Controlling growth and morphogenesis of the industrial enzyme producer Streptomyces lividans

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Chapter 3

Apical synthesis of an extracellular polysaccharide involved in aerial growth and hyphal attachment of *Streptomyces lividans* depends on CslA and GlxA

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ABSTRACT

Extracellular polysaccharides are produced by many microorganisms and play pivotal roles in various aspects of their biology. The cellulose synthase-like protein (CslA) synthesizes a β-(1,4)-glycan at hyphal tips in the filamentous bacterium *Streptomyces coelicolor*. Here we show that the downstream located *glxA*, which encodes a radical copper oxidase, acts in conjunction with *cslA* in the production of a β-(1,4) glycan in *Streptomyces lividans*, and is required for its apical localization. The extracellular glycan becomes microscopically visible as fibrillar structures upon binding to a chimeric eGFP-chitin-binding domain fusion protein, suggesting that it consists at least in part of chitin. Perhaps as a consequence of the failure to produce the glycan polysaccharide, colonies of *S. lividans cslA* and *glxA* mutants could not grow invasively into the agar and were developmentally arrested. In liquid-grown cultures, *cslA* and *glxA* mutants did not form pellets but rather produced open mycelial networks. Taken together, our data demonstrate the involvement of *cslA* and *glxA* in the control of mycelial architecture in liquid- and solid-grown cultures.

INTRODUCTION

Streptomycetes are filamentous soil-dwelling bacteria, which establish branched networks of vegetative hyphae. When nutrients become limiting, a developmental program is initiated, producing on solid media erect aerial hyphae that eventually differentiate to generate chains of spores, which are then dispersed. Unlike unicellular bacteria, which grow by lateral wall extension, growth of *Streptomyces* hyphae occurs at the hyphal tips, and exponential growth is achieved by combination of tip growth and branching. A pivotal role in the control of apical growth is exerted by the polar-localized protein DivIVA, which orchestrates cell wall synthesis (Flärdh et al. 2012). DivIVA is part of a larger so-called tip-organizing center (TIPOC) and in recent years, several proteins and complexes have been identified that are (transiently) localized at the hyphal tip. These include the cytoskeletal protein Scy (Holmes et al. 2013), the Tat secretion system (Willemse et al. 2012) and the cell wall remodeling protein SsgA (Noens et al. 2007). Furthermore, new chromosomes are also replicated near the tip in so-called replisomes (Wolánski et al. 2011). This complex TIPOC likely ensures that all apical processes, such as DNA replication and cell wall synthesis, are carried out in coordinated fashion, while cell damage is prevented (Ditkowski et al. 2013; Fuchino et al. 2013).

The TIPOC protein CslA, which stands for cellulose synthase-like protein (Bentley et al. 2002), plays multiple roles in growth and development; mutants lacking the *cslA* gene fail to form aerial hyphae and are affected in attachment to solid surfaces (Xu et al. 2008; de Jong et al. 2009). Furthermore, CslA plays an important role in the morphology of mycelial clumps in liquid cultures (Xu et al. 2008; van Veluw et al. 2012). CslA is classified as a family 2 glycosyltransferase (GT2, http://www.cazy.org/). This family does include cellulose synthases but also chitin synthases and other polysaccharide synthases. The polymers produced by these synthases have many different functions, often directed at providing structural integrity.

Cellulose is the most abundant natural polymer on earth and present in plants, where it provides strength to the cell wall (Bringmann et al. 2012), and

in bacteria (Römling 2002), where it contributes to the formation of biofilms and plays a role during infection (Beloin et al. 2008). For instance, *Rhizobiaceae* produce cellulose to adhere to plants (Smit et al. 1992), while *Salmonella* spp. and *Escherichia coli* require cellulose for virulence (Zogaj et al. 2003; White et al. 2003; Saldaña et al. 2009). Like cellulose, chitin typically also has a structural role (Martinez et al. 2009). In fungi chitin is a major component of the cell wall, where it plays crucial roles in tip growth, cytokinesis, spore formation and also pathogenicity (Latgé 2007; Lenardon et al. 2010). It is also produced in pathogenic amoebae, in parasitic nematodes and is abundantly present in the exoskeletons of invertebrates (Merzendorfer 2011). In *Streptomyces*, chitin is found among others in the spore coat (Smucker and Pfister 1978; Gomes et al. 2008).

The polysaccharide synthesized by CslA has not yet been characterized. Typically, genes involved in bacterial cellulose synthesis are organized in an operon, containing the *bcsABCD* genes (Römling 2002). However, *Streptomyces* lacks a c-di-GMP binding protein (BcsB), which is essential for the synthesis of cellulose according to the currently accepted model (Ross et al. 1991). Interestingly, *cslA* is translationally coupled to *glxA*, which encodes a radical copper oxidase that requires for its activity the Cu cofactor and the formation of a characteristic Tyr-Cys covalent bond. GlxA has weak homology to galactose oxidases and does not utilize galactose efficiently as substrate (Whittaker and Whittaker 2006). Besides in streptomycetes, this particular gene organization is only found in the myxobacterium *Stigmatella aurantiaca*, where deletion of either *fbfA* or *fbfB* hampers fruiting body formation following starvation (Silakowski et al. 1996). While *cslA* and *glxA* mutants of the model streptomycete *Streptomyces coelicolor* are blocked in development on rich media (Liman et al. 2013), the functional correlation of these two genes has not yet been clarified.

In this work, we show that GlxA cooperates with the synthase CslA in forming a β-(1,4)-glycan, presumably chitin-like, that is deposited at the hyphal surface fulfilling multiple roles in growth and development.

RESULTS

Conserved gene synteny around cslA-glxA *in streptomycetes*

The overlapping *cslA* and *glxA* genes of *S. coelicolor* encode the cellulose synthase-like protein CslA (SCO2836) and the radical copper oxidase GlxA (SCO2837), respectively. In *S. lividans*, the equivalent gene cluster consists of SLI_3187-3189 (Cruz-Morales et al. 2013) (Fig. 1).

Transcriptome analysis performed in our laboratory (Chapter 4) shows that *cslA* and *glxA* have similar expression levels in both *S. coelicolor* and in *S. lividans*, supporting the idea that *cslA-glxA* form an operon. *cslA, glxA* and SCO2838-SLI3190, encoding a polysaccharide hydrolase, are likely functionally linked and present in nearly all streptomycetes. Interestingly, some of them, including *S. griseus* and *S. albus*, have a second copy of the *cslA-glxA* operon elsewhere on the genome, with 54 and 66% end-to-end amino acid identity between the paralogous of CslA and GlxA proteins in *S. griseus*, respectively (not shown).

In terms of broader gene synteny, it is interesting to point at the following genes that are found in close proximity to *glxA-cslA* in nearly all streptomycetes: *chb* (SCO2833/SLI_3182), for a chitin binding protein that is part of the chitinolytic system of *S. coelicolor* (Schrempf 2001; Colson et al. 2007), SCO2834/SLI_3183 for a flotillin domain protein implicated in localizing multiprotein complexes to the membrane, SCO2835/SLI_3184 for a peptidoglycan binding protein, SCO2841/SLI_3192 for sortase E and SCO2843/SLI_3194 for a sugar hydrolase related to N-acetylglucosamine deacetylase (Table 1). Thus, at least two genes near *cslA-glxA* correlate to chitin or its subunit N-acetylglucosamine.

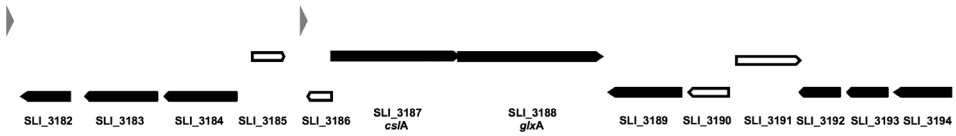

Figure 1. Gene organization around the *cslA-glxA* operon.
The gene context of the chromosomal locus around *cslA* and *glxA* i. The filled arrows represent genes that are conserved in nearly all streptomycetes. The open arrows represent genes that are not conserved, while the grey arrowheads represent tRNA loci.

Table 1. Annotation and gene numbers in *S. lividans* and *S. coelicolor.*

S. lividans gene #	S. coelicolor gene #	Annotation
SLI_3182	SCO2833	Chb, Copper-dependent lytic polysaccharide monooxygenase (LPMO); chitin binding domain; AA10 family1
SLI_3183	SCO2834	Penicillin acylase/amidohydrolase, flotillin
SLI_3184	SCO2835	Protein with C-terminal putative peptidoglycan binding domain
SLI_3185		doubtful ORF
SLI_3186		doubtful ORF
SLI_3187	SCO2836	CslA, Glycosyl Transferase Family 21
SLI_3188	SCO2837	GlxA, Radical copper oxidase
SLI_3189	SCO2838	Glycosyl Hydrolase Family 6
SLI_3190	SCO2839	Lipoprotein
SLI_3191	SCO2840	LysR-family transcriptional regulator
SLI_3192	SCO2841	Peptidase C60 Family, Sortase E
SLI_3193	SCO2842	Putative substrate of SCO28412
SLI_3194	SCO2843	Sugar (GlcNAc-6P) phosphatases of the HAD superfamily

The cslA *and* glxA *mutants are stalled in development and are hampered in agar invasion*

To study the role of *cslA* and *glxA* in growth and morphogenesis of *S. lividans*, deletion mutants were constructed (see Material and Methods) and grown on different media (Fig. 2). Unlike *S. coelicolor* A3(2), *S. lividans* 1326 is delayed in development when grown on R5 agar plates, but the development can be stimulated by adding 2-10 μM Cu(II) to the medium (Fig. 2A and (Keijser et al. 2000). Null mutants of *cslA* or *glxA* in *S. lividans* failed to form aerial hyphae on R5 agar plates with 10 μM copper. In

contrast, development of the mutants was comparable to that of the parental strain when grown on MS agar plates. Development of the mutant was restored when a copy of the respective genes, expressed from the *cslA* promoter, was re-introduced (Fig 2A). This shows that the mutant phenotypes were specifically caused by absence of *cslA* or *glxA*.

Notably, the mycelia of *cslA* and *glxA* mutants had lost the ability to adhere to and grow into the agar (Fig. 2B). As a result, colonies of the mutant strains could easily be removed from the agar surface. Introduction of a copy of the respective wild-type genes restored the ability of colonies of the *cslA* mutant and (although to a somewhat lesser extent) of the *glxA* mutant to grow into the agar (Fig. 2B).

CslA and GlxA control pellet architecture in liquid-grown cultures

The *S. coelicolor cslA* mutant was previously shown to form significantly smaller pellets than the wild-type strain (Xu et al. 2008; van Veluw et al. 2012). To see if the same was true for a mutant lacking the *glxA* gene, the morphology of the *cslA* and *glxA* null mutants was compared to that of the parental strain *S. lividans* 1326 in YEME and TSBS liquid-grown cultures (Fig. 2C). This revealed that like *cslA* mutants, *glxA* mutants also formed less dense pellets in YEME. The most striking effect was observed in TSBS medium, wherein the mutants formed open mycelial networks (mycelial mats) rather than the clumps typical of the parental strain. These results indicate a role for CslA and GlxA in pellet architecture. In addition, the similarity between the phenotypes of the *cslA* and *glxA* mutants in liquid-grown cultures further supports functional linkage between the two genes.

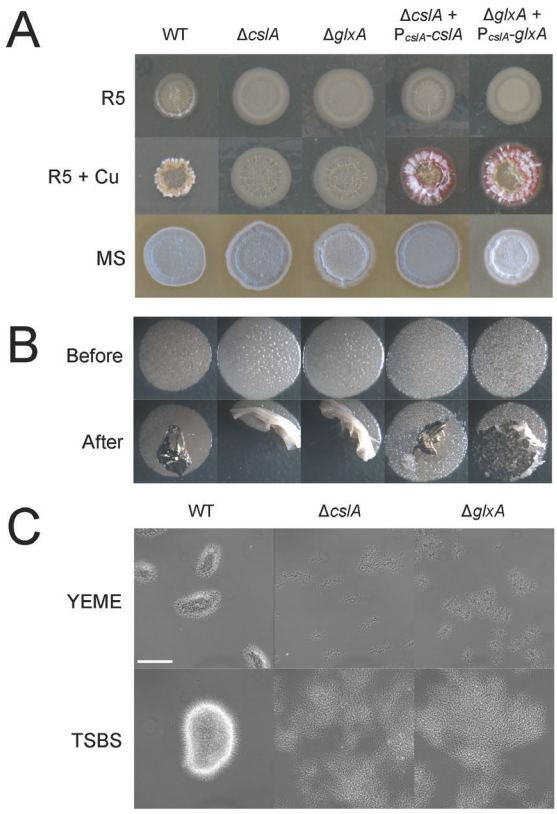

Figure 2. Morphology, copper dependency and hyphal attachment of *cslA* and *glxA* mutants in *S. lividans*.
(A) The wild type, *cslA*, *glxA* mutants and the mutants complemented with a plasmid expressing the corresponding gene (pGMMP3 and 4) were grown for 4 days on R5, R5 supplemented with 10μM Cu(II) and MS. Drops of 10 μL containing 1000 spores were spotted on the plates followed by incubation at 30 ^{0}C. Both the mutants and the complemented strains are bald on R5, while the wild type strain is entering aerial growth (top row). Supplementing the R5 medium with 10μM Cu(II) does stimulate development in the wild type and complemented strains, but not in the mutants (middle row). Note that on both R5 and R5 supplemented with Cu(II) the wild type grows in a more compact manner than the mutants and the complemented mutants. All the strains do produce spores on MS medium (bottom row).
(B) The ability of the mycelium to invade the agar resulting in attachment of the mycelium to the agar is demonstrated on Nutrient Agar. The top row shows the growth of the spotted spores before the attempt to lift the mycelium with a flat toothpick. The bottom row shows the result after the lifting attempt. The wild type is growing into the agar, while the two mutants do not and their mycelium can be lifted/removed as an intact sheet. Growth inside the agar is fully restored in the complemented *cslA* mutant but only partially in the complemented *glxA* mutant. The same effect was observed on R5 media (data not shown).
(C) The wild type and the *cslA* and *glxA* mutants show very different mycelium morphology in liquid cultures after 24 hrs of growth. The two mutants are growing in smaller and more open clumps in YEME (top) and they show a completely open phenotype in TSBS (bottom). The wild type on the other hand shows the familiar dense mycelium structure. Complemented mutants have wild type pellet size and morphology (data not shown). Bar size: 150 μm

GlxA and CslA colocalize in two distinct positions in hyphal tips

To localize CslA and GlxA, CslA-eGFP and GlxA-eGFP fusion proteins were studied by fluorescence microscopy, using a low autofluorescent *S. lividans* derivative, 1326-FM, as a host. This strain was isolated by a similar selection procedure as for the low autofluorescent derivative of *S. coelicolor* (Willemse and van Wezel 2009). To create the chimeric genes, either gene was cloned without its stop codon upstream of the gene for eGFP in low-copy number shuttle vector pGFP-strep, behind the *cslA* promoter for *cslA-egfp* or the *ftsZ* promoter for *glxA-egfp* (see Materials and methods).

CslA is a predicted membrane protein, with six membrane-spanning domains and a cytoplasmic catalytic domain (http://www.cbs.dtu.dk/services/TMHMM/). Consistent with previous results, CslA-eGFP localized in bright foci at the hyphal tips of *S. lividans* 1326-FM (Fig. 3A). In addition to the bright foci at the tips, many additional but less intense foci were always observed throughout the hyphae. Furthermore, its localization was not dependent on GlxA, as a similar localization pattern was observed in a *glxA* mutant derivative of 1326-FM. However, around six times more foci of the CslA-eGFP fusion were observed in the *glxA* null mutant (Fig. 3B). In agreement, semi-quantitative RT-PCR analysis confirmed that the number of *cslA* transcripts was some 7.5-fold enhanced when *glxA* was deleted (Fig. 3C).

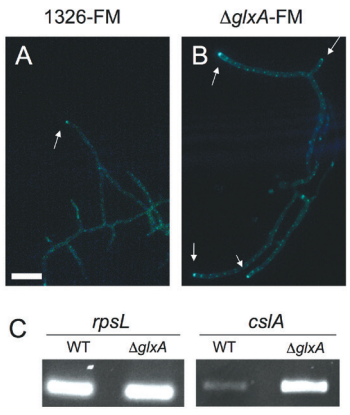

Figure 3. Hyphal tip localization of CslA-eGFP. Localization of CslAeGFP in *S. lividans* 1326-FM (A) and in *glxA*-FM (B) was studied with fluorescence microscopy after the strains were grown for 24 hrs on MS. CslA localizes at the tips of the hyphae (arrows) and does not require GlxA. (C) RT-PCR analysis of the amount of *cslA* transcripts in the wild type and Δ*glxA* strain grown for 24 hrs on MS. The rpsL gene encoding ribosomal protein S9 is used as an internal control. In the *glxA* mutant, 7.5 times more mRNA of *cslA* is detected. This is consistent with the amount of fluorescence observed with the CslA-eGFP construct in the *glxA* mutant which is approximately six-fold up. Bar, 5 μm.

GlxA contains a predicted N-terminal transmembrane helix that likely acts as a membrane anchor. Mycelium fractionation followed by Western blot analysis with anti-GlxA antibodies showed that about half of GlxA resides in the insoluble fraction, whereas the other half is soluble (Fig. 4A). It was previously suggested that a putative C-terminal sortase sequence could anchor GlxA to the peptidoglycan layer (Whittaker and Whittaker 2006), and a gene for sortase E is in close proximity in all streptomycetes. To establish the role for the putative sortase signal in anchoring of GlxA, a recombinant GlxA protein lacking the C-terminal 35 residues, including the putative sortase sequence, was expressed, but the proportion of the truncated protein found in the insoluble fraction was similar as for wild-type GlxA (Fig. 4A).

Like CslA-eGFP, GlxA-eGFP localized as bright foci at apical sites, and also for GlxA-eGFP many less intense foci were observed further away from the tips. Apical sites also stained intensely with fluorescent wheat germ agglutinin (f-WGA), which binds to N-acetylglucosamine subunits from among others precursors of peptidoglycan or chitin (Wright et al. 1991; see arrow in Fig. 4B). Further away from hyphal tips, at subapical sites that are by definition the older parts of the hyphae, GlxA-eGFP foci and f-WGA staining frequently did not overlap (arrowhead in Fig. 4B). To analyze the relative spatial localization of CslA and GlxA foci, their distances from the hyphal tip were assessed, relative to the f-WGA staining (Fig. 4C). Analyzing at least 40 tip located foci revealed that both CslA-eGFP and GlxA-eGFP localize in two distinct positions, one precisely in the hyphal tip, and a second at some distance (around 0.5 μm) behind the tip.

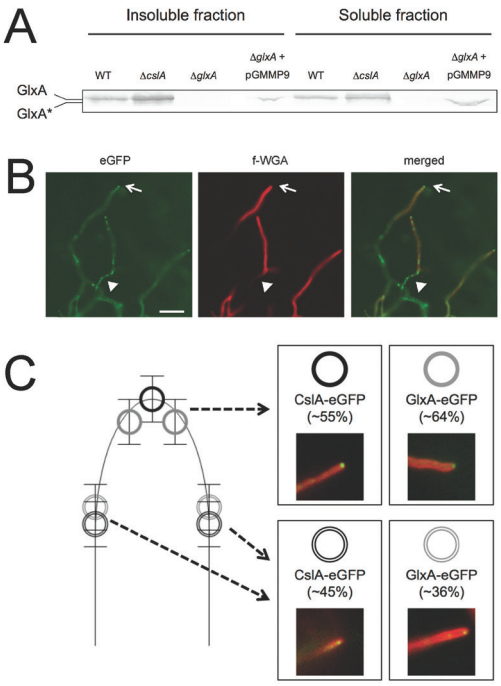

Figure 4. Localization of GlxA in *S. lividans*.
(A) Western blot analysis with anti-GlxA antibodies on the insoluble and soluble fraction of mycelial extracts. Strains were *S. lividans* 1326, its *cslA* and *glxA* mutants and the *glxA* mutant harboring pGMMP9. The latter plasmid contains the gene for a truncated GlxA (designated GlxA*) lacking the C-terminal 35 residues. The strains were grown for 24 hrs on solid MS. GlxA (around 71 kDa) is present in the insoluble and soluble fractions of the wild type strain and in the *glxA* mutant harboring pGMMP9, enhanced in the *cslA* mutant, but absent in the *glxA* mutant. Sample loading was adjusted to the wet weight of the mycelium and the cytosolic fraction is two-fold diluted as compared to the membrane fraction.
(B) Localization of GlxA-eGFP in *S. lividans* 1326-FM was studied with fluorescence microscopy, and in conjunction with staining with the lectin fluo-WGA (f-WGA, middle column). Right column shows the merged images. GlxA localizes at the tips of the hyphae (arrows). f-WGA staining is also observed at the tips, as well as further away from apical sites. Hyphal sections with GlxA-eGFP foci further away from the apical site (arrowheads) are not always stained with f-WGA. Bar size: 5 μm
(C) Fluorescence micrographs and derived cartoon of the localization of CslA-eGFP (black circles) and GlxA-eGFP (grey circles) in *S. lividans*. Both proteins show localization at the tip (60 ± 5% of the foci) or localization away from the tip (40 ± 5% of the foci). For each of the fusion proteins 40 foci were analyzed.

CslA and GlxA ensure the production of a polysaccharide at hyphal tips

It was shown previously that CslA is required for the accumulation of a β-(1,4) polysaccharide at apical sites during vegetative growth, identified by the fluorescent dye calcofluor white (CFW) (Xu et al. 2008; de Jong et al. 2009). This dye binds to a range of β-(1,4)-linked polysaccharides including cellulose and chitin (Herth and Schnepf 1980). Staining of wild-type mycelium of *S. lividans* revealed fluorescence of CFW in approximately 60% of the hyphal tips. Notably, stained hyphal tips were invariably swollen (see arrows in Fig. 5). Accumulation of CFW, as well as tip swelling, was completely absent in the *S. lividans cslA* and *glxA* null mutants grown on solid medium (Fig 5). However, while accumulation of CFW at hyphal tips was absent in the *glxA* mutant, bright fluorescence was frequently detected at subapical sites (arrowheads). Similar results were obtained in liquid-grown cultures (not shown). These results show that GlxA is involved in the biosynthesis and/or modification of the same β-(1,4) polysaccharide produced by CslA at apical sites.

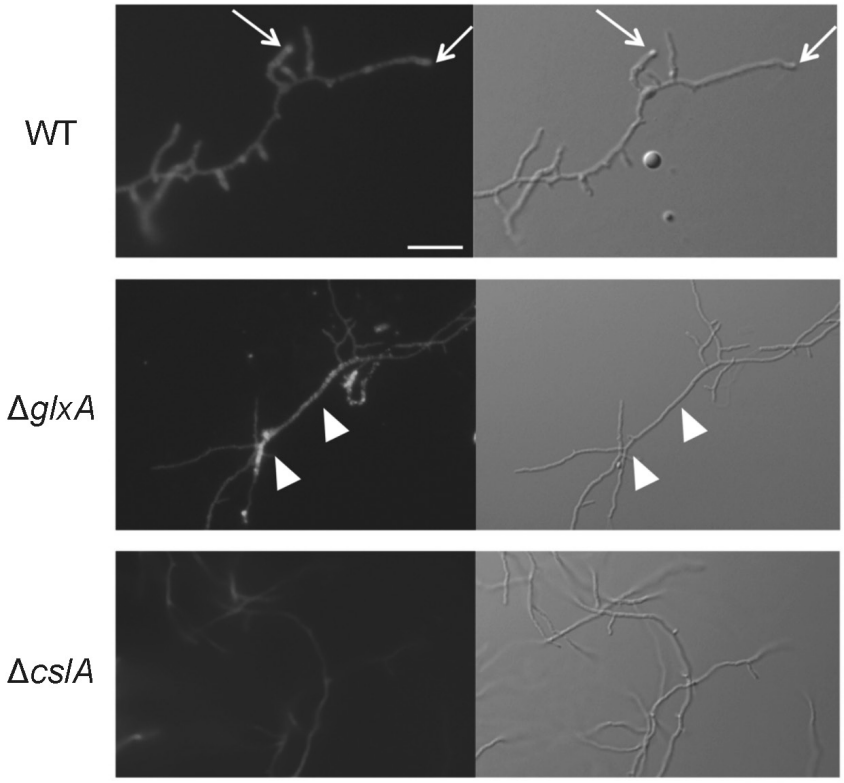

Figure 5. Visualization of β-(1,4) glycan production by calcofluor white (CFW) staining.
The wild type strain (top), and its *glxA* (middle) and *cslA* (bottom) mutants were grown for 24 hrs on MS agar plates and subsequently stained with CFW. Left panel, fluorescence micrograph; right panel, bright field image. CWF revealed accumulation of polysaccharides at the apical sites of the wild-type strain. In *glxA* null mutants, CFW failed to stain the tips, and instead accumulated in older sections of the hyphae (arrowheads). No CFW staining (other than occasional background fluorescence) was observed in the *cslA* mutant. Note the widening of apical sites of wild-type hyphae (arrows) due to the binding of CFW, indicative of CFW-induced lysis, which was not seen in either mutant incubated in precisely the same manner. The same results were observed on R5 media (data not shown). Bar size: 5 μm.

The CslA-dependent polymer becomes visible upon binding to a chitin-binding protein

The presence of genes related to chitin binding and N-acetylglucosamine metabolism around the *cslA-glxA* operon were reason to analyze whether CslA together with GlxA in fact synthesizes a polymer consisting perhaps in part of N-acetylglucosamine. Therefore, the sequence for a His_6-tagged version of a chitin-binding domain (CBD) derived from chitinase A1 from *Bacillus circulans* WL-12 (Watanabe et al. 1994) was fused to the C-terminal end of the gene for eGFP, expressed in *E. coli* and purified. To assess its affinity for chitin, we incubated the eGFP-CBD fusion protein with chitin beads (Fig. 6A). The strong fluorescence of the chitin beads after binding of the fusion protein confirms its affinity for chitin polymers. Strikingly, fibril-like structures were identified when the purified eGFP-CBD fusion protein was added to mycelium of the wild-type strain (Fig 6B). After binding by eGFP-CBD, the long fibrils were also observed by light microscopy, and were often detected in close proximity to the hyphae (Fig. 6F). This strongly suggests that the chitin binding protein resulted in bundling of the fibrils. Notably, these bundled fibrils could also be stained with CFW, whose fluorescence co-localized with that of the eGFP-CBD fusion (Fig 6E-F). Similar fibrils were also occasionally observed in *glxA* mutants, but were invariably absent in the *cslA* mutant (data not shown). Taken together, these experiments identify an extracellular glycan whose production is dependent on CslA and to a lesser extent on GlxA, and which under these conditions is loosely associated to the mycelium.

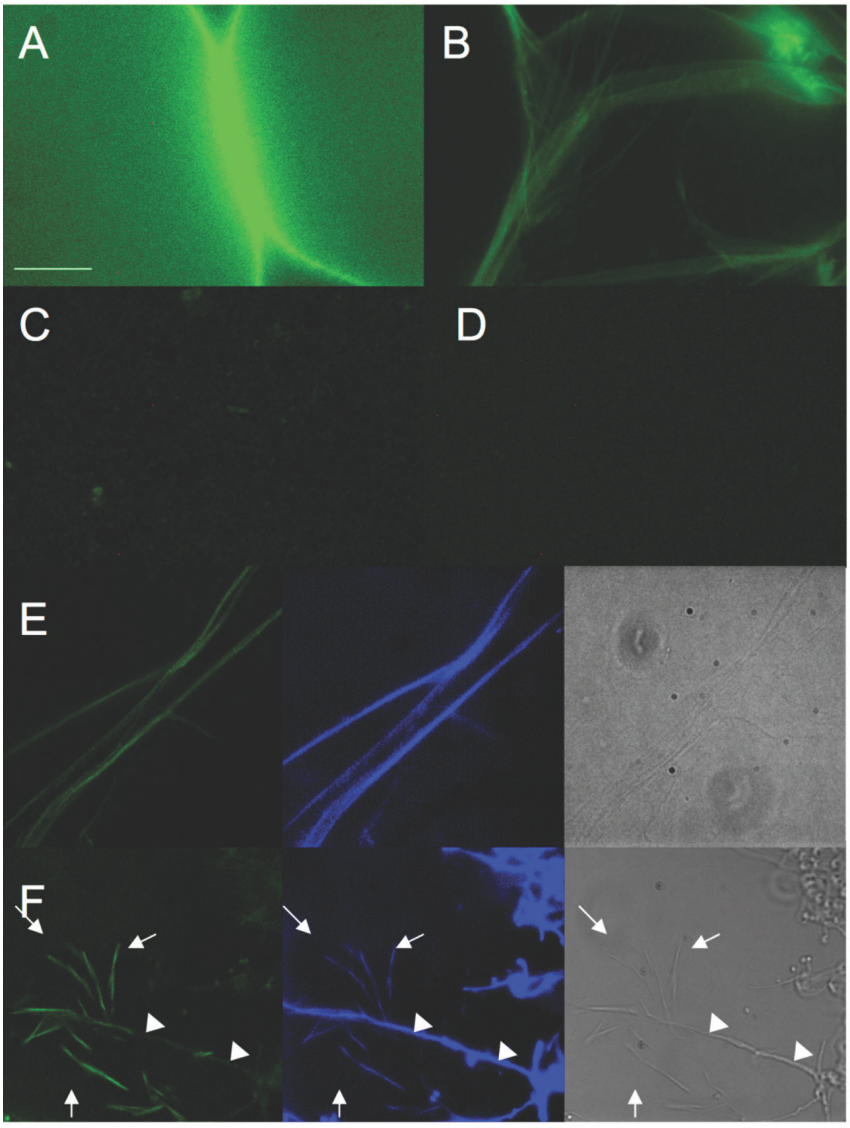

Figure 6. Visualization of extracellular fibrils using a chitin-binding domain fused to eGFP.
Chitin beads become fluorescent upon binding to the eGFP-CBD fusion protein (A). Long, extracellular structures were visualized in *S. lividans* 1326 following incubation of mycelia grown for 16 hrs on MS agar with eGFP-CBD (B). Control experiments showed that no background fluorescence was visible for the protein alone (C) or for the MS agar plates alone (D). The long fibrils bound by eGFP-CBD are stained by CFW and are also visible with light microscopy (E). These fluorescent fibrils were observed in close proximity to the mycelium of the wild type strain (F). Arrows: fibrils. Arrowheads: hyphae, as assessed by fluorescence microscopy. Bar size: 5 μm.

DISCUSSION

Streptomycetes are mycelial microorganisms that resemble filamentous fungi, with apical growth of the hyphae and similar challenges associated with growth in the soil. In particular, the apical sites are constantly being remodeled during elongation of the hyphae and this likely requires an additional surface layer to protect the hyphal tips from damage. We here show that in streptomycetes, the synthase CslA and the modifying oxidase GlxA together produce an extracellular β-(1,4) glycan that plays a critical role in growth and morphological development. Considering the presence of a CESA domain in CslA and the fact that CslA-dependent fibrils can be stained with calcofluor white (CFW), it was suggested previously that this glycan might consist of cellulose (Xu et al. 2008). However, CFW is not specific for cellulose and also efficiently binds to chitin. Additionally, *Streptomyces* lacks the c-di-GMP binding protein that is conserved in cellulose-producing organisms and that is required for cellulose synthesis (Ross et al. 1991; Römling 2002). We provide evidence that CslA and GlxA cooperate to produce an extracellular polysaccharide that may consist at least in part of chitin. This is suggested by experiments that showed that the CslA-dependent fibrils are efficiently bound by a chitin-binding protein, which presumably causes aggregation of smaller submicroscopic fibrils into larger structures that are easily discerned by light microscopy. Additionally, gene synteny analysis indicates that the *cslA-glxA* region (SCO2836-2837/SLI_3187-3188) is conserved in streptomycetes and contains *chb* (SCO2833/SLI_3182) for chitin binding protein Chb, which is part of the chitinolytic system (Schrempf 2001; Colson et al. 2007) and an N-acetylglucosamine metabolism-related enzyme (SCO2843/SLI_3194). Finally, the lectin f-WGA, which stains apical sites, binds to N-acetylglucosamine, allowing detection of peptidoglycan precursors as well as chitin oligosaccharides.

In filamentous fungi, polar growth requires chitin synthesis at apical sites (Bowman and Free 2006). The cell wall of filamentous fungi contains chitin (Lenardon et al. 2010) and other carbohydrate polymers (mostly β-(1,3) glucans and mannans), interspersed with glycoproteins (Levitz 2010), whereby a highly cross-linked, yet dynamic structure is formed. Chitin synthases transfer N-acetylglucosamine subunits

to the growing chitin chain, which is simultaneously exported to the extracellular environment. The chitin polymers are then bundled to form protective crystalline structures, or further processed to chitosan, the deacetylated form of chitin, or cross-linked to other cell-wall polysaccharides (Davis and Bartnicki-Garcia 1984).

We previously showed that CslA might play a role in attachment by forming fibrillar glycan structures that are associating with chaplins to form so-called fimbriae (de Jong et al. 2009). These fibrillar structures could be removed by incubation with fungal extracts containing cellulases, which suggested the presence of cellulose in the fibrils. However, analysis of the crude enzyme preparation by MALDI-ToF mass spectrometry revealed that, in addition to cellulase, the extract contained a number of other polysaccharide hydrolases that act on β-(1,4) glucans (not shown). Chemical characterization of the polysaccharide structure produced by CslA and GlxA in *Streptomyces* should provide the precise nature of the β-(1,4) glycan. The composition of the polymer produced by CslA and GlxA may in fact be different depending on the growth condition. Indeed, the bacterium *Gluconacetobacter xylinus,* which has served as a model organism for bacterial cellulose biosynthesis, can also incorporate glucosamine and/or N-acetylglucosamine in addition to glucose (the preferred substrate) into the growing polysaccharide chains. The net result is heteropolymers consisting of mixed sugar moieties, revealing the apparent promiscuous nature of the synthase (Lee et al. 2001).

Multiple roles in growth and development for the CslA- and GlxA-dependent glycan

Calcofluor white staining revealed that CslA and GlxA are both required for the synthesis of the β-(1,4) glycan at apical sites. Interestingly, CFW induced widening of apical sites in *S. lividans*. This is most likely indicative of the initial stages of lysis, consistent with the complete absence of such widening in either mutant. Such tip swelling was also observed in filamentous fungi grown in the presence of CFW (Damveld et al. 2005). In *cslA* null mutants, no CFW staining was found, explained by absence of the synthase CslA, while in *glxA* null mutants CFW staining was

observed, but in older sections of the hyphae, rather than at apical sites. This suggests that the immature glycan is produced by CslA, but does not remain associated with apical sites. We propose that the oxidase GlxA - which is a predicted to be mycelium associated through a N-terminal transmembrane segment - is involved in the subsequent maturation and apical attachment of the glycan.

Previous and current work indicates that this extracellular polysaccharide plays an important role during growth and development; the polysaccharide polymers facilitate the emergence of aerial hyphae during osmotic stress (Xu et al. 2008; Liman et al. 2013; this work) and contribute to invasion of and attachment to solid substrates, such as soil particles or (in the laboratory) the surface of agar plates. Interestingly, the putative chitin-like glycan dependent on CslA and GlxA is also involved in the architecture of mycelial pellets in liquid-grown cultures. Pellet formation is observed in many streptomycetes, although their specific sizes can be quite different, dependent on pH and other media-related factors as well as temperature, but also cell-wall related genes (van Veluw et al. 2012). Indeed, polymers such as hyaluronic acid but perhaps also extracellular DNA (eDNA) contribute to pellet integrity in *S. coelicolor* (Kim and Kim 2004). The chitin-related glycan produced by CslA and GlxA may play a role in formation of these structures. The open mycelial networks formed in the absence of the glycan fibrils suggest a role for these fibrils in surface adhesion so as to connect the hyphae, thus leading to the compact pellet structure. In this respect, mycelial pellets may be reminiscent of biofilm-like structures, which are embedded in an extracellular matrix (Flemming and Wingender 2010). Notably, such matrices also often contain amyloid proteins (Gebbink et al. 2005). It is interesting to note that the conserved *Streptomyces* chaplin proteins, which can assemble into amyloid fibrils (Claessen et al. 2003) are also required for maintaining pellet integrity (M.L.C. Petrus and D. Claessen, unpublished).

Besides the fundamental implications for understanding hyphal growth and development in *Streptomyces*, the open mycelial structures formed by *cslA* and *glxA* null mutants in liquid-grown cultures could also have implications in industrial processes. Pellet morphology directly relates to viscosity and mass-transfer issues

(efficiency of nutrient and oxygen uptake), and thus to the efficiency in protein or secondary metabolite production and secretion (van Wezel et al. 2006).

GlxA and the Cu-dependence of S. lividans *development*

Copper-dependent morphological development is common in *Streptomyces* and is most pronounced in *S. lividans*. At low Cu levels, *S. lividans* displays a delayed development, while the removal of all the available metal results in vegetative growth arrest (Ueda et al. 1997; Keijser et al. 2000). This dependency is related to the *ram* (rapid aerial mycelium) genes, which produce the secreted RamS protein, eventually processed to the lantibiotic-type molecule SapB (Willey et al. 1991; Kodani et al. 2004). Indeed, the copper dependence of *S. lividans* development can be complemented by the introduction of an extra copy of *ramSAB* (Keijser et al. 2000). The reason for this is unknown, as none of these genes has a direct correlation with copper. Strains harboring an extra copy of the *ram* cluster have strongly enhanced transcription of *ramS* . An interesting hypothesis to test is is whether more SapB is sufficient to provide compensation for the too low amounts of the CslA/GlxA synthesized glycan in the matrix under limited copper conditions .

Development of *S. lividans* requires the copper chaperone Sco1 and the deletion of the gene leads to vegetative arrest, which can be restored by the addition of 10 μM Cu (Fujimoto et al. 2012; Blundell et al. 2013). What then is the acceptor of the copper atom offered by Sco1, which could explain the copper dependence of *S. lividans* development? The cytochrome c oxidase (CcO), which requires Sco1 for its maturation, is not the effector, as a *cco* deletion mutant still showed full development, suggesting that Sco1 has yet another target (Blundell et al. 2013). In this work, we show that *glx*A null mutants cannot develop on R5 agar plates, and do not attach nor invade the agar surface, which in fact is the same phenotype as observed for *sco1* mutants (Worrall & Vijgenboom, unpublished). Since the phenotype of the *glxA* null mutant cannot be rescued by copper, GlxA could be the Sco1-dependent enzyme that is responsible for the Cu-dependence of the development of *S. lividans*. However, this awaits further evidence.

Localization of GlxA

In agreement with previous observations, GlxA and CslA localise to apical sites. We observed very bright foci for both proteins at apical sites, and in addition many less intense foci distributed throughout the hyphae. It is known from previous studies that CslA is most likely membrane-associated and localizes at apical sites independent from the radical copper oxidase GlxA (Liman et al. 2013) and interacts with the apically situated DivIVA (Xu et al. 2008). GlxA has a predicted secretion signal, but was found at least partially in the membrane fraction (Whittaker and Whittaker 2006). A putative C-terminal sortase signal might anchor the protein to the peptidoglycan layer (Whittaker and Whittaker 2006), but we show here that deletion of the C-terminal 35 residues of the protein has no effect on the localization of GlxA, consistent with a recent study that argues against a covalent attachment to the cell surface (Liman et al. 2013). How secreted GlxA remains associated with the hyphae remains to be established.

CslA and GlxA were fused to mCherry C-terminally, so as to allow colocalization of CslA-eGFP with GlxA-mCherry and *vice versa*, but the fluorescence of the mCherry fusions was not bright enough to allow their visualization. Therefore, the localization of the foci at the hyphal tips was assessed as the relative distance from the cell wall stained by f-WGA. In 60% of the cases, CslA-eGFP and GlxA-eGFP localized immediately at the tip, while 40% was found close to but not immediately in the apex. Whether the two proteins fully colocalize awaits further analysis.

Model

Our data are consistent with a model of polysaccharide synthesis and attachment at the hyphal tips of *Streptomyces* requiring the action of GlxA and CslA (Fig. 7). The mature form of GlxA migrates at the hyphal tips, where it is secreted and associated with the membrane by a yet unknown mechanism. GlxA colocalizes with its functional partner CslA and they cooperate to produce a glycan-type polysaccharide - such as cellulose as previously suggested or perhaps chitin, or even a combination thereof - at the hyphal apices. CslA is the synthase, while GlxA presumably modifies the glycan

through oxidation, allowing its extracellular association with the mycelium. The glycan then becomes part of an extracellular matrix and likely acts in concert with other matrix components and proteins, such as the chaplins, rodlins and/or SapB. The drastic changes in morphology and absence of hyphae capable of invading agar media in both the *cslA* and *glxA* mutant highlight the important and intriguing role of matrix components in the control of (liquid) morphology, growth and development of streptomycetes.

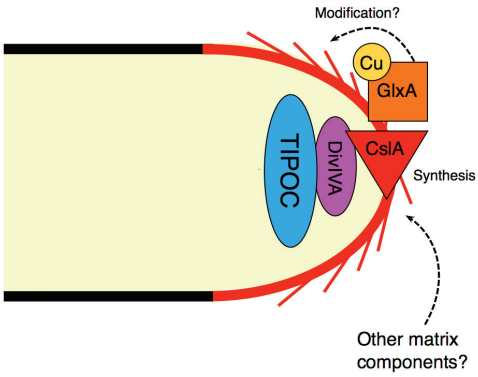

Figure 7. Summarizing model for the production of extracellular polysaccharides by CslA and GlxA.
GlxA localizes at the hyphal tips, where it is involved in the modification and accumulation of the polysaccharide produced by CslA. The polysaccharide may function together with other matrix components, such as the chaplins or SapB, in mediating its function in growth and development. Note that CslA interacts with DivIVA, which is an important member of the tip-organizing center (TIPOC) that controls polarized growth.

MATERIAL AND METHODS

Strains and media

All mutant strains were constructed in *S. lividans* 1326 (*S. lividans* 66, stock number 1326 from the John Innes Centre; (Hopwood et al. 1985). The low fluorescent *S. lividans* strain 1326-FM was isolated as described (Willemse and van Wezel 2009). *Escherichia coli* JM109 was used for routine cloning and plasmid amplification (Messing et al. 1981). All strains used and constructed are presented in Table S1. Soy flour mannitol (MS) agar plates were used for the isolation of spores from *Streptomyces* strains. For growth and phenotypical characterizations, R5 agar plates with or without 10 μM Cu(II), tryptic soy broth with 10% sucrose (TSBS) and yeast extract-malt extract medium (YEME) were used (Kieser et al. 2000). *E. coli* strains were routinely grown on Luria-Bertani medium (LB). Nutrient agar was obtained from Difco.

Constructs for gene replacement and deletion mutants

All the plasmids used in this work are listed in Table S2. The strategy for creating knock-out mutants is based on the unstable multi-copy vector pWHM3 (Vara et al. 1989) as described previously (van Wezel et al. 2005). For the deletion of *cslA*, the -1260/+78 and +1828/+3263 regions relative to the start of *cslA* were amplified by PCR, using primer pairs *cslA*-1260F/ *cslA*+78RV and *cslA*+1828F/*cslA*+3263RV, respectively (Table S3). Fragments were cloned into pWHM3, and the engineered XbaI site was used for insertion of the apramycin resistance cassette *aac(3)IV* flanked by *loxP* sites between the flanking regions. The constructed plasmid was called pGMMP1. Using essentially the same strategy as for pGMMP1, we constructed plasmid pGMMP2 for the single gene replacement of *glxA*. pGMMP2 contains the -1498/+59 and +1917/+3428 regions relative to the start of *glxA*. The antibiotic cassettes were subsequently removed from the mutants by the Cre-lox recombinase using plasmid pUWLcre (Fedoryshyn et al. 2008). This resulted in the *cslA* and *glxA* null mutants, which had the corresponding genes replaced by a scar *loxP* site flanked

by *Xba*I restriction sites. The mutants were complemented by plasmids pGMMP3 and pGMMP4, respectively carrying *cslA* and *glxA* under the control of P_{cslA} in plasmid pHJL401 (Larson and Hershberger 1986). This plasmid is ideally suited for complementation experiments for its very high stability and low copy number (van Wezel et al. 2000b). All plasmids were introduced through protoplast transformation (Hopwood et al. 1985). The primers used for PCR amplification are listed in Table S3.

Construction of eGFP fusion proteins

To create eGFP fusions, we used a low-copy number shuttle vector pGFP-strep, which is based on pHJL401, and carried a version of the gene for eGFP codon-optimized for expression in *Streptomyces*, which is transcribed from the *ftsZ* promoter region. This construct showed good results in other experiments and was therefore selected (J. Willemse, personal communication). For localization of CslA, the *cslA* gene with 500 bp of the upstream promoter region was cloned in pGFP-strep, whereby the *ftsZ* promoter was removed (pGMMP5). To localize GlxA, the *glxA* gene was cloned into pGFP-strep (pGMMP6). In this construct, expression of the fusion is under control of P_{ftsZ} (pGMMP6). The fusion constructs were introduced in 1326-FM and/or Δ*glxA*-FM.

Microscopy

Light microscopy was used to check the phenotypes of the strains in liquid culture, using a Carl Zeiss Standard 25 microscope and a 20x lens. The agar invasion capacity of strains was tested on various agar media and recorded with a Leica DFC295 stereomicroscope. The GFP fusion proteins were observed with a fluorescence microscope (Axioscope A1) and analyzed with the Axio Vision software to assess localization using filter set 38HE (470/40 nm excitation, 495 nm dichroic, 525/50 nm emission). The spores were inoculated at the edge of glass slides inserted at an angle of 45º in MS plates and grown for 24 hrs, generating a sample of vegetative mycelium. For CFW staining, mycelium was grown for 24 hrs on glass slides and MS plates as described above. The mycelium was stained with 5µl of a 10x diluted solution of a 1:1

mixture of CFW:10% KOH and analyzed at the fluorescent microscope (Axioscope A1). Alternatively, spores were inoculated in 10 ml liquid TSBS and grown for 24 hrs. A sample of 5 µl of the culture was mixed with 5 µl of a 10x diluted solution of a 1:1 mixture of CFW:10% KOH and analyzed. For CFW staining filter set 49 was used (excitation 365 nm, dichroic 395 nm, emission 445/50 nm).

For fluorescently conjugated wheat germ agglutinin staining (f-WGA), spores were inoculated on glass slides and MS plates as discussed above and grown for 24 hrs, incubated with 5µl of a 10x diluted solution of f-WGA in glycerol and analyzed with filter set 63HE (572/25 nm excitation, 590 nm dichroic, 629/62 nm emission).

To calculate distances between the fluorescent proteins and the WGA-stained cell wall, ImageJ was used (Schneider et al. 2012). All images were background-corrected using Adobe Photoshop CS4.

Protein methods

For the preparation of protein samples MS plates were overlaid with cellophane disks and inoculated confluently with 10^5 spores. After 24 hrs, the mycelium was harvested and the wet weight determined. Mycelium was resuspended in 10 mM Tris-HCl (pH7; volume adjusted according to wet weight, 20µl per mg of protein) and sonicated. After centrifugation, the supernatant was removed and mixed with an equal volume of SDS-PAGE loading buffer containing 1% β-mercaptoethanol. The pellet was directly resuspended in loading buffer, resulting in a 2x concentrated sample compared to the soluble fraction. Following electrophoresis in a 10% SDS PAGE gel, the proteins were blotted on nitrocellulose filters. Standard Western blot protocols and polyclonal antibodies against GlxA (1 µL per 10 mL) were used for detection with GARAP as the secondary antibody.

Analysis of GlxA anchoring by sortases

To assess the role of sortases in anchoring of GlxA, a truncated version of the *glxA* gene was cloned in pHJL401 under the control of the *cslA* promoter, generating plasmid

pGMMP9. The resulting recombinant protein lacks the C-terminal 35 amino acids, which would include the putative sortase signal (Whittaker and Whittaker 2006).

RT-PCR Analysis

Total RNA for transcript analysis was isolated from cultures grown on MS plates for 24 hrs, purified using the Kirby-mix protocol (Kieser et al. 2000) and additionally treated with DNase I to remove any traces of DNA. RT-PCR was carried on as described previously (Noens et al. 2007) using 200 ng of RNA as a template. Quantification of the amplified RNA on gel was carried out with a Bio-Rad Gel Doc EZ Imager and Image Lab software. The primers used for PCR amplification are listed in Table S3 of the Supporting Information.

Visualization of fibrils using a chitin-binding domain

The *eGFP* gene was amplified using pGFP as template DNA and cloned in pTYB3 (New England Biolabs), upstream of the intein domain and the chitin binding domain (CBD) of chitinase A1 from *Bacillus circulans* WL-12, generating plasmid pGMMP7. The chitin binding domain of chitinase A1 is known to have a strong preference for chitin binding (Watanabe et al. 1994; Chong et al. 1997).

The sequence encoding the eGFP-CBD fusion was amplified by PCR and cloned in pET28a (Novagen), thereby introducing an N-terminal His tag (plasmid pGMMP8). Standard procedures were followed to isolate the eGFP-CBD fusion protein on HisPur™ Cobalt resin (Thermo Scientific). Ultrafiltration (Amicon Ultra, 30 kDa cut off) was used to concentrate the protein. Several wash steps with 50 mM NaPi (pH 8) were performed to reduce the concentration imidazole and NaCl below 0.1 mM. About 3 ng of protein was used to detect the polysaccharide associated with the mycelium of the wild-type, *cslA* and *glxA* mutant strains that had been grown for 16 hrs on cover slips inserted in MS plates.

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SUPPLEMENTAL MATERIAL

Table S1. Strains used in this study.

Strains	Description	Reference
S. lividans		
1326	*S. lividans* 1326 wild type strain	Hopwood et al., 1985
1326-FM	derivative of *S. lividans* 1326 with reduced autofluorescence	This work
cslA	*cslA* deletion mutant in 1326-FM	This work
glxA	*glxA* deletion mutant in *S. lividans* 1326	This work
glxA-FM	*S. lividans glxA* mutant derivative with reduced autofluorescence	This work
E. coli		
JM109	*E.coli* K12 strain used for routine subcloning	Messing et al, 1981

Table S2. Plasmids used and constructed in this study

Name	Description	Reference
pWHM3	pIJ486 derived *E.coli-Streptomyces* shuttle vector, Tsr^R, Amp^R	(Vara et al., 1989)
pET28a	*E.coli* expression vector, Kan^R	Novagen
pGMMP1	pWHM3 containing the flanking regions of the *S. lividans cslA* gene, interspersed by the apraloxP_XbaI insert	This work
pGMMP2	pWHM3 containing the flanking regions of the *S. lividans glxA* gene, interspersed by the apraloxP_XbaI insert	This work
pUWLcre	pUWLoriT derivative containing the gene for the Cre-lox recombinase under the control of P_{ermE}	(Fedoryshyn et al., 2008)
pHJL401	*E.coli-Streptomyces* shuttle vector, Tsr^R, Amp^R	(Larson and Hershberger, 1986)
pGMMP3	pHJL401 containing the *cslA* gene under the control of the *cslA* promoter	This work
pGMMP4	pHJL401 containing the *glxA* gene under the control of the *cslA* promoter	This work
pGFP	pHJL401 containing a codon-optimized version of the *eGFP* gene under the control of P_{ftsZ}	This work
pGMMP5	pGFP derivative containing a chimeric *cslA-eGFP* gene under the control of P_{cslA} cloned as an EcoRI-XbaI fragment	This work

pGMMP6	pGFP derivative containing a chimeric *glxA-eGFP* gene under the control of P_{ftsZ} cloned as an NcoI-XbaI fragment	This work
pGMMP7	pTYB3 containing the *eGFP* gene from pGFP, cloned as an NcoI-XhoI fragment thereby creating an eGFP-intein-CBD fusion.	This work
pGMMP8	eGFP-intein-CBD domain from pGMMP7 cloned into pET28a as an NdeI-EcoRI fragment	This work
pGMMP9	pHJL401 containing the *cslA* promoter upstream of a truncated version of the *glxA* gene, which encodes GlxA lacking its 35 amino acids at its C-terminus.	This work

Table S3. Oligonucleotides used in this study.

Name	5'-3' sequence	Restriction site(s) (underlined in sequence)
cslA-1260F	GCGGAATTCGGCGTAGGGCGTGTCGAGCTTC	EcoRI
cslA+78RV	CGCTCTAGACCGGTGCCCGGGCACCCT	XbaI
cslA+1828F	GCGTCTAGAGGGTCCCGCCGCAGCAGG	XbaI
cslA+3263RV	CGCAAGCTTCTTCGGGTCGTCCGCCTTGAGGT	HindIII
cslA F	GCGGAATTCCTCACACTCCCGGTCGGCAGG	EcoRI
cslA RV	CGCGGATCCATATGTCATTCCCCCCACACGCGGGTC	NdeI
cslA RV 2	CGCTCTAGATTCCTTACGTCCCCCAAGTCCAC	XbaI
cslA RV 3	CGCTCTAGAGCATCATTCCTTACGTCCCCCAAGTCC	XbaI
glxA-1498F	GCGGAATTCCGAGACCGGCACCAAGGTCGC	EcoRI
glxA+59RV	GCGTCTAGACGTGCCTATCGCGAAGCGACG	XbaI
glxA+1917F	GCGTCTAGAGAGTGGGTGCGAGTTCCGTAGCG	XbaI
glxA+3428	GCGAAGCTTGAGTCGGCCGGCAAGCTGTGG	HindIII
glxA F	GCGCCATGGAAGACCGTGCCGGCCG	NcoI
glxA RV	CGCTCTAGACGGCACCCGCACCCACTC	XbaI
glxA F 2	GCGGAATTCCATATGAAAGACCGTGCCGGCCGC	EcoRI-NdeI
glxA RV 2	CGCAAGCTTGGCGCTACGGAACTCGCACC	HindIII
GFP F	CGCGAATTCCCATGGGCAAGGGCGAGGAGCTGTTC	EcoRI-NcoI
GFP RV	GCGAAGCTTCTCGAGCTTGTACAGCTCGTCCATGCC	HindIII-XhoI
pET28 F	CGCAAGCTTCATATGAGCAAGGGCGAGGAGCTGTTCAC	HindIII-NdeI
CBD RV	GCGGAATTCTCCTGCAGTCATTGAAGCTGCCACAAG	EcoRI
glxA-105nt RV	CGCTCTAGACACCGTCAGCTTGTCCCCGTC	XbaI
*rpsL*_for	GAGACCACTCCCGAGCAGCCGC	none
*rpsL*_rev	GTAGCGGTTGTCCAGCTCGAGCA	none
*cslA*_for	TGGGCGTGCACCACTTCTCC	none
*cslA*_rev	CGCGGTACCGGAAGGTGCCGAACA	none