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Contribution of the β-glucosidase BglC to the onset of the pathogenic lifestyle of *Streptomyces scabies*

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SUMMARY

Common scab disease on root and tuber plants is caused by *Streptomyces scabies* and related species which use the cellulose synthase inhibitor thaxtomin A as the main phytotoxin. Thaxtomin production is primarily triggered by the import of cello-oligosaccharides. Once inside the cell, the fate of the cello-oligosaccharides is dichotomized: (i) the fuelling of glycolysis with glucose for the saprophytic lifestyle through the action of β-glucosidase(s) (BGs); and (ii) elicitation of the pathogenic lifestyle by the inhibition of CebR-mediated transcriptional repression of thaxtomin biosynthetic genes. Here, we investigated the role of *scab57721*, encoding a putative BG (BglC), in the onset of the pathogenicity of *S. scabies*. Enzymatic assays showed that BglC was able to release glucose from cellobiose, cellotriose and all other cello-oligosaccharides tested. Its inactivation resulted in a phenotype opposite to that expected, as reduced production of thaxtomin was monitored when the mutant was cultivated on medium containing cello-oligosaccharides as unique carbon source. This unexpected phenotype could be attributed to the highly increased activity of alternative intracellular BGs, probably as a compensation for *bglC* inactivation, which then prevented cellobiose and cellotriose accumulation to reduce the activity of CebR. In contrast, when the *bglC* null mutant was cultivated on medium devoid of cello-oligosaccharides, it instead constitutively produced thaxtomin. This observed hypervirulent phenotype does not fit with the proposed model of the cello-oligosaccharide-mediated induction of thaxtomin production, and suggests that the role of BglC in the route to the pathogenic lifestyle of *S. scabies* is more complex than currently presented.

Keywords: β-glucosidase, CebR, cello-oligosaccharides, common scab disease, thaxtomin.

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INTRODUCTION

Streptomyces scabies is the causative agent of common scab on tuber and root plants via the production of the phytotoxin thaxtomin A amongst other virulence factors (Bignell *et al.*, 2010; Lerat *et al.*, 2009; Loria *et al.*, 2008). The onset of thaxtomin A is triggered on transport of the cello-oligosaccharides cellobiose [$(Glc)_2$] and cellotriose [$(Glc)_3$], which involves the ATP-binding cassette (ABC) transporter system CebEFG-MsiK (Jourdan *et al.*, 2016). Once inside the cell, mainly $(Glc)_2$, but also $(Glc)_3$, can interact with the cellulose utilization repressor CebR, preventing it from binding to its operator sequences associated with the thaxtomin biosynthetic gene cluster, and therefore allowing the production of the phytotoxin (Francis *et al.*, 2015). Adjacent to the *cebR-cebEFG* divergon and 146 nucleotides downstream of *cebG*, *scab57721* (*bglC*) encodes a putative β-glucosidase (BG) of the glycosyl hydrolase (GH) GH1 family, which is expected to catalyse the hydrolysis of terminal, non-reducing β-D-glucosyl residues, with the release of β-D-glucose from β-D-glucosides and oligosaccharides (Henrissat, 1991; ENZYME entry: EC 3.2.1.21). The presence of a gene coding for an intracellular GH within the cluster of a sugar ABC transporter is a common feature which allows the co-transcription of the genes required for carbohydrate import and their subsequent enzymatic degradation in the cytoplasm. The use of molecules that are also common (most likely the most recurrent) soil carbohydrate nutrients for the onset of pathogenicity is very intriguing (Jourdan *et al.*, 2017). In non-pathogenic *Streptomyces*, the coordinated expression of genes for BG and cello-oligosaccharide transport is appropriate for feeding the glycolysis pathway with glucose (Fig. 1). However, as stated earlier, in the plant pathogen *S. scabies*, $(Glc)_2$ and $(Glc)_3$ are not only perceived as nutrients used during the course of saprophytic behaviour, but are, above all, signalling molecules eliciting its pathogenic lifestyle (Johnson *et al.*, 2007; Jourdan *et al.*, 2016; Wach *et al.*, 2007). Enzymes with a BG activity could thus potentially play an important role in controlling the onset of the virulence of

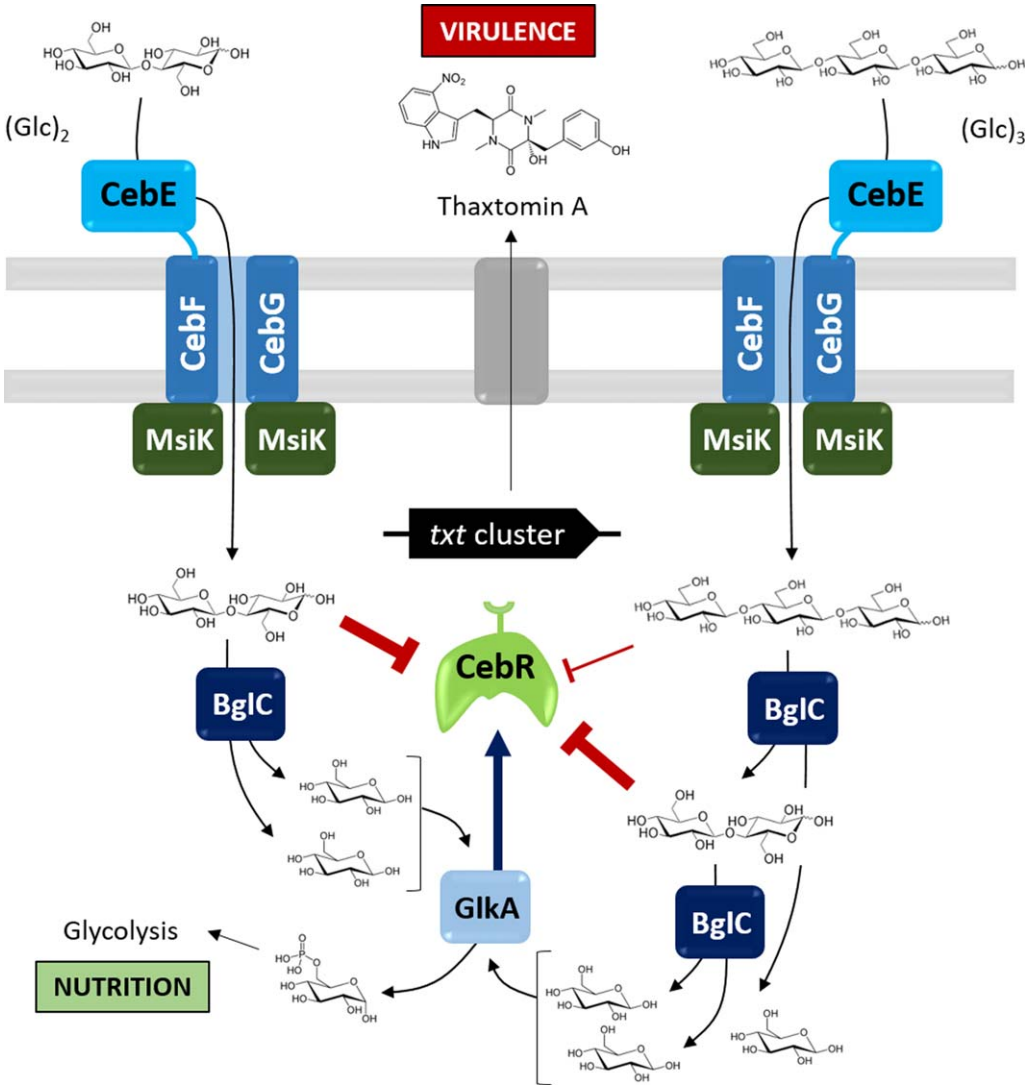

Fig. 1 Position of β-glucosidase activity on the modelled metabolic pathways from cellobiose [$(Glc)_2$] and cellotriose [$(Glc)_3$] transport to glycolysis and thaxtomin A production. When $(Glc)_2$ and $(Glc)_3$ are transported into the cytoplasm through the CebEFG-MsiK transporter, they both prevent the DNA-binding ability of the repressor CebR [$(Glc)_2$ does this much more efficiently than $(Glc)_3$; Francis *et al.*, 2015], thus allowing the expression of CebR-controlled genes, including the thaxtomin biosynthetic genes (*txt* cluster), *cebEFG* and *bglC*. Once expressed, BglC cleaves both the imported $(Glc)_2$ and $(Glc)_3$. $(Glc)_2$ hydrolysis leads directly to two glucose molecules, whereas $(Glc)_3$ hydrolysis generates first glucose and $(Glc)_2$, the latter being the best allosteric effector of CebR. $(Glc)_3$ uptake therefore inhibits CebR-mediated repression more effectively than does $(Glc)_2$ uptake. The glucose generated by BglC activity is further metabolized by entering glycolysis, with phosphorylation by the glucose kinase GlkA to glucose-6-phosphate as the first step.

S. scabies by limiting the intracellular accumulation of signals triggering thaxtomin A biosynthesis (Fig. 1). As a consequence, intracellular BG(s) of *S. scabies* might have evolved to display specific/unique properties to ensure that the microorganism adopts the appropriate behaviour—saprophytic versus pathogenic—according to the environmental conditions (Fig. 1). In this work, we define the enzymatic properties, assess the expression control mechanism and investigate the role of *scab57721* (*bglC*) in thaxtomin A production, and therefore in the onset of the virulence of *S. scabies*.

RESULTS AND DISCUSSION

Enzymatic properties of BglC of *S. scabies*

The gene s*cab57721* encodes a 480-amino-acid peptide orthologous to the well-characterized intracellular GH1 family BG BglC of *Thermobifida fusca* (53% and 67% amino acid identity and similarity, respectively) which also lies downstream of the *cebEFG* operon (Spiridonov and Wilson, 2001). BglC of *S. scabies* contains the MYVTENGAA sequence (amino acids 376–384) which

Table 1 Primers and plasmids used and generated in this study.

Primers	Sequences (5′–3′)*	Application
scab_57721_+3_NdeI	TT*CATATG*CCTGAACCCGTGAATCCGG	PCR for cloning *scab57721* in pET28a
scab_57721_+1458_HindIII	TT*AAGCTT*TGGTCCGTCGCTGCCCTACG	
imf298	CCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG**ATTCCGGGGATCCGTCGACC**	*scab57721* (*bglC*) redirect deletion cassette
imf299	GCTCCCCGGCCCCCGGCTCCGTGGTCCGTCGCTGCCCTA**TGTAGGCTGGAGCTGCTTC**	
imf300	ATGCTGATGTTCGTGCAGAC	PCR verification of Δ*scab57721*
imf301	GAAGACGACGGTGAGGAAGC	
imf302	GACCTTTCCTCCCGCCTTC	*scab57721* (*bglC*) expression analysis
imf303	GTGTGACTGAAGGTGTCCCA	
imf381	**AAA***TCTAGA*ATGCTGATGTTCGTGCAGAC	Complementation of Δ*scab57721*
imf382	**AAA***TCTAGA*GAAGACGACGGTGAGGAAGC	
Plasmids, cosmids	Description†	Source or reference
pJET1.2/blunt	*E. coli* plasmid used for high-efficiency cloning of PCR products (Amp^R)	Thermo Scientific, St. Leon-Rot, Germany
pET28a	Expression vector used to produce N-terminal His-tagged protein in *E. coli* (Kan^R)	Novagen, Darmstadt, Germany
pSAJ021	pJET1.2 derivative containing the scab57721 (*bglC*) coding sequence (Amp^R)	This study
pSAJ022	pET28a derivative containing the scab57721 (*bglC*) coding sequence inserted into *Nde*I and *Hind*III restriction sites (Kan^R)	This study
pIJ790	λ Red plasmid (t^S, Cml^R)	Gust *et al.* (2003)
pUZ8002	Supplies transfer functions for mobilization of *oriT*-containing vectors from *E. coli* to *Streptomyces* (Kan^R)	Kieser *et al.* (2000)
pIJ773	Template for the REDIRECT© PCR targeting system, contains the [*aac(3)IV*+*oriT*] disruption cassette (Amp^R, Apr^R)	Gust *et al.* (2003)
Supercos1	SuperCos1 derivative containing the *S. scabies* 87-22 cellobiose utilization regulator CebR locus (Kan^R, Amp^R)	Stratagene, Amsterdam, The Netherlands
Cosmid 833	SuperCos1 derivative containing the *S. scabies* 87-22 cellobiose/cellotriose ABC transporter locus (Kan^R, Amp^R)	Francis *et al.* (2015)
pCR™-BluntII-TOPO	Cloning vector for PCR products (Kan^R)	Invitrogen, Carlsbad, Canada
pAU3–45	pSET152 derivative, integrates into the ΦC31 *attB* site in *Streptomyces* (Apr^R, $Thio^R$)	Bignell *et al.* (2005)
pIMF001	pAU3–45 derivative containing *scab57721* and its upstream region cloned into the *Xba*I site	This study

*Non-homologous extensions are shown in bold and engineered restriction sites are indicated in italics.
†Amp^R, ampicillin resistance; Apr^R, apramycin resistance; Cml^R, chloramphenicol resistance; Kan^R, kanamycin resistance; $Thio^R$, thiostrepton resistance; t^S, temperature sensitive.

matches the GH1 family active site signature [LIVMFSTC]-[LIVFYS]-[LIV]-[LIVMST]-E-N-G-[LIVMFAR]-[CSAGN] (PROSITE accession number PS00572). In order to assess the substrate specificity and enzymatic properties of the predicted intracellular BG, s*cab57721* (*bglC*) was cloned into pET-28a (Table 1) for heterologous expression in *Escherichia coli* with a six histidine tag fused to the N-terminus part of the protein (6His-BglC). Purification through Ni^{2+}-nitrilotriacetic acid (Ni-NTA) affinity chromatography enabled the recovery of 6His-BglC with an apparent molecular weight (MW) of ~54 kDa which corresponds well to the calculated MW of 54.121 kDa (Fig. 2A).

The kinetic parameters of 6His-BglC were determined by measuring the initial rate of $(Glc)_2$ hydrolysis (glucose release) at various concentrations of $(Glc)_2$. The maximum rate of the reaction (V_{max}) was 7.3 μmol/min/mg. The K_m and k_{cat} values were 0.77 mM and 400 min^{-1}, respectively (Fig. 2B). The activity of 6His-BglC at different temperatures (from 20 to 55 °C) and pH values (pH 5–10) was measured using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPβG) as substrate [mimicking $(Glc)_2$]. The activity of the enzyme gradually increased from 20 to 30 °C, remained constant up to 37 °C and declined abruptly to 10% of the maximal activity at 42 °C (Fig. S1, see Supporting Information). The optimal pH of BglC is around pH 7.5 as the enzyme maintained high activity between pH 6.5 and pH 8.5, and declined rapidly to 30% and 50% of its optimum at pH 5.5 and pH 9, respectively (Fig. S1).

To determine the substrate specificity of BglC, the recombinant protein was incubated with $(Glc)_2$, various cello-oligosaccharides ranging from $(Glc)_3$ to cellohexaose $[(Glc)_6]$ and different disaccharides unrelated to cellulose degradation (lactose, saccharose, maltose, trehalose and turanose). Samples collected after increasing incubation times were spotted on a thin layer chromatography plate which revealed that 6His-BglC was able to generate glucose from $(Glc)_2$ and all other cello-oligosaccharides tested (Fig. 2C). 6His-BglC was not able to release glucose from disaccharides unrelated to cellulose, except for lactose, but with much lower efficiency relative to $(Glc)_2$ or any of the other cello-oligosaccharides (data not shown). If BglC displayed activity *in vitro* against $(Glc)_4$, it was unlikely to occur inside the cytoplasm, as the extracellular ABC transporter component CebE of

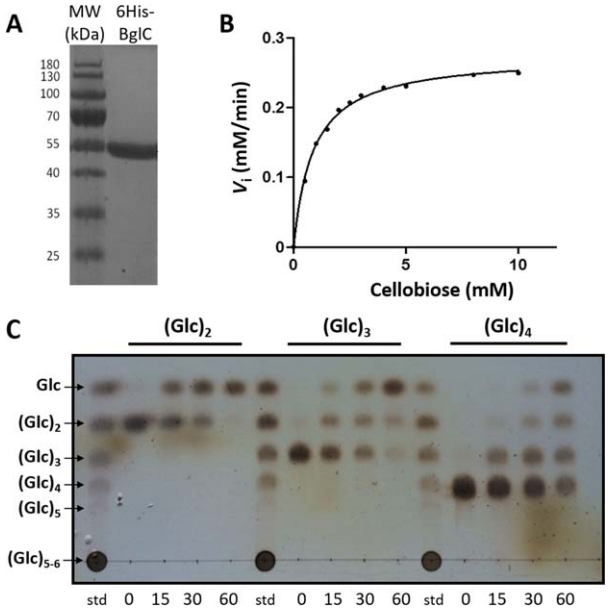

Fig. 2 *scab57721* encodes a β-glucosidase. (A) Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing the level of purity of 6His-BglC used for enzymatic assays. Lane 1, molecular weight marker; lane 2, purified 6His-BglC whose migration size (54 kDa) corresponds well to its predicted calculated size (54.121 kDa). (B) Initial velocity (V_i) of 6His-BglC as a function of the cellobiose concentration. Rates of cellobiose degradation were obtained by measuring the glucose released at the beginning of the hydrolysis reaction performed in 50 mM HEPES buffer, pH 7.5, at 25 °C. Data were fitted to the Henri–Michaelis–Menten equation using GraphPad Prism 5 software in order to obtain V_{max}, K_m and k_{cat}. (C) Substrate specificity of 6His-BglC for cello-oligosaccharides. Cello-oligosaccharides (6.25 mM) were incubated with pure 6His-BglC (0.4 μM) at 30 °C for 0, 15, 30 and 60 min. std, standard cello-oligosaccharides; Glc, glucose; $(Glc)_2$, cellobiose; $(Glc)_3$, cellotriose; $(Glc)_4$, cellotetraose.

S. scabies only displayed a high binding affinity to $(Glc)_2$ and $(Glc)_3$ (Jourdan *et al.*, 2016).

bglC expression is repressed by CebR and induced by $(Glc)_2$

In order to ascertain that BglC is indeed involved in the catabolism of $(Glc)_2$ and $(Glc)_3$ *in vivo*, we assessed whether its expression/production in *S. scabies* is under the control of the cellulose utilization repressor CebR. Quantitative reverse transcription-polymerase chain reaction (qPCR) was performed on RNA extracted from the wild-type strain of *S. scabies* 87-22, and its *cebR* deletion mutant Δ*cebR*, grown on International *Streptomyces* Project medium 4 (ISP4). This revealed that the deletion of *cebR* resulted in an 85-fold (wild-type 0.025 vs. *cebR* mutant 2.13) overexpression of *bglC* (Fig. 3A). In addition, targeted liquid chromatography-multiple reaction monitoring (LC-MRM) analysis allowed the evaluation of the effect of the deletion of *cebR*, as well as the presence of $(Glc)_2$, on BglC production in *S. scabies*. Quantitative analyses of two specific tryptic peptides of BglC (LVDELLAK and TDPVASLR) showed that the protein was more abundant in the total intracellular protein extracts of the Δ*cebR* mutant (2.3-fold more abundant relative to 87-22), as well as in extracts of the *S. scabies* wild-type strain grown in $(Glc)_2$-containing medium [2.9-fold more abundant relative to the condition without $(Glc)_2$] (Fig. 3B). The observed transcriptional repression exerted by CebR and the $(Glc)_2$-dependent induction of *bglC*/BglC are mediated through direct binding of CebR to the CebR-binding site (TGGaAGCGCTCCCA) identified at position −14 nucleotides upstream of *bglC* (Fig. 3C). The results deduced from the targeted proteomic approach are in agreement with the early and constitutive overall intracellular BG activity measured as a consequence of *cebR* deletion, whereas the *S. scabies* 87-22 wild-type only displayed measurable BG activity when grown in the presence of $(Glc)_2$ (Fig. 3D).

Inactivation of *bglC* results in reduced thaxtomin A production when *S. scabies* is grown with cello-oligosaccharides as the sole carbon source

As we demonstrated that *bglC*/BglC is induced by cello-oligosaccharides and displays BG activity against $(Glc)_2$ and $(Glc)_3$, we finally assessed whether the catabolic activity of BglC influenced the production levels of thaxtomin A and, as a consequence, the virulence of *S. scabies*. We generated a *bglC* null mutant (Δ*bglC*) by replacing *orf scab57721* with the apramycin resistance cassette, as performed previously for *cebR*, *cebE* and *msiK* (Francis *et al.*, 2015; Jourdan *et al.*, 2016). Semi-quantitative analysis by high-performance liquid chromatography (HPLC) revealed that the Δ*bglC*/Δ*57721* mutant produced only 37% and 9% of the thaxtomin levels produced by the wild-type strain when cultivated in liquid minimal medium (MM) with $(Glc)_2$ or $(Glc)_3$ as sole carbon source, respectively (Fig. 4). This result was unexpected, as the deletion of *bglC* should normally lower the catabolism of cello-oligosaccharides for glycolysis, and therefore would result in their higher intracellular accumulation as allosteric inhibitors of CebR and activators of *txtR* expression.

To tentatively explain the reduced thaxtomin A production as a result of the inactivation of *bglC*, we monitored $(Glc)_2$ and $(Glc)_3$ consumption, as well as the total BG activity, of the Δ*bglC* strain. For this purpose, *S. scabies* wild-type 87-22 and its *bglC* null mutant were grown for 24 h in MM supplemented with 500 μM of $(Glc)_2$ or $(Glc)_3$ as sole carbon source. The concentration of cello-oligosaccharides remaining in the culture supernatant was measured by HPLC at 1.5-h intervals post-inoculation (Fig. 5A). Full consumption of $(Glc)_2$ and $(Glc)_3$ by the wild-type strain 87-22 was accomplished at 3 and 4.5 h post-inoculation (hpi), respectively, whereas the *bglC* mutant was impaired in both $(Glc)_2$ and $(Glc)_3$ utilization, as the total consumption of these cello-

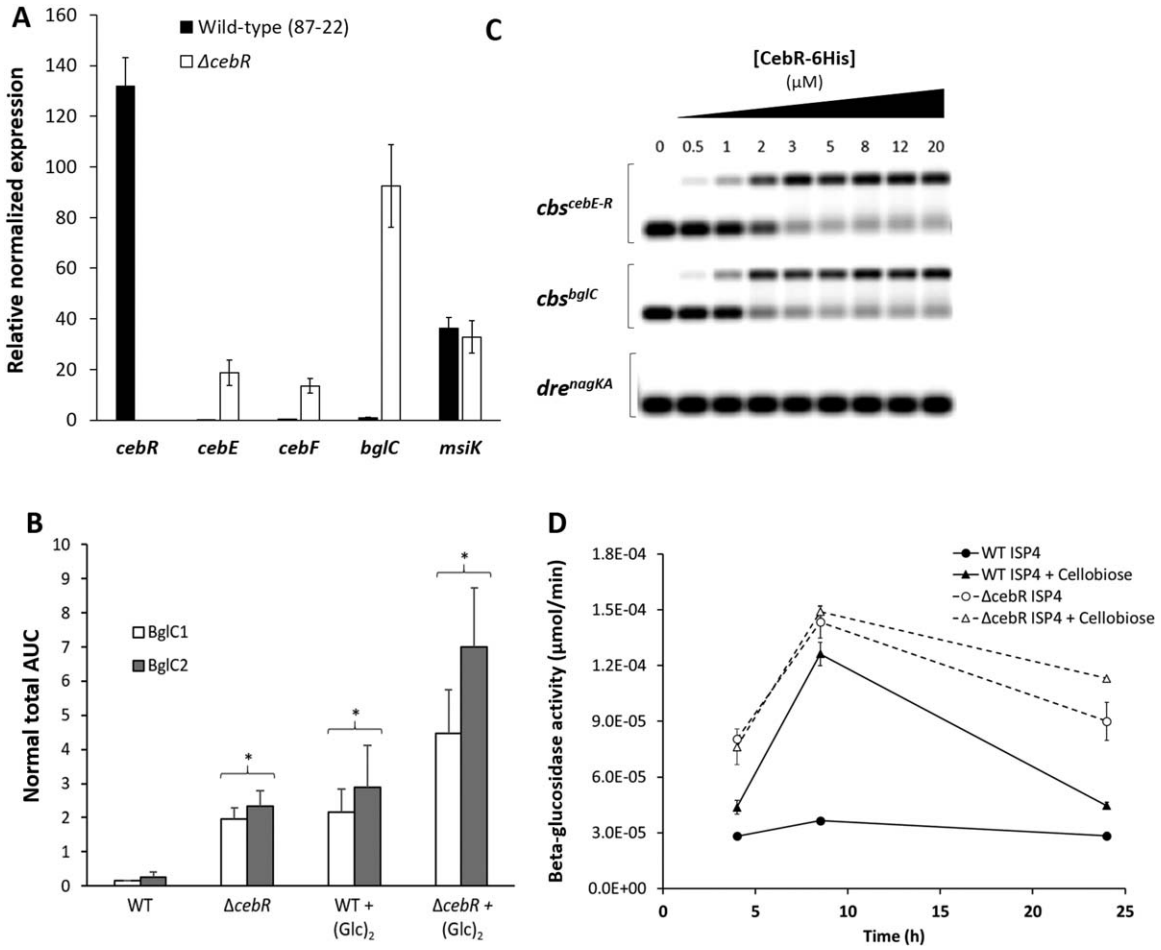

Fig. 3 Expression of *bglC* is repressed by CebR and induced by cellobiose. (A) Quantitative reverse transcription-polymerase chain reaction (qPCR) analysis of *bglC* expression levels in *Streptomyces scabies* 87-22 and in the Δ*cebR* strain. Data were normalized using the *gyrA* and *murX* genes as internal controls and the *cebE*, *cebF* and *cebR* genes as CebR repressed genes. Mean normalized expression levels (± standard deviations) from three biological repeats analysed in triplicate are shown, with the *bglC* expression level in the wild-type normalized to one-fold. (B) Relative normalized abundance of BglC peptides in response to the deletion of *cebR* (Δ*cebR*) and/or cellobiose supply, determined by liquid chromatography-multiple reaction monitoring (LC-MRM) mass spectrometry (MS) on tryptic digests of protein extracts. Target peptides for BglC: LVDELLAK (BglC1) and TDPVASLR (BglC2). *Significant quantitative peptide overproduction ($P < 0.05$) compared with the wild-type (WT) strain grown in International *Streptomyces* Project medium 4 (ISP4) without cellobiose supply. Statistical significance was assigned by performing two-sided Student's *t*-tests and assuming groups of equal variances. AUC, area under the curve. (C) Electrophoretic mobility shift assays (EMSAs) showing the specific interaction of CebR with the *cbs* (CebR-binding site) element at position −14 nucleotides upstream of *bglC*. Probes with the DasR-responsive element (*dre*) upstream of *nagKA* (Tenconi *et al.*, 2015) and with the *cbs* upstream of *cebE* were used as negative and positive controls, respectively. (D) Overall β-glucosidase activity of *S. scabies* 87-22 and its *bglC* null mutant grown in liquid ISP4 with or without cellobiose (0.5 mM) supply.

oligosaccharides required about 3 h longer than for the wild-type (Fig. 5A). This delayed import and consumption could possibly postpone the production of thaxtomin A in the *bglC* mutant, but should not be responsible for the observed massive reduction in the production of the phytotoxin.

Concomitant with the measurement of cello-oligosaccharide consumption, we assessed the intracellular and extracellular relative BG activity to evaluate to what extent the loss of *bglC* impacted the overall BG activity (Figs 5B,C and S2, see Supporting Information). Each soluble fraction (intra- and extracellular) was assessed at five different time points in both wild-type and Δ*bglC* strains using *p*-NPβG as substrate. Very low extracellular BG activities were obtained for both strains and under MM with $(Glc)_2$ or $(Glc)_3$ culture conditions (Fig. S2). Assessment of the intracellular BG activity against *p*-NPβG revealed that, although the activities measured in *S. scabies* wild-type and Δ*bglC* were similar in $(Glc)_2$-containing medium at the beginning of culture, the activity of the mutant strain increased dramatically after 3 hpi (Fig. 5B). At the end of the experiment, the wild-type strain presented only a slight increase in BG activity, reaching merely one-

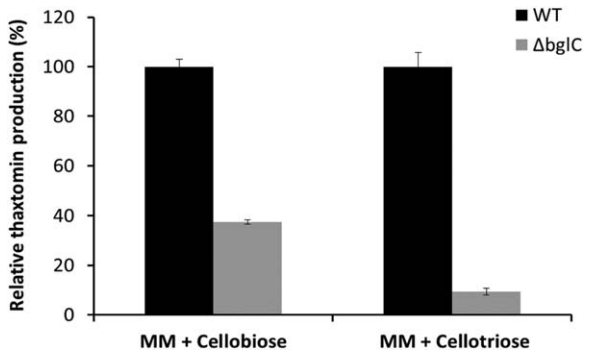

Fig. 4 Effect of *bglC* deletion on the cello-oligosaccharide-mediated induction of thaxtomin A production. *Streptomyces scabies* 87-22 and its *bglC* null mutant were grown in liquid minimal medium (MM) supplemented with 0.5 mM cellobiose or cellotriose. Thaxtomin production was quantified by high-performance liquid chromatography (HPLC) at 24 h post-inoculation and wild-type production levels in each condition were fixed to 100%.

third of the overall BG activity displayed by the *bglC* null mutant (Fig. 5B). The corresponding activities measured in $(Glc)_3$-containing medium were more similar for both strains at the beginning of the culture, but the *bglC* null mutant presented a BG activity about four-fold higher than that of the wild-type at 24 hpi (Fig. 5C). This delay in the response of BG might be a consequence of the delay in $(Glc)_3$ consumption observed for the Δ*bglC* strain (Fig. 5A), but also because $(Glc)_3$ is a much weaker allosteric effector of CebR relative to $(Glc)_2$ (Francis *et al.*, 2015). These observations demonstrate that BglC is not the only functional BG in *S. scabies* to catabolize cello-oligosaccharides. The fact that the mutant displayed BG activity points to the presence of one or several additional/alternative BGs which are apparently overproduced, or for which the biosynthesis is awakened, when $(Glc)_2$ or $(Glc)_3$ is provided as the sole carbon source. The nature and pathway associated with the induction of the alternative BG(s) are currently unknown, but might involve CebR, as the response differed according to $(Glc)_2$ or $(Glc)_3$ supply. The contribution of BglC to the overall BG activity of the wild-type is another pending question.

The fact that the *bglC* null mutant displayed a much higher overall BG activity would result in a more rapid depletion of the incorporated thaxtomin-inducing cello-oligosaccharides, thus providing a possible explanation for the unexpected decreased thaxtomin A production of *S. scabies* Δ*bglC* compared with the wild-type when cello-oligosaccharides are provided as the only carbon source. Similarly reduced thaxtomin A production levels were also observed when assays were performed on solid MM. When inoculated on MM with $(Glc)_2$ as sole carbon source (TDMc, Fig. 6), the Δ*bglC* mutant displayed a growth delay during the first 24 h consistent with the absence of a major $(Glc)_2$ hydrolysing enzyme. When incubated for a longer period, growth recovered, but the Δ*bglC* strain could not reach the level of thaxtomin produced by the wild-type in TDMc, as described previously in liquid MM (Fig. 4).

Inactivation of *bglC* results in overproduction or constitutive production of thaxtomin A when cello-oligosaccharides are not the only carbon source

The capability of the mutant to produce thaxtomin A was also monitored on a series of solid media, including complex oat bran agar (OBA) medium, which naturally contains cello-oligosaccharides and other carbon sources (Johnson *et al.*, 2007; Fig. 6). When grown on OBA, the Δ*bglC* mutant overproduced thaxtomin A compared with the wild-type strain (Fig. 6B). On this medium, the addition of $(Glc)_2$ neither decreased nor further increased thaxtomin production, suggesting that the *bglC* mutant could have partially lost its capacity to respond to $(Glc)_2$ when other carbon sources are available (Fig. 6B). Surprisingly, the *bglC* mutant also overproduced thaxtomin A when inoculated on ISP4 medium deprived of cello-oligosaccharides as nutrient sources

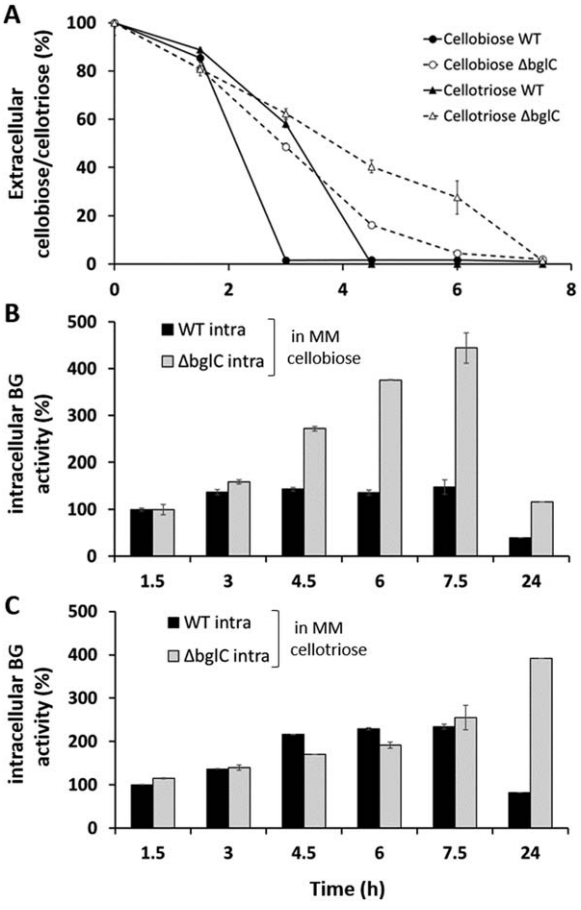

Fig. 5 Consumption of the cello-oligosaccharides cellobiose and cellotriose (A), and correlation with the intracellular β-glucosidase (BG) activity of *Streptomyces scabies* wild-type and the *bglC* null mutant (B, C). The BG activity of the wild-type at the first time point was set to 100%.

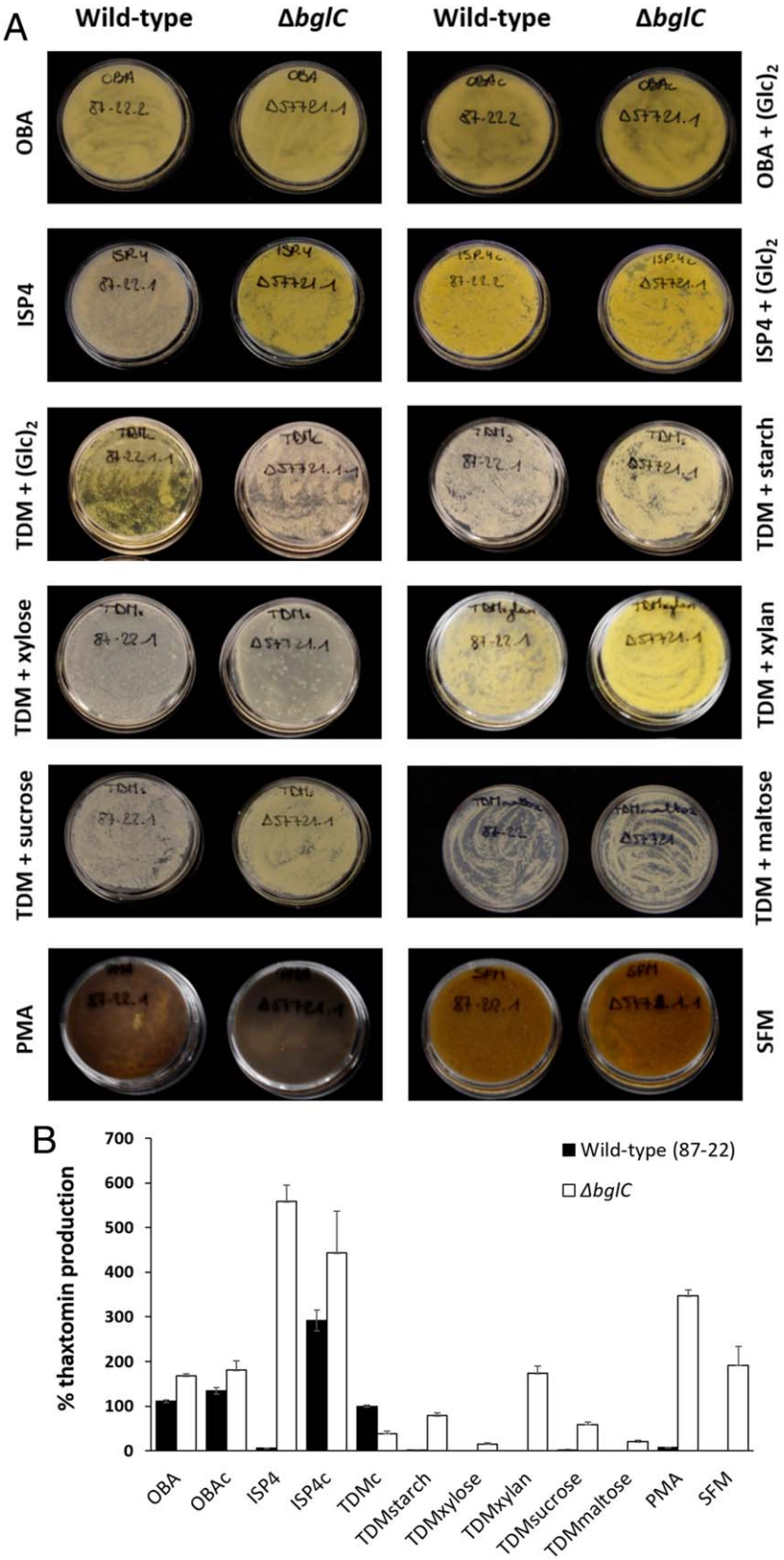

Fig. 6 Thaxtomin A production by *Streptomyces scabies* wild-type (87-22) and the *bglC* null mutant grown on various minimal and complex solid media. (A) Photographs of media inoculated with *S. scabies* 87-22 and its *bglC* null mutant. Thaxtomin A production can be seen by its distinct yellow pigmentation. (B) Quantification of thaxtomin A extracted from plates shown in (A) after incubation for 7 days at 28 °C. Means and standard deviations were calculated on three biological replicates. The wild-type production level in thaxtomin defined medium + cellobiose [TDM + $(Glc)_2$] was fixed to 100%. OBA, oat bran agar; PMA, potato mash agar; SFM, soy flour mannitol.

(Fig. 6). In order to ascertain the validity of this unexpected phenotype, the mutant was complemented by introducing plasmid pIMF001 (Table 1) containing the *bglC* gene with its promoter into the Δ*bglC* mutant isolates. Complementation of Δ*bglC* restored the wild-type phenotype when bacteria were streaked out on ISP4 (Fig. S3, see Supporting Information), demonstrating that the observed alteration in thaxtomin production was indeed caused by the deletion of the *bglC* (*scab_57721*) gene and not by a possible unspecific event, such as a spontaneous mutation. The thaxtomin A overproduction phenotype was further confirmed on other media tested, regardless of the presence of $(Glc)_2$ or other cello-oligosaccharides (Fig. 6).

As Δ*bglC* showed the constitutive production of thaxtomin A, its virulence capacity was evaluated on *Arabidopsis thaliana* and radish seedlings. No different outcome was observed between radish seedlings infected with the wild-type or the mutant (Fig. 7A). However, as the outcome of the radish assay is mostly influenced by the effect of thaxtomin on the plant's growth and development, and thaxtomin is active in nanomolar concentrations (King *et al.*, 2001), it is hard to determine any difference between the production levels of the wild-type and a potential thaxtomin overproducer using radish as host. Assays were also performed using slightly older seedlings (48 h instead of 30 h after sowing) or a lower inoculum [200 μL of a mycelial stock with an optical density at 600 nm (OD_{600}) of 0.1 instead of OD_{600} of 1.0], but no difference could be observed. Yet, when thaxtomin was extracted from the agar–water support with the radish seedlings, a significantly higher concentration of thaxtomin A was measured for the assays performed with the Δ*bglC* isolates compared with the wild-type strain (Fig. S4, see Supporting Information). *Arabidopsis thaliana* (ecotype Col-0) as the plant model was more suitable than radish seedlings for the monitoring of hypervirulent phenotypes, as observed previously for the *cebR* mutant which also overproduces thaxtomin A (Francis *et al.*, 2015). *Arabidopsis thaliana* seeds grown on Murashige–Skoog (MS) agar were inoculated with spores of *S. scabies* 87-22 (wild-type) and its Δ*bglC* mutant. After 7 days of growth, seedlings inoculated with the *bglC* mutant presented stronger growth and developmental defects compared with those inoculated with the wild-type strain (Fig. 7B). Closer inspection of individual plants revealed stronger root and shoot stunting as a consequence of the *bglC* deletion (Fig. 7B).

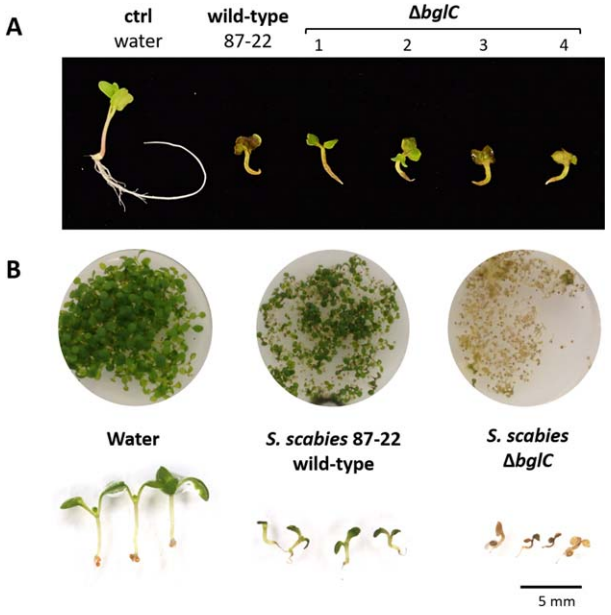

Fig. 7 Effect of *bglC* deletion on the virulence of *Streptomyces scabies*. (A) Phenotypes of representative radish seedlings treated with water, the wild-type strain 87-22 and *bglC* mutant isolates at 6 days post-infection. (B) Phenotype of *Arabidopsis thaliana* grown for 7 days in the presence of *S. scabies* 87-22 (wild-type) and its *bglC* null mutant, with a close-up of the representative plants grown on Murashige–Skoog (MS) plates shown in the top panel.

CONCLUSION AND PERSPECTIVES

In this work, we have demonstrated that the protein encoded by the gene *scab57721* located downstream of the *cebEFG* operon is a BG active against different cello-oligosaccharides, including the best inducers of thaxtomin A production, i.e. $(Glc)_2$ and $(Glc)_3$. Expression of *bglC* is also repressed by CebR, the master regulator of pathogenicity in *S. scabies*, and is induced by $(Glc)_2$. As $(Glc)_2$ and $(Glc)_3$ consumption by *S. scabies* correlates with an intracellular increase in BG activity, we assumed that BglC (and any other enzyme with BG activity) would play an essential role in controlling the pool of imported elicitors to trigger the CebR regulon, and therefore thaxtomin production, as proposed in the model illustrated in Fig. 1. In line with the current model of the cello-oligosaccharide-mediated induction of thaxtomin A production, we were expecting that the inactivation of *bglC* would simply result in an increased or prolonged production of thaxtomin under culture conditions supplemented with $(Glc)_2$ or $(Glc)_3$, as these CebR allosteric molecules would remain longer in the cytoplasm. However, surprisingly, we observed that the presence of $(Glc)_2$ and $(Glc)_3$ as sole carbon source reduced the production levels of thaxtomin A, probably as a consequence of the awakening of alternative BG(s) encoded in the genome of *S. scabies* as compensation for the loss of BglC. The identification of the protein(s) responsible for the high BG activity in the *bglC* mutant is currently under investigation.

Finally, the most striking phenotype observed for the Δ*bglC* strain was the loss of $(Glc)_2$-dependent induction of thaxtomin, and thus constitutive thaxtomin production, in complex media devoid of eliciting cellulose-related sugars (Fig. 6). That this mutant is able to produce thaxtomin without the presence of inducing molecules is difficult to explain based on the current model of the induction pathway of thaxtomin production, and

suggests that the role of BglC in the induction of *S. scabies* pathogenicity involves mechanisms that remain to be uncovered.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

Escherichia coli strains [DH5α and Rosetta™ (DE3)] were cultured in Luria–Bertani (LB) medium at 37 °C. *Streptomyces* strains (wild-type 87-22 and mutant strains Δ*57721*/Δ*bglC*) were routinely grown at 28 °C in tryptic soy broth (TSB; BD Biosciences, Detroit, MI, USA) or on ISP4 (BD Biosciences). When 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). $(Glc)_2$ and cello-oligosaccharides were purchased from Megazyme (Bray, Ireland). For the BG activity assays and the thaxtomin production assays, the *Streptomyces* strains were grown on the complex media OBA (Johnson *et al.*, 2007), soy flour mannitol (SFM; Kieser *et al.*, 2000), potato mash agar (PMA; 12.5 g potato flakes and 5 g agar per litre), as well as the MM thaxtomin defined medium (TDM), modified from Johnson *et al.* (2007) by omitting sorbose and using a final concentration of 1% of the carbon source of choice.

Heterologous expression and purification of His-tagged BglC

The open reading frame encoding SCAB57721 (BglC) was amplified by PCR using the primers scab_57721_+3_*Nde*I and scab_57721_+1458_*Hind*III (see Table 1 for primer sequences). The PCR product was subsequently cloned into the pJET1.2/blunt cloning vector, yielding pSAJ021. After DNA sequencing to verify the correct amplification of *scab57721*, an *Nde*I-*Hind*III DNA fragment was excised from pSAJ021 and cloned into pET-28a digested with the same restriction enzymes, leading to pSAJ022. *Escherichia coli* Rosetta™ (DE3) cells carrying pSAJ022 were grown at 37 °C in 250 mL of LB medium containing 50 μg/mL of kanamycin until the culture reached an absorbance at 600 nm (A_{600}) of 0.6. The production of 6His-tagged BglC (6His-BglC) was induced overnight (~20 h) at 16 °C by the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and ruptured by sonication in lysis buffer (100 mM Tris-HCl buffer, pH 7.5, 250 mM NaCl and 20 mM imidazole) supplemented with ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitor cocktail (Roche, Indianapolis, USA). Soluble proteins were loaded onto a pre-equilibrated Ni-NTA-agarose column (bed volume, 5 mL), and 6His-BglC was eluted within the range 100–150 mM imidazole. Fractions containing the pure protein were pooled (Fig. 2A) and dialysed overnight in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5.

Construction of the *bglC* mutant in *S. scabies* 87-22 and its genetic complementation

The deletion of the *bglC* coding region was created as described previously (Francis *et al.*, 2015; Jourdan *et al.*, 2016). Specific primers used to generate and verify the gene deletion and to complement the *bglC* null mutant are listed in Table 1. A fragment containing the *bglC* coding region and the upstream region (379 bp) harbouring the promoter was generated by PCR using primers with engineered *Xba*I sites (Table 1), and cloned into pCR™-BluntII-TOPO (Invitrogen, Carlsbad, Canada). After sequence confirmation, fragments were retrieved through an *Xba*I restriction digest, gel purified and cloned into an *Xba*I-linearized pAU3–45 (Bignell *et al.*, 2005), resulting in plasmid pIMF001 (Table 1). Complementation constructs, as well as the empty pAU3–45 plasmid, were introduced into three *bglC* mutant isolates through intergeneric conjugation, similar to the gene deletion process as described previously (Francis *et al.*, 2015; Jourdan *et al.*, 2016).

qPCR

RNA was prepared from 72-h-old mycelia grown on ISP4 plates at 28 °C using the RNeasy minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Verification of the absence of contaminating genomic DNA, cDNA synthesis and qPCR were performed as described previously (Francis *et al.*, 2015; Jourdan *et al.*, 2016). The *bglC*-specific internal primers imf302 and imf303 were used to quantify the expression levels of the *bglC* gene (Table 1). The *murX*, *hrdB* and *gyrA* genes were used to normalize the amount of RNA in the samples (Joshi *et al.*, 2007). Each measurement was performed in triplicate with three different *cebR* mutant isolates.

Targeted proteomics

Streptomyces scabies 87-22 and its *cebR* null mutant were grown on ISP4 plates with or without 0.7% $(Glc)_2$. The mycelium was collected after 48 h of incubation at 28 °C, and resuspended in 50 mM NH_4HCO_3 buffer (pH 7.5). Crude intracellular extracts were obtained after sonication of the mycelium as described previously (Jourdan *et al.*, 2016). Sample preparation for LC-MRM analysis and the LC-MRM analysis procedure were performed as described previously (Jourdan *et al.*, 2016).

BG activity assays

The relative enzyme activity was determined using *p*-NPβG as substrate. The reaction mixture (200 μL) containing 50 mM HEPES buffer (pH 7.5), 0.2 μM of purified 6His-BglC and the tested reagent was incubated for 10 min at 25 °C before the addition of 1 mM *p*-NPβG. The reaction was carried out at 25 °C for 2 min and stopped by the addition of 100 μL of 2 M Na_2CO_3. All assays were performed under these conditions, unless otherwise indicated. The release of *p*-nitrophenol (*p*-NP) was measured at 405 nm with a TECAN infinite® 200 PRO (Männedorf, Switzerland).

Kinetic analysis

Kinetic parameters of BglC (K_m and k_{cat}) were determined by measuring the glucose released at various $(Glc)_2$ concentrations in 50 mM HEPES buffer, pH 7.5, at 26 °C. A reaction time of 7 min was chosen to ensure initial rates of hydrolysis. The glucose released was determined using the D-Glucose HK Assay Kit from Megazyme. Data were fitted to the Henri–Michaelis–Menten equation using GraphPad Prism 5 software.

Hydrolysis of disaccharides and oligosaccharides

The cleavage ability of BglC was tested against different cello-oligosaccharides [$(Glc)_2$, $(Glc)_3$ and cellotetraose (Megazyme)] or different

disaccharides (lactose, saccharose, maltose, trehalose and turanose). Reaction mixtures (100 μL) containing 50 mM HEPES buffer, pH 7.5, 0.4 μM of purified 6His-BglC, 6.25 mM of cello-oligosaccharides or 12.5 mM of disaccharides were incubated at 30 °C. Samples (15 μL) of each were collected at 0, 15, 30 and 60 min, and heated at 98 °C for 5 min to stop the reaction. Each sample was spotted onto an aluminium-backed silica gel plate (Sigma, Steinheim, Germany). The plates were run with chloroform–methanol–acetic acid–water solvent (50 : 50 : 15 : 5, v/v), air dried, dipped in 5% H_2SO_4 in ethanol and heated over a hot plate until visualization of the carbohydrate spots, as described by Gao and Wakarchuk (2014).

Monitoring of $(Glc)_2$ and $(Glc)_3$ consumption and glucose production

Glucose, $(Glc)_2$ and $(Glc)_3$ consumption measurements were performed by HPLC (Alliance, Waters, Milford, MA, USA) on a lead-form Aminex HPX-87P column (300 mm × 7.8 mm; particle size, 9 μm; supplied by Bio-Rad, Richmond, Canada) in combination with two Micro-Guard columns (deashing refill cartridge, 30 mm × 4.6 mm, supplied by Bio-Rad) heated to 80 °C with Milli-Q (18.2 MΩ cm) distilled–deionized H_2O in an isocratic mode (flow rate, 0.6 mL/min). Peaks were detected by a refractive index detector (Waters 2414) and processed with Empower 3 software (Waters).

Thaxtomin production assays

Thaxtomin production assays were performed as described previously (Francis *et al.*, 2015; Jourdan *et al.*, 2016). Briefly, plates were inoculated with equal amounts of mycelial suspensions of the *S. scabies* 87-22 wild-type and its *bglC* null mutant, and incubated for 7 days at 28 °C. Thaxtomin was extracted from the agar and quantification by reverse-phase HPLC was performed as described previously (Francis *et al.*, 2015; Jourdan *et al.*, 2016). For liquid cultures, thaxtomin was extracted from 1 mL of the culture supernatant with 0.3 mL of ethyl acetate, and quantified by HPLC using a NUCLEODUR® 100-5 C18ec column (Macherey-Nagel, Duren, Germany). Samples were eluted at a flow rate of 0.8 mL/min, and A_{400} was monitored using a Multi λ fluorescence detector (2475, Waters). All experiments were repeated using three different biological and technological replicates for each *S. scabies* strain.

Virulence assays

Virulence assays on *Arabidopsis* seedlings were performed as follows. Seeds of the Col-0 ecotype were surface sterilized for 15 min in bleach solution (40% v/v bleach, 0.05% v/v Tween-20), thoroughly rinsed with sterile H_2O and stratified for 3 days at 4 °C in the dark before sowing. *Arabidopsis* seeds (300–400) were sown in each well of a six-well plate containing half-concentrated MS medium (Sigma M5513) supplemented with 1% sucrose. Each well was inoculated with 250 μL of spore suspension of the *S. scabies* 87-22 wild-type and *bglC* mutant (5×10^4 spores/μL), or sterile water as the control. The plates were incubated at 25 ± 0.5 °C under a 16-h photoperiod for 7 days.

Virulence phenotypes on radish seedlings were performed as described previously (Jourdan *et al.*, 2016). Thaxtomin was extracted from the combination of the radish seedlings and the water–agar medium by cutting the material into small pieces and soaking in 15 mL of methanol for 10 min. The liquid phase was dried down and resuspended in 1 mL of methanol. These samples were analysed by HPLC as described above.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Effect of temperature and pH on BglC activity. The optimal temperature was determined by measuring the relative enzyme activity of BglC (0.2 μM) in HEPES (50 mM, pH 7.5) at 20, 25, 30, 37 and 42 °C. The effect of pH on the relative activity of BglC was assessed in the ranges pH 5.0–6.5 (50 mM 2-(N-morpholino)ethanesulfonic acid [MES] buffer), pH 7.0–8.5 (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer) and pH 9–10 (50 mM N-Cyclohexyl-2-aminoethanesulfonic acid [CHES] buffer) at 25 °C.

Fig. S2 Weak overall extracellular β-glucosidase activity of *Streptomyces scabies* compared with its overall intracellular β-glucosidase activity. Overall intra- and extracellular β-glucosidase activities of *S. scabies* wild-type and *bglC* null mutant grown in minimal medium (MM) supplemented with cellobiose (A) and cellotriose (B).

Fig. S3 Complementation of the *bglC* mutant. The *bglC* mutant complemented with plasmid pIMF001 carrying the *Streptomyces scabies bglC* gene and its upstream region restored thaxtomin production to a similar level as produced by the wild-type, demonstrating that the phenotype of the mutant is indeed caused by the chromosomal deletion of the *S. scabies bglC* gene.

Fig. S4 Thaxtomin A production of *Streptomyces scabies* wild-type and the *bglC* mutant when inoculated on radish seedlings. High-performance liquid chromatography (HPLC) analysis of thaxtomin extracted from the corresponding radish assays showing that, although there is no visual difference in virulence on radish between wild-type and mutant strains (Fig. 7A), the *bglC* mutant isolates produced significantly more thaxtomin than the wild-type bacteria when inoculated on plants.