From stress to success: how actinobacteria exploit life witout a cell wall

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Chapter 6

YidC2 is required for insertion of the holin-endolysin system during phage infection in *Streptomyces coelicolor*

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Abstract

Bacteriophage infection has been extensively studied, but the molecular mechanism of a successful infection cycle remains largely unknown. Here, we show that phage CF3 needs the host membrane protein insertase YidC2 to successfully complete its infection cycle in *Streptomyces coelicolor*. Adsorption assays and cryo-electron microscopy revealed that phage CF3 could attach and inject its DNA in a strain lacking YidC2, but was unable to subsequently induce lysis of the bacteria. Additionally, robust interactions between the holin-endolysin system of the phage and the YidC2 protein of *S. coelicolor* were observed and the block in phage release in the *yidc2* mutant could be overcome by Sec-dependent endolysin secretion. Therefore, we propose that the holin-endolysin system of phage CF3 exploits the membrane insertase YidC2 to complete the phage infection cycle. Additionally, our findings suggest that YidC2 is critical for maintenance of cell wall-deficiency following phage infection. Altogether, our work contributes to a deeper understanding of the intricate dynamics between bacteria and bacteriophages, shedding light on the molecular mechanisms of phage infections.

Introduction

Bacteriophages, or phages in short, are viruses that can infect bacteria. They represent the most abundant biological entities in nature and play an instrumental role in all ecosystems [19]. The interaction between phages and their hosts has been extensively studied, but mostly in unicellular model organisms like *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* [13, 14, 145-147]. The molecular mechanism behind the phage infection cycle in non-model bacteria remains largely unknown. The multicellular lifestyle of *Streptomyces* is unique in the bacterial realm and starts when a spore enters a favorable environment. This causes spores to germinate, leading to the formation of vegetative hyphae. These hyphae elongate and branch, forming a dense interconnected network of filaments, called a mycelium. Upon nutrient scarcity, *Streptomyces* undergoes a process of differentiation, which involves the formation of specialized hyphae that grow into the air to form spores again [4]. Concomitant with this morphological differentiation, chemical differentiation is initiated, leading to the production of antibiotics and other specialized metabolites [104, 148-150]. Previous research has shown that phages cannot infect spores, while young germinating spores are the most vulnerable developmental stage for phage infection [112]. In addition, recent research showed that aerial hyphae and spores are formed in response to phage infection to suppress further spread of phages [17].

Most research on *Streptomyces*-phage interactions has been focused on the characterization of defense systems against phage attack, like chemical defenses [51, 151], the phage-growth limitation system [91] and many more [152]. One recently discovered escape mechanism against phage attack is the ability of *Streptomyces* to become cell wall-deficient (CWD) [93]. Without their cell wall, the usual receptor molecules like peptidoglycan, teichoic acids or proteins associated with the cell wall are lost and bacteriophages can no longer infect the bacterial cell. After evasion of phage predation, the cell wall-deficient cells of *Streptomyces* can revert to their canonical mycelial mode-of-growth [93, 153].

During lytic phage infection, the host cell's metabolism is exploited and directed towards supporting the production of new progeny phages. Phages

can encode their own proteins to carry out key cellular functions, or rely on the host metabolism to complete their infection cycle [154]. A critical step following the assembly of new phages is lysis of the host to release progeny phages in the environment. Therefore, phages use so-called holins that are inserted in the bacterial membrane. These holins enable the passage of endolysins across the membrane that subsequently cleave the peptidoglycan layer. Especially in multicellular bacteria, little is known about the host machinery required for phage production and bacterial lysis. However, if a phage is fully dependent on host proteins, the host might mutate or eliminate proteins that the phage requires, to prevent successful phage reproduction.

Here, we show that YidC2 of *Streptomyces coelicolor* plays a key role during infection of phage CF3, which is a temperate phage classified in the genus *Camvirus*. YidC2 is predicted to be a membrane protein insertase required for inserting proteins into the membrane [155, 156]. We found that phage CF3 was unable to complete its infection cycle in a Δ*yidc2* mutant. Adsorption assays and cryo-electron microscopy revealed that phage CF3 could still attach and infect *S. coelicolor* Δ*yidC2*, but progeny phages remained stuck inside the bacteria. Additionally, we found interactions between the holin-endolysin system and the YidC2 protein with bioinformatics and a bacterial two-hybrid assay. Notably, the block in phage release in the Δ*yidc2* mutant could be overcome by secretion of additional endolysins in a Sec-dependent manner. Altogether, we hypothesize that the holin-endolysin system of phage CF3 requires YidC2 for proper insertion in the bacterial membrane to complete the phage infection cycle and subsequently, lyse the bacteria.

Material and methods

Strains and growth conditions

All bacterial strains and phages used in this study are described in Table S1. *Streptomyces* strains were grown for five days on Soy Flour Mannitol (SFM) at 30 °C to collect spores. For growth of *Streptomyces* in liquid, Difco Nutrient Broth (DNB) or L-phase broth (LPB) were used [11].

Construction of *S. coelicolor* Δ*yidC2*

All plasmids and primers used in this study are listed in Table S2 and Table S3, respectively. To delete *yidC2* in *S. coelicolor*, PCR targeted mutagenesis was performed [157]. In short, *Escherichia coli* BW25113/pIJ790 containing cosmid E20 (containing *yidC2,* SCO2851) was transformed with an integrating PCR product carrying an apramycin resistance cassette. This PCR product was generated by using primers E20_KOF and E20_KOR. After introduction of the PCR product and recombination, *E. coli* colonies with the mutated cosmid were selected by being resistant to both apramycin and kanamycin. The mutated cosmid was isolated and transformed into *E. coli* ET12567/pUZ8002, which was then used for conjugation to *S. coelicolor*. Ex-conjugants that were apramycin resistant and kanamycin sensitive were picked and verified by southern hybridization for loss of the *yidC2* gene [158].

Construction of Sec-endolysin and Sec-eGFP secretion strains

Genomic DNA of *Streptomyces coelicolor* M145 or phage CF3 and plasmids pCB-1 [159] or pGWS758 [160] were used as templates to amplify coding sequence of Sec signal peptides (SPs)/YidC2, the coding sequence of the holin-endolysin of phage CF3, the cumate-inducible promoter and the coding sequence of eGFP, respectively. All genes were expressed using the integrative plasmid pIJ82Cu harboring a cumate-inducible promoter. To create pIJ82Cu, the promoter region of pCB-1 was amplified using primers IDProm-F and IDProm-R creating a 5' NdeI restriction enzyme site. The amplified product was then inserted into the plasmid pIJ82 that has been linearized with BamHI via Gibson assembly.

To create *S. coelicolor* strains expressing Sec-secreted variants of the endolysin of phage CF3 and eGFP, both genes were placed behind the sequence encoding the N-terminal SP of SCO5376 [161] under control of the cumate-inducible promotor [162]. Therefore, primer pairs SecGFP-F1/SecGFP-R1 and SecGFP-F2/TatGFP-R2 primers were used to amplify the SP of SCO5376 and eGFP, respectively. For creation of the Sec-secreted endolysin variant, primer pairs SecGFP-F1/SecEndo-R1 and SecEndo-F2/TatEndo-F2 were used to amplify the SP of SCO5376 and endolysin, respectively. Subsequently, Gibson assembly was use to insert the amplified product pairs into plasmid pIJ82Cu that had been linearized with NdeI digestion, generating plasmids, pIJ82Cu-SecGFP and pIJ82Cu-SecEndo.

To express the endolysin and eGFP signal sequence under the control of the cumate-inducible promoter, both genes were amplified with GFP-F/TatGFP-R2 and Endo-F/TatEndo-F2, respectively. The amplified products were subsequently inserted into pIJ82Cu that has been linearized with NdeI digestion via Gibson assembly, yielding pIJ82Cu-GFP and pIJ82Cu-Endolysin, respectively. Conjugation [130] and heat-shock transformation [163] were used to introduce plasmids into *Streptomyces* and *E. coli* cells, respectively.

Bacteria-phage interactions

Phage stocks were isolated as described previously [111]. Briefly, bacteriophages were added to DNB cultures containing *S. coelicolor* spores at Multiplicity of Infection (MOI) = 0.1. After overnight incubation at 30 °C (200 RPM), phages were filtered through a 0.22 µm filter for phage stocks of ~10^7 phages ml^{-1}.

Plaque assays were used to determine if phages could infect the *S. coelicolor* mutant strains. To this end, phages were serial diluted in DNB medium and 3 µl was spotted in duplicate on a DNB double agar overlay plate containing the strain of interest. After overnight incubation at 30 °C, individual plaques were quantified. To test the interaction in liquid, bacteria and phages were inoculated at MOI=1 in 10 ml DNB or LPB and incubated overnight at 30 °C, while shaking at 200 RPM. To test whether *S. coelicolor* Δ*yidC2* could make chemically induced CWD cells, 0.2 mg ml^{-1} lysozyme or 0.5 mg $µl^{-1}$ mitomycin C was added to the spores in LPB medium.

Bacterial two-hybrid assay

To assess protein interactions, a bacterial two-hybrid assay was used, which was essentially performed as described [164]. Interactions of LpmP and CslZ were used as the positive control [164]. Primer pairs ThYidC2-F/ThYidC2-R, ThHolin-F/ThHolin-R and ThEndo-F/ThEndo-R were used to amplify the genes encoding YidC2, holin and endolysin, respectively, using genomic DNA of *S. coelicolor* or phage CF3 as the template. Amplified products were ligated into either plasmids pKT25 or pUT18C using XbaI and EcoRI restriction enzyme sites, generating pKT25-YidC2, pKT25-Holin, pKT25-Endolysin, pUT18C-YidC2, pUT18C-Holin and pUT18C-Endolysin, respectively.

Conjugation [130] and heat-shock transformation [163] were used to introduce plasmids into *Streptomyces* and *E. coli* cells, respectively.

Membrane fluidity

Membrane fluidity was measured as previously described [165]. In short, three biological replicates of 20 ml of 16 hour-old mycelium were centrifuged for 10 minutes at 2,500 RPM and washed three times in 5 ml PBS buffer. The pellet was resuspended in 100 µl PBS containing 1 mM Laurdan (6-Dodecanoyl-N, N-dymethyl2-naphthylamine, Sigma) dissolved in dimethylformamide (DMF) and allowed to incubate in the dark for 10 minutes at 30 °C, 200 RPM. The stained mycelia were collected and washed twice in pre-warmed PBS with 1% DMF and subsequently resuspended in 100 µl pre-warmed PBS buffer. The dye was excited at 350 nm and fluorescence was measured at 435 and 490 nm using a Zeiss LSM900 Airyscan 2 microscope with the climate control chamber set at 30 °C. Images of the hyphal tips were used to calculate membrane fluidity with the corresponding generalized polarization (GP) value as described before [166]. Statistical analysis was performed using a Student's *t*-test.

Adsorption assay

To determine if phage CF3 could attach to the bacterial hosts, adsorption assays were performed. In short, 10^7 spores were inoculated in 10 ml DNB broth in triplicate and allowed to germinate for four hours for *S. coelicolor* and seven hours for *S. coelicolor* Δ*yidC2* at 30 °C, while shaking at 200 RPM. At timepoint zero, 10^5 phages ml^{-1} of phage CF3 were added to the cultures and every ten minutes, 100 µl samples were collected and immediately filtered through a 0.22 µm filter. Plaque assays were performed on strain MBT61 and after overnight incubation at 30 °C, plaque-forming units (PFU) were calculated and normalized to 100% free phages at timepoint zero. The effect of penicillin G (0.4 mg ml^{-1}), rifamycin (50 µg ml^{-1}), or lysozyme (0.2 mg ml^{-1}) on phage release in the *yidC2* mutant was evaluated by adding these compounds at T=0. The adsorption assay with the Δ*yidC2* Sec-endolysin strain was performed similarly, but after 60 minutes of infection with phage CF3, the release of endolysin through the Sec-secretion pathway was induced by adding 100µM of cumate.

Microscopy

To image the formation of cell wall-deficient cells, a Zeiss Axio Lab A1 upright microscope, equipped with an Axiocam 105 colour (resolution of 5 megapixel) camera was used to take bright field images. eGFP expression after cumate induction was imaged using a Zeiss LSM900 Airyscan 2 microscope at excitation and emission wavelengths of 488 nm and 509 nm, respectively. Images were adjusted using OMERO software [167].

To examine the morphology of phage CF3, transmission electron microscope (TEM) was used. To prepare a single grid, 3 µl of phage CF3 diluted in DNB medium (~10^4 phages ml^{-1}) was pipetted on a glow-discharged 200 mesh carbon coated copper grid (EMS). After 30 seconds, excess liquid was removed with filter paper. Next, the phages were stained with 2% uranyl acetate for 45 seconds, after which excess staining was removed and the grids were air-dried for 5 minutes. Negative staining images were recorded using a single tilt specimen holder inside a 120 kV Talos L120C TEM with a Lab6 electron source and Ceta detector at the Netherlands Center for Nanoscopy (NeCEN, Leiden).

Cryo-EM was used to investigate *S. coelicolor* Δ*yidC2* after infection with phage CF3. In short, spores were allowed to germinate for seven hours in DNB broth. Afterwards, the germlings were centrifuged for 10 minutes at 5,000 RPM and the bacterial pellet was dissolved in 100 µl lysate of phage CF3 (10^7 phages ml^{-1}). After 30 minutes of infection at room temperature, the culture was resuspended with 10 nm gold fiducial markers (Cell Microscopy Core, UMC Utrecht) at 1:25 ratio. Of this sample, 3 µl was added to a glow discharged R2/2 200 mesh holey carbon EM grid (Quantifoil). After 30 seconds of incubation, the sample was blotted for one second and immediately plunged in liquid ethane with the Leica GP automated freeze-plunger. The grids were observed inside the 120 kV Talos L120C TEM with a Lab6 electron source and Ceta detector at the Netherlands Center for Nanoscopy (NeCEN, Leiden).

Bioinformatic analysis

Protein structures and interactions between proteins were predicted by Alphafold 3.0 [168] and protein membrane topology and transmembrane domains were predicted by DeepTMHMM [169]. Sequence alignments were made with NCBI MSA sequence viewer [170].

Results

YidC2 plays an important role in phage infection

The molecular mechanism of phage infection in bacteria remains largely unknown. To identify host components required for successful completion of the phage infection cycle in *Streptomyces*, we systematically screened a large collection of *S. coelicolor* mutants for their susceptibility to our *Streptomyces* phage collection (Fig. S1). In short, 46 phages were spotted in duplicate on plaque assays with 28 different *S. coelicolor* strains lacking distinct cell wall-associated proteins. When lysis zones looked visually different compared to the WT plaque assay, serial dilutions were performed. Most phages did not show a difference in lysis zone formation in the mutants. However, when phage CF3 was serial diluted and spotted onto a lawn of *S. coelicolor* Δ*yidC2* in a double agar overlay assay (Fig. 1A), it was unable to form plaques. This suggests an interruption in the phage infection cycle. To validate these observations, we introduced phage CF3 to both the *yidC2* mutant and the parental strain in osmoprotective LPB medium, known to cause the formation of CWD cells during phage infection [93]. While the parental strain generated CWD cells following infection, the *yidC2* mutant persisted as mycelium (Fig. 1C), underscoring the necessity of YidC2 for completing the infection cycle.

Since *S. coelicolor* Δ*yidC2* could still be infected by phage CD8, we tested if this phage could still induce the shedding of the bacterial cell wall in LPB. However, *S. coelicolor* Δ*yidC2* did not form CWD cells after infection in osmoprotective medium with this phage. Interestingly, mitomycin C, a chemical that induces the lytic cycle of prophages in bacteria, could also not induce the formation of CWD cells in the *yidC2* mutant, unlike the parental strain that did produce CWD cells upon mitomycin C treatment. Notably, the *yidC2* mutant could produce CWD cells when treated with lysozyme (Fig. 1C). Taken together, these results show that YidC2 is required for completing the infection cycle as well as the formation of CWD cells upon phage infection.

YidC2 is a second paralogue of YidC, which acts as a protein membrane insertase in bacteria. *S. coelicolor* possesses two orthologues (YidC1 and YidC2) of *E. coli* YidC, which also shows significant homology to *B. subtilis* SpoIIIJ (Fig. S2). However, the C-terminus of YidC1 has an extension compared to YidC2, while YidC from *E. coli* has a relatively long N-terminal extension that is absent in YidC2. Additionally, YidC1 and YidC2 are highly divergent and only share around 23% amino acid sequence identity. Knocking-out YidC in *E. coli* results in a non-viable mutant, since the export of many proteins are dependent on this protein [155], but little is known about the function of YidC1 and YidC2 in *S. coelicolor*. Knocking-out YidC1 in *S.coelicolor* resulted in a non-viable mutant as well (data not shown), indicating that YidC1 is likely essential, in contrast to YidC2.

To predict the protein structure of YidC2 in *S. coelicolor*, Alphafold3 was used [168]. Additionally, membrane topology and transmembrane domains were predicted by InterPro [169]. As displayed in Figure 1B, YidC2 is predicted to have six transmembrane domains. Since depletion of YidC is in accordance with membrane-related defects in *E. coli* [171], we tested the difference in membrane fluidity between the *S. coelicolor* Δ*yidC2* and WT strain. The lipid dye $DiIC_{12,}$ with a high affinity for fluid membranes was used [172], but no differences in membrane fluidity were found between the both strains (Fig. S3). Additionally, no obvious differences in morphology between both strains could be found though visual inspection, but the *yidC2* mutant germinates approximately 3 hours slower in liquid compared to *S. coelicolor* (Fig. S4).

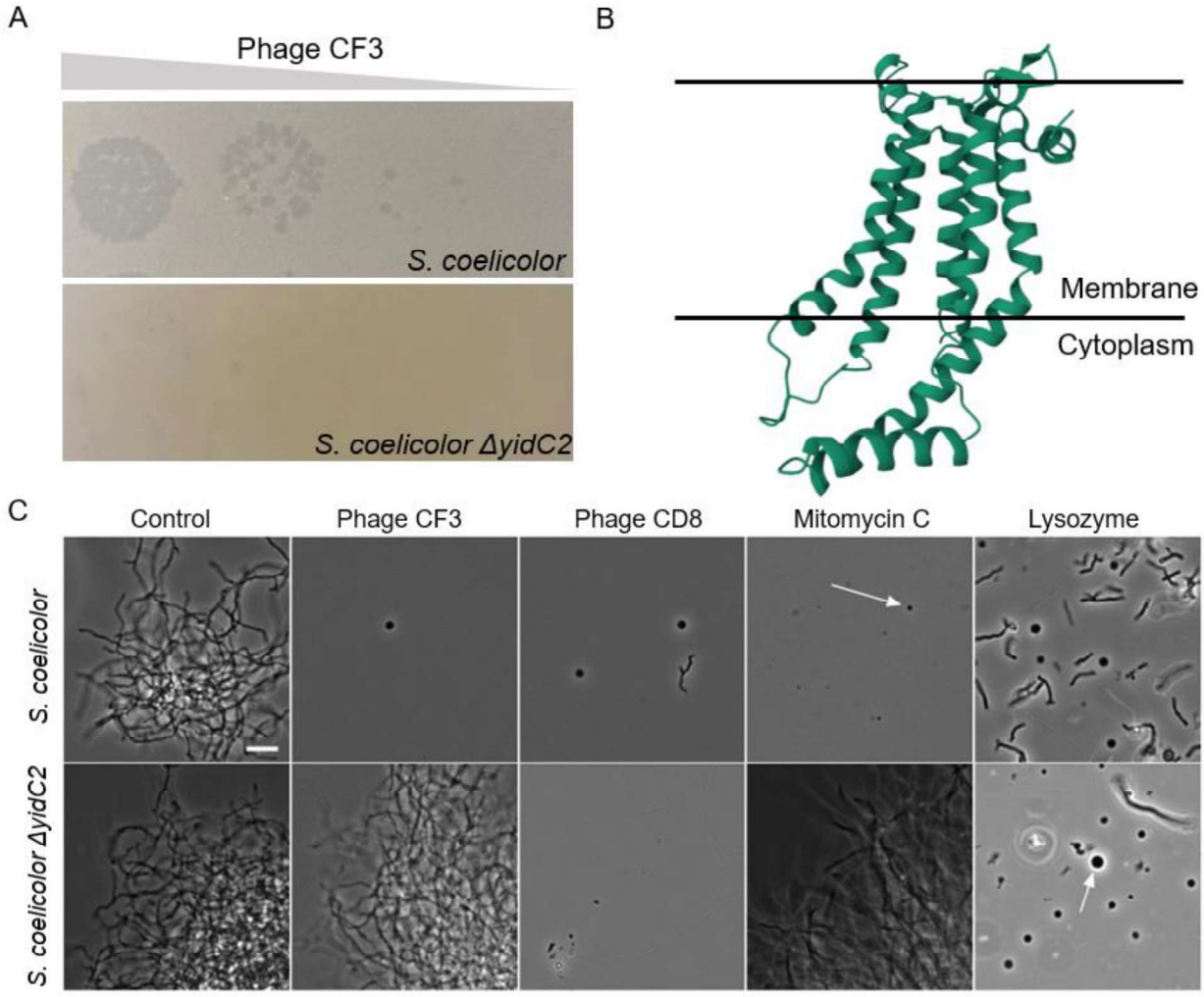


Figure 1. Phage CF3 cannot lyse *S. coelicolor* Δ*yidC2.* (A) Plaque morphology of serial diluted phage CF3 on *S. coelicolor* and *S. coelicolor* Δ*yidC2*. (B) Alphafold3 prediction of the structure of the YidC2 protein in *S. coelicolor*. The hypothetical membrane protein insertase YidC2 has six predicted transmembrane domains. (C) In osmoprotective medium, *S. coelicolor* forms CWD cells after infection with phage CF3, unlike the *yidC2* mutant that retains its mycelial morphology. In contrast, exposure of the *yidC2* mutant to phage CD8 leads to complete lysis without the formation of CWD cells. Mitomycin C, which induces the lytic cycle of prophages, can induce the CWD state in *S. coelicolor,* but not in *S. coelicolor* Δ*yidC2*. Interestingly, cell wall-deficiency can be induced by lysozyme in both strains.

YidC2 is required for the release of phages after infection

To examine the morphology of phage CF3, electron microscopy was used (Fig. 2A). This revealed that phage CF3 is morphologically classified as a siphovirus. Sequencing revealed that phage CF3 is a species in the genus

Camvirus, closely related to *Streptomyces* phage Vanseggelen [173]. These phages were previously shown to attach to the cell wall of *Streptomyces* via receptor proteins on their tail fibers. To test if phage CF3 could still adsorb to the *yidC2* mutant, an adsorption assay was performed. Since the *S. coelicolor* Δ*yidC2* has a reduced growth speed (Fig. S4), we allowed the mutant spores to germinate for seven hours, contrary to the spores of the parental strain, which were germinated for four hours. These different incubation times were selected, as they resulted in a comparable length of the germ tubes. Phage CF3 can adsorb to the parental strain and new progeny phages are released into the environment after 30 minutes (Fig. 2B). While the phages could still adsorb to the *yidC2* mutant as well, no new progeny phages were detected even after 90 minutes of infection. Since phages can adsorb to the parental and the mutant strain, it is unlikely that YidC2 serves as the receptor for CF3 or inserts the receptor in the bacterial membrane.

To gain further understanding of the specific stage at which the infection cycle was blocked, we infected 7-hour-old germlings of the *yidC2* mutant with phage CF3. Thirty minutes after the phage infection, we plunged the samples in liquid ethane and conducted imaging using cryo-EM. As seen in Figure 2C, we observed phages with both full and empty capsid heads inside the bacterium. The phages do not have a coherent size and capsid heads without DNA seem to gather at the cell membrane. This indicates that the phage successfully injected its DNA into the host and initiated the assembly of new phages. Consequently, it seems that the infection cycle was stalled during the release of the phage particles from the host.

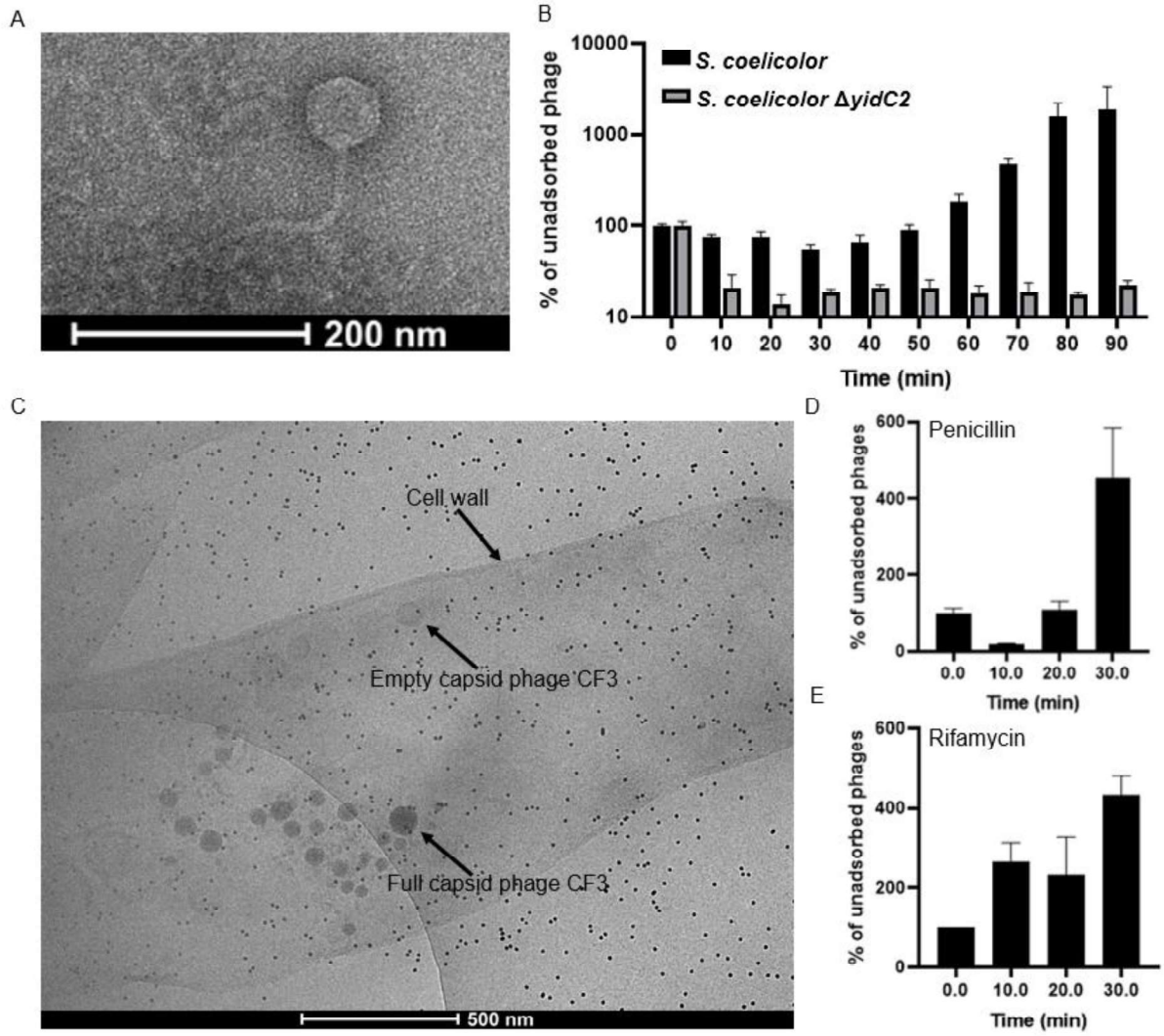


Figure 2. Phage CF3 cannot release progeny phages after infection in *S. coelicolor* Δ*yidC2*. (A) Representative TEM image of phage CF3 stained with 2% uranyl acetate. (B) Adsorption assay showing that phage CF3 can still adsorb and replicate in *S. coelicolor* but does not replicate in *S. coelicolor* Δ*yidC2*. Note that *S. coelicolor* Δ*yidC2* was allowed to germinate for seven hours instead of four hours, since this strain germinates slower. (C) Cryo-EM image of *S. coelicolor* Δ*yidC2* 30 minutes after infection with phage CF3. (D) Adsorption assay of phage CF3 with additional penicillin G in *S. coelicolor* Δ*yidC2* showing an increase in phage titer after 20 minutes. (E) Adsorption assay of phage CF3 with additional rifamycin in *S. coelicolor* Δ*yidC2* showing an increase in phage titer already after 10 minutes.

To verify if progeny phages were stuck inside the bacteria, we performed additional adsorption assays, while adding antibiotics that cause lysis of the host. When penicillin G, which inhibits cross-linking in the peptidoglycan [174], was introduced in the adsorption assay, we observed that progeny phages

were released into the medium of the *yidC2* mutant after 20 minutes (Fig. 2D). This aligns with our hypothesis that functional phages are assembled, but unable to exit the host. A similar response was observed with rifamycin (Fig. 2E), which leads to a suppression of RNA synthesis and ultimately cell death of the bacteria [175]. We also tested if lysozyme could lyse the bacteria from the outside to aid phage CF3 to get out of the bacteria, but phage CF3 could not survive an adsorption assay with additional lysozyme (Fig. S5).

YidC2 is predicted to interact with the holin-endolysin system of phage CF3

Since YidC2 is a predicted membrane protein insertase, we hypothesized that phage CF3 utilizes this host protein to putatively insert some of its own proteins in the membrane. One of the phage machineries that interacts with the bacterial membrane is the holin-endolysin system. The holin and endolysin of phage CF3 could be predicted by a BlastP search of all proteins of phage CF3 to the Actinobacteriophage database [108]. This holin is a type III holin, which has one transmembrane domain [176], and does not have a secretion peptide, indicating that it requires insertion into the membrane by another protein.

To predict whether YidC2 interacts with the holin-endolysin system of phage CF3, predicted aligned error (PAE) plots were made using Alphafold3 (Fig. 3). We observed a strong predicted interaction between YidC2 and the holin protein (Fig. 3A), while YidC2 is also predicted to interact with the N-terminus of the endolysin protein (Fig. 3B). The endolysin interacts strongly with itself, but also with the C-terminus of the holin protein (Fig. 3C). These results were partially confirmed with a bacterial two-hybrid assay in Figure 3D, but the strong interaction between YidC2 and the holin protein as predicted by Alphafold3 was not observed here. However, these results indicated that the holin-endolysin system of phage CF3 probably makes a robust interaction with the YidC2 protein from *S. coelicolor*.

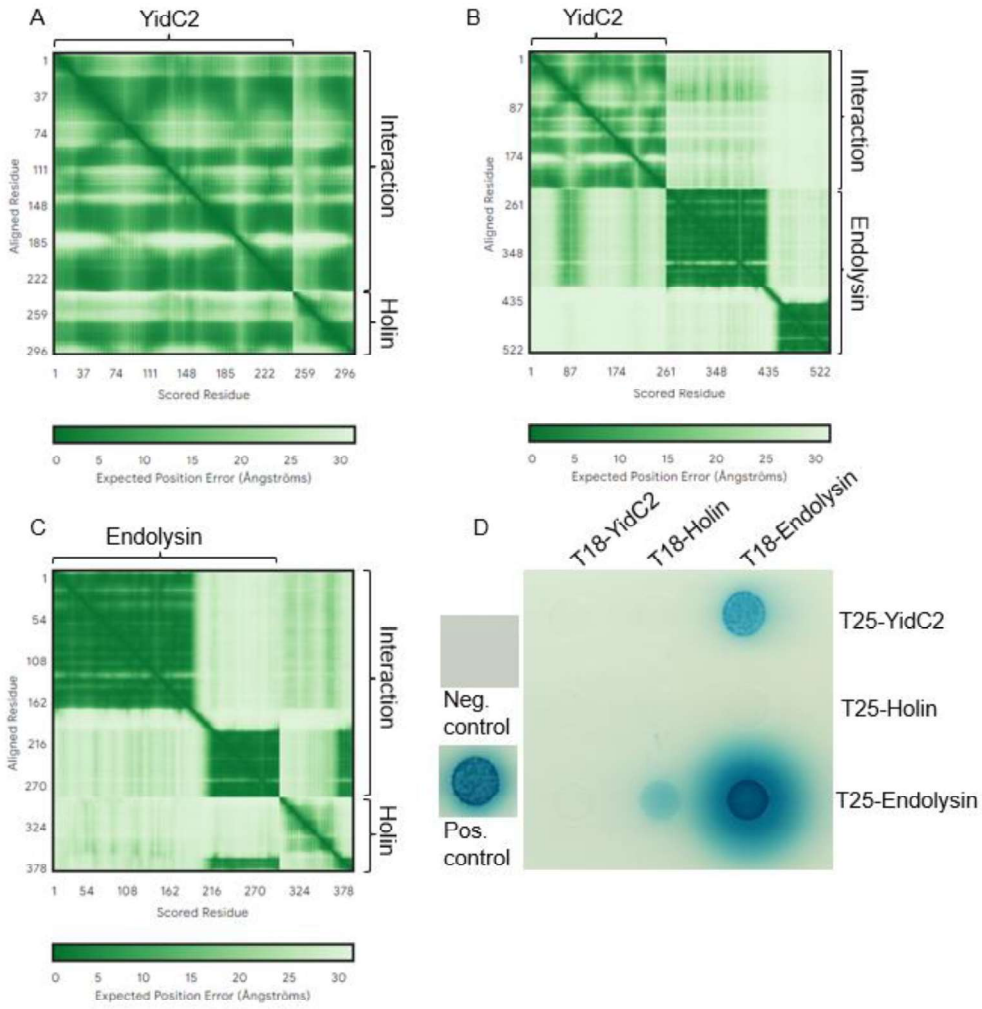


Figure 3. Predictions of holin, endolysin and YidC2 interactions. Predicted aligned error (PAE) plots made with Alphafold3, where a dark green color indicates higher confidence in an interaction between the two proteins. An interface predicted template modeling (ipTM) score <0.6 likely suggest a failed structure, and >0.8 represents confident high quality predictions, while a predicted template modeling (pTM) score >0.5 indicates that the overall predicted fold is highly similar to the true structure [168]. Note that TM scores are very strict for small proteins, like the holin protein. (A) PAE plot of the YidC2 protein with the holin protein from phage CF3. The dark green color in the interaction section indicates that an interaction between the two proteins over their entire length is highly likely. Scores of ipTM = 0.61 and pTM = 0.67. (B) PAE plot of the YidC2 protein with the endolysin protein of phage CF3. The endolysin is highly likely to interact with itself, and a weak interaction is predicted between YidC2 and the endolysin. Scores of ipTM = 0.21pTM = 0.42 (C) PAE plot of the endolysin protein and holin protein of phage CF3. An interaction is predicted between the C-terminal of the endolysin and the C-terminal of the holin protein. Scores of ipTM = 0.32 and pTM = 0.49. (D) Bacterial two-hybrid assay. Blue color indicates predicted interaction. Endolysin strongly interacts with itself, which was also seen in the Alphafold3 predictions. Additionally, the holin and endolysin of phage CF3 have a predicted weak interaction, and the endolysin interacts with the YidC2 protein.

All our data suggested that YidC2 may be required for the insertion of the holin-endolysin system of phage CF3 into the host membrane, facilitating the transport of endolysins across the membrane. To corroborate these findings, we engineered a strain with the endolysin protein placed behind a Sec-secretion signal and expressed from a cumate-inducible promotor. This setup ensures that upon cumate induction, endolysins are synthesized and secreted independent of the YidC2 protein. To test our setup, we first created a cumate inducible Sec-eGFP *S. coelicolor* strain, leading to secretion of eGFP to the exterior of the cell upon cumate induction. Indeed, as seen in Figure 4A, the eGFP signal is not retained inside *S. coelicolor* when the inducer was added to the Sec-eGFP strain, indicating that the Sec-secretion signal leads to efficient transport of eGFP across the membrane into the environment. When we infected the inducible Sec-endolysin parental and Δ*yidc2* strain with phage CF3, we observed clearer lysis zones upon cumate induction compared to the uninduced plaque assays (Fig. 4B). However, the efficiency of plating was still lower in the *yidc2* mutant compared to the wildtype. Therefore, an additional adsorption assay was performed using the Sec-endolysin Δ*yidc2* strain, to verify if phage CF3 could escape the bacteria after cumate induces the release of additional endolysins 60 minutes after phage infection (Fig. 4C). Importantly, we observe that phage CF3, upon induction, could escape from the bacteria after 120 minutes. These results show that the block in phage release in *S. coelicolor* Δ*yidc2* can be overcome by ectopic expression and Sec-dependent secretion of the endolysins.

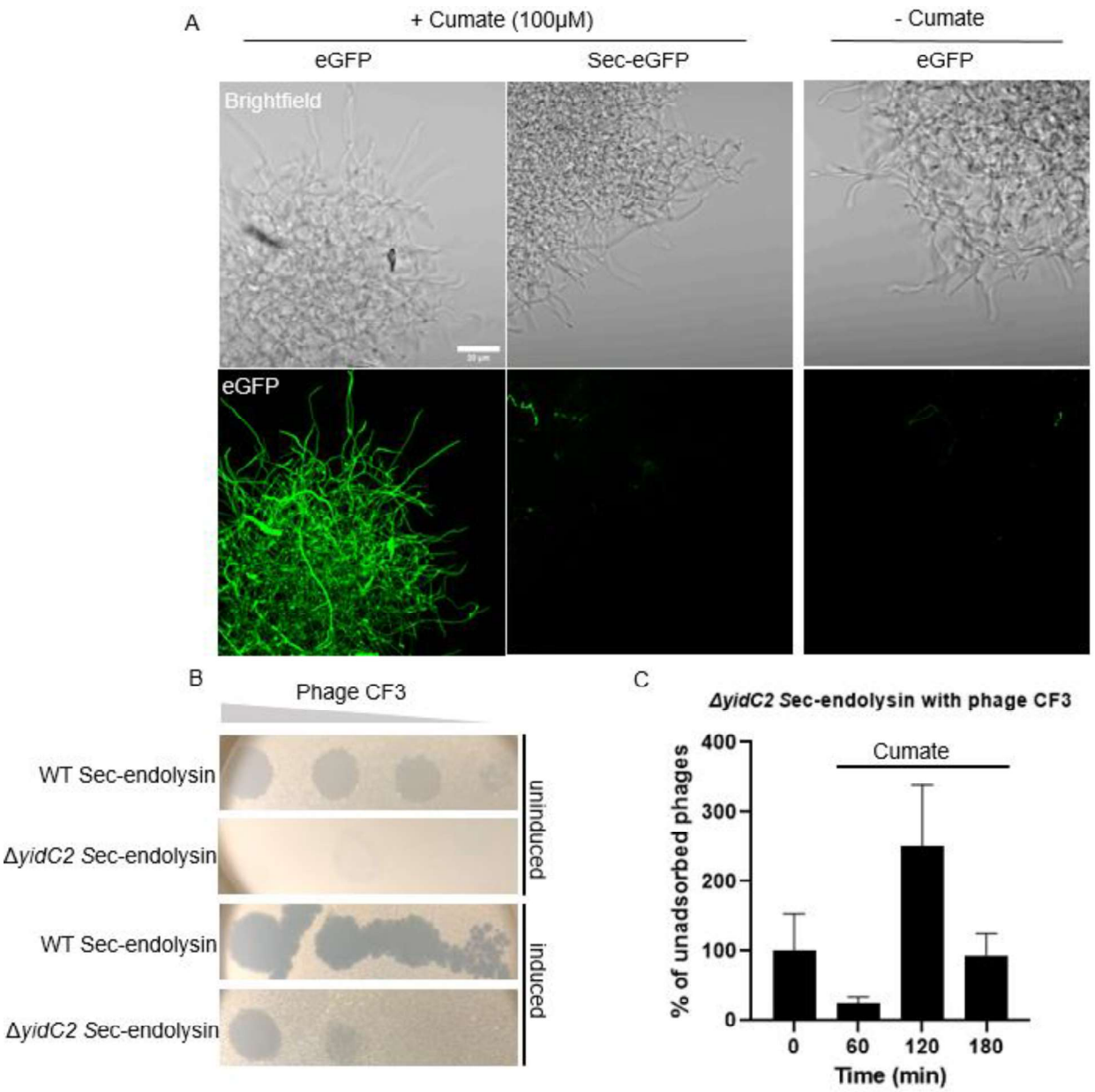


Figure 4. YidC2-independent secretion of the endolysin rescues release of phage CF3. (A) An inducible Sec-dependent eGFP protein is secreted outside of the cells after induction with 100µM cumate. Without cumate, the cells are not fluorescent. Scale bar is 20µm. (B) The WT and Δ*yidC2* strain with Sec-secreted endolysin proteins show clearer lysis zones when the bacterial strains are induced with 100µM cumate and infected with a serial dilution of phage CF3. (C) Adsorption assay of the Δ*yidC2* Sec-endolysin strain with phage CF3. Phage CF3 was added to young germlings at T=0 and after an hour of phage infection, the strain was induced with 100µM cumate to release endolysins in a Sec-dependent manner, which causes an increase in free phages after 120 minutes.

Summary and discussion

Our knowledge on the molecular mechanism of completing a successful phage infection is generally unknown. Once a phage has managed to eject its genome into a suitable bacterium, the phage has to redirect the metabolic activities of the host towards progeny phage production. Most phages depend on the host cell to supply essential amino acids, energy and machinery for replication. However, the strategies of a successful phage infection and thus a successful host-takeover are different for each particular phage-host pair [177]. After phage replication, holin proteins disrupt the bacterial membrane, allowing the fully-folded endolysin that accumulated in the cytosol to access the peptidoglycan, causing cell lysis and release of progeny phages [178]. Here, we showed that the membrane protein insertase YidC2 of *S. coelicolor* has a crucial interaction with the holin-endolysin system of phage CF3. Additionally, YidC2 might also be involved during cell wall shedding in these multicellular bacteria. Interestingly, these results could also be relevant for completion of the phage infection cycle in unicellular bacteria, as shown before [93].

Plaque assays and phage infection assays in liquid showed that *S. coelicolor* Δ*yidC2* was no longer susceptible to infection by phage CF3. Interestingly, no morphological differences were found between the wildtype and the *yidC2* mutant. Additionally, adsorption assays and cryo-EM revealed that phage CF3 could still attach and infect *S. coelicolor* Δ*yidC2*, but progeny phages could not lyse the host and accumulated inside the bacteria. Therefore, we hypothesized that the holin-endolysin system of phage CF3 interacts with the putative membrane protein insertase YidC2, which subsequently inserts the phages' holin protein in the bacterial membrane.

We predicted the interaction between the holin-endolysin system and the YidC2 protein with Alphafold3. Here, we showed that YidC2 has a strong predicted interaction with the holin protein and interacts with the N-terminus of the endolysin protein, while the holin interacts with the C-terminus of the endolysin. These results were also confirmed with an *in vivo* bacterial two-hybrid assay, suggesting an holin-endolysin interaction, an endolysin/YidC2 interaction and a strong dimerization of the endolysin protein. However, no interaction was observed between the holin and YidC2 protein in the bacterial

two-hybrid assay. The interaction interfaces of the holin could be blocked by the N- or C-terminal fusions or the holin protein could not fold properly during the assay, leading to a false-negative result [179]. When the endolysin protein of phage CF3 was synthesized and secreted independently of YidC2 in cumate-inducible Sec-endolysin Δ*yidC2* strain, we observed that the progeny phages could be released into the environment. Plaque assays showed clearer lysis zones when additional endolysins were induced from inside the Sec-endolysin Δ*yidC2* strain, but the infection could not be restored to the wildtype lysis zone morphology. Perhaps more proteins of phage CF3 rely on a functional YidC2 for release of healthy progeny phages. Since release of additional endolysins via the Sec-pathway is not enough to entirely restore the infection during plaque assays, perhaps lysis and subsequent progeny phage release is not completely holin-independent or the catalytic activity of the endolysins needs to be enhanced by holin interactions or various other processes [180, 181].

Notably, the term “holin” might be misleading, since not all holins form a hole or channel that let the endolysin pass through and many holins are uncharacterized at the molecular level [182]. Previous research has shown that phage-derived holins can be used for non-lytic endolysin translocation, by permeabilizing the bacterial cytoplasmic membrane and subsequent passage of secreted endolysins [183]. Correct insertion of the type III holin into the bacterial membrane exposes the C-terminus region, allowing the endolysin to associate. The membrane is then destabilized by multimeric patches of holins, resulting in local thinning of the membrane. The multimeric holin patches are triggered by an interaction with the single transmembrane helix and the holin flips irreversibly in a transmembrane orientation, moving the associated endolysin through the membrane. The endolysin will be released from the holin by the unstable transmembrane-oriented transition state. Subsequently, it will enzymatically degrade the peptidoglycan layer of the host bacterium [183]. Interestingly, this might also explain why we see the formation of CWD cells in WT *S. coelicolor* after phage infection, since the holins do not form the typical pore, and therefore the membrane remains intact in osmoprotective medium (Fig. 5).

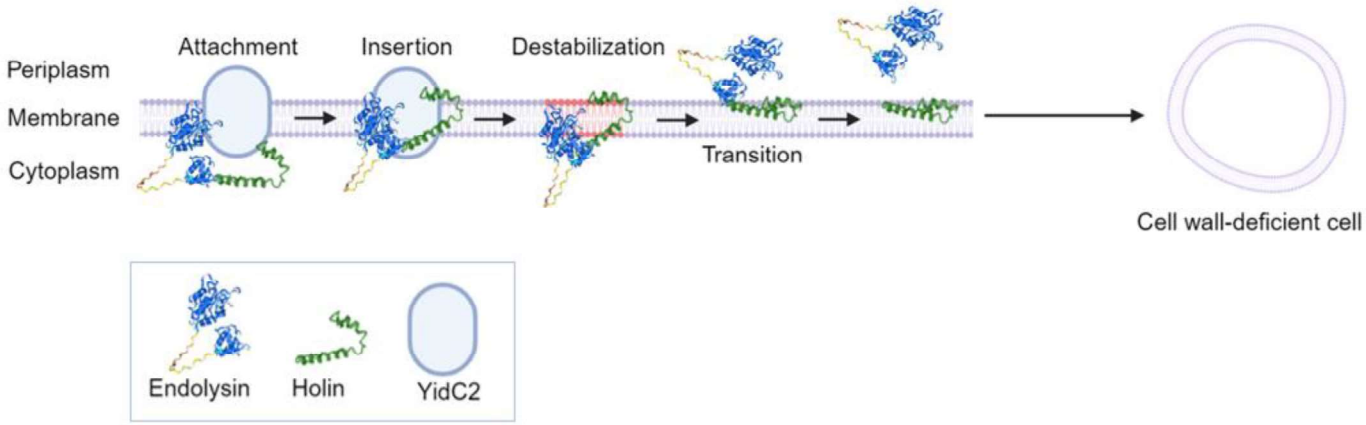
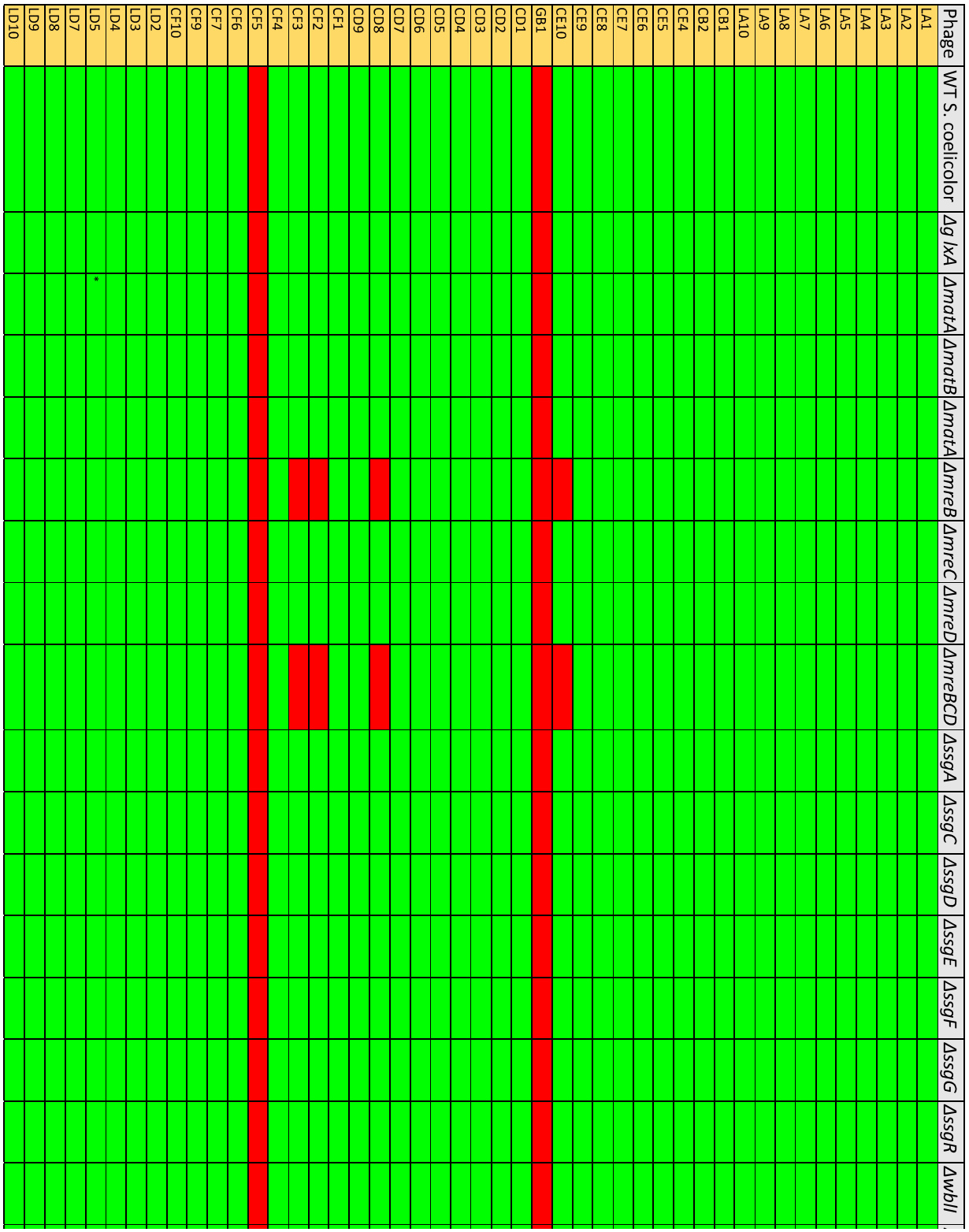


Figure 5. Hypothetical model of the function of YidC2 during infection with phage CF3. In *S. coelicolor*, YidC2 is a predicted membrane protein that integrates the phages' holin into the membrane. The endolysin interacts with the YidC2 protein, while the holin interacts with the C-terminus of the endolysin and probably also with YidC2. Then the endolysin switches conformation by interacting with itself and the holin is integrated in the membrane by YidC2. The holin destabilizes the membrane and switches to a transition state, subsequently "flipping" the endolysin through the membrane, so it can break down the peptidoglycan layer. Since the holin does not make a pore, the membrane remains intact, allowing the cell to shed the cell wall and switch to a CWD state. When YidC2 is knocked-out, the phage's endolysin cannot interact with YidC2 and the holin cannot be properly inserted in the membrane. Therefore, the progeny phages remain "stuck" inside the bacteria. Created with BioRender.com.

Taken together, this work suggests a molecular interaction between the holin-endolysin system of phage CF3 and the bacterial protein YidC2 from *S. coelicolor*. This further expands our knowledge on how phages takeover and manipulate the hosts metabolism. Additionally, we discovered that YidC2 plays a role during shedding of the bacterial cell wall after phage infection in *Streptomyces*. Altogether, these results form the basis to further explore the molecular mechanism of phage infection in *Streptomyces*, that are often used for antibiotic production in industry.

Supplementary data



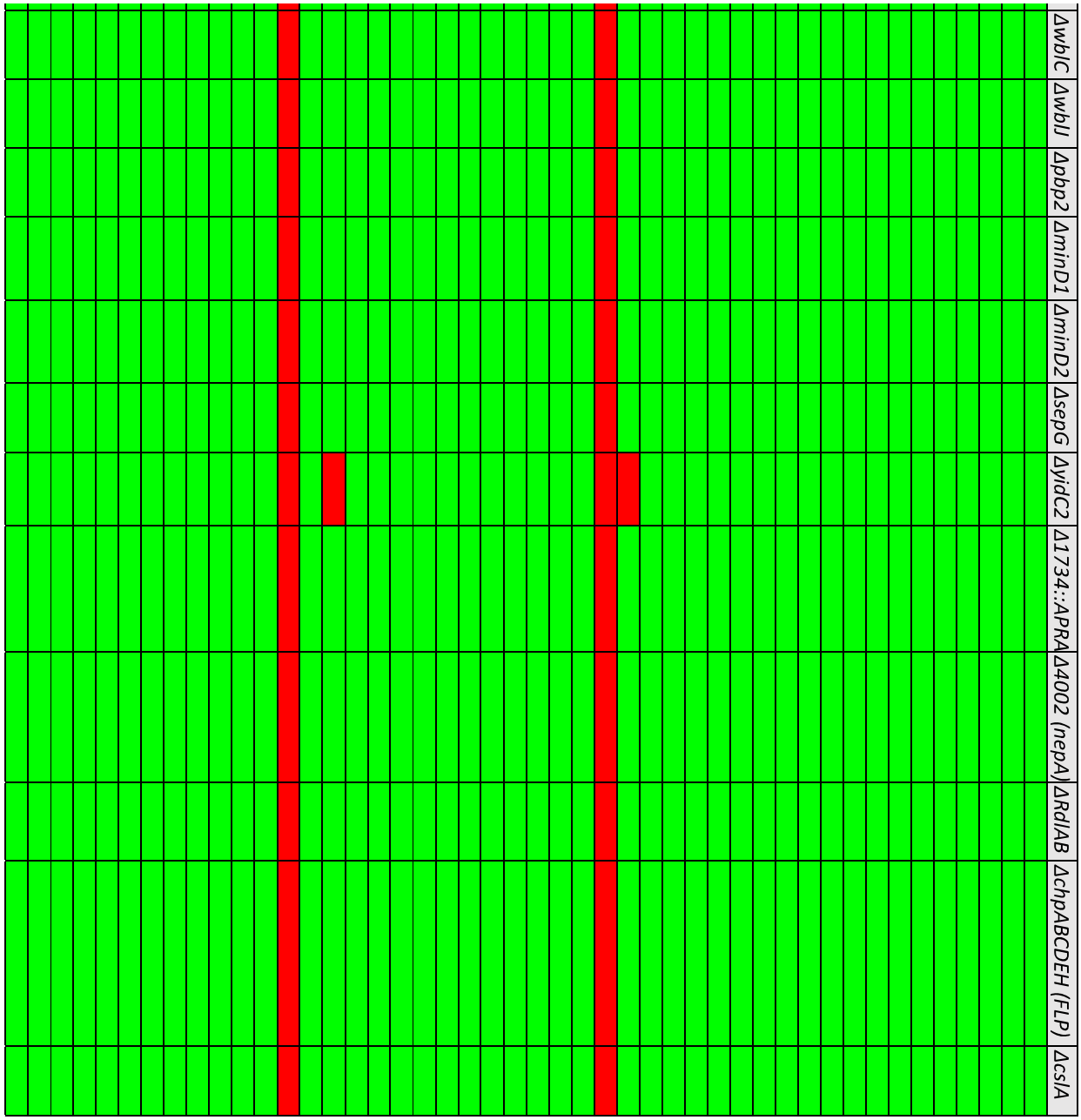
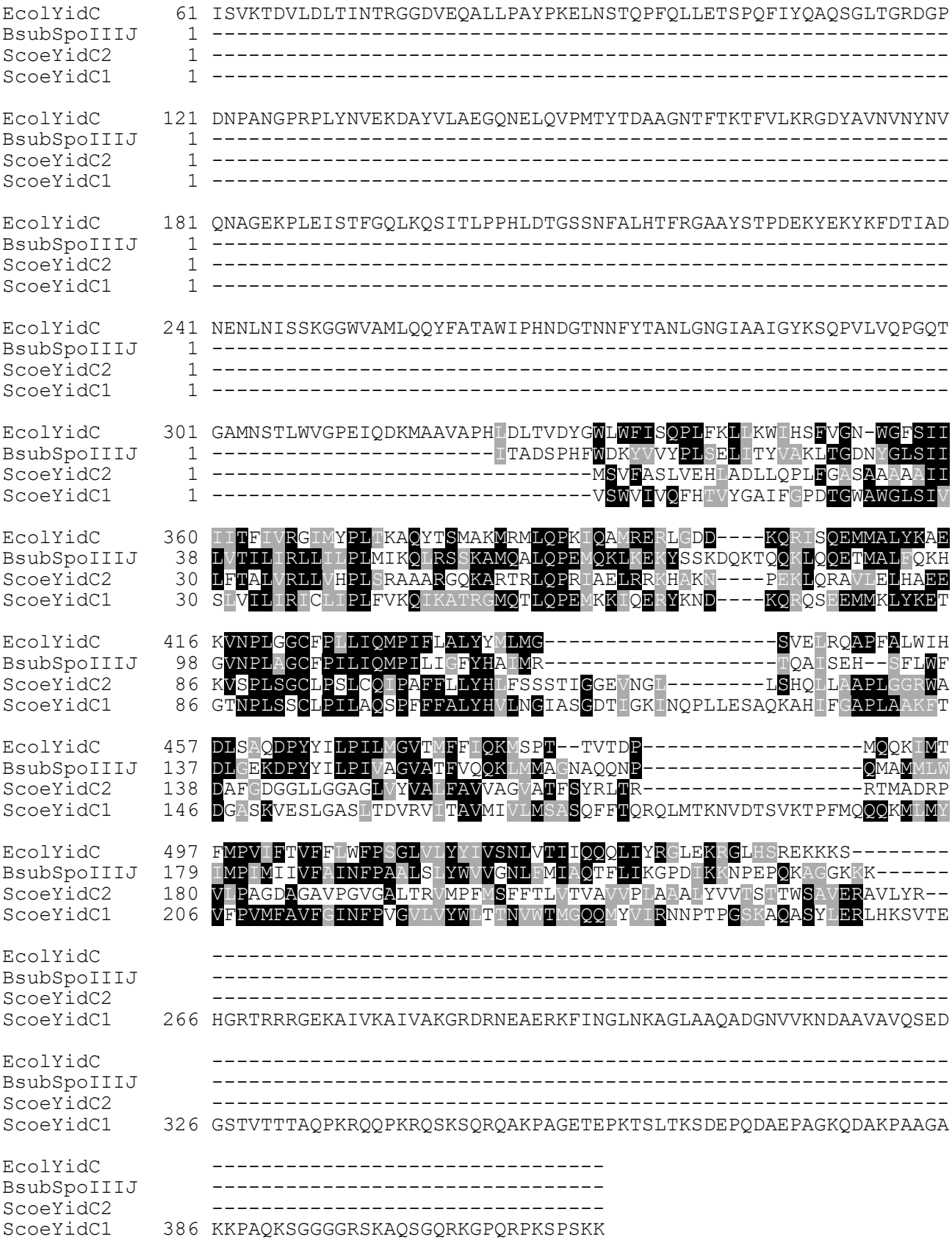


Figure S1. Phage-host interactions of *S. coelicolor* mutants against our phage library. Bacteria-phage interactions are depicted as plaque formation, both turbid and clear (green) or no visible plaque formation (red). Note that not all phages have been sequenced and we therefore suspect that phage CF3, CF2, CD8 and CE10 have a similar lifecycle.


EcolYidC 61 ISVKTDVLDLTINTRGGDVEQALLPAYPKELNSTQPFQLLETSPQFIYQAQSGLTGRDGP
BsubSpoIIIJ 1
ScoeYidC2 1
ScoeYidC1 1
EcolYidC 121 DNPANGPRPLYNVEKDAYVLAEGQNELQVPMTYTDAAGNTFTKTFVLKRGDYAVNVNYNV
BsubSpoIIIJ 1
ScoeYidC2 1
ScoeYidC1 1
EcolYidC 181 QNAGEKPLEISTFGQLKQSITLPPHLDTGSSNFALHTFRGAAYSTPDEKYEKYKFDTIAD
BsubSpoIIIJ 1
ScoeYidC2 1
ScoeYidC1 1
EcolYidC 241 NENLNISSKGGWVAMLQQYFATAWIPHNDGTNNFYTANLGNGIAAIGYKSQPVLVQPGQT
BsubSpoIIIJ 1
ScoeYidC2 1
ScoeYidC1 1
EcolYidC 301 GAMNSTLWVGPEIQDKMAAVAPHLDLTVDYGWLWFISQPLFKLLKWIHSFVGN-WGFSII
BsubSpoIIIJ 1 ITADSPHFWDKYVVYPLSELITYVAKLTGDNYGLSII
ScoeYidC2 1 MSVFASLVEHLADLLQPLFGASAAAAAII
ScoeYidC1 1 VSWVIVQFHTVYGAIFGPDTGWAWGLSIV
EcolYidC 360 IITFIVRGIMYPLIKAQYTSMAKMRMLQPKIQAMRERLGDD----KQRISQEMMALYKAE
BsubSpoIIIJ 38 LVTILIRLLILPLMIKQLRSSKAMQALQPEMQKLKEKYSSKDQKTQQKLQQETMALFQKH
ScoeYidC2 30 LFTALVRLLVHPLSRAAARGQKARTRLQPRIAELRRKHAKN----PEKLQRAVLELHAEE
ScoeYidC1 30 SLVILIRICLIPLFVKQIKATRGMQTLQPEMKKIQERYKND----KQRQSEEMMKLYKET
EcolYidC 416 KVNPLGGCFPLLIQMPIFLALYYMLMG----------SVELRQAPFALWIH
BsubSpoIIIJ 98 GVNPLAGCFPILIQMPILIGFYHAIMR----------TQAISEH--SFLWF
ScoeYidC2 86 KVSPLSGCLPSLCQIPAFFLLYHLFSSSTIGGEVNGL--------LSHQLLAAPLGGRWA
ScoeYidC1 86 GTNPLSSCLPILAQSPFFFALYHVLNGIASGDTIGKINQPLLESAQKAHIFGAPLAAKFT
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BsubSpoIIIJ 137 DLGEKDPYYILPIVAGVATFVQQKLMMAGNAQQNP----------QMAMMLW
ScoeYidC2 138 DAFGDGGLLGGAGLVYVALFAVVAGVATFSYRLTR----------RTMADRP
ScoeYidC1 146 DGASKVESLGASLTDVRVITAVMIVLMSASQFFTQRQLMTKNVDTSVKTPFMQQQKMLMY
EcolYidC 497 FMPVIFTVFFLWFPSGLVLYYIVSNLVTIIQQQLIYRGLEKRGLHSREKKKS--------
BsubSpoIIIJ 179 IMPIMIIVFAINFPAALSLYWVVGNLFMIAQTFLIKGPDIKKNPEPQKAGGKKK------
ScoeYidC2 180 VLPAGDAGAVPGVGALTRVMPFMSFFTLVTVAVVPLAAALYVVISITWSAVERAVLYR--
ScoeYidC1 206 VFPVMFAVFGINFPVGVLVYWLTTNVWTMGQQMYVIRNNPTPGSKAQASYLERLHKSVTE
EcolYidC
BsubSpoIIIJ
ScoeYidC2
ScoeYidC1 266 HGRTRRRGEKAIVKAIVAKGRDRNEAERKFINGLNKAGLAAQADGNVVKNDAAVAVQSED
EcolYidC
BsubSpoIIIJ
ScoeYidC2
ScoeYidC1 326 GSTVTTTAQPKRQQPKRQSKSQRQAKPAGETEPKTSLTKSDEPQDAEPAGKQDAKPAAGA
EcolYidC
BsubSpoIIIJ
ScoeYidC2
ScoeYidC1 386 KKPAQKSGGGGRSKAQSGQRKGPQRPKSPSKK

Figure S2. Alignment of *E. coli* YidC (NP_418161), *B. subtilis* SPOIIIJ (Z14225), *S. coelicolor* YidC1 (Y16311; SCO3883) and YidC2 (NP_627080; SCO2851). Black boxes indicate identical aa residues, while grey boxes indicate conserved aa changes. Note that amino acids 1-60 of *E. coli* YidC are not shown.

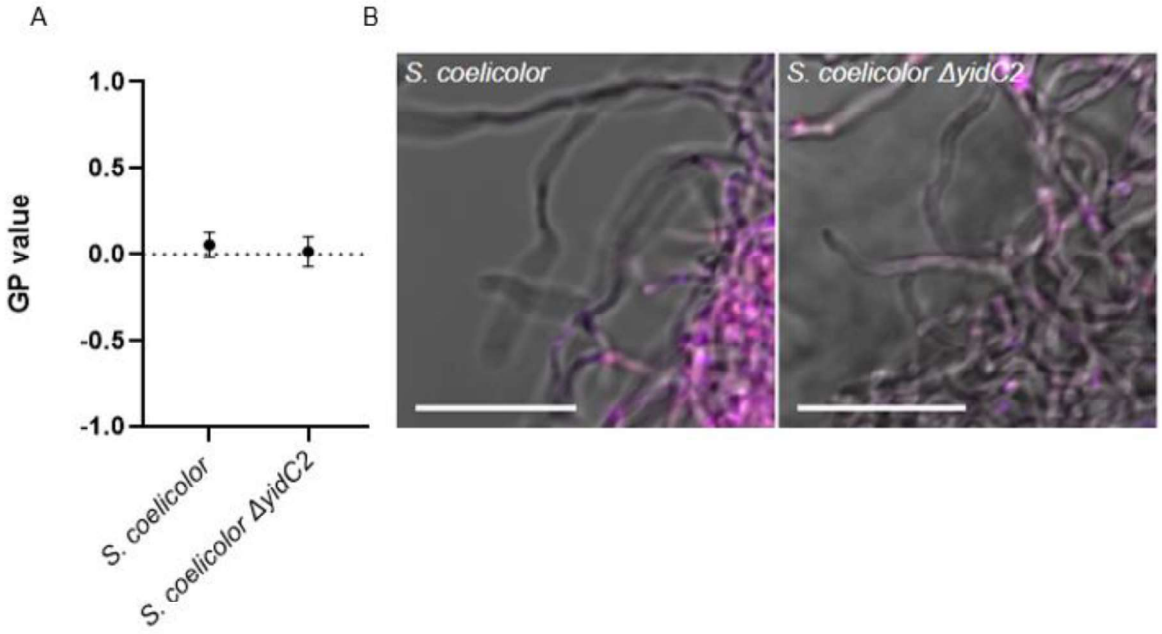

Figure S3. Membrane fluidity of *S. coelicolor* Δ*yidC2*. (A) Analysis to measure membrane fluidity using Laurdan staining. 24-hour old mycelia of both strains were labeled with 1mM 100µM Laurdan. The generalized polarization (GP) values range from +1 (less fluid) to -1 (more fluid). No significance in membrane fluidity of *S. coelicolor* Δ*yidC2* was observed. (B) Representative images of the Laurdan staining. Scalebar represents 10 µm.

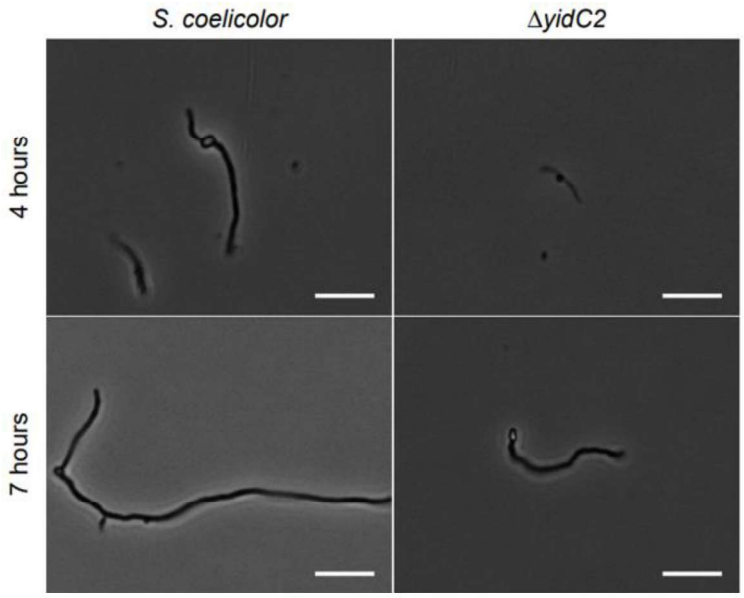

Figure S4. Growth speed. The *yidC2* mutant has a reduced growth speed in DNB medium at 30°C compared to the parental strain. After 7 hours of incubation, the mutant strains has developed germ tubes of approximately the same length as the parental strain at 4 hours.

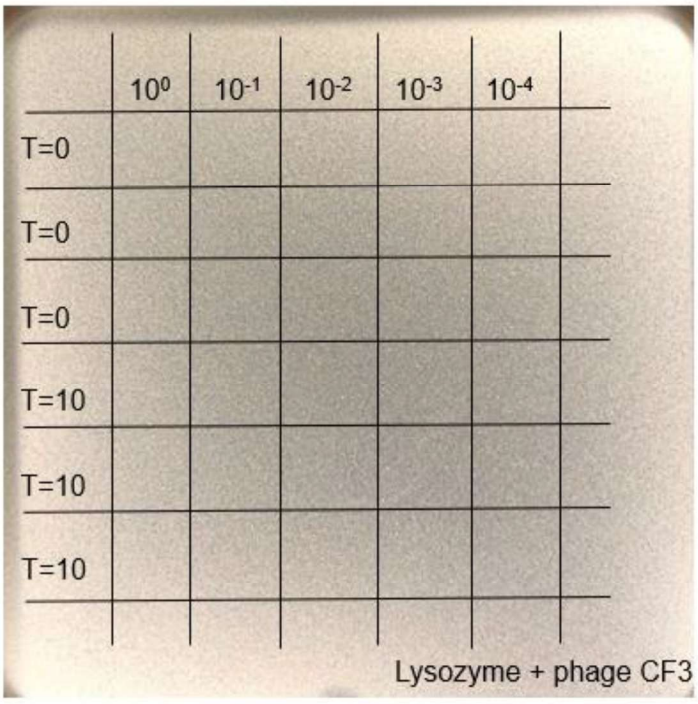

Figure S5. Phage CF3 does not survive an adsorption assay with lysozyme. When an adsorption assay of the *yidC2* mutant was supplemented with 0.2 mg ml^{-1} lysozyme and phage CF3, the phage did not give any plaques on *Streptomyces* strain MBT61, even at timepoint zero.

Table S1. Bacterial strains and phages used in this study.

Strains	Description	Reference
Bacterial strains		
Streptomyces coelicolor M145	Wildtype	Lab collection [94]
Streptomyces coelicolor Δ*yidC2*	Δ*yidC2*::aac(IV)3	This work
Streptomyces sp. MBT61	Wildtype	Lab collection [94]
Escherichia coli BW25113/pIJ790	K12 derivative: Δ*araBAD*, Δ*rhaBAD*	Lab collection [157]
Escherichia coli ET12567/pUZ8002	*dam, dcm, hsdS, cat, tet*	Lab collection [157, 184]
Phages		
Phage CF3	Wildtype, host: *Streptomyces coelicolor*	This work
Phage CD8	Wildtype, host: *Streptomyces coelicolor*	This work

Table S2. Plasmids used in this study.

	Description	Reference
Plasmids		
pIJ790	λ-RED (*gam, bet, exo*), *cat, araC, rep101*^{ts}	[157]
pUZ8002	*tra, neo*, RP4	[185]
S. coelicolor cosmid	Carb^{R}, Kan^{R}	[157]
pCB-1	addgene, #210387	[159]
pGWS758	Used to amplify coding sequence of eGFP	[160]

pIJ82	Streptomyces integrative plasmid	[130]
pIJ82Cu	pIJ82 containing the cumate-inducible promoter	This study
pIJ82Cu-SecGFP	pIJ82 plasmid expressing *signal peptide of SCO5376-eGFP* fragment from cumate inducible promoter	This study
pIJ82Cu-SecEndo	pIJ82 plasmid expressing *signal peptide of SCO5376-endolysin* fragment from cumate inducible promoter	This study
pIJ82Cu-GFP	pIJ82 plasmid expressing eGFP from cumate inducible promoter	This study
pIJ82Cu-Endo	pIJ82 plasmid expressing endolysin from cumate inducible promoter	This study
pKT25	pSU40 derivative expressing the T25 fragment of CyaA (amino acids 1–224)	[186]
pUT18C	pUC19 derivative expressing the T18 fragment of CyaA (amino acids 225–339)	[186]
pKT25-YidC2	pKT25 containing nucleotides 1–714 of YidC2	This study
pKT25-Holin	pKT25 containing nucleotides 1–279 of holin	This study
pKT25-Endolysin	pKT25 containing nucleotides 1–861 of endolysin	This study
pUT18C-YidC2	pUT18C containing nucleotides 1–714 of YidC2	This study
pUT18C-Holin	pUT18C containing nucleotides 1–279 of holin	This study
pUT18C-Endolysin	pUT18C containing nucleotides 1–861 of endolysin	This study

Table S3. Primers used in this study.

	Description
Primers	
E20_KOF	ACGTACTGGACCGTAGGCACGCACCGGACCGGCCCGTGTTCAATTCCGGGGATCCGT
E20_KOR	CTTGCCCGGCACGACGAGACCCTCGGAGGGCTCACCCACCCATGTAGGCTGGAGCTGCTTC

IDProm-F	AATTCGATATCGCGCGCGGCCGCGAACAATAAGGCCTCCCTAACGG
IDProm-R	GGCTGCAGGTCGACTCTAGACATATGGACACTCCTTACTTAGAATAATAC AAACAG
SecGFP-F1	TTATTCTAAGTAAGGAGTGTCCATATGCGCTTCAGACACAAAGCCGCGG CACTCGCAGC
SecGFP-F2	CGAGCCCGGCCCAGGCGGCCACGATGGTGAGCAAGGGCGAG
GFP-F	TTATTCTAAGTAAGGAGTGTCCATATGGTGAGCAAGGGCGAG
TatGFP-R2	GGCTGCAGGTCGACTCTAGAGCATATTACTTGTACAGCTCGTCCATGCCG AG
TatEndo-F2	CACCCGCCGCTCATGCCGCAGACGTGAGTGCAGTGCAGAAGGTCATC
SecEndo-R1	ATGACCTTCTGCACTGCACTCACCGTGGCCGCCTGGGCCGGGCTC
SecEndo-F2	CGAGCCCGGCCCAGGCGGCCACGGTGAGTGCAGTGCAGAAGGTCATC
Endo-F	TTATTCTAAGTAAGGAGTGTCCATGTGAGTGCAGTGCAGAAGGTCATC
ThYidC2-F	gccTCTAGAaATGTCCGTTTTCGCCAGCCTGGTCGAGCAC
ThYidC2-R	cggGAATTCtcaTCAGCGGTACAGCACGGCCCGC
ThHolin-F	gccTCTAGAaATGGGCCGACACACCGCAC
ThHolin-R	cggGAATTCtcaTCAGCCCTCGTAGGCCGACTCG
ThEndo-F	gccTCTAGAaGTGAGTGCAGTGCAGAAGGTCATC
ThEndo-R	cggGAATTCtcaTCAGCGACGACCACACTGCTTC