The DyP-type peroxidase DtpA is a Tat-substrate required for GlxA maturation and morphogenesis in Streptomyces

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The filamentous bacterium Streptomyces lividans depends on the radical copper oxidase GlxA for the formation of reproductive aerial structures and, in liquid environments, for the formation of pellets. Incorporation of copper into the active site is essential for the formation of a cross-linked tyrosyl-cysteine cofactor, which is needed for enzymatic activity. In this study, we show a crucial link between GlxA maturation and a group of copper-related proteins including the chaperone Sco and a novel DyP-type peroxidase hereinafter called DtpA. Under copper-limiting conditions, the sco and dtpA deletion mutants are blocked in aerial growth and pellet formation, similarly to a glxA mutant. Western blot analysis showed that GlxA maturation is perturbed in the sco and dtpA mutants, but both maturation and morphology can by rescued by increasing the bioavailability of copper. DtpA acts as a peroxidase in the presence of GlxA and is a substrate for the twin-arginine translocation (Tat) translocation pathway. In agreement, the maturation status of GlxA is also perturbed in tat mutants, which can be compensated for by the addition of copper, thereby partially restoring their morphological defects. Our data support a model wherein a copper-trafficking pathway and Tat-dependent secretion of DtpA link to the GlxA-dependent morphogenesis pathway.

1. Introduction

Streptomyces are multicellular bacteria with a complex developmental life cycle. Following the germination of spores, a network of interconnected filaments is established, which is called a vegetative mycelium. This mycelium feeds on nutrients in the soil until they become depleted. This nutrient scarcity triggers the onset of a developmental programme, leading to the lysis of the vegetative mycelium, and the formation of aerial hyphae that erect from the colony surface into the air, and which gives the colony a white, fluffy appearance [1,2]. Differentiation of these reproductive structures leads to the synchronous production of millions of grey-pigmented spores that easily disperse. At the onset of aerial mycelium formation, streptomycetes produce a richness of secondary metabolites, including numerous antibiotics, anti-tumour compounds and anthelmintic agents that make them of interest for pharmaceutical purposes [3,4]. Additionally, owing to their competence to directly secrete proteins in the culture broth, streptomycetes hold promise as hosts for the heterologous production of enzymes [5,6].

Metabolite and enzyme production typically occurs in large bioreactors. Growth under these conditions is characterized by the formation of large, biofilm-like aggregates of mycelium, called pellets [7,8]. Like in biofilms, formation and integrity of these structures depends on the synthesis of extracellular glycans [9–11]. Recently, reverse engineering of a non-pelleting strain of
Streptomyces lividans indicated a crucial role for the newly identified mut gene locus, putatively involved in synthesis of an extracellular glycan needed for pellet formation [12]. Deletion of the mut genes leads to a dispersed mycelium with a 60% increase in growth rate and productivity of S. lividans [12]. A second extracellular glycan involved in pellet formation is produced by enzymes encoded by the cslA–glxA locus [13–15]. The cslA gene encodes a protein belonging to family 2 of the glycolyltransferases, which contains cellulose and chitin synthases, among others [16]. CslA synthesizes a β-(1,4)-glycan at hyphal tips, which is thought to provide protection during the ongoing cell wall remodelling at these sites [15]. Mutation of cslA not only abolishes pellet formation in liquid-grown cultures, but also blocks aerial growth [13,15]. The cslA gene is located in an operon with the downstream located glxA gene. The cslA–glxA operon is probably acquired via horizontal gene transfer and is conserved among all streptomycetes, with some species having two copies [17]. In most streptomycetes, this gene cluster also contains a third gene downstream of glxA, called cslZ, which encodes an endoglucanase [15,17]. Like in the absence of cslA, deletion of glxA blocks development and abolishes pellet formation, coinciding with the loss of glycan deposition at hyphal tips [14,17]. This is consistent with a model in which both proteins cooperatively function in glycan deposition.

GlxA has been recently characterized [14]. The X-ray crystal structure revealed a unique tertiary structure with an active site consisting of a mononuclear copper (Cu) ion and a tyrosyl-cysteine redox cofactor, bearing resemblance to the Cu active site in fungal galactose oxidases (Gox) [18]. Enzymes of this family carry out the two-electron oxidation of primary alcohols to aldehydes with the reduction of dioxygen to hydrogen peroxide [19]. Unlike Gox, the active site Cu and putative substrate binding pocket is buried in GlxA, but can be accessed through channels leading down from three separate surface locations [14]. Notably, no significant in vitro enzymatic activity with D-galactose or a range of mono- or disaccharide substrates that are turned over by Gox was detected [14,20]. However, GlxA was able to turnover glycolaldehyde, the smallest molecule to contain both an aldehyde and a hydroxyl group. Thus, it is likely that the substrate specificity of GlxA is different from that of Gox.

Streptomyces lividans strongly depends on Cu to initiate the morphological switch from vegetative to aerial growth [21,22]. Our previous work provided clues for the existence of a Cu-trafficking pathway involved in this process. One of the proteins in this pathway, the Cu chaperone Sco, is required for morphogenesis under conditions of low Cu availability. Notably, morphogenesis of the sco mutant is restored by the addition of Cu to the medium [23]. Sco receives its Cu ion from the extracytoplasmic Cu chaperone ECUc [24], and, in turn, delivers Cu to the CuA site of an aa3-type cytochrome c oxidase (CcO) and to a second target, possibly the copper-enzyme GlxA, that is required to trigger aerial growth [14,23,24]. In contrast to the sco mutant, the glxA phenotype (on solid media or in solution) cannot be rescued by the addition of exogenous Cu [14].

sco (SLI_4214) and ecuc (SLI_4213) are the first two genes of an operon that also contains genes for a putative Cu transport protein (SLI_4212) and for a secreted protein with a putative twin-arginine translocation (Tat) signal sequence (SLI_4211) [25] and a dye-decolorizing peroxidase (DyP)-type domain [23,26,27]. DyPs are a new class of monohaem peroxidases that are widely distributed among bacteria and fungi, but their physiological role remains unclear [28,29]. Here, we show that SLI_4211, hereinafter called dtpA (for DyP-type peroxidase A), encodes a protein that functions as a peroxidase in the presence of GlxA and is required for executing a crucial enzymatic step in the cascade of the GlxA-dependent morphogenesis pathway. Deletion of the dtpA gene leads to an arrest in development owing to impaired GlxA maturation and function, which can be overcome by the extracellular addition of Cu to the medium. Extracellular complementation with Cu also restores GlxA maturation issues and development in tat mutants, thereby connecting Tat-dependent secretion of DtpA to GlxA-dependent morphogenesis. We propose an integrated model for how a Cu-trafficking pathway and Tat secretion ultimately link to the GlxA-dependent morphogenesis pathway.

Table 1. Streptomyces lividans strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or Source</th>
</tr>
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<tbody>
<tr>
<td>1326</td>
<td>Wild-type S. lividans 1326</td>
<td>[30]</td>
</tr>
<tr>
<td>ΔcslA</td>
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<td>[14]</td>
</tr>
<tr>
<td>ΔglxA</td>
<td>1326 lacking glxA (marker-less)</td>
<td>[14]</td>
</tr>
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<td>ΔcslZ</td>
<td>1326 lacking cslZ (marker-less)</td>
<td>this work</td>
</tr>
<tr>
<td>Δsco</td>
<td>1326 lacking sco (marker-less)</td>
<td>[23]</td>
</tr>
<tr>
<td>Δecuc</td>
<td>1326 lacking ecuc (marker-less)</td>
<td>[24]</td>
</tr>
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<td>ΔdtpA</td>
<td>1326 lacking dtpA (marker-less)</td>
<td>this work</td>
</tr>
<tr>
<td>ΔSLI_4212</td>
<td>1326 lacking SLI_4212 (marker-less)</td>
<td>this work</td>
</tr>
<tr>
<td>Δaxx</td>
<td>SLI_2481-2482::aac(3)IV</td>
<td>[23]</td>
</tr>
<tr>
<td>TK24</td>
<td>S. lividans TK24</td>
<td>[31]</td>
</tr>
<tr>
<td>ΔtatA</td>
<td>TK24 tatA::aac(3)IV</td>
<td>[32]</td>
</tr>
<tr>
<td>ΔtatB</td>
<td>TK24 tatB::aac(3)IV</td>
<td>[33]</td>
</tr>
<tr>
<td>ΔtatC</td>
<td>TK24 tatC::neo</td>
<td>[34]</td>
</tr>
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2. Methods

2.1. Bacterial strains and plasmids

All Streptomyces strains used in this study are presented in table 1. Mutants were constructed in S. lividans 1326 (S. lividans 66, stock number 1326 from the John Innes Centre) [30]. The tat mutants, kindly provided by Dr J. Anné and Dr L. Vannellaert (Katholieke Universiteit Leuven), were created in the S. lividans TK24 background [32–34]. Escherichia coli JM109 was used for routine cloning purposes [35]. Vectors and constructs were summarized in table 2.

2.2. Growth conditions and media

Streptomyces strains were grown at 30°C [31]. Streptomyces spores were isolated from soy flour-mannitol (MS) agar plates [38]. For phenotypical characterizations, ±10⁶ spores
were plated in square 2 × 2 cm patches on R5 agar plates, supplemented with 10 μM CuSO₄, FeSO₄, MnSO₄, ZnSO₄ or Co(NO₃)₂ if necessary. Photographs of plates were taken daily with a compact digital camera (Canon Ixus). For morphology in liquid-grown cultures, tryptic soy broth with 10% sucrose (TSBS) was used, which was supplemented daily with a compact digital camera (Canon Ixus). 

were inoculated with 10⁶ spores ml⁻¹. Samples from liquid-grown cultures were analysed by light microscopy with a Zeiss standard 25 microscope and sequencing. The mutants, which were then verified by PCR amplification and sequencing. The dtpA mutant was complemented by integration of plasmid pMLCP1 (table 2), which contains the dtpA gene under the control of the sco promoter. All primers used in this work are shown in table 3.

Table 2. Vectors and constructs used in this study.

<table>
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<th>plasmid</th>
<th>description</th>
<th>reference</th>
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</tr>
<tr>
<td>pWHM3</td>
<td>Streptomyces/E. coli shuttle vector</td>
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<td>pΔcsIZ</td>
<td>pWHM3 derivative containing the flanking regions of the S. lividans csIZ gene (SLI_3189) interspersed by the apra-loxP cassette</td>
<td>this work</td>
</tr>
<tr>
<td>pΔdtpA</td>
<td>pWHM3 derivative containing the flanking regions of the S. lividans dtpA gene (SLI_4211) interspersed by the apra-loxP cassette</td>
<td>this work</td>
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<tr>
<td>pΔSLI_4212</td>
<td>pWHM3 derivative containing the flanking regions of the S. lividans SLI_4212 gene interspersed by the apra-loxP cassette</td>
<td>this work</td>
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<td>pTDW46</td>
<td>pSET152 derivative containing the dagA gene, where the sequence corresponding to the original DagA signal peptide is replaced by aadA, the streptomycin resistance gene</td>
<td>[25]</td>
</tr>
<tr>
<td>pTDW47</td>
<td>pTDW46 containing a fragment encoding the DagA signal peptide</td>
<td>[25]</td>
</tr>
<tr>
<td>pMLCP1</td>
<td>pSET152 derivative with dtpA under control of the sco promoter</td>
<td>this work</td>
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<td>pMLCP2</td>
<td>pTDW46 derivative containing a fragment encoding the putative DtpA signal sequence</td>
<td>this work</td>
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<td>pMLCP3</td>
<td>pTDW46 derivative containing a fragment encoding the CslZ signal sequence</td>
<td>this work</td>
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<tr>
<td>pMLCP4</td>
<td>pTDW46 derivative containing a fragment encoding the putative EcUc signal sequence</td>
<td>this work</td>
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<td>pMLCP5</td>
<td>pTDW46 derivative containing a fragment encoding the putative GlxA signal sequence</td>
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<tr>
<td>pET3188</td>
<td>pET28a vector with N-terminal His-tag, containing the glxA (SLI_3188) gene encoding residues 35-645, restricted between the Ndel and HindIII sites</td>
<td>[14]</td>
</tr>
</tbody>
</table>

2.3. Construction of the csIZ, dtpA and SLI_4212 mutants

The csIZ, dtpA and SLI_4212 null mutants were created in S. lividans 1326 in a two-step process using the unstable pWHM3 plasmid and the Cre-loxP system as described [39]. In the csIZ null mutant, nucleotides +15 to +1011 relative to the start codon of SLI_3188 were replaced with the loxP-apra cassette, whereas in the dtpA mutant nucleotides +19 to +1208 relative to the start of SLI_4212 and in the SLI_4212 mutant nucleotides −18 to +1932 relative to the start of SLI_4212 were replaced. The Cre recombinase was used to remove the loxP-apra cassette from the obtained mutants, which were then verified by PCR amplification and sequencing. The dtpA mutant was complemented by integration of plasmid pMLCP1 (table 2), which contains the dtpA gene under the control of the sco promoter. All primers used in this work are shown in table 3.

2.4. Cloning and site-directed mutagenesis of dtpA and glxA

The SLI_4211 gene encoding DtpA was amplified from the genomic DNA of S. lividans strain 1326 by PCR using a forward primer (4211-F) with a flanking 5’-Ndel restriction site and a reverse primer (4211-R) with a flanking HindIII restriction site (table 3). The resulting PCR product (1134 bp) was ligated into the Ndel and HindIII sites of a pET28a (Kan⁺) vector (Novagen) to create an N-terminal His₆-tagged construct (pET4211). The C121G variant of GlxA was created using a QuickChange mutagenesis approach using the C121G-F and C121G-R primers and the pET3188 vector as template (table 3).

2.5. Over-expression and purification of DtpA

pET4211 (Kan⁺) vector was transformed into E. coli BL21 (DE3) cells. Overnight precultures (low salt LB medium;
Melford) were successively used to inoculate 1.4 l of high salt LB medium (10 g tryptone, 10 g sodium chloride, 5 g yeast extract per litre) with 50 mg ml⁻¹ kanamycin and grown at 37°C, 180 r.p.m. At an OD₂₆₀ of 1.2, 5-aminolevulinic acid (1 mM, 30 min, 4°C) and the cell pellet resuspended in 10 mM Tris/HCl, 500 mM NaCl, pH 7. A major peak eluted at approximately 77 ml consistent with a monomer species with bound CO and imidazole followed by application to an S200 Sephadex column (GE Healthcare) equilibrated with 20 mM NaPi, 100 mM NaCl, pH 7. A major peak eluted at approximately 77 ml consistent with a monomer species with fractions assessed by SDS–PAGE then concentrated and stored at −20°C. DtpA concentrations were determined by UV–visible (vis) spectroscopy (Varian Cary 60 UV–vis spectrophotometer) using an extinction coefficient (ε) at 280 nm of 37 470 M⁻¹ cm⁻¹.

### 2.6. Over-expression and purification of holo- and apo-GlxA proteins

Wild-type (wt) GlxA (residues 354–645) and the C121G variant were over-expressed in *E. coli* BL21 (DE3) using the pET3188 vector (table 2) and purified as previously reported [14]. For the C121G variant, no CuSO₄ was added before and after cell lysis so as to produce the apo-form of the variant. The production of apo wt GlxA required the adoption of an autoinduction procedure [40] using the medium 8ZY-4LAC-SUC [41]. All plastic and glassware was soaked in 0.1 M EDTA and rinsed extensively with doubly deionized water prior to use to ensure as metal-free conditions as possible. LB precultures were used to inoculate 400 ml 8ZY-4LAC-SUC

<table>
<thead>
<tr>
<th>primer name</th>
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<td>SLI_4211.P1</td>
<td>GATCGAATTCCTGCGGCGGCTGTTACAC</td>
<td>EcoRI</td>
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<td>SLI_4211.P4</td>
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<td>HindIII</td>
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</table>

Table 3. Primers used in this study. The used restriction sites are highlighted in italics.
cultures in 2 l baffled flasks with shaking at 180 r.p.m. 25°C for 48 h. Cells were then harvested and apo-GlxA was purified as previously described [14]. GlxA protein concentrations were determined using an ε_{280} of 78 730 M\(^{-1}\) cm\(^{-1}\).

2.7. Complex object parametric analyser and sorter measurements

Particle analyses using the complex object parametric analyser and sorter (COPAS) Plus profiler were performed as described previously [13,42]. Briefly, pellets were fixed with 4% formaldehyde for 30 min on ice, washed twice with phosphate-buffered saline, and stored at −20°C until further use. Samples were analysed using a COPAS Plus profiler equipped with a 1-mm nozzle (Union Biometrica, Holliston, MA). Pellets pass the laser beam over their longitudinal axis and data will be collected on the extinction (EXT) and time of flight (TOF) for all objects with a minimum TOF of 40. All experiments were performed in triplicate, and at least 2500 pellets were analysed per sample. The mean TOF values of the mutant strains were compared with that of the wt strain, which was set to 100%.

2.8. Western blot analyses

Mycelium was harvested from either TSBS liquid-grown cultures after 24 h of growth, or from solid R5 agar plates that had been overlaid with Track Edge membranes (Millipore) after 2 days of growth. The mycelia were washed with 10 mM Tris/HCl pH 7 buffer, and resuspended in 300 μl of the same buffer, followed by sonication on ice using a Bioruptor Plus (Diagenode). Complete lysis was checked by microscope, after which the lysed mycelium was separated into a supernatant and pellet fraction by centrifugation at 16 000 g (4°C). Bradford analysis was used to determine the protein concentrations in the supernatant fraction, and 10 μg of protein was used for separation by SDS–PAGE on precast 7.5% mini-buffer, followed by sonication on ice using a Bioruptor Plus equivalent of H2O2 followed by addition of 0.2 M glycolaldehyde (Sigma); 20 mM GlxA and 0.2 M glycolaldehyde; 0.2 M glycolaldehyde; 1 equivalent of H2O2 followed by 1 equivalent of ferrocyanide ([Fe(CN)₆]³⁻). Catalytic turnover was measured using a coupled assay whereby production of H₂O₂ by GlxA in the presence of the substrates glycolaldehyde (0.2 M), d-galactose (0.6 M), d-glucose (0.6 M) and N-acetyl-d-glucosamine (0.1 M) (all from Sigma) was tested for in the presence of DtpA or horse radish peroxidase (HRP) and the subsequent oxidation of ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma). Samples were prepared in 1 ml cuvettes containing 20 mM NaPi, 100 mM NaCl, pH 7.0, 30 mM ABTS, 20 μM GlxA, 5 μM DtpA or 1 μl of HRP (10 mg ml⁻¹) and the respective GlxA substrate, with reactions started by addition of GlxA. Oxidation of ABTS was monitored at 436 nm using a Hewlett-Packard 8453 diode-array spectrophotometer scanning between 190 and 1100 nm and thermostatted at 30°C. Turnover rate (k, s⁻¹) was calculated from ((ΔA_{436}/[ABTS]/t)/[GlxA]), where ΔA_{436} is the absorbance change at 436 nm upon ABTS oxidation, ε_{ABTS} is the extinction coefficient of the ABTS cation radical oxidation product taken as 29.3 mM⁻¹ cm⁻¹, t is the time in s and [GlxA] is the total millimolar concentration of GlxA in the assay.

2.10. Tat-dependent secretion assay

Analysis of Tat-dependent protein secretion was performed as described [25,44]. PCR fragments encoding the candidate signal peptides were cloned as Ndel–BamHI fragments into pTDW46, which contains the agarase gene lacking its original Tat signal sequence, expressed from the dagA promoter [44]. The empty plasmid pTDW46 was used as a negative control, whereas pTDW47 carrying a fragment for the original DagA signal peptide was used as a positive control. Candidate signal sequences were those from CslZ, DtpA, ECuC and GlxA (the putative signal peptide sequences are shown in table S). All constructs were transformed into S. lividans TK24 and S. lividans TK24 ΔnatC. The agarase assay was performed by spotting each strain (1000 spores in a 10 μl drop) on MM-C medium, which contains agar as the sole carbon source [25]. After 5 days of growth, plates were overlaid with Lugol solution (Sigma), and staining was recorded by taking digital images after 45 min. Average diameters of clearing zones were calculated from 10 replicates per strain.

2.11. Bioinformatics

SLI database numbers refer to the genome of S. lividans 66 (alternatively known as 1326 [45]). To study the conservation of gene order, we used the synteny web service SyntTax [46]. Signal sequence predictions were carried out using PRED-TAT [47], and prediction of the Tat-motif and scores for the peptidase cleavage sites were obtained using TatP [48].

3. Results

3.1. Identification and characterization of copper-related morphogens in Streptomyces lividans

The cslA and glxA genes are conserved in streptomycetes and are organized in a larger gene cluster that also contains the cslZ gene (electronic supplementary material, figure S1). In Frankia species, this gene cluster is located adjacent to a sco homologue. Also in certain Burkholderia species, Gox-encoding genes co-occur with sco genes, suggestive of a direct functional
correlation (electronic supplementary material, figure S1). In the genome of *S. lividans* 66 (also referred to as *S. lividans* 1326 [45]), *sco* is located elsewhere on the chromosome as a member of an operon containing the *ecuc*, *SLI_4212* and *dtpA* genes (electronic supplementary material, figure S1). Given the putative correlation, deletion mutants lacking the majority of the coding sequences of either *cslZ*, *SLI_4212* or *dtpA* were created as described in the Methods section, and compared with the previously generated mutants lacking *cslA*, *glxA*, *ecuc* or *sco* (figure 1). In line with earlier work, the *cslA* and *glxA* mutants failed to produce aerial hyphae on R5 agar plates, and development could not be restored to the mutants by the addition of 10 μM exogenous CuSO₄ (figure 1). In contrast, the *cslZ* and *SLI_4212* null mutants were identical to the parental strain and formed sporulating aerial hyphae after 3 days of growth. Deletion of *ecuc* led to a slight delay in aerial hyphae formation when compared with the parental strain (figure 1). However, in contrast to *sco* mutants, the *ecuc* mutant progressed through development after 6 days, whereas no aerial hyphae were formed by the *sco* mutant (electronic supplementary material, figure S2). Notably, the addition of 10 μM CuSO₄ to the medium rescued the morphological defects in the *ecuc* and *sco* mutants (figure 1 and electronic supplementary material, figure S2). The lack of development of these mutants is not a result of decreased CeO activity because the *cox* mutant, lacking CeO, develops normally (figure 1 and electronic supplementary material, figure S2 [23]). Interestingly, deletion of *dtpA*, encoding a putative haem peroxidase, also stalled development, which again could be restored by the addition of 10 μM CuSO₄ to the medium (figure 1 and electronic supplementary material, figure S2) or by reintroduction of the gene (electronic supplementary material, figure S3). Development could not be restored to any of the mutants by the addition 10 μM of FeSO₄, ZnSO₄, MnSO₄ or Co(NO₃)₂, showing that it is a specific effect mediated by the addition of Cu (electronic supplementary material, figure S4).

Given the defects of *cslA* and *glxA* mutants in pellet formation, we analysed mycelial morphology of the other mutants lacking copper-related morphogenes both microscopically and quantitatively using a COPAS [13–15]. This revealed that the average size of mycelia of the *cslA* and *glxA* mutants decreased to 28% and 31%, respectively, of those of the parental strain, and this was not affected by the addition of 10 μM CuSO₄ (figures 1b and 2). The *cslZ* and *SLI_4212* mutants formed similarly sized pellets as the parental strain in both the presence and absence of additional Cu (figures 1b and 2). Interestingly, like in the *cslA* and *glxA* mutants, mycelia of the *sco* mutant were less dense and more open in TSBS-grown cultures (figure 1b). Their average size was reduced to 29% of the size of wt mycelia (figure 2). In contrast to *cslA* and *glxA* mutants, pellet formation was restored to *sco* mutants by extra Cu (figure 1b and 2). Delelton of *ecuc* had a relatively minor effect, with the average pellet size reduced to 65% of that of wt pellets, whereas pellet sizes slightly increased to 75% of wt values when Cu was added to the cultures (figures 1b and 2). Like in the absence of *cslA*, *glxA* or *sco*, mycelial pellets were also much smaller in *dtpA* mutants, yielding mycelia whose average size was reduced to 36% of the size of wt mycelia. In the presence of elevated levels of Cu, pellet morphology and size were similar to those of wt pellets (figures 1b and 2). Taken together, these data indicate that development and pellet morphology strongly depend on *cslA*, *glxA*, *sco* and *dtpA* and the bioavailability of Cu. Furthermore, it suggests an interdependence of the four proteins.

### 3.2. The absence of Sco, ECuC or DtpA affects GlxA maturation

GlxA functionality requires the formation of a Tyr–Cys cross-link and the incorporation of a Cu ion [14]. Previous studies with fungal Gox have indicated that Cu is required to initiate

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![Figure 1](http://rsob.royalsocietypublishing.org/)
the formation of the Tyr–Cys cross-link. This processed form migrates faster on an SDS–PAGE gel than the immature form (without the Tyr–Cys cross-link) \[49\]. To assess whether this maturation affects GlxA mobility, purified apo-, holo-GlxA and a C121G variant were run on an SDS–PAGE gel and migration patterns detected by GlxA polyclonal antibodies. From figure 3, it is apparent that apo-GlxA (prepared under Cu-starved conditions) and the C121G variant, in which the cross-linking Cys residue is replaced by a Gly, migrate slower than the holo-GlxA (prepared under Cu-replete conditions). This indicates that the Tyr–Cys cross-link absent in both the apo-GlxA and the C121G variant accounts for this retardation of electrophoretic mobility. Interestingly, both the mature and the immature forms of GlxA were detected in mycelia of \textit{S. lividans} 1326 grown in TSBS cultures, with the mature form being more prominent (figure 4a). We then investigated whether the absence of Sco would influence the maturation of GlxA. In the absence of Sco, GlxA was exclusively found in its immature form, unlike in the parental strain (figure 4a). Interestingly, growth of the \textit{SCO} mutant in the presence of 10 \mu M CuSO\textsubscript{4} led to the accumulation of the mature form of GlxA (figure 4a), consistent with the restored formation of pellets (see above). Given the changes in pellet morphology in the \textit{ecuc} and \textit{dtpA} mutant strains, we also verified the maturation pattern of GlxA in these mutants. In the \textit{ecuc} mutant strain, only a small fraction of GlxA was in the mature form, with the majority being immature (figure 4a). However, no mature GlxA was identified in the \textit{dtpA} null mutant. Like in the \textit{SCO} mutant, the mature form of GlxA reappeared in the \textit{ecuc} and \textit{dtpA} null mutants grown in TSBS supplemented with 10 \mu M CuSO\textsubscript{4} (figure 4a). Deletion of \textit{CSLZ}, \textit{SLI}_4212 or \textit{CcO} (electronic supplementary material, figure S5A). Altogether, these data implicate Sco, ECuC and DtpA in the GlxA maturation pathway and in pellet morphology under low levels of Cu in liquid-grown cultures.

We also analysed whether the developmental block on solid medium related to GlxA maturation defects. The amount of the mature form of GlxA was strongly decreased in the absence of Sco and DtpA, and could be increased by the addition of 10 \mu M CuSO\textsubscript{4} to the medium (figure 4b). No major changes in the abundance of the mature form of GlxA were observed in the absence of ECuC, CSLZ, SLI_4212 and CcO (electronic supplementary material, figure S5B).

### 3.3. DtpA acts as a peroxidase in the presence of GlxA

Purified DtpA gave a UV–vis spectrum with absorption maxima at 406 (soret band), 502, 635 nm and a shoulder at 540 nm, typical of a resting state ferric (Fe\textsuperscript{III}) haem peroxidase.
Figure 4. Immunoblot analysis of GlxA maturation in the wt strain and Δsco, Δecuc and ΔdtpA mutants grown for 24 h in TSBS medium (a) or grown for 2 days on RS medium (b) in the presence (+) or absence (−) of 10 μM CuSO4. The two bands indicated with the solid arrows represent GlxA, the upper band being immature GlxA (no Tyr–Cys cross-link) and the lower band being mature GlxA (with Tyr–Cys cross-link). The band indicated with a dashed arrow indicates EF-Tu, which serves as a control for the total amount of protein loaded on the gel. Molecular weight markers are indicated in kDa.
reporter system that makes use of secretion of the agarase protein DagA, which is strictly Tat-dependent [25,44]. If secreted, then DagA will degrade agar into its oligosaccharides, visible as a halo surrounding the colony after staining with iodine. Control colonies of *S. lividans* TK24 expressing DagA with its native N-terminal signal sequence were surrounded by a zone of clearing of 1.09 ± 0.02 cm. Zones of clearing were also observed when the N-terminal signal sequences of CslZ (0.72 ± 0.03 cm) or DtpA (0.92 ± 0.02 cm) were fused to DagA, consistent with the *in silico* prediction (figure 6b). In contrast, no halos were found with the putative Tat signal sequences of GlxA and ECuC (figure 6b). Furthermore, when DagA fused behind the signal sequences of DtpA or CslZ was expressed in the tatC null mutant of *S. lividans*, no zones of clearance were detected (figure 6b). Taken together, these results demonstrate that the N-termini of CslZ and DtpA are bona fide Tat signal
sequences and that secretion of these two proteins depends on the Tat secretion pathway.

### 3.5. The addition of Cu restores GlxA maturation and morphogenesis to tatA and tatC mutants

Considering that the Tat substrate DtpA influences development in a Cu-dependent manner, we speculated that the previously described morphological defects of tat mutants [32,33] might be restored by the addition of 10 μM CuSO₄ (figure 7a). Surprisingly, this addition to R5 agar plates was sufficient to restore aerial growth in the tatA, tatB and tatC mutants. Addition of 10 μM ZnSO₄, MnSO₄ or Co(NO₃)₂ did not restore the formation of aerial hyphae when the strains were grown on R5 agar plates, whereas 10 μM FeSO₄ only slightly improved aerial growth in the tatB mutant (electronic supplementary material, figure S7). Notably, Western blot...
analysis revealed that the addition of 10 \( \mu M \) CuSO\(_4\) led to an increase in the mature form of GlxA in the \( \Delta tatA \) and \( \Delta tatC \) mutants, similarly as observed for the \( sco \) and \( dtpA \) mutants on R5 agar plates (figures 4b and 8a). In contrast, mature GlxA was detected in the \( \Delta tatB \) mutant irrespective of the presence of 10 \( \mu M \) CuSO\(_4\).

We also analysed pellet formation and GlxA maturation in liquid-grown TSBS cultures in the presence or absence of additional Cu. As expected, the three \( tat \) mutants grew as dispersed mycelia in liquid-grown TSBS cultures without added Cu. As on R5 agar, the mature form of GlxA was present in the \( \Delta tatB \) mutant in TSBS-grown cultures without additional Cu, in contrast to the \( \Delta tatA \) and \( \Delta tatC \) mutants (figure 8b). The mature form of GlxA reappeared in the \( \Delta tatA \) and \( \Delta tatC \) mutants grown in the presence of 10 \( \mu M \) CuSO\(_4\), and, importantly, restored pellet formation to the \( \Delta tatC \) mutant (figure 7b).

4. Discussion

Morphological differentiation in streptomycetes is a complex process that depends on environmental conditions and extensive extracellular signalling between hyphae [1,11,54]. Over the last decades, a large number of so-called \( bld \) genes were identified, which are required for development and in particular on the reference media, namely R2YE (or R5) agar plates. In most cases, the precise function of these genes is not clear. Recently, it was demonstrated that several of the so-called ‘classical’ \( bld \) mutants are disturbed in desferrioxamine (DFO) biosynthesis [55]. DFO is a chelator that recruits iron (Fe) from the extracellular environment [56,57]. Development in some of these \( bld \) mutants, and notably \( bldJ \) and \( bldK \), is restored by the addition of exogenous Fe to the culture, thereby bypassing the requirement for this chelator. Work from our and other groups has shown that in addition to Fe, Cu also plays a crucial role in morphogenesis [21,22,24,58]. The work described in this paper provides further molecular insights into the importance of significant levels of Cu for development, as we show here that many of the genes relating to what we have dubbed the Cu-trafficking pathway are required for aerial hyphae formation—and hence also for reproductive sporulation—when the bioavailability of Cu becomes limiting.

A key member of this pathway is GlxA, which requires Cu for formation of a cross-linked Tyr–Cys cofactor and enzymatic activity [14]. We provide evidence that the novel \( bld \) gene \( dtpA \) encodes a Tat substrate that is involved in Cu-dependent morphological development. DtpA is required for GlxA maturation, together with the Cu chaperone Sco, and as a haem-containing peroxidase DtpA also provides an interesting link between the copper- and iron-dependent pathways leading to morphogenesis.

The \( dtpA \) gene is located in a cluster of \( bld \)-related genes that is present not only in streptomycetes but also in the remotely related actinomycetes \textit{Frankia} sp. \textit{Ccl3}, \textit{Thermobifida fusca}, \textit{Nocardiopsis dassonvillei} and \textit{Catenulispora acidiphila}. The clustering of \( sco \), \( ecuc \) and \( dtpA \) in these organisms infers functional linkage between their gene products. Indeed, our work demonstrates that in streptomycetes these genes are all required for morphogenesis under conditions of Cu limitation, which is probably common in many laboratory media and in nature [59]. Based on past and current data, we propose a model for how the Cu chaperones Sco and ECuC function together with DtpA in the GlxA maturation pathway (figure 9). Eventually, mature GlxA functions together with CslA in the production and modification of an extracellular glycan that plays a crucial role in morphogenesis [14,15].

The absence of Sco has a dramatic effect on GlxA maturation, which can be compensated for by the addition of Cu to the medium. This connects well to our earlier work suggesting that Sco acts as the chaperone that provides Cu to GlxA [14]. Based on genomic context, this may also be true in other species, as previously suggested [60]. Sco in turn receives its Cu from ECuC [24], and our data indicate that the absence of \( ecuc \) also affects the correct maturation

Figure 8. Immunoblot analysis of GlxA maturation in \textit{S. lividans} TK24 and \( \Delta tat \) mutant strains grown for for 2 days on R5 medium (a) or grown for 24 h in TSBS medium (b) in the presence (+) or absence (−) of 10 \( \mu M \) CuSO\(_4\). The two bands indicated with the solid arrows represent GlxA, the upper band being immature GlxA (no Tyr–Cys cross-link) and the lower band being mature GlxA (with Tyr–Cys cross-link). The band indicated with a dashed arrow indicates EF-Tu, which serves as a control for the total amount of protein loaded on the gel. Molecular weight markers are indicated in kDa.
of GlxA, in particular under oxidizing conditions like in shaken liquid-grown cultures. However, the activity of ECuC is not essential for morphogenesis as the ecuc mutant formed a substantial aerial mycelium after prolonged incubation, and also formed pellets, albeit smaller, in liquid-grown cultures. This implies a role for ECuC in ensuring optimal Cu trafficking, but also indicates that Sco can obtain Cu in an ECuC-independent manner depending on the redox state of the environment. Something similar is true for GlxA, which in the absence of both Sco and ECuC can reach its mature conformation by the addition of Cu. How Cu is sequestered and transferred in the absence of these chaperones is not known and is under further investigation.

Our work clearly indicates that the maturation of GlxA depends on DtpA. We hypothesize that DtpA oxidizes Sco-bound Cu(I) to Cu(II). This would not only explain why GlxA maturation is impaired in both dtpA and sco mutants, but also why the CeO activity is reduced in the dtpA mutant ([26] and our unpublished data, 2015). Sco proteins bind both Cu(I) and Cu(II), and Cu transfer to acceptor proteins may depend on the oxidation state of the metal [60]. A role for DtpA in oxidizing metal ions would thus be very similar to the function of the Bacillus subtilis DyP-type peroxidase EfeB, which oxidizes Fe(II) to Fe(III) before uptake [61]. S. lividans also possesses an efeB homologue (SLI_2602), which is located in a gene cluster that contains genes encoding a lipoprotein (SLI_2601/efeO) and an iron transporter (SLI_2603/efeU). This organization is analogous to the dtpA gene cluster that contains genes for the lipoproteins Sco and ECuC and also for a metal transporter, in this case, the putative Cu transporter SLI_4212. Our data indicate that the dtpA gene cluster is tailored towards Cu trafficking, whereas the efeB gene cluster more likely influences iron homeostasis. Notably, the EfeO protein is reported to have a cupredoxin domain and to bind both Cu and Fe [62], again inferring cross-talk between both metals in pathways that are crucial for morphogenesis.

Our data demonstrate that GlxA produces H2O2 in the presence of glycolaldehyde, which is so far the best substrate determined for GlxA [14]. The H2O2 is then used by DtpA, thus contributing to protection of the hyphal tip from oxidative damage. Removal of the H2O2 leads to the formation of DtpA compound I. The conversion of compound I to compound II is not observed in our assay because of the reducing nature of excess glycolaldehyde leading to the slow formation of an oxyferrous species. However, compound II is detected in DtpA through the controlled reduction of compound I (as shown in figure 5a), indicating that DtpA is behaving as a true peroxidase and that the peroxidation mechanism is operable. Lack of a true substrate for GlxA therefore hampers a fuller investigation into the events occurring after compound I formation, but the formation of the latter clearly indicates a synergy between GlxA and DtpA. Cooperation between an oxidase and peroxidase has been demonstrated in some fungi, for instance between the glyoxal oxidase and manganese peroxidase in Phanerochaete chrysos [63,64]. Therefore, the discovery of DtpA might help unravelling the substrate that is converted by GlxA, a crucial
step towards discovering the composition and structure of the
glycan produced by CsaA and GlxA.

4.1. Can the Tat substrate DtpA explain the
morphological defects of tat mutants?

The Tat secretion pathway is a major route for protein export in
streptomycetes in comparison with most other bacteria [25,33],
and resides at the hyphal tip [65]. *Streptomyces* mutants lacking
tatA, tatB or tatC have morphological defects in liquid-
grown environments, and also fail to develop a robust aerial
mycelium [25,32–34]. Owing to the large number of predicted
Tat substrates (between 145 and 189 [25,48,66,67]), no obvious
candidates could be held responsible for these defects, which
are undoubtedly caused by multiple missing proteins. However,
our work shows that the Tat-secreted protein DtpA is a
crucial substrate that, at least in part, explains some of the
morphological defects observed in the tat mutants.

Adding Cu remarkably improved aerial mycelium for-
mation by *S. lividans* tat mutants, which was also sufficient to
restore the formation of pellets, albeit small, in liquid-grown
cultures of *tatC* mutants. Western blot analysis indicated that
the levels of mature GlxA in the tatA and tatC mutants were
increased by the addition of Cu to the medium. These results
are consistent with a model in which the TatA and TatC com-
ponents of the Tat translocation machinery facilitate secretion
of DtpA at the hyphal tip, where it contributes to the CsaA–
GlxA-dependent pathway of morphogenesis (figure 9). The
addition of Cu also stimulated aerial growth in the tatB
mutant, but this appears to be unrelated to the maturation
status of GlxA. Given that in organisms that have a TatB protein
it is also required for efficient Tat-mediated translocation
[68–70], we suspect that, like in tatA and tatC mutants, DtpA
is also not secreted in *tatB* mutants. However, besides its role
in protein translocation, TatB may have an additional function,
which is not shared with TatA and TatC. This would also be
consistent with the observed differences in growth and mor-
phology of the tat mutants (figure 7a). Such differential
phenotypes were also observed for components of another
important tripartite transport system, namely that of the PTS
sugar transport system in *Streptomyces*, where mutants in
ptsH have a phenotype that is distinct from that of *ptsI* and
crr mutants [71]. Also, in this case, no additional function is
known for any of these genes. We hypothesize that the
developmental rescue of the *tatB* mutant by Cu is mediated
by an unknown Cu protein that does not necessarily relate to
the GlxA-dependent pathway described here. The observed
variations in phenotype and GlxA maturation between the
different tat mutants thus form an interesting starting point
for further analysis of possibly specific roles of the individual
Tat proteins in streptomycetes.

Our model suggests that the proteins involved in apical
polymer synthesis may be organized in a larger complex.
CsaA is an integral membrane protein, whereas GlxA has an
N-terminal membrane anchor and is shown to be mem-
brane located [14]. Indeed, both proteins were shown to be
tip-localized [15,17]. The chaperones Sco and ECuC are pre-
dicted lipoproteins [72], whereas DtpA may also remain
anchored to the membrane considering the presence of a
transmembrane helix that overlaps with the Tat signal
sequence. Given the weak signal peptidase recognition site,
this offers the option that DtpA remains anchored to the
membrane after transport, which is particularly important
in liquid environments where the protein could otherwise
diffuse away from its proposed functional site. Interestingly,
the gene adjacent to *sco* encodes a copper-responsive protein
with a so-called cohesin domain. Such domains are important
for assembly of large macromolecular complexes, most nota-
bly the cellulosome of *Clostridium thermocellum* [73]. Whether
this protein is involved in assembly of a large protein com-
plex involved in hyphal tip glycan deposition is under
current investigation.

Data accessibility. The datasets supporting this article have been
uploaded as part of the electronic supplementary material.

Authors’ contributions. M.L.C.P., E.V. and A.K.C. carried out the labora-
designed the experiments and wrote the manuscript; all authors
helped draft the manuscript, and gave their final approval for
publication.

Competing interests. We declare we have no competing interests.

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