4256 were rarely found in non-eCIS encoding regions.
Structurally characterized eCIS\textsuperscript{5,13,19} and contractile phage tails\textsuperscript{1} have fiber proteins attached to
their baseplates. In phages, these fibers mediate host cell attachment. In most contractile-tailed
phage genomes and in the genomes of characterized eCIS\textsuperscript{8,11,13,14}, fiber proteins are encoded
immediately downstream of genes encoding the baseplate wedge proteins, BW2 and BW3. In the
Sco eCIS region, the protein product of the gene in this position (sco4242) (Fig. 5a) was found to
be a component of the eCIS particle (Supplementary Table 1). A BLAST search with this protein
revealed closely related homologues in 75\% of class IId eCIS regions in Streptomyces, and in the
class IId eCIS of other Actinomycete species (Fig. 6c; Supplementary Data Sheet 6). Alignment
of these proteins revealed a conserved C-terminal region approximately 150 residues long, which
is preceded by a strongly predicted transmembrane motif (Fig. 6c). The C-terminal domain has
strong similarity to carbohydrate-degrading domains, as predicted by HHpred (Supplementary
Data Sheet 7). Since receptor binding proteins of phages, of which fibers are one group, often
bind to or degrade carbohydrates on the bacterial cell surface, these predicted functions of the
Sco4242 protein are consistent with its serving as a bacterial cell surface binding protein.
Conserved domain 2, comprising approximately 50 residues immediately preceding the
transmembrane segment is Cys-rich and may be a Zn-binding domain as predicted by HHpred
(Supplementary Data Sheet 7). This region might mediate attachment of these proteins to the
baseplate. In summary, our bioinformatic data (summarized in Supplementary Data Sheet 1)
show that most Streptomyces type IId eCIS share related putative fiber and toxin proteins,
supporting a common function for eCIS in this genus.
Discussion

eCIS particles are produced by bacterial species in widely divergent bacterial clades. The dispersed occurrence of eCIS and their demonstrated roles in mediating toxic activities against eukaryotic cells has led to a view of these systems as defense mechanisms, useful under specific conditions but not central to normal cellular function. The results presented here introduce an entirely distinct role for eCIS in *Sco* where they appear to participate in the developmental process of this species. In particular, we have shown that *Sco* produces eCIS particles as part of its normal growth cycle and that strains lacking functional eCIS particles exhibit pronounced alterations in their developmental program. These changes included perturbation of antibiotic production during growth on solid and in liquid media (Fig. 4a; Supplementary Fig. 6), and accelerated development of aerial hyphae on solid media (Fig. 4b). Most intriguingly, eCIS-deficient mutants displayed significantly reduced levels of cell death and altered morphology during liquid growth (Fig. 3). These results suggest that *Sco* eCIS function by inducing intraspecies lethality, which may play a role in the developmental process.

In contrast to a previous study on *Streptomyces lividans*, we did not detect eCIS mediated inhibition of *S. cerevisiae* growth by *Sco*\textsuperscript{21}. Given that the eCIS systems in these two species are very closely related, we would expect them to perform the same function. It is possible that we did not detect the growth inhibitory activity of the *Sco* eCIS because we used a different assay that may not be as sensitive as that used in the other study. In addition, since we observed complicated changes in antibiotic production by the eCIS-deficient strains, it is possible that indirect effects on antibiotic production caused the effects seen in the previous study. This idea is supported by the absence of *S. cerevisiae* growth inhibition induced by strain M1152 (Fig. 2), which does not produce the four major *Sco* antibiotics.

While intracellular *Sco* lysates displayed mostly fully sheathed eCIS structures with typical appearance (Fig. 1a), extracellular samples almost exclusively contained empty contracted sheath species (Fig. 1c). This stark difference between the intracellular and extracellular pools of eCIS-derived structures suggested that eCIS were not escaping from cells through generalized lysis, a conclusion also supported by the absence of ActR, a cytoplasmic protein, in the extracellular fractions containing sheath proteins (Supplementary Fig. 1b). A model that could account for the above observation is that in intact mycelia the tail-like structures are membrane-bound. Upon
encountering the appropriate stimulus, the eCIS may extend through the membrane to contact a neighboring cell, resulting in contraction and release of the contracted sheath. The possibility of the Sco eCIS being attached to the cell membrane is supported by the conserved presence of a strongly predicted transmembrane helix in its putative fiber protein (Fig. 6c). In recent structural studies on the eCIS of Anabaena, a filamentous cyanobacterium, the eCIS structures were shown to be anchored to the thylakoid membrane by a transmembrane helix in a fiber-like protein that was attached to the baseplate. Although we did not observe fibers on the Sco eCIS particles, we did detect the putative fiber protein in our purified samples by mass spectrometry (Supplementary Table 1). The conditions of cell lysis during purification would likely disrupt membrane interactions leaving the fibers in variable conformations that might be difficult to distinguish by TEM.

One potential limitation of our study is that some key experiments, such as those investigating cell death, were performed in liquid cultures in which Sco does not sporulate. However, the developmental program with respect to gene expression has been shown to be similar in liquid and solid medium. Thus, we expect to see related differences during growth on solid media where cell death has also been shown to occur.

Vegetative hyphal degradation during development has long been recognized as a feature of Streptomyces development and more recently programmed cell death has been postulated to occur as part of the shift from vegetative to aerial mycelial growth. It is thought that a coordinated stage of cell death limited to a subset of the vegetative hyphal population during development may serve to provide additional nutrients to the growing reproductive aerial mycelium and dead hyphae may also serve a structural role or facilitate nutrient transport.

Consistent with a role at this stage in the Sco lifecycle, we have shown that eCIS expression peaks late in the vegetative growth phase in a temporal pattern similar to the antibiotic regulator gene, redD. The requirement of BldA, a master regulator of the morphogenetic switch, for the expression of the Sco eCIS genes also supports a role in this process. The apparent requirement for bldA for expression of eCIS genes in many Streptomyces species as well as the conservation of putative effector and fiber proteins among many Streptomyces eCIS regions suggest that these eCIS may share a conserved function in programmed cell death. This shared function in the Streptomyces lifecycle would explain the very frequent occurrence of eCIS in this clade.
Interestingly, a role in intra-strain cell death was recently proposed for the eCIS of *Anabaena*, a filamentous cyanobacteria with a complex lifestyle\(^{13}\). We found that the eCIS of diverse Actinomycetes and other filamentous Cyanobacteria encode homologues of the Sco4256 putative effector protein, providing another connection between eCIS function in these two filamentous groups of bacteria.

To conclude, this study has revealed a previously unknown function for eCIS in mediating intra-strain cell lethality that impacts the developmental process of *Streptomyces*. The possibility that this function is conserved among diverse species opens up exciting avenues for future research.

**References**


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Methods

Bacterial strains, plasmids, and media

Bacterial strains, plasmids and cosmids used in this chapter are listed in Supplementary Table 3. All Streptomyces strains used were derivatives of Streptomyces coelicolor A3(2). Sco strains were grown at 30 °C on maltose-yeast extract-malt extract (MYM) agar medium for 3 days or in yeast extract-malt extract (YEME) liquid medium with continuous agitation at 180 rpm for 3 days, unless indicated otherwise. Other bacterial strains were grown in Lysogeny Broth (LB) medium or agar (pH 7.0) at 37 °C unless indicated otherwise. Saccharomyces cerevisiae were grown in YPD medium or agar (pH 7.0) at 30 °C.

Construction and testing of Lux reporter strains

The eCIS promoter sequences were amplified from wild-type Sco M145 genomic DNA using primers SCO_eCISp1p2GEN and SCO_eCISp3p4GEN (Supplementary Table 4). The resulting PCR fragments were used as a template to introduce an EcoRV blunt cut site using SCO_eCISp1p2EcoRV and SCO_eCISp3p4EcoRV primers. The PCR products were cloned into the EcoRV site of the pFlux plasmid both in the forward and the reverse orientation for the ability to test both transcriptional directions of the divergent promoter regions. All plasmids were verified by sequencing. The resulting reporter plasmids pFlux-eCISp1-p4 were electroporated into methylation-deficient E. coli ET12567/pUZ8002 cells. Plasmids were introduced into wild-type S. coelicolor by intergenic conjugation and selection on apramycin (50 µg/mL).

For luminescence assays 10^4 spore forming units (SFU) of each Sco reporter strain were resuspended in 10 μL of saline and plated in triplicate onto 200 μL of soya-mannitol (MS) agar in 96-well polystyrene plates. Plates were incubated for 7 days at 30°C and luminescence was measured three times daily using a PerkinElmer Victor X multilabel plate reader.

Generation of gene replacement mutants

To create genomic replacements of the eCIS tail sheath, baseplate and ATPase encoding genes the REDIRECT method for PCR targeting in Streptomyces was employed as described. The aac(3)(IV)-oriT cassette which confers resistance to apramycin, was amplified using SCO4253_Disp, SCO_BP_Disp or SCO4259_Disp primer pairs, respectively (Supplementary Table 4). The amplified cassettes were used to replace the respective eCIS genes in cosmid StD8a. The resulting cosmids (listed in Supplementary Table 3) were introduced by conjugation from the non-methylating E. coli strain ET12567/pUZ8002 into the wild-type strain M145 to obtain apramycin-resistant exconjugants. To select for double cross-over exconjugants the apramycin resistant colonies were screened for kanamycin resistance. Three Apr^R Kan^R exconjugant strains were selected for each gene, resulting from separate conjugation events. Mutant strains were confirmed by PCR using oligonucleotides; StD8aΔTS_aac(3)IV, StD8aΔBP_aac(3)IV or StD8aΔATP_aac(3)IV (Supplementary Table 4), respectively and by DNA sequencing.

Actinorhodin production assay

Wild-type Sco M145 or Sco Δsco4253 (ΔTS) were inoculated in experimental triplicate into R2YE liquid media at a final concentration of 1.5x10^6 SFU/mL. Cultures were grown with
agitation for 22 hours at 30 °C. All cultures were standardized to an OD$_{450}$ of 0.5. Cells were recovered by centrifugation at 3500 x g for 10 min, resuspended in saline and centrifuged again at 3500 x g for 10 minutes. Cell pellets were resuspended in 50 mL of fresh RG2 minimal media. The cultures were incubated at 30 °C with continuous shaking for 94 h. For visual comparison of pigment production, images of the growing cultures were taken using a NIKON D3000 camera at a fixed position within a light box at times of 0, 18 hr, 24 hr, 41 hr, 49 hr, 65 hr, 70 hr and 94 hr. For quantification of total actinorhodin production, 480 µl samples were collected at the time points indicated above. 120 µl of 5M KOH was added to a final concentration of 1M, and samples were vortexed and centrifuged at 3000 x g for 5 minutes. The absorbance of the supernatant at 640 nm was read using a polystyrene 96 well plate. Each assay was standardized to the weight of the pellet.

**Aerial mycelium formation assay**

10⁵ SFU of *S. coelicolor* wild-type or eCIS-deficient mutant strains in biological duplicate were plated on R2YE agar plates and grown at 30 °C. Starting from 24 hr post germination, sterile glass cover slips were gently applied to the top surface of each bacterial lawn at approximately 12-hour intervals. Cover slips were sealed on top of a microscope glass slide and examined using a Zeiss transmitted light microscope at 40 X magnification.

**Inter-species growth inhibition assays**

Spores (10⁵ SFU suspended in 5 µL of 0.85% saline) of wild-type *S. coelicolor* M145, M1152 or the eCIS mutant strains ΔTS or M1152 ΔTS were spotted on R2YE agar plates. Plates were incubated for 3 days at 30°C before being overlaid with 5 mL molten, extra-soft LB agar (0.5% agar) containing organisms to be tested (at a final OD$_{600}$ ranging from 0.01-0.1). Plates were incubated at 37 °C overnight and antimicrobial activity was assessed by measurement of zones of clearance in the indicator lawn. Assays against *Streptomyces* strains were conducted by overlaying a 3-day colony plate with 1 mL 0.85% saline solution containing 5 µL of concentrated *Streptomyces* spores. Plates were incubated for 3 days at 30 °C before antimicrobial activity was assessed. Assays against *Saccharomyces cerevisiae* were conducted by overlaying a 4-day colony plate with 5 mL molten, extra-soft YPD agar containing *S. cerevisiae* (at a final OD$_{600}$ ranging from 0.01-0.05). Plates were incubated at 30 °C overnight.

**Generation of antibodies against TS and BW1 proteins**

The TS and BW1 proteins were expressed and purified from *E. coli*. Genes encoding the BW1 protein (*sco4245*) and the TS protein (*sco4253*) were cloned from *Sco* genomic DNA into the BseR1 site of p15TV-L vector using the In-fusion HD cloning kit (Clonetech) using primers listed in Supplementary Table 4. Verified plasmids were transformed into *E. coli* strain BL21 (DE3), grown at 37 °C with appropriate antibiotics to an OD$_{600}$ of 0.6 and protein expression was induced with 1 mM IPTG. Cultures were grown for 16-18 hours at 17 °C. Cells were collected by centrifugation and resuspended in binding buffer (20 mM Tris-HCl pH 7.5; 200 mM NaCl; 1 mM imidazole, 5 mM β-mercaptoethanol (βME)). Cells were lysed by sonication. Lysates were cleared by centrifugation at 17,000 rpm for 20 minutes at 4 °C. Proteins were affinity-purified by incubation with 2 mL of Ni-NTA agarose resin (Invitrogen) for 30 minutes at 4 °C. The mixture was passed through a column at room temperature and washed extensively with binding buffer containing 30 mM imidazole. Bound proteins were eluted with binding buffer containing 300
mM imidazole and dialyzed overnight at 4 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% (vol/vol) glycerol, 1 mM DTT. Samples were concentrated using Vivaspin filter concentrators (Sigma-Aldrich) and further separated using Superdex200 16/60 size exclusion column on an ÄKTA fast performance liquid-chromatography system (GE Healthcare). Relevant fractions were analyzed and verified by SDS-PAGE and concentrated to a final concentration of 0.1 - 0.25 mg/ml.

Mouse polyclonal antibodies were generously generated by the Gray-Owen lab at the University of Toronto. Fresh purified protein at a concentration of 0.05-0.25 mg/mL was mixed with 100 µl Emulsigen adjuvant and injected into mice. A second booster dose was administered 21 days later. Antisera were collected on day 35. Antisera specificity was tested by Western blotting with appropriate controls.

eCIS particle purification

Sco strains were grown on MYM agar at 30 °C for 4 days. 1 cm² squares of agar containing mycelium and spores were excised from the plate and directly transferred into 50 mL YEME liquid medium. Cells were incubated at 30 °C for the indicated various times with shaking. Extracellular sample (E) was taken from the supernatant. Pellets were washed in 10% sucrose and centrifuged again, resuspended in 16 mL of buffer P8 supplemented with 2 mg/mL lysozyme and incubated at 30 °C for 1 hour. Cells were spun down and resuspended in Buffer C (50 mM Tris pH 7.5, 150 mM NaCl, 15% vol/vol glycerol, 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich-one tablet per 50 mL of buffer)), and sonicated. Lysed cells were centrifuged at 12,000 rpm for 20 minutes and the supernatant was then filtered through a 0.45 µm filter. Filtered samples were spun at 150,000 × g for 3 hours at 4 °C. The pellet was resuspended in 5 mL of ice cold 1XPBS. The samples were loaded onto columns containing 5 mL of DEAE Sepharose anion exchange resin (Sigma-Aldrich), washed extensively with PBS and eluted using 0.5M NaCl in PBS buffer. Samples were ultracentrifuged at 150,000 × g for 90 minutes at 4 °C and pellets were resuspended in 200 µL ice-cold PBS and stored at 4 °C for up to two weeks.

Western blotting

Samples were mixed in a 1:1 ratio with 2 X SDS loading buffer (100 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 200 mM β-mercaptoethanol (βME); 0.2% Bromophenol blue). Proteins were separated on a 12% polyacrylamide SDS Tris-Tricine gels and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour in 5% skim milk in Tris-buffered saline with 1% Tween (TBS-T). When tested with α-eCIS specific antibodies antiserum was added at 1:5000 dilution in TBS-T with 5% milk. Alternatively, α-FLAG primary antibody (Sigma-Aldrich) was added at 1:10,000 dilution. Membranes were incubated overnight at 4 °C. After washing, the membranes were probed with horseradish peroxidase conjugated goat anti-mouse antibody (Santa Cruz Biotechnology 1:2000 dilution in TBS-T) for 1 hour at room temperature and developed using chemiluminescent reagents (GE Healthcare).

Transmission electron microscopy

Purified eCIS samples were briefly centrifuged to remove impurities and 5 µL of supernatant was applied onto freshly glow discharged carbon-coated copper grids (CF400-CU, 6 nm, 400 mesh, Electron Microscopy Sciences). Samples were allowed to adsorb for 2 minutes before
being blotted off by filter paper and grids were washed twice with filtered distilled water. The
grids were negatively stained with 15 µL of 2% uranyl acetate for 15 seconds. Grids were
imaged with a JEM-1011 (JEOL USA, INC.), digital CDD camera (5 megapixels XR50S, AMT,
USA).

Live/Dead assay

*S. coelicolor* wild-type or eCIS mutant strains were inoculated into 40 mL of YME culture
medium from fresh spores at a density of 10⁷/mL and incubated at 30 °C with shaking. Samples
of 1 mL were centrifuged for 5 min at 12,000 rpm, washed twice and resuspended in 1 mL of
distilled water. LIVE/DEAD Bac-Light bacterial viability kit (L7012; Invitrogen) was used to
stain cells. The bacterial suspension was mixed with 3 µl of SYTO 9 and propidium iodide (PI)
nucleic acid stains, premixed at a 1:1 ratio. The cell suspension and nucleic acid stains were
mixed by vortex and left to stand at room temperature for 10 min in the dark. 10 µl of the
suspension was deposited on a clean slide and covered with an 18 mm square cover slip. Images
were acquired within 30 min of incubation using a Zeiss Axio imager 2, at an excitation of 488
nm and 568 nm and emission of 530 nm (green) or 630 nm (red). Images were analyzed using
Zeiss Zen Blue software and statistical analysis was performed on GraphPad Prism 9.

Mass spectrometry analysis of purified *Sco* eCIS samples

eCIS samples were purified as described above. Extracellular samples were similarly purified
from cell-free supernatants following the initial centrifugation step at 7,500 rpm. Purified
samples of 30-150 µg total protein were reduced with DTT, alkylated with iodoacetamide, and
subjected to tryptic digest. Liquid chromatography tandem-mass spectrometry spectra were
collected on a linear ion-trap instrument (ThermoFisher) (SPARC BioCentre, The Hospital for
Sick Children, Toronto, Canada). Proteins were identified using Mascot (Matrix Science,
London, UK) and analyzed in Scaffold version 3.0 (Proteome Software Inc., Portland, OR,
USA). The cut off for protein identification was set at a confidence level of 95%.

Figure Legends

**Fig. 1** eCIS-derived particles can be visualized in cell lysates and extracellular media. *a-c,*
Transmission electron micrographs represent wild-type *Sco* grown in liquid YME media for 72
hr post inoculation. *a,* eCIS were purified from *Sco* lysates as described in Methods and
concentrated to 30X the concentration of the original culture. Images were taken at 100,000 X
magnification. White arrow heads point to empty sheaths, present alongside with fully assembled
eCIS. *b,* A single assembled eCIS particle in its uncontracted conformation is shown. The white
arrow points to the baseplate, and the black arrow shows the tail terminator complex. *c,*
Extracellular fraction. Cell-free media was ultracentrifuged and concentrated to 30X the
concentration of the original sample Images taken at 80,000X magnification. White arrows point
to typical emptied contracted tail sheath particles. Scale bar represents 100 nm in all images. *d,*
Representative TEM images of *Sco* eCIS-derived structures from early stages of liquid growth
are shown. All images are of purified lysate samples of wild-type *Sco* grown in liquid YME
media for 48 hr post inoculation. Tails of different aberrant forms are shown. White arrows point to abnormal contraction or breaks in the sheath coating and black arrows point to the exposed internal tail tube. Images were taken at 100,000X magnification. Scale bar represents 100 nm. e, Average length and width of the eCIS-derived particles described in (a-c) (n=80 for fully assembled uncontracted intracellular particles, n=215 for extracellular contracted empty sheath particles). Error bars represent standard deviation of the mean. f, Schematic illustration of an eCIS particle. The diagram shows the conserved core structural components of an eCIS tail. Proteins are indicated by abbreviations (Tr = tail terminator; TT = tail tube; TS = tail sheath; BS = baseplate). W, BH1, BH2 = baseplate hub components; BW1, BW2, BW3 = baseplate wedge components; M1152 harboring a tail sheath knockout (ΔTS), ΔTS M1152 (a mutant that does not produce the four major Sco antibiotics), or M1152 harboring a tail sheath knockout mutation (M1152 ΔTS). Cells were grown for 3 days before being overlaid with species to be tested as indicated in each panel. Zones of clearing around the Sco colonies indicate lethality or growth inhibitory activity against the indicator lawn. All of the results shown for a tested species strain were obtained from the same agar plate. Images are representative examples of many replicates (n > 3).

Fig. 2 Interspecies growth inhibition induced by WT and eCIS-deficient Sco strains. Assays were performed by spotting on agar plates 1x10⁵ SFU of Sco wild-type (WT), tail sheath knockout (ΔTS), M1152 (a mutant that does not produce the four major Sco antibiotics), or M1152 harboring a tail sheath knockout mutation (M1152 ΔTS). Cells were grown for 3 days before being overlaid with species to be tested as indicated in each panel. Zones of clearing around the Sco colonies indicate lethality or growth inhibitory activity against the indicator lawn. All of the results shown for a tested species strain were obtained from the same agar plate. Images are representative examples of many replicates (n > 3).

Fig. 3 Sco eCIS mutants exhibit reduced cell death during hyphal differentiation. Sco wild-type (WT), tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) strains were inoculated into 50 ml of YEME media at a final concentration of 10⁵ SFU/mL and grown at 30 °C. At a, 48 hr, b, 54 hr, or c, 74 hr post-inoculation, 1 mL samples were collected, washed in saline, and stained with equal proportions of SYTO 9, a membrane permeant nucleic acid stain, and propidium iodide, which stains only dead or damaged cells. Therefore, dead and live cells are visualized by red and green fluorescence, respectively. Samples were imaged using a fluorescent microscope within 30 minutes of staining. The ratio of live/dead cells was calculated from total fluorescence intensity values per image. One-way ANOVA and Dunnett’s multiple comparisons test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs. Diameters of individual mycelial pellets were manually measured from each collected image (images measured for WT, ΔTS and ΔBP, respectively; at 48 hr – n=99, 110, 130, at 54 hr – n=173, 114, 138 and at 74 hr – n= 73, 70, 71). One-way ANOVA and Dunnett’s multiple comparisons test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs. Representative images are shown here. Experiments were carried out at least three times for each strain.

Fig. 4 eCIS mutants display accelerated development and early spore formation on solid medium. a, 10⁸ SFU of Sco wild-type (WT), two independently isolated tail sheath knockout strains (ΔTS_1 and ΔTS_2) or a baseplate knockout strain (ΔBP) were plated on R2YE agar. Top panel shows representative images of secondary metabolite development in lawns grown for 48 hours; bottom panel shows aerial hyphae development on the same plates as above, at 72 hours of growth. b, Representative transmission light micrographs of surface imprints on cover
slips of agar-grown WT Sco, or ΔTS or ΔBP mutant strain lawns 42 and 55 hr post germination. The aerial hyphae and spores stick to the cover slip, but not the vegetative hyphae. Two fields are shown for each sample. Results are representative of three independent experiments with two biological replicates per strain.

**Fig. 5** The eCIS cluster of *S. coelicolor* is expressed during vegetative growth right before the metabolic switch. **a**, Schematic representation of the eCIS cluster of Sco. Open reading frames and their transcriptional direction are represented by block arrows and are drawn to scale. Gene name is shown under the first and last genes in the cluster, spanning from sco4242-sco4263. The encoded eCIS function is shown as an abbreviation above each ORF. The Afp eCIS annotation, which is used in some other publications, is shown inside the arrows (Afp1-16\(^{18}\)). The two divergent promoter regions are shown as red blocks. Forward and reverse transcriptional direction shown in blue arrows above the cluster. **b**, A fusion construct of each eCIS promoter (eCISp1-p4, corresponding to promoters P1-P4 shown in (a)) with luxCDABE was conjugated with Sco and grown on 96-well MS agar plates for 7 days. hrdBp and redDp reporter strains were used as controls. Luminescence was measured three times a day. Activity of the weaker promoters: eCISp1, eCISp2 and eCISp3 is shown in enlarged view in the top right insert. Three biological replicates were tested for each assay (n = 3). The mean normalized to empty vector is shown with error bars representing standard deviation of the mean.

**Fig. 6** Conservation of the putative toxin and fiber of the Sco eCIS. **a**, A diverse selection of hits from a BLAST search initiated with Sco4256, the putative toxin, were aligned and analyzed by HHpred. Sequences shown are from *Streptomyces* species (unshaded), other Actinomycete species (shaded gray), Cyanobacteria (shaded green), and Chorflexi (shaded yellow). All proteins are associated with type IId systems and all genera are filamentous except for *Cyanothece*. **b**, A schematic of the conserved domains of proteins related to Sco4256 is shown. **c**, A diverse selection of hits from a BLAST search initiated with Sco4242, the putative fiber, were aligned. Sequences shown are from *Streptomyces* species (unshaded), other Actinomycete species (shaded gray). These proteins are found in type IId eCIS. All alignments were performed with MUSCLE\(^{59}\) as implemented in Jalview\(^{60}\). The clustal coloring scheme was used.

**Supplementary Figures**

**Supplementary Fig. 1** eCIS proteins are detected by specific polyclonal antibodies in lysates of WT Sco. **a**, Sco wild-type (WT) or a tail sheath knockout strain (ΔTS) were grown in liquid R2YE media to the indicated time points. Whole cell lysates were analyzed by Western blot using α-TS sera. Heterologously expressed and purified 6XHis-tagged-tail sheath (His-TS) was used as a positive control. **b**, WT Sco cells were grown in liquid YEME media, cells were collected at the indicated time points and an extracellular “E” sample was taken from the cell-free supernatant. Cells were lysed and an intracellular total lysate “T” sample was collected. The lysate was ultracentrifuged for 3 h at 150,000 X g. Samples of the supernatant “S” and pellet “P” were analyzed. Samples were probed using α-TS antibodies. **c**, Sco wild-type (WT) or a baseplate knockout (ABP) were grown and fractionated as in (b). Samples were probed by Western blot using α-BW1 antibodies.. For the Sco strain expressing FLAG-tagged ActR from a
plasmid, expression was induced with 30 µg/mL final concentration of thiostrepton, added 45 minutes before cells were harvested by centrifugation.

**Supplementary Fig. 2** The Sco ATPase mutant produces both mature eCIS and empty sheath particles, detectable in the lysate and extracellular media. All images represent Sco ΔATPase grown in YEME liquid media for 48 hr (a), 54 hr (b) or 72 hr (c) post inoculation. a, Representative images of purified eCIS particles from cell lysate concentrated to 30 X original culture after 48 hr of growth. The majority of particles at this time are in their extended conformation. b, The extracellular fraction after 54 hr of growth is shown. White arrows point to typical emptied contracted tail sheath particles. c, Purified lysate from cells grown for 72 hr. emptied sheath particles are indicated with white arrows. Scale bar represents 100 nm in all images. Images are representative of results obtained for three separate biological replicates.

**Supplementary Fig. 3** Growth inhibition of other Streptomyces species by WT and eCIS-deficient Sco strains. a, A representative TEM image of extracellular cell-free media collected from Sco M1152 strain grown in rich liquid media for 72 hr. Empty contracted sheath particles, visually identical to ones produced by Sco WT M145 strain are observed. These particles is indicative of production of normal eCIS particles that can contract. No particles were detected in a M1152 strain harboring a tail sheath knockout mutation (M1152 ΔTS). Scale bar indicates 100 nm. b, Assays were performed by spotting on agar plates 1x10^5 SFU of Sco wild-type (WT) or tail sheath knockout (ΔTS) strains. Cells were grown for 3 days before being overlaid with species to be tested as indicated in each panel. Zones of clearing around the Sco colonies indicate lethality or growth inhibitory activity against the indicator lawn. The results shown for a tested species strain were obtained from the same agar plate. Images are representative examples of many replicates (n > 3).

**Supplementary Fig. 4** The Sco ATPase deletion mutant strain exhibits reduced cell death during hyphal differentiation. a-c Sco wild-type (WT) or an ATPase knockout (ΔATPase) strains were inoculated into 50 ml of YME media at a final concentration of 10^7 SFU /mL and grown at 30 °C. At 48 hr (a), 54 hr (b) or 74 hr (c) post-inoculation 1 mL samples were collected, washed in saline, and stained with equal proportions of propidium iodide and SYTO 9. Total (live and dead) cells are stained with SYTO9 and are visualized by green fluorescence. Cells with damaged membranes (dead cells) are stained only with PI and are visualized by red fluorescence. Samples were imaged within 30 minutes of staining. The ratio of SYTO9 to PI (Live/dead ratio) was calculated from total fluorescence intensity values per image. Diameters of individual hyphal pellets were manually measured from each collected image (n > 30). Single factor ANOVA and Welch’s t-test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs.

**Supplementary Fig. 5** Growth curve for WT Sco and eCIS-deficient mutant strains. 10^8 SFU of Sco WT, tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) were inoculated in R2YE liquid media and grown for 70 hours. Optical density (OD_600) of each sample was taken at the indicated time points. ΔTS_1, ΔTS_2 and ΔBP_1, ΔBP_2 represent two biological replicates.
of each mutant. The mean is shown with error bars representing standard error of the mean (n=3).

Supplementary Fig. 6I eCIS mutants exhibit decreased actinorhodin production in minimal media. a, Comparison of wild-type Sco (left) and ΔTS mutant (right) development in liquid media. Three experimental replicates of RG2 liquid media cultures were inoculated to an OD of 0.5 and incubated with shaking at 30 °C for 94 hours. Images of each culture flask were taken at the indicated time points. b, For quantification of total actinorhodin, samples of 0.5 ml were taken at the same time points as in (a). KOH was added to a final concentration of 1 M to extract the actinorhodin and the sample was centrifuged. The optical density (OD) of the supernatant was measured and normalized to wet pellet weight. The mean is shown with error bars representing standard deviation of the mean (n=3). c, Equal amounts of Sco wild-type (WT), tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) spores were streaked out on minimal Soy Mannitol (MS) agar plates and grown at 30 °C for 4 days. The agar plate shown is representative of multiple independent assays with 3 biological replicates (strains ΔTS 1-3, ΔBP 1-3, each obtained by a separate conjugation event).

Supplementary Fig. 7I Sco eCIS promoter regions. The DNA sequence of the eCIS promoter regions of Sco is shown as diagrammed in Fig. 5a. The two divergent promoter regions are indicated by red blocks. The translational start codons (for sco4248 and sco4249 on the top and sco4253 and sco4254 on the bottom) are underlined in red. Primers used to clone the promoter regions into the luxCDABE reporter plasmid are highlighted in yellow and indicated by arrows above the sequence.
## Supplementary Tables

### Supplementary Table 1: Summary of LC-MS/MS analysis of purified *Sco* eCIS samples.

<table>
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<tr>
<th>eCIS protein</th>
<th>WT Intracellular</th>
<th>ΔBP Intracellular</th>
<th>WT Extracellular</th>
<th>ΔBP Extracellular</th>
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<td>TR (DUF4255)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>Tail tube</td>
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</tr>
<tr>
<td>BH1</td>
<td></td>
<td></td>
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<tr>
<td>BH2/Spike</td>
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<td></td>
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<tr>
<td>BW1</td>
<td></td>
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<tr>
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<tr>
<td>ATPase</td>
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Hits detected using LC-MS/MS analysis in three separate purifications are highlighted in dark grey. Hits identified in fewer than 3 experiments are highlighted in light grey. Only proteins with E-values better than $10^{-5}$ were considered a hit.
### Supplementary Table 2: Detailed results of LC-MS/MS analysis of WT eCIS samples.

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<th>Gene name</th>
<th>Protein accession</th>
<th>Function</th>
<th>log(e)</th>
<th>log(I)</th>
<th>% Coverage</th>
<th>Total spectra</th>
<th>Molecular mass</th>
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<td>Tail sheath</td>
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The table contains eCIS encoded proteins detected in lysate purified eCIS samples by LC-MS/MS in three separate experiments. Values shown are from one representative run. eCIS from wild-type Sco were purified as described in Methods. The samples were reduced, trypsin digested and C18 purified before LC-MS and data processing.

- **Log(e):** the base-10 log of the expectation that any particular protein assignment was made at random.
- **Log(I):** the base-10 log of the sum of the fragment ion intensities in the tandem mass spectra used to make this assignment.
- **% Coverage:** the amino acid coverage of the protein in this assignment / the coverage corrected for peptide sequences that are unlikely to be observed using normal proteomics methods.
- **Total spectra:** the total number of tandem mass spectra that can be assigned to this protein.
### Supplementary Table 3: Strains and plasmids used in this study.

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<th>Strains</th>
<th>Description</th>
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<td><strong>Streptomyces coelicolor strains</strong></td>
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**Plasmids**

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<td>eCIS promoter region P2 (Sco4248-4249 forward)</td>
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<td>pFlux-eCISp3</td>
<td>eCIS promoter region P3 (Sco42453-4254 reverse)</td>
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<td>pFlux-eCISp4</td>
<td>eCIS promoter region P4 (Sco42453-4254 forward)</td>
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<td>pIJ7331</td>
<td>pBluescript KS (+), aac(3)IV, oriT (RK2)</td>
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<tr>
<td>p15TV-L</td>
<td>T7 expression vector, derivative of pET15b, AmpR, (GenBank ID: EF456736.1)</td>
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<tr>
<td>p15-SC4245</td>
<td>6XHis BW1 expression plasmid</td>
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<tr>
<td>p15-SC4253</td>
<td>6XHis tail sheath expression plasmid</td>
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**Cosmids**

<p>| | |</p>
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<tr>
<td>StD8a</td>
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<td>StD8aΔsco4253::apra</td>
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# Supplementary Table 4: Oligonucleotides used in this study.

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<td>SCO_eCISp1p2EcoRV_F</td>
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<td><strong>Recombinant protein cloning</strong></td>
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<tr>
<td>p15TV-L SCTS_R</td>
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**a** S. coelicolor Intracellular eCIS 72 hr

**b**

**c** Extracellular media 72 hr

**d**

<table>
<thead>
<tr>
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<th>Intracellular mature particles</th>
<th>Extracellular empty sheaths</th>
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<td>Length</td>
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<td>132</td>
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<tr>
<td>Width</td>
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**e**

Tr, TT, TS, BH2, BH1, BW1, BW2, BW3, BS, Fiber

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**Fig. 1** eCIS-derived particles can be visualized in cell lysates and extracellular media.  
*a-c*, Transmission electron micrographs represent wild-type *Sco* grown in liquid YEME media for 72 hr post inoculation.  
*a*, eCIS were purified from *Sco* lysates as described in Methods and concentrated to 30X the concentration of the original culture. Images were taken at 100,000 X magnification. White arrow heads point to empty sheaths, present alongside with fully assembled eCIS.  
*b*, A single assembled eCIS particle in its uncontracted conformation is shown. The white arrow points to the baseplate, and the black arrow shows the tail terminator complex.  
*c*, Extracellular fraction. Cell-free media was ultracentrifuged and concentrated to 30X the concentration of the original sample Images taken at 80,000X magnification. White arrows point to typical emptied contracted tail sheath particles. Scale bar represents 100 nm in all images.  
*d*, Representative TEM images of *Sco* eCIS-derived structures from early stages of liquid growth are shown. All images are of purified lysate samples of wild-type *Sco* grown in liquid YEME media for 48 hr post inoculation. Tails of different aberrant forms are shown. White arrows point to abnormal contraction or breaks in the sheath coating and black arrows point to the exposed internal tail tube. Images were taken at 100,000X magnification. Scale bar represents 100 nm.  
*e*, Average length and width of the eCIS-derived particles described in (a-c) (n=80 for fully assembled uncontracted intracellular particles, n=215 for extracellular contracted empty sheath particles). Error bars represent standard deviation of the mean.  
*f*, Schematic illustration of an eCIS particle. The diagram shows the conserved core structural components of an eCIS tail. Proteins are indicated by abbreviations (Tr = tail terminator; TT = tail tube; TS = tail sheath; BH1, BH2 = baseplate hub components; BW1, BW2, BW3 = baseplate wedge components; BS = tail spike).
**Fig. 2** Interspecies growth inhibition induced by WT and eCIS-deficient Sco strains. Assays were performed by spotting on agar plates 1x10^5 SFU of Sco wild-type (WT), tail sheath knockout (ΔTS), M1152 (a mutant that does not produce the four major Sco antibiotics), or M1152 harboring a tail sheath knockout mutation (M1152 ΔTS). Cells were grown for 3 days before being overlaid with species to be tested as indicated in each panel. Zones of clearing around the Sco colonies indicate lethality or growth inhibitory activity against the indicator lawn. All of the results shown for a tested species strain were obtained from the same agar plate. Images are representative examples of many replicates (n > 3).
**Fig. 3** *Sco* eCIS mutants exhibit reduced cell death during hyphal differentiation. *Sco* wild-type (WT), tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) strains were inoculated into 50 ml of YEME media at a final concentration of $10^7$ SFU/mL and grown at 30 °C. At **a**, 48 hr, **b**, 54 hr, or **c**, 74 hr post-inoculation, 1 mL samples were collected, washed in saline, and stained with equal proportions of SYTO 9, a membrane permeant nucleic acid stain, and propidium iodide, which stains only dead or damaged cells. Therefore, dead and live cells are visualized by red and green fluorescence, respectively. Samples were imaged using a fluorescent microscope within 30 minutes of staining. The ratio of live/dead cells was calculated from total fluorescence intensity values per image. One-way ANOVA and Dunnett’s multiple comparisons test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs. Diameters of individual mycelial pellets were manually measured from each collected image (images measured for WT, ΔTS and ΔBP, respectively; at 48 hr – n=99, 110, 130, at 54 hr – n= 173, 114, 138 and at 74 hr – n= 73, 70, 71). One-way ANOVA and Dunnett’s multiple comparisons test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs. Representative images are shown here. Experiments were carried out at least three times for each strain.
Fig. 4| eCIS mutants display accelerated development and early spore formation on solid medium. **a**, 10⁸ SFU of Sco wild-type (WT), two independently isolated tail sheath knockout strains (ΔTS_1 and ΔTS_2) or a baseplate knockout strain (ΔBP) were plated on R2YE agar. Top panel shows representative images of secondary metabolite development in lawns grown for 48 hours; bottom panel shows aerial hyphae development on the same plates as above, at 72 hours of growth. **b**, Representative transmission light micrographs of surface imprints on cover slips of agar-grown WT Sco, or ΔTS or ΔBP mutant strain lawns 42 and 55 hr post germination. The aerial hyphae and spores stick to the cover slip, but not the vegetative hyphae. Two fields are shown for each sample. Results are representative of three independent experiments with two biological replicates per strain.
Fig. 5. The eCIS cluster of *S. coelicolor* is expressed during vegetative growth right before the metabolic switch. **a**, Schematic representation of the eCIS cluster of *Sco*. Open reading frames and their transcriptional direction are represented by block arrows and are drawn to scale. Gene name is shown under the first and last genes in the cluster, spanning from *sco4242*-*sco4263*. The encoded eCIS function is shown as an abbreviation above each ORF. The Afp eCIS annotation, which is used in some other publications, is shown inside the arrows (Afp1-16). The two divergent promoter regions are shown as red blocks. Forward and reverse transcriptional direction shown in blue arrows above the cluster. **b**, A fusion construct of each eCIS promoter (eCISp1-p4, corresponding to promoters P1-P4 shown in (a)) with *luxCDABE* was conjugated with *Sco* and grown on 96-well MS agar plates for 7 days. *hrdBp* and *redDp* reporter strains were used as controls. Luminescence was measured three times a day. Activity of the weaker promoters: eCISp1, eCISp2 and eCISp3 is shown in enlarged view in the top right insert. Three biological replicates were tested for each assay (n = 3). The mean normalized to empty vector is shown with error bars representing standard deviation of the mean.
Fig. 6 | Conservation of the putative toxin and fiber of the Sco eCIS. a, A diverse selection of hits from a BLAST search initiated with Sco4256, the putative toxin, were aligned and analyzed by HHpred. Sequences shown are from *Streptomyces* species (unshaded), other Actinomycete species (shaded gray), Cyanobacteria (shaded green), and Chorflexi (shaded yellow). All proteins are associated with type IId systems and all genera are filamentous except for *Cyanothece*. b, A schematic of the conserved domains of proteins related to Sco4256 is shown. c, A diverse selection of hits from a BLAST search initiated with Sco4242, the putative fiber, were aligned. Sequences shown are from *Streptomyces* species (unshaded), other Actinomycete species (shaded gray). These proteins are found in type IId eCIS. All alignments were performed with MUSCLE as implemented in Jalview. The clustal coloring scheme was used.
Figure a: Western blot analysis showing the expression of α-TS in different strains of E. coli. The expression is assessed at 24 hr, 48 hr, and 96 hr post-inoculation.

Figure b: Western blot analysis of α-FLAG and α-TS proteins. Time points include 42 hr, 72 hr, and 24 hr post-inoculation.

Figure c: Western blot analysis showing the expression of α-BW1 and Non-specific, cytoplasmic protein. The blot also highlights Sco4245 BW1.
Supplementary Fig. 1| eCIS proteins are detected by specific polyclonal antibodies in lysates of WT Sco. a, Sco wild-type (WT) or a tail sheath knockout strain (ΔTS) were grown in liquid R2YE media to the indicated time points. Whole cell lysates were analyzed by Western blot using α-TS sera. Heterologously expressed and purified 6XHis-tagged-tail sheath (His-TS) was used as a positive control. b, WT Sco cells were grown in liquid YEME media, cells were collected at the indicated time points and an extracellular “E” sample was taken from the cell-free supernatant. Cells were lysed and an intracellular total lysate “T” sample was collected. The lysate was ultracentrifuged for 3 h at 150,000 X g. Samples of the supernatant “S” and pellet “P” were analyzed. Samples were probed using α-TS antibodies. c, Sco wild-type (WT) or a baseplate knockout (ΔBP) were grown and fractionated as in (b). Samples were probed by Western blot using α-BW1 antibodies. For the Sco strain expressing FLAG-tagged ActR from a plasmid, expression was induced with 30 µg/mL final concentration of thiostrepton, added 45 minutes before cells were harvested by centrifugation.
Supplementary Fig. 2 | The Sco ATPase mutant produces both mature eCIS and empty sheath particles, detectable in the lysate and extracellular media. All images represent Sco ΔATPase grown in YEME liquid media for 48 hr (a), 54 hr (b) or 72 hr (c) post inoculation. **a**, Representative images of purified eCIS particles from cell lysate concentrated to 30 X original culture after 48 hr of growth. The majority of particles at this time are in their extended conformation. **b**, The extracellular fraction after 54 hr of growth is shown. White arrows point to typical emptied contracted tail sheath particles. **c**, Purified lysate from cells grown for 72 hr. Emptied sheath particles are indicated with white arrows. Scale bar represents 100 nm in all images. Images are representative of results obtained for three separate biological replicates.
Supplementary Fig. 3| Growth inhibition of other *Streptomyces* species by WT and eCIS-deficient *Sco* strains. **a**, A representative TEM image of extracellular cell-free media collected from *Sco* M1152 strain grown in rich liquid media for 72 hr. Empty contracted sheath particles, visually identical to ones produced by *Sco* WT M145 strain are observed. The presence of these particles is indicative of production of normal eCIS particles that can contract. No particles were detected in a M1152 strain harboring a tail sheath knockout mutation (M1152 ΔTS). Scale bar indicates 100 nm. **b**, Assays were performed by spotting on agar plates 1x10^5 SFU of *Sco* wild-type (WT) or tail sheath knockout (ΔTS) strains. Cells were grown for 3 days before being overlaid with species to be tested as indicated in each panel. Zones of clearing around the *Sco* colonies indicate lethality or growth inhibitory activity against the indicator lawn. The results shown for a tested species strain were obtained from the same agar plate. Images are representative examples of many replicates (n > 3).
Supplementary Fig. 4: The Sco ATPase deletion mutant strain exhibits reduced cell death during hyphal differentiation. a-c Sco wild-type (WT) or an ATPase knockout (ΔATPase) strains were inoculated into 50 ml of YEME media at a final concentration of $10^7$ SFU/mL and grown at 30 °C. At 48 hr (a), 54 hr (b) or 74 hr (c) post-inoculation 1 mL samples were collected, washed in saline, and stained with equal proportions of propidium iodide and SYTO 9. Total (live and dead) cells are stained with SYTO9 and are visualized by green fluorescence. Cells with damaged membranes (dead cells) are stained only with PI and are visualized by red fluorescence. Samples were imaged within 30 minutes of staining. The ratio of SYTO9 to PI (Live/dead ratio) was calculated from total fluorescence intensity values per image. Diameters of individual hyphal pellets were manually measured from each collected image (n > 30). Single factor ANOVA and Welch’s t-test were performed to calculate statistical significance. Adjusted P-values are shown above the relevant graphs.
Supplementary Fig. 5l Growth curve for WT Sco and eCIS-deficient mutant strains. 10^8 SFU of Sco WT, tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) were inoculated in R2YE liquid media and grown for 70 hours. Optical density (OD_{600}) of each sample was taken at the indicated time points. ΔTS_1, ΔTS_2 and ΔBP_1, ΔBP_2 represent two biological replicates of each mutant. The mean is shown with error bars representing standard error of the mean (n=3).
**Supplementary Fig. 6** eCIS mutants exhibit decreased actinorhodin production in minimal media. 

**a,** Comparison of wild-type Sco (left) and ΔTS mutant (right) development in liquid media. Three experimental replicates of RG2 liquid media cultures were inoculated to an OD$_{450}$ of 0.5 and incubated with shaking at 30 °C for 94 hours. Images of each culture flask were taken at the indicated time points. 

**b,** For quantification of total actinorhodin, samples of 0.5 ml were taken at the same time points as in (a). KOH was added to a final concentration of 1 M to extract the actinorhodin and the sample was centrifuged. The optical density (OD$_{640}$) of the supernatant was measured and normalized to wet pellet weight. The mean is shown with error bars representing standard deviation of the mean (n=3). 

**c,** Equal amounts of Sco wild-type (WT), tail sheath knockout (ΔTS) or baseplate knockout (ΔBP) spores were streaked out on minimal Soy Mannitol (MS) agar plates and grown at 30 °C for 4 days. The agar plate shown is representative of multiple independent assays with 3 biological replicates (strains ΔTS 1-3, ΔBP 1-3, each obtained by a separate conjugation event).
Supplementary Fig. 7l Sco eCIS promoter regions. The DNA sequence of the eCIS promoter regions of *Sco* is shown as diagrammed in Fig. 5a. The two divergent promoter regions are indicated by red blocks. The translational start codons (for *sco*4248 and *sco*4249 on the top and *sco*4253 and *sco*4254 on the bottom) are underlined in red. Primers used to clone the promoter regions into the luxCDABE reporter plasmid are highlighted in yellow and indicated by arrows above the sequence.