The Cellobiose Sensor CebR Is the Gatekeeper of *Streptomyces scabies* Pathogenicity

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**ABSTRACT** A relatively small number of species in the large genus *Streptomyces* are pathogenic; the best characterized of these is *Streptomyces scabies*. The pathogenicity of *S. scabies* strains is dependent on the production of the nitrated diketopiperazine thaxtomin A, which is a potent plant cellulose synthesis inhibitor. Much is known about the genetic loci associated with plant virulence; however, the molecular mechanisms by which *S. scabies* triggers expression of thaxtomin biosynthetic genes, beyond the pathway-specific activator TxtR, are not well understood. In this study, we demonstrate that binding sites for the cellulose utilization repressor CebR occur and function within the thaxtomin biosynthetic cluster. This was an unexpected result, as CebR is devoted to primary metabolism and nutritive functions in nonpathogenic streptomycetes. In *S. scabies*, cellobiose and cellotriose inhibit the DNA-binding ability of CebR, leading to an increased expression of the thaxtomin biosynthetic and regulatory genes txtA, txtB, and txtR. Deletion of *cebR* results in constitutive thaxtomin A production and hypervirulence of *S. scabies*. The pathogenicity of *S. scabies* is thus under dual direct positive and negative transcriptional control where CebR is the cellobiose-sensing key that locks the expression of *txtR*, the key necessary to unlock the production of the phytotoxin. Interestingly, CebR-binding sites also lie upstream of and within the thaxtomin biosynthetic clusters in *Streptomyces turgidiscabies* and *Streptomyces acidiscabies*, suggesting that CebR is most likely an important regulator of virulence in these plant-pathogenic species as well.

**IMPORTANCE** What makes a microorganism pathogenic is not limited to the genes acquired for virulence. Using the main causative agent of scab lesions on root and tuber crops as an example, our work identified the subtle but essential genetic changes that generate the cis-acting elements necessary for proper timing of the expression of the cluster of genes responsible for the biosynthesis of thaxtomin A, the primary virulence factor in plant-pathogenic streptomycetes. These data illustrate a situation in which a regulator associated with primary metabolism in nonpathogens, CebR, has been coopted as a master regulator of virulence in pathogenic species. Furthermore, the manipulation of CebR-mediated control of thaxtomin production will facilitate overproduction of this natural and biodegradable herbicide for commercial purposes. Our work thus provides a concrete example of how a strictly theoretical and computational work was able to elucidate a regulatory mechanism associated with the virulence of a plant pathogen and to generate solutions to purely agro-industrial concerns.

*S. scabies* is a large genus of Gram-positive, high-G+C-content bacteria that are largely nonpathogens and well known for the production of pharmaceutically and agriculturally important secondary metabolites (1). Although several hundred species are known to date, only a few are phytopathogenic (2). The best-studied pathogens are *Streptomyces scabies*, *Streptomyces acidiscabies*, *Streptomyces turgidiscabies*, and *Streptomyces ipomoeae*, which cause raised or pitted scab lesions on economically important root and tuber crops, like potato, radish, beet, peanut, and sweet potato. The primary virulence determinant of *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* is the phytotoxin thaxtomin A (3). It is a member of a family of nitrated diketopiperazines formed by nonribosomal peptide synthases out of the main components 4-nitro tryptophan and phenylalanine (4). The biosynthetic pathway is unusual in that 4-nitro tryptophan is derived via a nitric oxide synthase from arginine (3, 5).

Plant-pathogenic streptomycetes are neither host nor tissue specific (3). In keeping with this broad host range, thaxtomin A has a highly conserved target—the plant cell wall. Thaxtomin affects the structure of the cell wall in dividing and expanding plant cells through an alteration of expression of cell wall biosynthesis-related genes and depletion of cellulose synthase complexes from the plasma membrane. This causes extensive cell wall remodeling characterized by reduced incorporation of crystalline cellulose into the plant cell wall, which is compensated for by an increased amount of pectins and hemicelluloses (6, 7).

The thaxtomin biosynthetic genes are highly conserved across plant-pathogenic streptomycetes and lie on a pathogenicity island.
that is mobilized in some species (3). However, acquisition of genes required for virulence is only the first step on the way to a pathogenic lifestyle. Subtle genetic changes are necessary to adapt the expression of newly acquired genes to the environment and the life cycle of the recipient microorganism. A limited number of mutations in regulatory regions and especially the distribution of cis-acting elements are key to developing a strain-specific transcriptional response. These DNA motifs are targeted by transcription factors that sense environmental signals through direct interaction with membrane sensors or indirect association with elicitor transporters. The production of thaxtomin A itself is under strict transcriptional control, including at least five global regulators belonging to the bld gene family, involved in morphological differentiation and/or the secondary metabolism of Streptomyces (8) in addition to the thaxtomin biosynthesis pathway-specific transcriptional activator TxtR (9). The multiplicity of global and specific regulators associated with thaxtomin production suggests that S. scabies may respond to multiple triggers that originate from plant material, such as xylan degradation products (10), suberin (11), and, more importantly, cellulose, a product of cellulose degradation and a well-known elicitor of thaxtomin biosynthesis (4, 10) (it directly targets TxtR [9]). In this work, we describe an additional level of control of S. scabies pathogenicity. By recruiting CebR, the cellulose utilization regulator (12–14), we illustrate how the plant pathogen causes a trans-acting factor to digress from its primary function in order to use it to lock the expression of thaxtomin biosynthesis genes, thaxtomin production, and, therefore, its virulent behavior.

RESULTS AND DISCUSSION

Identification of CebR-binding sites associated with thaxtomin biosynthetic genes. The transcription factors involved in the control of thaxtomin A production were identified by scrutinizing the genome of S. scabies for the occurrence of putative cis-acting elements of well-characterized DNA-binding proteins. Position weight matrices were generated from the compilation of DNA motifs bound by a series of regulators that were selected due to their notorious direct or indirect (implied) role in secondary-metabolite biosynthesis and/or morphological differentiation in streptomycetes. The PREDetector software (15) identified two 14-bp almost-perfect palindromic sequences highly similar to CebR boxes (henceforth called cbs for CebR-binding sites; see Fig. S1 in the supplemental material and reference 14) which are associated with the thaxtomin biosynthetic genes 

![FIG 1](image-url)

CebR binding to cbs associated with thaxtomin biosynthetic genes is relieved by cellobiose. (A) Sequences and positions of CebR-binding sites identified in the chromosome region of the thaxtomin biosynthetic genes in S. scabies. Numbers associated with genes/ORFs are SCAB numbers from the annotated genome of S. scabies 87–22. The lowercase letters indicate nucleotides that do not match with the cbs consensus sequence. (B) EMSA demonstrating CebR binding to DNA motifs identified in the txtRA intergenic region (cbs<sup>txtR-A</sup>) and in txtB (cbs<sup>txtB</sup>). Numbers 1 to 14 refer to increasing concentrations of pure CebR-His<sub>6</sub>, i.e., 0 (free probe, 30 nM), 80, 160, 240, 320, 400, 480, 560, 640, 720, 960, 1,200, 1,600, and 3,200 nM, respectively. (C) EMSAs demonstrating cellobiose as the best allosteric effector of CebR. Numbers 1 and 2 refer to EMSAs with free probes (6 nM) and with probes incubated with CebR-His<sub>6</sub>, respectively. Numbers 3 to 7 refer to EMSAs with CebR-His<sub>6</sub> preincubated with oligosaccharides, i.e., cellobiose (lane 3), cellotriose (lane 4), cellotetraose (lane 5), cellopentaose (lane 6), or cellohexaose (lane 7). Quantification of the shifted bands revealed that cellobiose (lane 3), cellotriose (lane 4), and cellohexaose (lane 7) were able to impair the CebR DNA-binding ability by approximately 56, 20, and 12%, respectively.
CebR was identified as the repressor of cellulose/cellobiose biosynthesis in streptomycetes (12–14), and in addition, this repressor has been shown to trigger morphogenesis in *Streptomyces griseus* (14). Binding of cellopentaose or cellobiose to CebR of *S. reticuli* or *S. griseus*, respectively, relieves binding of CebR to cbs and allows transcription of the downstream genes (12, 14). Interestingly, cellobiose and cellotriose are also known as the best elicitors of thaxtomin A production in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* (9, 10), which strengthens the possible role of CebR in controlling thaxtomin biosynthesis.

Electromobility gel shift assays (EMSAs) performed with double-stranded Cy5-labeled probes centered on the putative cbs sequence showed that *cbs*<sup>txtR-A</sup> and *cbs*<sup>txtB</sup> are strongly bound by CebR (Fig. 1B; Fig. S2). Under the same conditions, we also observed strong CebR binding to *cbs*<sup>txtA</sup>, which was used as a positive control because it is known to be specifically bound by CebR (12, 14) (Fig. 1B). EMSAs performed with CebR preincubated with cellobiose or cellooligosaccharides [(glucose)<sub>3</sub> to (glucose)<sub>6</sub>]<sup>14</sup> (Fig. 1B). EMSAs performed with CebR preincubated with cellobiose to CebR of *S. scabies* also known as the best elicitors of thaxtomin A production in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* (9, 10), which strengthens the possible role of CebR in controlling thaxtomin biosynthesis.

Deletion of *cebR* results in constitutive production of thaxtomin A. In order to uncover the role of CebR in controlling the expression levels of the thaxtomin biosynthetic genes *txtA* and *txtB*, we generated a *cebR* null mutant in *S. scabies* (*ΔcebR* mutant). Quantitative real-time reverse transcription-PCR (qPCR) on RNA extracted from wild-type *S. scabies* (87-22) and its *cebR* null mutant grown on International *Streptomyces* Project medium 4 (ISP-4) showed that the deletion of *cebR* results in overexpression of *txtA*, *txtB*, and *txtR*, respectively (Fig. 2). CebR thus acts as a transcriptional repressor of the thaxtomin biosynthetic genes in *S. scabies* as it represses the expression of the cellulolytic system in *S. griseus*. This result confirms that, despite the unusual positions of *cbs*<sup>txtR-A</sup> and *cbs*<sup>txtB</sup> further upstream of the target *txtA* and *txtR* genes and within the coding region of *txtB* (Fig. 1A), respectively, the identified DNA motifs are truly functional cis-acting elements.

Moreover, the search for similar sequences in other thaxtomin A-producing *Streptomyces* pathogens confirmed the occurrence of *cbs* in their thaxtomin biosynthetic regions. In *S. acidiscabies*, three *cbs* signatures were identified within the thaxtomin biosynthetic cluster, i.e., one upstream of *txtA* (nt −787), one within *txtA* (nt +32), and one within *txtB* (nt +1559). In *S. turgidiscabies*, we found one *cbs* signature within *txtA* (nt +32) and one within *txtB* (nt +1508). A third *cbs* is found at nt position −1444 upstream of *txtR*. No *cbs* associated with thaxtomin production could be identified in *S. ipomoeae*, which produces mainly thaxtomin C, a phytotoxic member of the thaxtomin family; thaxtomin C production is not responsive to cellobiose or other oligosaccharides (16).
Therefore, the occurrence of cbs upstream of or within the txt genes correlates with the observed cellulbiose-dependent production of thaxtomin A in characterized pathogenic Streptomyces species.

We also assessed the thaxtomin production levels of both the S. scabies wild type and the cebR null mutant on oat bran agar (OBA), an undefined complex medium known to induce thaxtomin production (17), and ISP-4. The cebR mutant overproduced thaxtomin, which is bright yellow, compared to the level of its production by the wild-type strain 87-22 on OBA (Fig. 3A). More strikingly, overproduction was also observed when the ΔcebR strain was streaked onto plates containing routinely used Streptomyces ISP-4, which contains soluble starch as a carbon source (Fig. 3A). Extraction of thaxtomin from the plates and analysis by high-performance liquid chromatography (HPLC) confirmed the production of the toxin by the cebR mutant on ISP-4 (Fig. 3B). Since the wild-type strain produced only trace amounts of thaxtomin on ISP-4, this result demonstrates that we generated a mutant able to produce thaxtomin A independently of the presence of the elicitor cellulbiose. Importantly, the ΔcebR mutant complemented with plasmid pRLIF8 carrying the S. scabies cebR gene and its upstream region restored the cellulbiose-dependent induction of thaxtomin (see Fig. S3 in the supplemental material), demonstrating that the phenotype of the mutant is caused only by the chromosomal deletion of the S. scabies cebR gene (SCAB_57761).

Deletion of cebR leads to hypervirulence in S. scabies. Since thaxtomin is the primary virulence determinant of the pathogenic streptomycetes, we evaluated the effect of cebR deletion on the virulence phenotype of S. scabies. Arabidopsis thaliana (ecotype Col-0) seeds grown on Murashige-Skoog (MS) agar were inoculated with spores of S. scabies 87-22 (wild-type) and its ΔcebR mutant (Fig. 4A). After 8 days of growth, seedlings inoculated with the cebR mutant presented stronger root and shoot stunting than those inoculated with the wild-type strain (Fig. 4B). In addition, we observed an increased capacity of S. scabies ΔcebR to induce pitting and necrosis of potato tuber slices compared to that of the wild-type strain 87-22 (Fig. 4C). Overall, the deletion of cebR resulted in a hypervirulent phenotype of S. scabies, confirming that CebR is the master repressor of plant pathogenicity through its impact on thaxtomin production.

New pieces of the puzzle for thaxtomin regulation. Regulation of virulence genes is critical for microbial pathogens and therefore often under strict control involving several layers of regulation. For thaxtomin production, one of the regulators involved is the pathway-specific TxtR protein, which acts as a transcriptional activator, inducing the expression of the thaxtomin biosynthetic genes (9). TxtR is an AraC family transcriptional regulator (AFTR) that binds cellulbiose and was thought to be the primary regulator of thaxtomin production and virulence. However, enhanced transcription of txtR and thaxtomin biosynthetic genes in the cebR deletion mutant, in the absence of cellulbiose, suggests a new paradigm for the molecular mechanism by which S. scabies and other thaxtomin A-producing streptomycetes utilize cellulbiose as a signal. Our data show that the ability of cellulbiose to induce thaxtomin production is implemented primarily through relief of CebR repression upon the transcription of the thaxtomin biosynthetic genes and that specific interaction of cellulbiose with TxtR is not required for it to function as a transcriptional activator (9). Though some AFTRs require ligand binding to activate the transcription of virulence genes, this is not always the case (18). It may be that the specific interaction of cellulbiose with TxtR provides a mechanism to fine-tune the regulation of thaxtomin production. However, results presented here reinforce the significance of cellulbiose as a signal for thaxtomin production and, therefore, virulence and demonstrate how minor intergenic and intragenic changes in the region coding for thaxtomin biosynthesis allow CebR to act as the “gatekeeper” of pathogenicity in S. scabies. Likewise, Streptomyces coelicolor recruited DasR, the N-acetylglucosamine utilization regulator, to control the production of the antibiotic actinorhodin and the prodiginines (19), as well as siderophores (20, 21), probably as a response to sensing its own cell wall degradation during programmed cell death (22). Though noteworthy, the CebR and DasR examples in S. scabies and S. coelicolor, respectively, are not sufficient for us to state that enrolling transcription factors normally devoted to primary carbon metabolism is a common feature of streptomycetes in regulating the onset of their secondary metabolism. Finding chemical elicitors and culturing conditions that trigger the production of the so-called cryptic secondary metabolite is presented as a challenging, expensive, and time-consuming task (23–25). By deleting cebR, we have been able to create a strain that produces much...
higher levels of thaxtomin than the natural strains produce, which will assist in getting thaxtomin onto the market. Our studies of S. coelicolor and S. scabies thus suggest that at least some actinomycete species, by having selected-by-products of the most abundant carbon and nitrogen sources on earth (cellulose and chitin or peptidoglycan) as signaling molecules, have evolved pathways simple to engineer as a means of awakening the sleeping antibiotics (26, 27).

MATERIALS AND METHODS

Bacterial strains, media, chemicals, and culture conditions. All strains and plasmids used in this study are described in Table S1 in the supplemental material. Escherichia coli strains were cultured in Luria-Bertani (LB) medium at 37°C. Streptomyces strains were routinely grown at 28°C in tryptic soy broth (TSB; BD Biosciences) or on International Streptomycetes Project medium 4 (ISP-4), 1 liter of which consists of soluble starch (10 g), K2HPO4 (1 g), MgSO4·7H2O (1 g), NaCl (1 g), (NH4)2SO4 (2 g), CaCO3 (2 g), FeSO4·7H2O (0.001 g), MnCl2·7H2O (0.001 g), ZnSO4·7H2O (0.001 g), agar (20 g) (BD Biosciences), and soy flour man- nitol (SMF) agar (28). Where required, the medium was supplemented with the antibiotics apramycin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), thiostrepton (25 μg/ml), and/or nalidixic acid (50 μg/ml). Cellulbiose and cellooligosaccharides were purchased at Megazyme (Ireland).

CebR regulon prediction in streptomycetes. The position weight matrix (PWM) was created and the CebR regulon in S. scabies was computationally predicted with the PREDetector software (15) as described previously (20). We used DNA motifs known to be bound by CebR in S. griseus (14) as a training set to generate the PWM (PWM-CebR) (Fig. S1) and scan the genomes of pathogenic Streptomyces species for similar DNA motifs using different types of threshold scores (29).

His-tagged CebR production in E. coli and protein purification. The open reading frame (ORF) encoding SCAB57761 was amplified by PCR using the primers SCAB_57761+3_Ndel and SCAB_57761+1056_EcoRI (see Table S2 in the supplemental material). The corresponding PCR product was subsequently cloned into the pET21b vector, yielding pSAJ001. After DNA sequencing, an Ndel−EcoRI DNA fragment was excised from pSAJ001 and cloned into pET22b digested with the same restriction enzymes. E. coli Rosetta(DE3) competent cells were transformed with the resulting construct, pSAJ002. E. coli cells carrying pSAJ002 were grown at 37°C in 250 ml LB medium containing 100 μg/ml of ampicillin until the culture reached an optical density at 600 nm (OD600) of 0.6. Production of His6-tagged CebR (CebR-His6) was induced overnight (≈20 h) at 16°C by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl buffer; pH 7.5, 1 mM dithiothreitol, 0.25 mM CaCl2, 0.5 mM MgCl2, 50 mM KCl, and 0.5 pmol of each gene-specific primer (Table S2), and the mixture was subjected to the following PCR protocol: 3 min at 95°C and 40 cycles of 30 s at 95°C, followed by 45 s at 60°C. A melting curve analysis (samples were heated from 60°C to 95°C) was performed after each qPCR run to verify specific amplification. The maxX, hnb, and gyrA genes (11) were used to normalize the amount of RNA in the samples. Each measurement was performed in triplicate with three different cebR mutant isolates.

Analysis of thaxtomin A production. Mycelial suspensions of S. scabies strains were prepared from 48- to 72-h-old TSB-grown cultures by pelleting the mycelia, washing them twice with sterile water, and resuspending them in 1 ml of sterile water to obtain an OD600 of 1.0. Samples of 50 μl were plated out on small petri dishes (5-cm diameter) containing OBA or ISP-4 medium. After incubation for 7 days at 28°C, the medium was dropped into small cubes and soaked in 8 ml of methanol for 10 min. The supernatant was filtered through a 0.2-μm polytetrafluoroethylene (PTFE) filter and analyzed via HPLC as described previously (16). All experiments were repeated using different biological replicates of the Streptomyces strains, with three technical replicates per strain.

Virulence bioassays. Arabidopsis thaliana (ecotype Col-0) seeds were surface sterilized with sodium hypochlorite and washed three times in deionized H2O. Seeds were suspended in 0.1% agarose and placed on Murashige-Skoog (MS) (33) agar medium supplemented with 1% sucrose. After an incubation period of one night at 4°C, seeds were inoculated with the wild-type or cebR spore suspension (10⁶ spores/ml). Plants were grown at 21 ± 2°C, with a 12-h photoperiod, for 5 to 6 days. Potato tuber slices were prepared as described previously (34) and inoculated with 5 mg of Streptomyces mycelium. Potato slices were then incubated at 23 ± 2°C in a dark and moist incubator for 4 days.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.02018-14/-/DCSupplemental. Figure S1, TIF file, 0.5 MB. Figure S2, TIF file, 0.1 MB. Figure S3, TIF file, 0.3 MB. Table S1, DOCX file, 0.01 MB. Table S2, DOCX file, 0.01 MB. Figure S3, TIF file, 0.3 MB. Table S2, DOCX file, 0.01 MB. ACKNOWLEDGMENTS

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