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Whole-cell biosensor of cellobiose and application to wood decay detection

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ABSTRACT

Fungal biodegradation of wood is one of the main threats regarding its use as a material. So far, the detection of this decaying process is empirically assessed by loss of mass, when the fungal attack is advanced and woody structure already damaged. Being able to detect fungal attack on wood in earlier steps is thus of special interest for the wood economy. In this aim, we designed here a new diagnostic tool for wood degradation detection based on the bacterial whole-cell biosensor technology. It was designed in diverting the soil bacteria *Streptomyces* CebR sensor system devoted to cellobiose detection, a cellulolytic degradation by-product emitted by lignolytic fungi since the onset of wood decaying process. The conserved regulation scheme of the CebR system among *Streptomyces* allowed constructing a molecular tool easily transferable in different strains or species and enabling the screen for optimal host strains for cellobiose detection. Assays are performed in microplates using one-day culture lysates. Diagnostic is performed within one hour by a spectrophotometric measuring of the cathecol deshydrogenase activity. The selected biosensor was able to detect specifically cellobiose at concentrations similar to those measured in decaying wood and in a spruce leachate attacked by a lignolytic fungus, indicating a high potential of applicability to detect ongoing wood decay process.

1. Introduction

Thanks to its intrinsic mechanical properties, wood is used since the Neolithic era as a material for construction, furnishing, heating or wood-derived products such as paper. Nowadays, there is also a regain of interest for this material as it is renewable and a source of biomass for conversion into bio-ethanol (Wang et al., 2013). As a material, wood is highly resistant to the environmental conditions such as rain, sun or other abiotic stresses and the main threat regarding its use comes mostly from the attack by lignolytic organisms. This wood bio-degradation represents a major economic problem. For instance, in the US only, it costs more than 5 billion USD each year to homeowners and almost 10% of the annual product of forest is used to replace the degraded products (Schultz and Nicholas, 2008). Being able to detect the early wood bio-degradation is thus crucial. During storage, it would avoid to use contaminated wood for construction. When the wood is already used as a material it would enable to apply early curative measures before any significant loss of mechanical properties. However, to our knowledge, there is no tool available to achieve this goal and wood degradation is so far only assessed by resistance testing and visual expertise only relevant and operating on wood decayed at an advanced level.

Wood bio-degradation in forest ecosystems is mainly the fact of diverse fungi (Rajala et al., 2012) classified according to the type of decay they cause (*e.g.* white-rots, brown-rots, soft-rots, see for review (Schwarze et al., 2000)). If rot-fungi have distinct specificities in term of ecology or degradation strategies, they nevertheless all degrade and use as a carbon source the cellulose that represents 40%–50% of the plant dry weight (Howard et al., 2004). Two main degradation strategies exist during the wood degradation by fungi:

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enzymatic pathways involving cellulases or oxidative mechanisms using Fenton reaction and polysaccharide monooxygenases (Lynd et al., 2002; Phillips et al., 2011). However, a canonical degradation pathway is common for all of them where the cellulose fiber is shortened into simpler forms: the cellobiose (two β-1,4 linked glucose units) or sometimes into cellodextrines (generally from three to six β-1,4-linked glucose units) (Langston et al., 2011; Lynd et al., 2002). These cellulose degradation by-products will be hydrolyzed later on into glucose by the action of β-glucosidases (Langston et al., 2011). Thus, the initial release and presence of cellobiose and cellodextrines is a common denominator and can be considered as a signature of the attack of wood by rot-fungi.

The *Streptomyces* are filamentous spore forming soil dwelling bacteria that are generally not able to degrade native wood, but are considered as essential to recycle biomass polymers in environment thanks to their large-enzymatic arsenal able to degrade wood by-products (Bontemps et al., 2013; Bruce et al., 2010). The detection of these compounds and the activation of the enzymatic pool to degrade them has been linked to the regulator CebR of the LacI family in *Streptomyces griseus* (Marushima et al., 2009), *Streptomyces reticuli* (Schlösser et al., 2000), *Streptomyces* sp. ActE (Takasuka et al., 2013). Since its characterization by Schlösser et al. (Schlösser et al., 2000), and Marushima et al. (Marushima et al., 2009), it is known that the CebR transcriptional repressor prevents gene expression from binding a conserved 22 bp hairpin motif (*cebR*-box) found in the transcriptional region of its targets. In presence of inducer molecules such as cellobiose or in some cases cellodextrines or cellulose, the CebR repression is alleviated and enables the expression of the controlled genes. So far, the complete CebR regulome is not well-known. However, a transcriptomic analysis of the *Streptomyces* sp. ActE (Sirex) (a symbiotic strain that helps the pine-boring woodwasp *Sirex noctilio* to deconstruct wood biomass) has shown that, in presence of wood derived compounds, the most up-regulated genes were under the control of the CebR-system. These genes were mostly involved in the uptake (ABC transporter system) or in the production of cellulolytic and hemicellulolytic enzymes (β-glucosidases, cellulases, cellobiohydrolases, mannosidases) (Takasuka et al., 2013). Moreover, CebR could induce other functions like the pathogenic factors of *S. scabies* in presence of cellobiose (Francis et al., 2015).

Since cellobiose is a key product of cellulose hydrolysis and indirectly indicates fungal wood degradation, we designed and developed a *cebR*-box based biosensor expressed by *Streptomyces* in order to detect the presence of cellobiose, demonstrated its sensibility and specificity and showed its applicability in a case of wood degradation detection.

2. Material and methods

2.1. Plasmids, strains and media

Plasmids and strains used in this work are presented in Table 1. *Escherichia coli* strains were grown in LB medium (Kieser et al., 2000) at 37 °C and *Streptomyces* at 30 °C either on solid SFM medium [20 g mannitol, 20 g soy flour and 20 g bacto-agar per liter] or in liquid modified HT medium (HT*) [1 g yeast extract, 1 g beef extract, 5 g mannitol, 2 g bacto-tryptone and 0.02 g $COCL_2$ per liter, pH = 7.3]. For the biosensor tests, cellobiose (Alfa Aesar, Karlsruhe, Germany) and cellodextrines (Elicityl, Crolles, France) were added after autoclaving in liquid modified HT at the appropriate concentration. Avicel Ph-101 (Sigma, St. Louis, USA), a crystalline form of cellulose, was added in liquid medium before autoclaving. Media were supplemented with apramycin (50 μg ml^{-1}) for the selection of strains transformed with the pIB139 derived plasmids or with ampicilline (100 μg ml^{-1}) for the selection of the *E. coli* transformed with pGemT-easy (Promega, Madison, USA).

2.2. Wood infection, leachate preparation and cellobiose quantification

Wood degradation was performed according the European standard EN 113, 1996 (EN 113, 1996) with some modifications. Spruce (*Picea abies* L.) wood specimens (40 × 15 × 5 mm) were sterilized at 103 °C for 2 days. Then wood specimens were placed under sterile conditions in petri dishes containing one-week old mycelium of the lignolytic *Poria placenta* fungus. Wood specimens were incubated at 22 °C and 70% relative humidity for 16 weeks. For leachate preparation, *P. placenta* mycelium was carefully removed from the wood by hand scratching. Eight wood specimens were impregnated under vacuum with 100 ml of PBS buffer pH 7.4 for 15 min. For cellobiose quantification, 20 μl of leachate were run on HPLC equipped with a refractometer. Sugars were separated on a Shodex sugar KS-803 column (Waters SAS, Guyancourt, France) at 80 °C with a flow of 0.8 ml min^{-1} using HPLC grade water as solvent. Cellobiose and glucose were detected after 12.4 min and 13.2 min of elution, respectively. The cellobiose concentration present in the leachate was estimated by peak area integration and comparison with a cellobiose standard calibration curve. The cellobiose calibration curve ranged from 2.92 mM to 29.3 μM. Specificity and detection of free glucose resulting from wood cell wall degradation was detected by comparison with a calibration curve build from glucose concentrations ranging from 5.56 mM to 55.6 μM.

2.3. DNA manipulations

Polymerase chain reactions were performed with the Dream Taq polymerase (ThermoFisher Scientific, Waltham, USA) for fragments under 1.5 kb in size or Taq polymerase Takara (Takara Bio Inc., Kusatsu, Japan) for larger fragments. PCR primers are listed in Table 2. Ligation reactions were carried out with T4 DNA ligase (ThermoFisher Scientific, Waltham, USA). DNA was digested and purified from gel matrix respectively with restriction enzymes and the GeneJet Gel Extraction Kit purchased from ThermoFisher Scientific (Waltham, USA). The kits were used according to supplier's recommendations. Alkaline lysis plasmid extractions, plasmid electroporations and DNA extractions were performed as described in (Sambrook et al., 2001).

2.4. Functional characterization of biosensors

Functional screenings of the biosensors efficiency were performed by streaking of 2 μl of spore suspension (ca 10^8–10^9 spores/mL) of each biosensor on HT* plates supplemented with 5 gL^{-1} of cellobiose and incubation at 30 °C for 2 days. Controls without cellobiose or with crystalline cellulose (Avicel) were performed in parallel. After growth, plates were sprayed with a 0.5 M catechol solution and incubated for 10 to 20 min in the dark. The visualization of a yellow color in the mycelium and in its surrounding medium attested the detection of cellobiose by the biosensor after confrontation with the control plates *i.e.* without cellobiose and supplemented with crystalline cellulose. A positive result in both latter cases would constitute false positives.

2.5. Biosensor microplate tests

Spore suspensions of the *Streptomyces* S4N27 biosensor strain were realized on SFM complemented with apramycin (Kieser et al., 2000) and stored in a 20% (v/v) glycerol solution at −20 °C. To perform biosensor qualitative and quantitative tests in presence of cellobiose, cellodextrines or wood leachates, 5.10^5 spores were

Table 1
Bacterial strains and vector used in this study.

Strain or plasmid	Relevant characteristic(s)	Reference
E. coli DH5α	*supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-11 relA1*	Hanahan (1983)
E. coli S17.1	*recA pro hsdR RP4-2-Tc:Mu-Km:Tn7*	Simon et al. (1983)
S. coelicolor KC900	*actI:KC900 hisA1 uraA1 strA1 pgl⁻ SCP1⁻ SCP2⁻*	Bruton et al. (1991)
S. ambofaciens ATCC 23877	Wild-type strain	Thibessard et al. (2015)
S. lividans TK24	Wild-type strain	Cruz-Morales et al. (2013)
S. coelicolor M145	*SCP1⁻ SCP2⁻*	Kieser et al. (2000)
Streptomyces sp. S9N29	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S4N27	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S6N6	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S2N2	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S9N14	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S1N3	Environmental wild-type strain	Bontemps et al. (2013)
Streptomyces sp. S4N22	Environmental wild-type strain	Bontemps et al. (2013)
pIB139	*oriT attP int aac(3)IV PermE**	Wilkinson et al. (2002)

Table 2
List of primers used in this study.

Name	Sequence	Restriction site
xylE_F	ATGAACAAAGGTGTAATGCG	–
xylE_R	TCAGGTCAGCACGGTC	–
Biosens_R	TCTAGACGAATTCTCAGGTCAGCACGGTC	XbaI-EcoRI
Biosens_F	CATATGGGATCCACTG*TGGGAGCGCTCCCA*CAAG**AAAGGAGG**TCTAGAATGAACAAAGGTGTAATGCG	NdeI-BamHI -XbaI
SP6	GATTTAGGTGACACTATAG	–
T7	TAATACGACTCACTATAGG	–

Restriction sites are underlined. The *cebR*-box in the Biosens_F primer is in italics and the RBS is in bold characters.

inoculated into 3 ml of modified HT medium cultures complemented with the sugars at the selected concentrations or with 1 ml of wood leachate. Cultures were incubated at 30 °C for 16–18 h under agitation (200 rpm). Cells were harvested by centrifugation at 4500 rpm for 5 min, washed with 1 ml of 20 mM phosphate buffer pH 7.2 and suspended in 1 ml of sample buffer (100 mM phosphate buffer pH 7.5, 20 mM NaEDTA pH 8, 0.1% Triton ×100, 10% acetone [v/v]). Cells were then lysed on ice by sonication (three cycles of 20 s in Bioruptor Standard device, Diagenode, New Orleans, USA). The cell lysate was harvested by centrifugation for 6 min at 7000 rpm at 4 °C and 20 μl were mixed with 300 μl of assay buffer (100 mM phosphate buffer pH 7.5, 0.2 mM cathecol) previously warmed at 30 °C. Assay buffer was prepared just before use by adding the catechol from a 20 mM stock solution (in water) to the pre-warmed phosphate buffer (Ingram et al., 1989).

The yellow catechol degradation signal resulting from XylE activity was quantified by spectrometry at OD 595 nm (Bioteck, Winooski, USA). The catechol dioxygenase activity was determined and normalized at 375 nm per min per milligram of protein and converted to milliunits per milligram (Ingram et al., 1989; Kieser et al., 2000). The protein concentration was estimated by the method of Bradford (Bradford, 1976) by using bovine serum albumin as a standard. For each condition, experiments were realized with at least three repeats. The result of each experiment was expressed as the expression of XylE in the assay relative to that observed in the controls (*i.e.*, in the absence of cellobiose) systematically run in parallel. The significance of the relative signal of the assay *versus* the controls was statistically assessed with a *t*-student test.

3. Results

3.1. In silico *analysis to assess the potential of* Streptomyces *as biosensor hosts*

CebR prevents gene transcription in binding a specific sequence called the *cebR*-box and this repression is alleviated by the recognition of the inducer molecules, *i.e.* the cellobiose (Fig. 1A). The CebR sensor system has been previously found and reported as a highly conserved regulatory system in some *Streptomyces* species (Marushima et al., 2009; Schlösser et al., 2000; Takasuka et al., 2013), notably in term of sequence identity of the *cebR*-box. The concept of the biosensor was to build a generic biosensor cassette that could be regulated in different genetic backgrounds, *i.e.* in *Streptomyces* strains potentially exhibiting different specificities or sensitivities towards the target molecules. In order to assess the range of utilization of this construction within the *Streptomyces* genus we investigated the distribution of the CebR system within the 42 available fully sequenced genomes. BlastP searches (NCBI) were done using as query the CebR sequence of the fully characterized *Streptomyces reticuli* system CebR (CUW28692) (Schlösser et al., 2000) with a cut-off of 50% of identity in amino acid. Conserved regulatory motif TGGGAGCGCTCCCA the so-called "*cebR*-box" was also searched. All genomes but five (*Streptomyces albulus* NK660, *Streptomyces albulus* ZPM, *Streptomyces albus* DSM41398 and *Streptomyces* sp. 769, *Streptomyces lydicus* A02) possessed of at least one CebR homologue with amino acid identity ranging from 63% to 96% and the *cebR*-box motif as well as conserved regulatory *cebR*-boxes. This analysis showed that the CebR-system is widespread in this genus and conserved enough to recognize similar *cebR*-boxes. Consequently, most of the *Streptomyces* strains (including presumably environmental ones) are likely to be able to regulate a construction based on a conserved *cebR*-box through their endogenous CebR protein and could be turned into biosensors.

3.2. Construction of the biosensor

The biosensor design is depicted in Fig. 1B. Briefly, the concept consists to put a reporter gene under the transcriptional control of CebR. The *xylE* gene from *Pseudomonas putida* which was successfully used in *Streptomyces* was chosen (Ingram et al., 1989; Kieser et al., 2000). It codes for a catechol dioxygenase that converts the colorless substrate catechol to an intensely yellow hydroxymuconic semialdehyde. The genomic DNA of *Streptomyces coelicolor* KC900 which includes a *xylE* gene was used as a PCR template

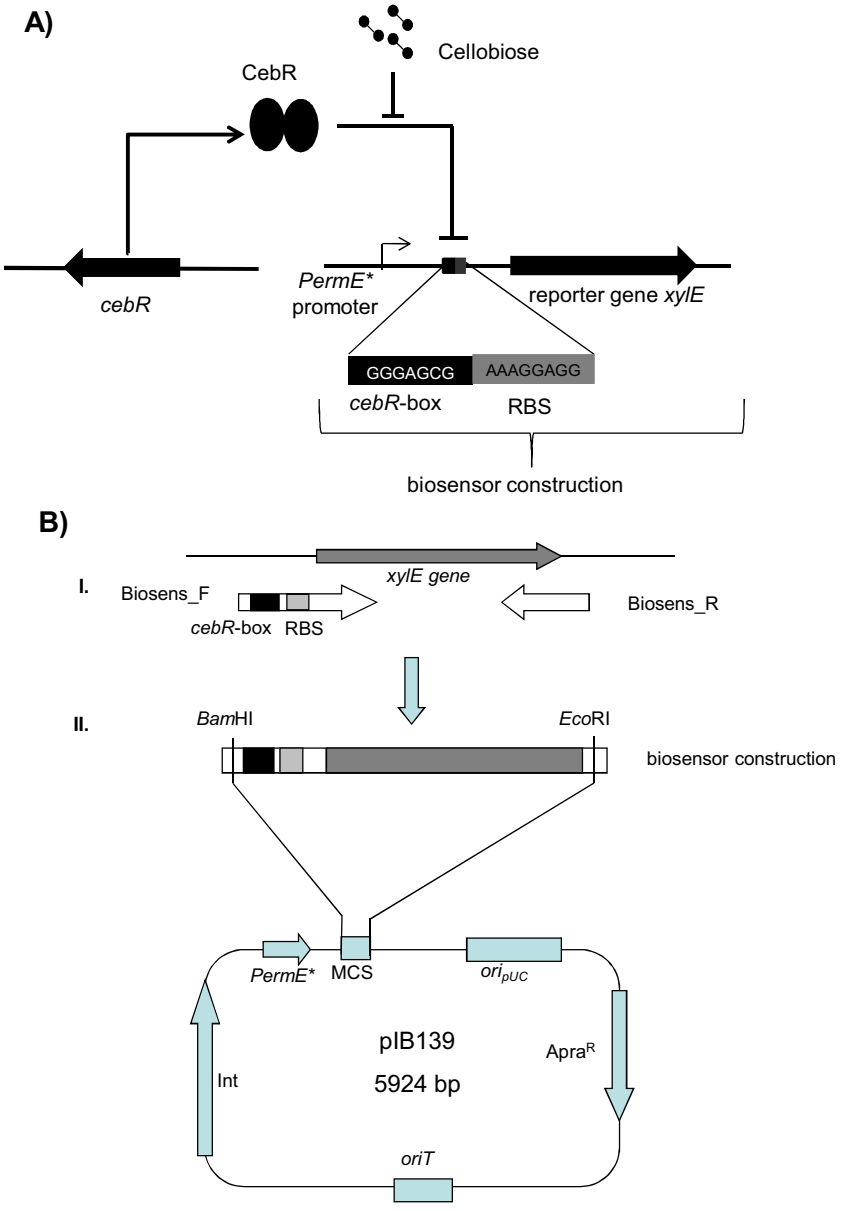

Fig. 1. Design and construction flowchart of the biosensor.
A) Schematic illustration of the cellobiose biosensor system. The biosensor construction consists in a *Streptomyces* conserved *cebR*-box sequence cloned between the constitutive promoter *PermE** and the reporter gene *xylE*. Once integrated in a *Streptomyces* genome harboring an endogenous CebR able to recognize the *cebR*-box, this transcriptional repressor will bind it and prevents the *xylE* expression. The presence of cellobiose alleviated the CebR binding on the *cebR*-box and enables the transcription of *xylE*. In presence of catechol, the XylE protein will emit a yellow compound revealing the presence of cellobiose. B) Flowchart of the biosensor construction. I. For the construction of the biosensor, the reporter gene *xylE* was amplified from *Streptomyces coelicolor* KC900 with the Biosens_F and Biosens_R primers (955 bp). The primer Biosens_F was designed from 5′ to 3′ with a *Bam*HI site as a future cloning site in pIB139. The black box symbolizes the *cebR*-box, the light grey box a Ribosome Binding Site (RBS) and the white arrow the beginning of the *xylE* gene. The reverse primer Biosens_R has from 5′ to 3′, an *Eco*RI site as future cloning site with pIB139 and the white arrow the end of the *xylE* gene. II. After amplification, the PCR product was cloned in the pIB139 plasmid that enables the integration of the construction into *Streptomyces* chromosome.
*PermE**: constitutive promotor; ori_{pUC}: replicative origin in *E. coli*; $Apra^R$: Apramycine gene resistance; *oriT*: transfert origin, Int: integrase gene; MCS: Multiple cloning site.

with the primer couple consisting of the forward Biosens_F and the reverse Biosens_R primers (Table 2, Fig. 1B.I). The Biosens_F primer sequence includes a *cebR*-box and a ribosome binding site (RBS). The amplification thus places the *cebR*-box and a RBS site upstream of the coding region of *xylE* (Fig. 1B.I). The 955 bp PCR product was subcloned in the pGEM-T Easy vector, introduced in *E. coli* DH5α and its sequence checked by sequencing. After releasing from pGEM-T Easy by a double restriction digestion *Bam*HI-*Eco*RI present in the Biosens_F and Biosens_R primer sequences respectively. This DNA fragment was further cloned directionally into the multicloning site of the pIB139 vector also digested with the same couple of endonucleases (Fig. 1B.II). This cloning step resulted in the positioning of the *xylE* gene downstream the strong constitutive promoter *PermE** (Bibb et al., 1994; Schmitt-John and Engels, 1992). This construct could then be mobilized into *Streptomyces* cells by intergeneric conjugation using the intermediary of *E. coli* S.17 host strain. The vector pIB139 was chosen for its site-specific integration system targeting the bacteriophage ΦC31 attachment site (Kieser et al., 2000). The integration into the chromosome secures the stability of the construct. A single copy is expected to be inserted.

Nine *Streptomyces* species were chosen as receptors and the insertion of the construct was selected by the presence of apramycin in the culture medium. The absence of endogenous catechol dioxygenase activity was checked on plate in presence

of catechol which is the XylE substrate (data not shown). Three of them were reference strains (*Streptomyces ambofaciens* ATCC 23877, *Streptomyces coelicolor* M145 and *Streptomyces lividans* TK23). The seven others were environmental strains (*Streptomyces* sp. S9N29, S2N2, S6N6, S9N14, S4N22, S1N3 and S4N27). These strains were chosen for their cellulolytic properties and their representativeness of the taxonomic diversity of a forest soil (Bontemps et al., 2013). The presence of CebR was expected and highly likely considering the wide distribution of the CebR system in sequenced genomes (see above). After conjugation with *E. coli* S17.1 harboring the biosensor construct, *Streptomyces* transconjugants were selected on SFM plates with apramycin and nalidixic acid which counter selects *E. coli*. For each *Streptomyces* strain, eight transconjugants were selected. The integration into the ΦC31 attachment site was checked by PCR for *S. ambofaciens* ATCC 23877. For environmental strains, the insertion of the construct was checked by PCR amplification of the *xylE* gene (data not shown).

3.3. Screening for optimal host strain for the biosensor

In order to confirm the efficiency of the constructed strains as biosensors, they were functionally tested for their ability to emit a yellow color on plates complemented with cellobiose (5 gL^{-1}) as an inducer molecule. In comparison with plates without cellobiose, all transformed clones for 5 strains (*S. ambofaciens* ATCC 23877, *S. coelicolor* M145, *S.lividans* TK24, *Streptomyces* S4N27 and *Streptomyces* S6N6) exhibited a bright yellow coloration of mycelium and in the surrounding solid medium, indicating that the cellobiose was able to unlock the CebR repression and to activate the expression of *xylE*. In order to choose the optimal host strain among them, several screenings were performed (Table 3) and the strain S4N27 was selected for the following reasons. First, it visually exhibited the highest yellow color on plate (Fig. 2) suggesting an optimal production of the XylE protein. Secondly, no detection signal was obtained in presence of crystalline cellulose (Avicel), indicating that the presence of none-degraded cellulose could not lead to false positive. Further specificity and sensitivity experiments were carried out on mycelium grown in liquid medium in order to ensure quantitative and reproducible assays. For that purpose, mycelium samples were sonicated to release the intracellular XylE content. Consequently, another selection criterion was the ability to lyse the mycelium. One of our candidate environmental *Streptomyces* strain, S4N27, was typified by a planktonic growth mode while other species grew as typical dense mycelium pellets difficult to lyse. For that reason, we finally selected strain S4N27 as the best host biosensor for further tests (Table 3).

3.4. Sensitivity and specificity of cellobiose detection

Tests were performed in liquid cultures to enable the quantification of the XylE activity of the biosensor S4N27 in presence of cellobiose. Its sensitivity was tested with cellobiose concentrations ranging from 1.5 μM to 15 mM and after 10 min of reaction. Controls without cellobiose were performed for each experiment and exhibited a background signal presumably corresponding to the basal *xylE* expression from the construction as no endogenous dioxygenase activity was observed in wild-type strains devoided to the construction. As the latter signal was equivalent in all controls, the *xylE* activity of the assays was expressed as a relative expression of this baseline and did not raise an issue regarding the interpretations of the results. A significant detection was observed starting from 15 μM of cellobiose (equivalent to 5 mgL^{-1} concentration). This is in the same range than a leachate produced from spruce wood in degradation (see below) as measured by HPLC. However, no significant signal could be detected at 1.5 μM (Fig. 3). An increasing signal was observed in response to cellobiose concentration to reach a plateau from 80 μM. The stability of the colorimetric reaction was assessed every two minutes for 1 h at 80 μM of cellobiose. Since 4 min and until the end of the experiment, all the measured intensity ratios were statically identical with a value close to 2.4 that showed there was no signal loss over time (data not shown). The chemical reaction was thus stable during this lap of time and biochemical instability of the reaction could not technically impair the liability of the measures.

At the same 80 μM concentration of cellodextrines (triose, tetraose, pentaose and hexose), no signal could be observed (data not shown) showing that cellobiose was the specific signal sensed by the CebR system in the S4N27 strain.

3.5. Application of the biosensor for wood degradation detection

In order to test whether our biosensor was able to detect the presence of cellobiose in a context of wood degradation, spruce sticks were infected by the lignolytic fungi *Poria placenta* during a period of 7 weeks to allow an effective fungal attack. Wood leachates were extracted and tested on the biosensor along with

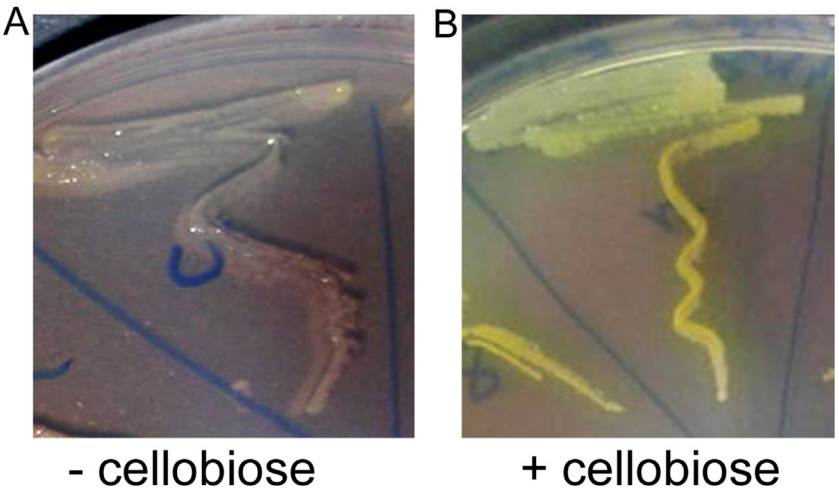

Fig. 2. Illustration of the detection of the cellobiose by the biosensor.
The S4N27 biosensor strain was grown for 2 days on HT* plates without cellobiose in A) or with cellobiose at 5 gL^{-1} in B). After catechol addition the yellow color is only observed in presence of cellobiose.

Table 3
Characteristics of strains screened for the biosensor construction.

Strain	XylE activities (yellow coloration)			Liquid growth
	HT*	HT*+ Avicel	HT* + Cellobiose	
Streptomyces sp. S4N27	–	–	+	Planktonic
S. ambofaciens ATCC23877	–	–	+	Pellets
S. lividans TK24	–	–	+	Pellets
S. coelicolor M145	–	–	+	Pellets
Streptomyces sp. S9N29	–	–	–	Pellets
Streptomyces sp. S2N2	–	–	–	Pellets
Streptomyces sp. S9N14	–	–	–	Pellets
Streptomyces sp. S1N3	–	–	–	Pellets
Streptomyces sp. S4N22	–	–	–	Pellets
Streptomyces sp. S6N6	+	–	+	Pellets

Eight independent clones for each biosensor strain were streaked on HT* medium complemented with the appropriate sugars at 5 gL^{-1}. After two days, a cathecol solution (0.5 M) was sprayed on plates and yellow colonies coloration was appreciated. Liquid phenotype growth was realized in HT* media after three days.

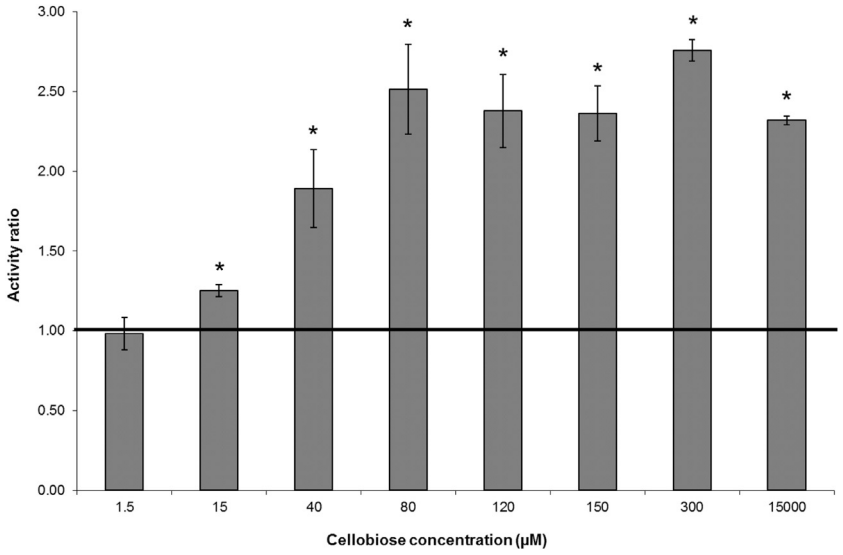

Fig. 3. Limit of detection of cellobiose by the biosensor.
The experiments were performed in HT* medium complemented with increasing cellobiose concentrations ranging from 1.5 µM to 15000 µM. For each concentration, the results are expressed as the ratio of the *xylE* activity measured in presence and in absence of cellobiose (background signal). An activity ratio of 1 (represented by the black horizontal line) corresponds to an activity equivalent to the background signal. Results for each concentration corresponded to three independent cultures. Error bars represent the standard deviation. Significant differences of signal intensity between the different conditions and the background signal are represented by asterisks and were determined with the Student's *t*-test ($P < 0.05$).

control leachates collected from uninfected spruce sticks. The *xylE* expression for infected spruce was 1.8 relative to the control in the same range than the ratio observed using pure cellobiose as inducer (Fig. 4). The cellobiose presence was checked and its concentration estimated in leachates by HPLC. While no cellobiose was detected in the uninfected control, a concentration of 15 µM was measured in the infected wood. This result confirmed that the biosensor was able to detect a low cellobiose concentration in a complex wood leachate and consequently enabled the detection of the fungal decaying process.

4. Discussion

The aim of this study was to create a biosensor dedicated to cellobiose detection. We based its construction on a microbial cellobiose sensor: the *Streptomyces* CebR system. Since, its characterization (Marushima et al., 2009; Schlösser et al., 2000), it is known that the CebR transcriptional repressor prevents gene expression from binding a conserved *cebR*-box. In presence of cellobiose it releases its action and enables the expression of the repressed genes such as genes coding cellulolytic enzymes (Marushima et al., 2009; Schlösser et al., 2000) or pathogenic factors in *S. scabies* (Francis et al., 2015). The presence of this system has been assessed in few *Streptomyces* so far (Francis et al., 2015; Marushima et al., 2009; Schlösser et al., 2000; Takasuka et al., 2013), but thanks to an *in silico* prospection of the CebR-system, we showed that it was actually highly prevalent in this genus. We also showed that the CebR harboring *Streptomyces* all had highly conserved *cebR*-boxes, suggesting that their CebR regulators were prone to recognize similar ones.

We took advantage of this conserved genetic scheme to design a whole-cell biosensor. Most microbial sensors are generally based on a specific promoter cloned upstream a reporting gene (Bereza-Malcolm et al., 2015; Park et al., 2013). Once integrated in the cell, the promoter initiates the transcription of the reporting gene when the cell comes in contact with the inducer signal. As CebR is a transcriptional repressor, we adapted this strategy in consequence and cloned a canonical *cebR*-box in the promoter region of a constitutively expressed reporter gene in order to regulate its expression by CebR. The *cebR*-box was cloned between the promoter and the transcription start of the gene as it is found in some *Streptomyces* (Marushima et al., 2009). Considering the widespread distribution of CebR among *Streptomyces*, we decided to integrate the construct in various *Streptomyces* and relied on the endoge-

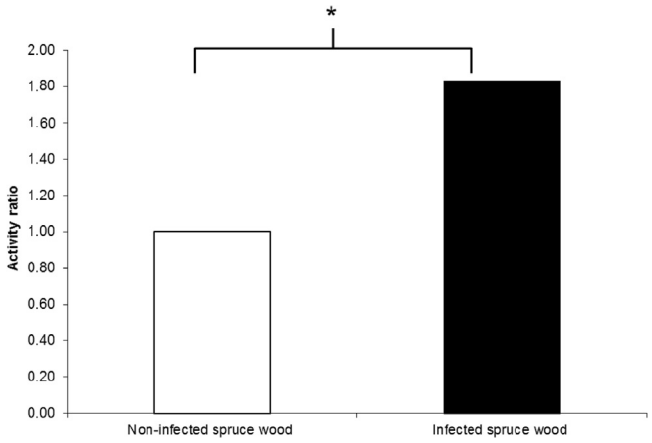

Fig. 4. Detection of the wood degradation by a lignolytic fungus.
Wood leachates were obtained from a spruce wood stick infected for 7 weeks by *Poria placenta* (black box) and from a non-infected control (white box). The results correspond to five independent biosensor assays and are expressed as the relative expression with the non-infected condition. Thus, the activity ratio of the non-infected spruce wood condition has a value of one and corresponds to the background signal. The significant difference between the two experimental conditions was attested by a Student's *t*-test ($P < 0.05$) and is symbolized by an asterisk.

nous CebR for its regulation. As these CebRs were naturally present in the strains they had the advantage to be perfectly adapted to the recipient strain and thus to offer an optimal regulation of the construct. The construct was successfully transferred and integrated with the pIB139 vector in different *Streptomyces* both model strains and environmental isolates, and its good regulation in presence of cellobiose was observed in one third of them. For the other strains, the absence of a visual signal during this screening could be due to various reasons such as a low *xylE* expression, a diffusion problem of the dioxygenase or non-recognition of cellobiose. All together, these results showed that our biosensor design was efficient and enabled to create a promiscuous construct that could be easily transferred and regulated in most *Streptomyces* to turn them into potential biosensors. More generally, this strategy that consists in diverting without *a priori* a conserved sensor system in different strains to control a same conserved molecular construction could be probably applied to other systems and in other bacteria. A main advantage is that it allows for a same construction the screening of different strains with different sensitivity or sensibility towards the molecule of interest and might ease the construction of whole-cell biosensors. A further development to our approach could be to introduce the construction in a strain that does not harbor the CebR system (*e.g. E. coli* or a *cebR*-free *Streptomyces*) along with a heterologously expressed *cebR* gene to control it. Previous biosensors, principally amperometric enzyme-based, were able to detect cellobiose (Bereza-Malcolm et al., 2015; Ludwig et al., 2010; Park et al., 2013). They generally used cellobiose dehydrogenase that are catalytic enzymes able to degrade cellobiose. However, these enzymes have a large spectrum of molecule recognition and the biosensors built with them were used to detect other compounds such as glucose, lactose or catechol, but never specifically and directly the cellobiose (Ludwig et al., 2010). Regarding the CebR system, some cross specificity for cellodextrines in addition to cellobiose has been identified in *S. griseus* (Marushima et al., 2009). Thus we did test the specificity of the *Streptomyces* S4N27 biosensor strain towards these compounds and got no positive detection, indicating a specificity of cellobiose recognition with our biosensor like in *S. reticuli* (Schlösser et al., 2000).

We designed the biosensor to detect wood degradation. Fungal attack is one of the main threats regarding the use of wood as a material. Generally, such attack is empirically discovered when the decaying process had already altered the wood mechanical properties. Even in the case of standardized tests aiming to assess the efficiency of protective treatments against fungal attack, professionals rely on 4 month experiments after infection where they measure the wood mass loss (EN 113, 1996). In order to assess whether our biosensor could bring a quicker answer in comparison to such tests, we performed a similar experiment as in the EN113 norm in infecting a spruce stick with *Poria placenta*, but for half of the normal time of the test. The application of the biosensor in this real context was successful and enabled to detect cellobiose in the wood attacked by the xylophagous fungus, but not in the uninfected control and by extension revealed the ongoing wood decaying process at this stage, dividing by two the time of the EN113 test. Such experiments are preliminary and despite further experiments are still needed to insure the full applicability of the biosensor in that context, this study led to a patent (WO2016/131665) regarding the exploitation of this biosensor. In future experiments, other types of wood-rots and kinds of wood should be tested but according to the fact that cellobiose remains a common denominator of fungal wood degradation process; it should be detected in most cases. Another aspect to be tested will be the kinetic of degradation to assess at which stage the biosensor would be applicable. Yet, as cellobiose is produced since the beginning of the decaying process, degradation of wood should be detected in the earlier steps.

In conclusion, we created here a whole-cell biosensor in diverting a widespread sensor of cellobiose in *Streptomyces*. According to the conservation of this system, we designed a construct optimized for being transferable and regulate by most *Streptomyces*. After selection of a biosensor strain, we have been able to detect cellobiose even in complex solutions such as a wood leachate. The application of this biosensor to detect wood degradation appears thus very promising for the wood industry.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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References

Bereza-Malcolm, L.T., Mann, G., Franks, A.E., 2015. Environmental sensing of heavy metals through whole cell microbial biosensors: a synthetic biology approach. ACS Synth. Biol. 4, 535–546, http://dx.doi.org/10.1021/sb500286r.

Bibb, M.J., White, J., Ward, J.M., Janssen, G.R., 1994. The mRNA for the 23S rRNA methylase encoded by the ermE gene of Saccharopolyspora erythraea is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. 14, 533–545.

Bontemps, C., Toussaint, M., Revol, P.-V., Hotel, L., Jeanbille, M., Uroz, S., Turpault, M.-P., Blaudez, D., Leblond, P., 2013. Taxonomic and functional diversity of Streptomyces in a forest soil. FEMS Microbiol. Lett. 342, 157–167, http://dx.doi.org/10.1111/1574-6968.12126.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bruce, T., Martinez, I.B., Maia Neto, O., Vicente, A.C.P., Kruger, R.H., Thompson, F.L., 2010. Bacterial community diversity in the Brazilian Atlantic forest soils. Microb. Ecol. 60, 840–849, http://dx.doi.org/10.1007/s00248-010-9750-2.

Bruton, C.J., Guthrie, E.P., Chater, K.F., 1991. Phage vectors that allow monitoring of transcription of secondary metabolism genes in streptomyces. Nat. Biotechnol. 9, 652–656, http://dx.doi.org/10.1038/nbt0791-652.

Cruz-Morales, P., Vijgenboom, E., Iruegas-Bocardo, F., Girard, G., Yáñez-Guerra, L.A., Ramos-Aboites, H.E., Pernodet, J.-L., Anné, J., Wezel, G.P., Barona-Gómez, van, 2013. The genome sequence of streptomyces lividans 66 reveals a novel tRNA-dependent peptide biosynthetic system within a metal-related genomic island. Genome Biol. Evol. 5, 1165–1175, http://dx.doi.org/10.1093/gbe/evt082.

EN 113, 1996. Wood Preservatives—Test Method for Determining the Protective Effectiveness Against Wood Destroying Basidiomycetes—Determination of the Toxic Values. European Committee for Standardization, Brussels.
Francis, I.M., Jourdan, S., Fanara, S., Loria, R., Rigali, S., 2015. The cellobiose sensor CebR is the gatekeeper of streptomyces scabies pathogenicity. mBio 6, e02018–14, http://dx.doi.org/10.1128/mBio.02018-14.
Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166, 557–580, http://dx.doi.org/10.1016/S0022-2836(83)80284-8.
Howard, R.L., Abotsi, E., Van Rensburg, E.J., Howard, S., 2004. Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr. J. Biotechnol. 2, 602–619.
Ingram, C., Brawner, M., Youngman, P., Westpheling, J., 1989. xylE functions as an efficient reporter gene in Streptomyces spp.: use for the study of galP1, a catabolite-controlled promoter. J. Bacteriol. 171, 6617–6624.
Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical Streptomyces Genetics. John Innes Foundation, Norwich.
Langston, J.A., Shaghasi, T., Abbate, E., Xu, F., Vlasenko, E., Sweeney, M.D., 2011. Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl. Environ. Microbiol. 77, 7007–7015.
Ludwig, R., Harreither, W., Tasca, F., Gorton, L., 2010. Cellobiose dehydrogenase: a versatile catalyst for electrochemical applications. ChemPhysChem 11, 2674–2697, http://dx.doi.org/10.1002/cphc.201000216.
Lynd, L.R., Weimer, P.J., Zyl, W.H. van, Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–577, http://dx.doi.org/10.1128/MMBR.66.3.506-577.2002.
Marushima, K., Ohnishi, Y., Horinouchi, S., 2009. CebR as a master regulator for cellulose/cellooligosaccharide catabolism affects morphological development in streptomyces griseus. J. Bacteriol. 191, 5930–5940, http://dx.doi.org/10.1128/JB.00703-09.
Park, M., Tsai, S.-L., Chen, W., 2013. Microbial biosensors: engineered microorganisms as the sensing machinery. Sensors 13, 5777–5795, http://dx.doi.org/10.3390/s130505777.
Phillips, C.M., Beeson, W.T., Cate, J.H., Marletta, M.A., 2011. Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by Neurospora crassa. ACS Chem. Biol. 6, 1399–1406, http://dx.doi.org/10.1021/cb200351y.
Rajala, T., Peltoniemi, M., Pennanen, T., Mäkipää, R., 2012. Fungal community dynamics in relation to substrate quality of decaying Norway spruce (Picea abies [L.] Karst.) logs in boreal forests. FEMS Microbiol. Ecol. 81, 494–505, http://dx.doi.org/10.1111/j.1574-6941.2012.01376.x.
Sambrook, J., Russell, D.W., et al., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Schlösser, A., Aldekamp, T., Schrempf, H., 2000. Binding characteristics of CebR, the regulator of the ceb operon required for cellobiose/cellotriose uptake in Streptomyces reticuli. FEMS Microbiol. Lett. 190, 127–132.
Schmitt-John, T., Engels, J.W., 1992. Promoter constructions for efficient secretion expression in Streptomyces lividans. Appl. Microbiol. Biotechnol. 36, 493–498.
Schultz, T.P., Nicholas, D.D., 2008. Introduction to developing wood preservative systems and molds in homes. In: Schultz, T.P., Militz, H., Freeman, M.H., Goodell, B., Nicholas, D.D. (Eds.), Development of Commercial Wood Preservatives: Efficacy, Environmental, and Health Issues. Chapter 1, American Chemical Society Symposium Series 982. Washington, DC., 2008, pp. 2–8.
Schwarze, F.W.M.R., Engels, J., Mattheck, C., 2000. Fungal strategies of wood decay in trees. Springer Sci. Bus. Media.
Simon, R., Priefer, U., Pühler, A., 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat. Biotechnol. 1, 784–791, http://dx.doi.org/10.1038/nbt1183-784.
Takasuka, T.E., Book, A.J., Lewin, G.R., Currie, C.R., Fox, B.G., 2013. Aerobic deconstruction of cellulosic biomass by an insect-associated Streptomyces. Sci. Rep. 3, 1030, http://dx.doi.org/10.1038/srep01030.
Thibessard, A., Haas, D., Gerbaud, C., Aigle, B., Lautru, S., Pernodet, J.-L., Leblond, P., 2015. Complete genome sequence of Streptomyces ambofaciens ATCC 23877, the spiramycin producer. J. Biotechnol. 214, 117–118, http://dx.doi.org/10.1016/j.jbiotec.2015.09.020.
Wang, L., Littlewood, J., Murphy, R.J., 2013. Environmental sustainability of bioethanol production from wheat straw in the UK. Renew. Sustain. Energy Rev. 28, 715–725, http://dx.doi.org/10.1016/j.rser.2013.08.031.
Wilkinson, C.J., Hughes-Thomas, Z.A., Martin, C.J., Böhm, I., Mironenko, T., Deacon, M., Wheatcroft, M., Wirtz, G., Staunton, J., Leadlay, P.F.0, 2002. Increasing the efficiency of heterologous promoters in actinomycetes. J. Mol. Microbiol. Biotechnol. 4, 417–426.