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ORIGINAL ARTICLE

Insights into bacterial cellulose biosynthesis by functional metagenomics on Antarctic soil samples

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In this study, the mining of an Antarctic soil sample by functional metagenomics allowed the isolation of a cold-adapted protein (RBcel1) that hydrolyzes only carboxymethyl cellulose. The new enzyme is related to family 5 of the glycosyl hydrolase (GH5) protein from *Pseudomonas stutzeri* (Pst_2494) and does not possess a carbohydrate-binding domain. The protein was produced and purified to homogeneity. RBcel1 displayed an endoglucanase activity, producing cellobiose and cellotriose, using carboxymethyl cellulose as a substrate. Moreover, the study of pH and the thermal dependence of the hydrolytic activity shows that RBcel1 was active from pH 6 to pH 9 and remained significantly active when temperature decreased (18% of activity at 10 °C). It is interesting that RBcel1 was able to synthetize non-reticulated cellulose using cellobiose as a substrate. Moreover, by a combination of bioinformatics and enzyme analysis, the physiological relevance of the RBcel1 protein and its mesophilic homologous Pst_2494 protein from *P. stutzeri*, A1501, was established as the key enzymes involved in the production of cellulose by bacteria. In addition, RBcel1 and Pst_2494 are the two primary enzymes belonging to the GH5 family involved in this process.
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Introduction

Metagenomics implies a series of laboratory manipulations leading to the isolation of DNA originating from (ideally) the entire diversity of microorganisms found in a specific sample (soil, water, tissues and so on), bypassing the necessity of dealing with culturing techniques (Handelsman, 2004; Streit and Schmitz, 2004). Moreover, despite the enormous diversity of microorganisms that inhabit our planet, it is estimated that more than 99% of them are not growing in standard culture conditions (Amann *et al.*, 1995; Rappe and Giovannoni, 2003), and thus await the development of new strategies that can disclose them, along with their wealth of resources (Riesenfeld *et al.*, 2004).

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The metagenomic approach requires the cloning of DNA fragments directly extracted from samples; these 'metagenomic libraries' are then screened to isolate phenotypically (by activity-driven screening) or genotypically (by sequence-driven screening) specific clones of interest (Gillespie *et al.*, 2002).

Therefore, it offers a powerful tool for accessing a range of almost unlimited possibilities for screening new activities from many different environments, by the isolation and cloning of DNA from microorganisms that have the ability to synthesize useful compounds, degrade waste products or even provide evidence to elucidate biological processes.

Using this approach, several groups isolated novel enzymes from various environments (Ranjan *et al.*, 2005; Rhee *et al.*, 2005; Feng *et al.*, 2007; Hardeman and Sjoling, 2007; Tirawongsaroj *et al.*, 2008), and many of them were found to offer a good potential as new tools for industrial applications, because their biophysical properties arise from improved adaptations to the conditions met in the environment during the sampling process. Although such

studies reported the characterization of metagenome-derived enzymes, insights into their physiological aspect are often lacking.

The study of cold-adapted enzymes, isolated from psychrophilic microorganisms, shows that they are characterized by a high specific activity at low and moderate temperatures associated with a rather high thermosensitivity that makes them valuable tools for biotechnology (Gerday *et al.*, 2000). Earlier isolated cold-adapted *Pseudoalteromonas haloplanktis* strains were studied as interesting hydrolytic enzyme producers, such as xylanase (EC 3.2.1.8) (Collins *et al.*, 2005), cellulase (EC 3.2.1.4) (Garsoux *et al.*, 2004), amylase (EC 3.2.1.1) (Feller *et al.*, 1999) and esterase (EC 3.1.1.1) (Aurilia *et al.*, 2007). Moreover, several genomes from isolated psychrophylic microorganisms have been completely sequenced (Rabus *et al.*, 2004; Médigue *et al.*, 2005; Methé *et al.*, 2005; Duchaud *et al.*, 2007), offering the possibility of discovering new phenotypes and of unraveling new cold-adapted metabolic pathways.

Besides the increasing number of enzymes produced by cultivable bacteria, the use of a metagenomic approach to study samples from cold environments could allow the isolation of new enzymes from psychrophilic, unbiased gene pools. In this regard, Antarctica represents one of the few places on earth that remains almost free of human-associated activity, and where the selective pressure is mainly exerted by environmental conditions. It is also an attractive place for the search of new 'cold-adapted' activities relevant for biotechnological and industrial applications.

The main goal of this study was to thoroughly describe new molecules with regard to both their biochemical and physiological properties to elucidate their biotechnological and physiological relevance. We provide the description of a small-insert metagenomic library (PP1) built after DNA extraction from an amended Antarctic soil sample. The library was directly screened for cellulolytic activity. The accurate characterization of a completely new gene encoding a cellulase, named RBcel1, is presented. In addition, its physiological function relating to bacterial cellulose synthesis (BCS) was elucidated. By this accurate description, we highlight a complex process dealing with cellulose production by bacteria. Such elucidation is of particular ecological relevance because of the bacterial cellulose impact on various processes such as plant–bacteria interactions (Branda *et al.*, 2005; Matthysse *et al.*, 2005) or biofilm initiation (Davey and O'Toole, 2000; Matthysse *et al.*, 2008). These complex structures put up various bacteria flourishing in their self-produced media and are observed in various humid ecosystems such as soil and river, and in human-associated samples such as teeth surface or wounds.

Both bacteria shifting their lifestyle from free organism to biofilm association and bacteria interacting with plant roots and/or sprout require cellulose production for attachment to solid surfaces (Davey and O'Toole, 2000; Matthysse *et al.*, 2008).

Materials and methods

Soil sample

The sample was collected from the surface of an unvegetated soil on Pointe Geologie archipelago, Terre Adélie, Antarctica (66°40′S–140°01′E) during the austral summer 1999–2000 expedition organized by IPEV (www.institut-polaire.fr/). The sample was picked from an Inipol Eap-22 (Elf Atochem)-amended soil ($\sim 5.5\,g\,kg^{-1}$). The bioremediation agent, Inipol, is a mixture containing 2-Butoxyethanol, Tri(laureth-4)-phosphate, and oleic acid, and is considered as a slow-release fertilizer usually used to stimulate the growth of heterotrophic microflora in oil-contaminated sea shores (Delille *et al.*, 2004). The temperature of the soil during the sampling process was 1.2 °C. The sample was kept at −70 °C until the construction of the metagenomic library.

Metagenomic library construction

A direct DNA extraction protocol developed by Henne *et al.* (1999) was used. Pure environmental DNA was partially digested using *Eco*RI (Fermentas, St Leon-Rot, Germany), and the resulting fragments were separated by overnight ultracentrifugation at 4 °C in a sucrose gradient (10–40%) at 27 000 *g*. DNA fractions with molecular sizes higher than 5 kb were cloned in the CopyControl pCC1BAC-*Eco*RI (Epicentre, Madison, WI, USA). The ligation mixture was used to transform electrocompetent *E. coli* TransforMax EPI300 cells (Epicentre), which were plated on Luria–Bertani agar media containing $12.5\,\mu g\,ml^{-1}$ chloramphenicol and different substrates (see below).

Metagenomic library characterization and screening for hydrolytic activities

To determine the total DNA contained in the metagenomic library, a fraction of the transformed cells were plated on Luria–Bertani agar media containing chloramphenicol ($12.5\,\mu g\,ml^{-1}$), isopropyl-beta-thio-galactoside (30 μM) and X-gal ($50\,\mu g\,ml^{-1}$) (Fermentas) to evaluate the efficiency of transformation (clones carrying insert-containing vectors versus self-ligated BAC). Moreover, to assess the average insert size, 40 BACs were isolated from randomly selected recombinant clones, and from *Eco*RI (Fermentas)-digested and DNA fragments separated by electrophoresis in 1% agarose gels.

Metagenomic library screening was carried out by plating recombinant *E. coli* clones on Luria–Bertani agar media supplemented with 0.5% carboxymethyl-cellulose (CMC) and 0.01% Trypan blue (Sigma, St Louis, MO, USA) to isolate cellulase-producing clones. Incubation was carried out for

16 h at 37 °C to grow the colonies. Thereafter, up to 5 days of additional incubation at a reduced temperature of 18 °C was carried out to allow cold-adapted phenotypes expression. Finally, positive clones surrounded by a pale-blue hydrolysis halo were isolated.

Cytoplasmic fractions from each clone expressing a cellulolytic phenotype were prepared using 5 ml of a 72 h culture at 18 °C. The cells were harvested and resuspended in 1 ml of 20 mM sodium phosphate buffer (pH 7.0) and sonicated. After centrifugation, supernatants were recovered and used for subsequent cellulolytic activity screening at various temperatures. Thermal denaturation was evaluated using crude cell extracts that were incubated for 30 min at different temperatures (from 4 to 80 °C) and tested for remaining activity at 20 °C by incubating the extract on CMC and Trypan blue-containing media.

Subcloning and DNA sequencing

BAC was isolated from the *E. coli* 39.5 clone and from *Eco*RI (Promega, Madison, WI, USA), digested to estimate the insert size. In addition, restriction fragments were gel purified and subcloned in an *Eco*RI-restricted pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). Screening for a specific cellulolytic phenotype and DNA sequencing were carried out to confirm the presence of active recombinant clones harboring the expected inserts.

DNA sequencing was carried out using universal M13 reverse and forward primers and a specific primer listed in Table 1, and their respective reverse complements, at the GIGA-sequencing platform of the University of Liège (Belgium). Comparative sequence analysis was carried out using the BLAST2.0 program to retrieve homologous protein sequences from protein and nucleotide database at the NCBI (National Centre for Biotechnology Information) (www.ncbi.nlm.nih.gov/BLAST/). Neighbor-joining tree construction was carried out using Geneious software (www.geneious.com) on protein sequences retrieved by BLASTp from the NCBI database. DNA sequences of the RBcel1-encoding gene and associated genes were deposited under the FJ647753 GeneBank accession number.

Expression and purification of RBcel1

RBcel1-start (5′-GAGGATCTACATATGAGACAGCC AGGCC-3′) and RBcel1-end (5′-CTCGAGTCATT CCGCCTTGGC1-3′) primers, containing *Nde*I and *Xho*I restriction sites (underlined), respectively, were used for PCR amplification of the RBcel1-encoding gene. The obtained amplicon was ligated in a *Nde*I/*Xho*I-digested pET22b plasmid (Novagen, San Diego, CA, USA). After confirmation of the sequence of the RBcel1-encoding gene, the pET22b:RBcel1 plasmid was introduced in competent *E. coli* BL21(DE3) cells (Novagen). Heterologous protein expression was carried out by induction with 0.1 mM isopropyl-beta-thio-galactoside. After overnight incubation at 18 °C under agitation, proteins from the periplasmic fraction were recovered as described earlier (Garsoux *et al.*, 2004). The RBcel1 cellulase was purified from the periplasmic fraction in three steps on an AKTA Prime Plus device (GE Healthcare, Uppsala, Sweden). The first purification step was carried out by ion exchange chromatography on a Q-HP Sepharose column (25 ml, GE Healthcare), pre-equilibrated in 20 mM Tris-HCl, pH 8.5. The proteins were eluted in a linear gradient of NaCl (0–500 mM) in the same buffer. The collected fractions were analyzed by testing the cellulolytic activity on CMC–Trypan blue agar media and positive fractions were further analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (15%). The selected fractions were pooled and dialyzed overnight against 10 volumes of 20 mM sodium phosphate buffer (pH 7.0). The second purification step was achieved by ion exchange chromatography on a 5 ml prepacked Q-HP Sepharose column (GE Healthcare), equilibrated in 20 mM sodium phosphate buffer (pH 7.0). Elution of the proteins was carried out in a linear gradient of NaCl from 0 to 500 mM. The fractions were analyzed as described before. Finally, the RBcel1-containing fractions were pooled and loaded on a Sephacryl HR100 column (GE Healthcare) equilibrated in 20 mM sodium phosphate buffer (pH 6.5).

Protein concentration and purity were determined by BCA-protein quantitation assay (Pierce, Rockford, IL, USA), using bovine serum albumin as standard, and by densitometry analysis on 15% SDS-PAGE gels, respectively. Purified protein was subjected to automatic Edman degradation for N-terminal amino acid sequence determination, using an Applied Biosystems 492 Protein Sequencer (Perkin Elmer, Waltham, MA, USA).

Table 1 List of the forward primers used for insert sequencing

Primer	*Sequence (5′–3′)*	*Location*
RBcel1int1(fwd)	CGGCTGACAAACTATGGCCTGCA	639–661
RBcel1int2(fwd)	GCCAATCCGCCGCCTGGCGT	1206–1225
RBcel1int3(fwd)	TGTCATCTACCAGCAAGATTT	1946–1966
RBcel1int4(fwd)	TGGTCTCGACGATTATCAGAT	2729–2749
RBcel1int5(fwd)	GATGATAGCGCTGTTGTTCTGA	3700–3721

Enzyme assay

Cellulase activity was measured by a modified dinitro-salicylic acid method (Ito *et al.*, 2005). The standard assay mixture contained 450 µl of substrate (2% CMC) in the adequate buffer (see below) and 50 µl of a suitably diluted enzyme. The reaction mixture was incubated in a thermostatized water bath and the reaction was stopped by adding 500 µl of dinitro-salicylic acid reagent. The mix was transferred for 5 min to a boiling water bath and

then chilled in ice-cold water. The amount of reducing ends (glucose equivalent) was determined by measuring the absorbance at 535 nm using glucose as standard.

The pH-dependence activity was determined by measuring the cellulase activity for 15 min at 37 °C, with 2% CMC as substrate in a 20 mM pH-adjusted solution (citrate-phosphate-CHES-CAPS) ranging from pH 3.5 to pH 11.

The thermal dependence of the activity was determined at pH 6.5 in sodium phosphate buffer using a thermostatized water bath from 4 to 80 °C.

Kinetic parameters were determined under initial rate conditions using a nonlinear regression analysis of the Michaelis–Menten equation. Hydrolysis was measured at 37 °C using CMC as substrate at concentrations ranging from 0.1% to 3% (w/v) in a 20 mM sodium phosphate buffer (pH 6.5). Activity on various polysaccharides (Avicel, soluble starch, Birchwood xylan and chitin; Sigma) was tested by standard assay using 0.9% (w/v) substrate concentration.

The influence of various chemicals on pure RBcel1 enzyme was investigated under standard conditions (Table 2). Inhibition and enhancement of the hydrolytic activity was measured in the presence of metal chloride salts (NaCl, $MgCl_2$ and $CaCl_2$) to test the divalent cation requirement and/or the RBcel1 halotolerance. The effect of NaEDTA (from 1 to 50 mM) was also tested for divalent cation requirement determination. In addition, with regard to the Inipol-containing sample, the effect of various alcohols was determined. Thereafter, protein stability in the presence of Triton X 100 and PEG 6000 was tested.

Table 2 Influence of solvents, detergents, EDTA and various metal chloride salts on the RBcel1 activity

Chemicals	*Concentration*	*Activity (%)*
Buffer		100
Methanol	25%	14 ± 6
Ethanol	25%	8 ± 2
1-Propanol	25%	1.5 ± 0.5
1-Butanol	25%	13 ± 2
NaEDTA	1 mM	40 ± 2
	2.5 mM	35 ± 3
	5 mM	30 ± 7
	10 mM	28 ± 3
	25 mM	9 ± 2
	50 mM	2 ± 1
Triton X 100	5%	82 ± 12
	1%	91 ± 8
PEG 6000	5%	95 ± 4
	1%	112 ± 5
MgCl2	10 mM	79 ± 3
CaCl2	10 mM	89 ± 4
NaCl	10 mM	102 ± 4
	100 mM	116 ± 14
	1 M	93 ± 3

Activity was measured using a standard assay and control activity was taken as 100%. Results are the mean values obtained from triplicate experiments.

Differential scanning calorimetry

Purified RBcel1 (2.1 mg) was dialyzed overnight against 1.5 l of 20 mM sodium phosphate buffer (pH 7). To prevent the aggregation of denatured proteins and precipitation at high temperatures, non-detergent sulfobetaine (3-(1-pyridino)-1-propanesulfonate, NDSB, Sigma) was used (Collins *et al*., 2006). Before the experiment, NDSB (3 M in dialysis buffer) was added to a final concentration of 0.75 M. Thermograms of protein unfolding were recorded in a thermal gradient from 30 to 80 °C at a scan rate of 1 °C min^{-1}, using a VP DSC (differential scanning calorimetry) microcalorimeter (MicroCal, Milton Keynes, UK). Data were analyzed using MicroCal Origin v7.0 Software.

Thin layer chromatography

Thin layer chromatography analysis was carried out on the degradation products of CMC. Glucose (G_1) and cello-oligosaccharides from cellobiose (G_2) to cellotetraose (G_4) (all purchased from Sigma) were used as standards. CMC enzymatic digestion was carried out at 37 °C in sodium phosphate buffer (pH 7), as described before. Aliquots were collected after various incubation times and 30 µl of each sample was spotted on a Partisil K6 Silica Gel 60A TLC plate (Whatman, Maidstone, UK). Migration was carried out in an ethylacetate:acetic acid:water mixture (2:1:1). Degradation products were showed by spraying an α-naphtol reagent (1.6 g naphtol, 51 ml ethanol, 4 ml water and 6.5 ml concentrated sulfuric acid) on the TLC plate and incubating it at 100 °C for 15 min.

Viscosimetric assay

Viscosimetric assays were carried out using a thermostatized (37 °C) Bohlin CS Rheometer (Malvern, Worcestershire, UK). Relative viscosity was measured during standard CMC hydrolysis and polymerization reactions were carried out with cellobiose as substrate. Sodium phosphate buffer (20 mM, pH 7) with or without CMC (2.7%) was considered equal to 100% and 0% relative viscosity, respectively.

Gel permeation chromatography of polymerized samples

Polymerization was carried out under the same conditions as that of hydrolysis (sodium phosphate buffer 20 mM pH 7), using cellobiose as the substrate (0.1 M). Samples were recovered after different incubation times (0–50 min). The reaction was stopped by incubating the reaction mix for 5 min in a boiling water bath. The enzyme was subsequently removed by centrifugation on a 10 kDa cutoff centrifugal filter (Millipore, Billerica, MA, USA), and the solution containing the transglycosylation products was freeze dried. The resulting polymers (60 mg) were

resuspended in 2 ml sodium nitrate (20 mM) and analyzed by aqueous GPC (gel permeation chromatography) using ultrahydrogel columns (Waters, Milford, MA, USA) that were earlier equilibrated in the same buffer. Product identification was carried out by a comparison of the elution profile to standard profiles of pure cello-oligosaccharides (from cellobiose to cellohexaose).

Scanning electron microscopy
The production of high molecular weight polysaccharides was investigated using scanning electron microscopy (SEM; Philips XL-30, Eindhoven, The Netherlands). SEM analysis was carried out on crude freeze-dried polymerization products without any treatment. Polymers were nickel sputtered over a carbon-coated aluminum stub. Observations were made on secondary electron using a 5–10 kV voltage for higher and lower magnitude.

Results

Metagenomic library characterization and screening
Environmental DNA was extracted from an Antarctic soil sample and cloned in the pCC1BAC cloning system, recovering 124 000 recombinant clones (4% of the tested clones do not possess any insert). The average insert size was estimated at 5.1 kb by analyzing BACs from 40 randomly selected recombinant colonies, the total genetic coverage of the library being around 636 Mb.

A total of 10000 clones were subjected to an activity-based screening, focusing on cellulose degradation. The process allowed the isolation of 11 positive recombinant clones. BACs were isolated and used to re-transform *E. coli* DH10B-competent cells for phenotypic confirmation.

Crude cell extracts from selected clones were prepared to test the thermal stability of the observed phenotypes. All extracts showed complete cellulolytic inactivation after incubation at 40 °C for 30 min, except for the extract from the *E. coli* 39.5 clone, which remained active after incubation at 55 °C (data not shown). Owing to its surprising phenotype, that is, cellulolytic activity at both low and high temperatures, this clone was selected for further study.

Sequence analysis
On sequencing the *E. coli* 39.5 clone, we found a fragment of 4.5 kb. BLASTn analysis of the sequence did not show any known homologous sequence. However, BLASTp analysis on hypothetical proteins deduced from all putative ORFs (open reading frames) located on the insert showed that there were relevant identities with putative proteins from *Pseudomonas stutzeri*, A1501. These ORFs encode putative endoglucanase, p-ribosyl-transferase, transferase/isomerase and glycosyl-transferase (Figure 1a). The protein sequence derived from the first ORF is related to the Pst_2494 locus from *P. stutzeri* A15011, corresponding to a putative endoglucanase. The mature forms of these two proteins share 47% identity and 67% similarity. This first ORF was then considered to be responsible for the production of the carboxymethylcellulase (CMCase), and was named *RBcel1*.

The existence of the observed synteny on the 39.5 insert and on the *P. stutzeri* genome links the environmental DNA fragment producing RBcel1 to a *Pseudomonodaceae*-like bacteria. Moreover, performing neighbor-joining tree construction, the RBcel1 enzyme seems to be a new subtype of an endoglucanase group (Figure 1b). In this group, enzymes for *Xanthomonodaceae*, *Gluconoacetobacter* and *Bacteroidaces* form different homogeneous subtypes (www.cazy.org) (Cantarel *et al.*, 2009).

It is interesting that the putative RBcel1 endoglucanase seems to be also related to the *Thermoascus aurantiacus* enzyme (accession number: 1GZJ), which is a thermophylic fungal endo-glucanase intensively studied in the past (Shepherd *et al.*, 1981; Lo Leggio *et al.*, 1997; Parry *et al.*, 2002; Van Petegem *et al.*, 2002; Hong *et al.*, 2003; Benko *et al.*, 2007). Amino acids sequence alignment of RBcel1, 1GZJ and Pst_2494 (Figure 2) highlights the presence of the conserved catalytic residues (E_{165} and E_{240}) and specific residues associated with the glycosyl hydrolase family 5 (GH5): R_{49}, H_{93}, N_{132}, H_{198} and Y_{200} (numbering referring to the *T. aurantiacus* endo-glucanase sequence).

Moreover, the lack of some important residues is also noteworthy: the W_{174} involved in substrate binding at the glycosyl (+3) group downstream of the cleavage site, or C_{212} and C_{249} implicated in a conserved disulfide bridge in 1GZJ and in many GH5 enzymes. The lack of this unique disulfide bridge could be related to an increasing flexibility in the cold-adapted proteins isolated from Antarctic soil samples (Narinx *et al.*, 1997; Asgeirsson *et al.*, 2003). In addition, as observed in several cellulases from the GH5 family, RBcel1 lacks a cellulose-binding domain.

The putative RBcel1 gene encodes a 351 amino acids protein with a theoretical molecular weight of 39.5 kDa and a pI of 6.12. In addition, a putative signal peptide comprising M_1 to A_{30} was predicted.

RBcel1 production and purification
The RBcel1-encoding gene was PCR amplified using modified primers and the PCR product was inserted in a pET22b plasmid. The recombinant plasmid was used to transform competent *E. coli* BL21(DE3) cells for protein heterologous expression. The expressed protein possesses its own natural signal peptide, and no modification of the amino acid sequence was attempted. Proteins from the periplasmic fraction extracted from a one-liter overnight culture at 18 °C were used to purify the recombinant RBcel1 enzyme. Pure cellulase was obtained after three

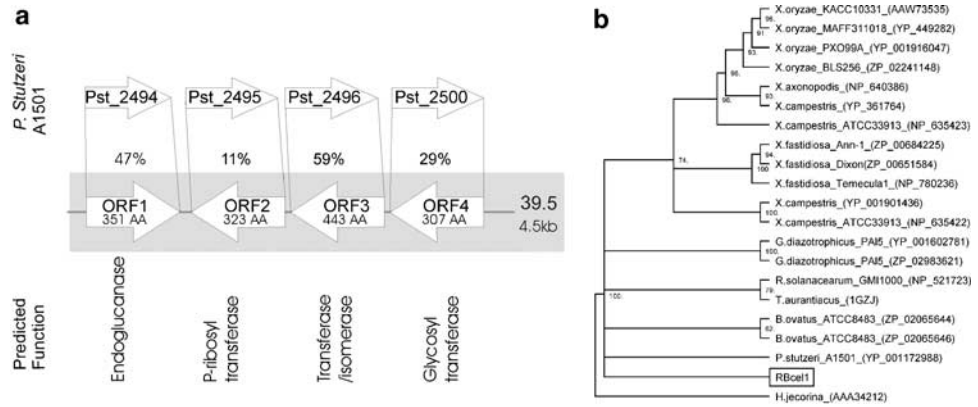

Figure 1 (**a**) Insert 39.5 map showing the ORFs localization and the amino acids identity scores obtained from sequences alignment of deduced amino acids sequences. Sequences retrieved from the *Pseudomonas stutzeri* A1501 genome were used as template. (**b**) Neighbour joining tree constructed using cellulase sequences retrieved from the BLASTp analysis.

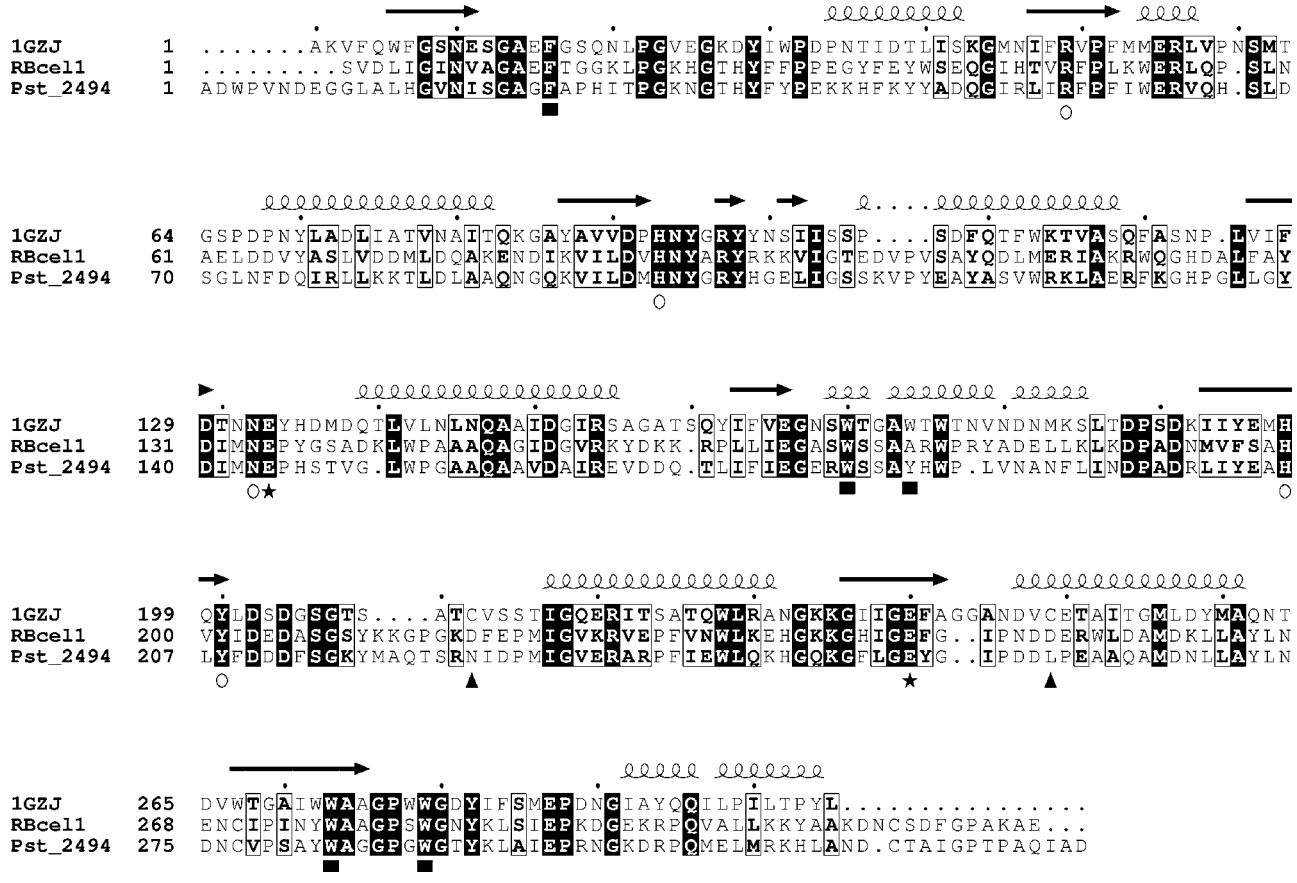

Figure 2 Alignment of cellulase sequences from *T. aurantiacus* (1GZJ), *P. stutzeri* (Pst_2494) and RBcel1 output by Espript (http://espript.ibcp.fr/ESPript/ESPript/index.php). Location of a helix and b sheets from the 1GZJ protein are presented. Remarkable residues are the two catalytic glutamic acids (*), the residues implied in the substrate binding (■), the GH5 conserved residues (○) and the two cysteins implicated in the formation of a disulfide bridge (▲).

purification steps consisting of two anion-exchange chromatographies (Q-HP Sepharose, GE Healthcare), followed by a gel filtration chromatography (Sephacryl HR100, GE Healthcare). Finally, 45 mg of pure protein was obtained. Signal peptide processing was assumed to be efficient as the predicted N-terminal amino acid sequence, SVDLI, was obtained as expected from bioinformatics tools.

pH and temperature dependence of the cellulolytic activity

pH activity dependence was investigated using purified RBcel1 and CMC (1%) as substrate in different buffers. Maximum activity was measured in the range of pH 7 (Figure 3a). No significant activity was recorded at pH values lower than 5 ($pKa_1 = 6.2$) and higher than 9 ($pKa_2 = 9.2$). Further experiments were carried out at pH 7.

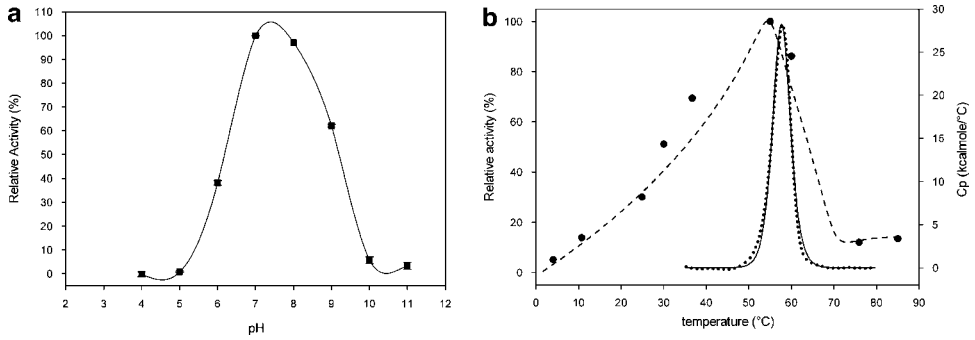

Figure 3 (**a**) Influence of the pH on the RBcel1 activity measured using standard assay mixtures using pH-adjusted polybuffer. The maximum measured activity at pH 7 was taken as 100%. Presented results are the mean values obtained from triplicate experiment. (**b**) Influence of temperature on the RBcel1 activity (dashed line) RBcel1 activity was measured using the standard assay mixture incubated from 4 to 86 °C. The maximum measured activity at 55 °C was taken as 100%. Thermal stability of RBcel1 measured by DSC (dotted line), the denaturation curve was fitted using the MicroCal Origin v7.0 software (continuous line).

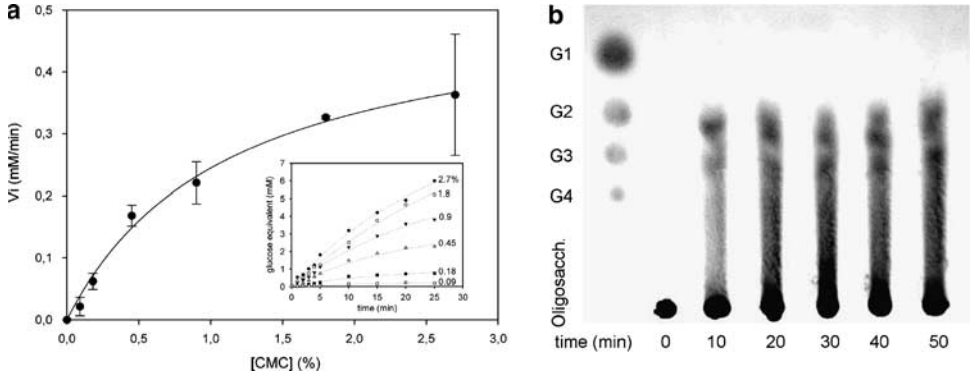

Figure 4 (**a**) Michaelis-Menten plot deduced from the initial rates measured for increasing CMC initial concentrations (graph insert). (**b**) Thin Layer Chromatography analysis of time course of the CMC hydrolysis derived products by RBcel1 at different incubation times. Standard mixture containing glucose (G1), cellobiose (G2), cellotriose (G3) and cellotetraose (G4).

Thermal dependence of the RBcel1 cellulolytic activity was determined under standard conditions. The activity increased from 4 to 55 °C, the latter corresponding to the apparent maximum activity, and then quickly decreased to reach a residual activity of 10% at 75 °C (Figure 3b).

Thermal stability of RBcel1 was investigated by DSC analysis in the presence of NDSB (Figure 3b). The protein seemed to be stable up to 50 °C. Above this temperature, the protein conformation quickly collapsed into an unfolded state. Analysis of the DSC scan was carried out according to a non-two-state model showing a single-stage transition at 57.7 °C (T_m). The protein-unfolding parameters, calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_{VH}), were found to be 159 ± 0.9 and 155 ± 1.1 kcal mol^{-1}, respectively. The $\Delta H_{cal}/\Delta H_{VH}$ ratio close to one indicates that RBcel1 unfolds according to a two-state process without significantly populated intermediates. Moreover, carrying out several denaturations, the protein unfolding seems to be partially reversible (30%, data not shown).

RBcel1 ($T_m = 57$ °C, $A_{max} \sim 55$ °C) seems to be more thermosensitive than does 1GZJ ($T_m = 76$–80 °C, $A_{max} \sim 70$ °C) (Parry *et al.*, 2002; Hong *et al.*, 2003). In addition, RBcel1 appears to remain active at low temperatures (18% of remaining activity at 10 °C), whereas 1GZJ activity quickly decreases below 50 °C (Hong *et al.*, 2003).

Stability on chemical compounds

The effect of various alcohols, detergents, metal ions and EDTA on RBcel1 was investigated under standard assay conditions using CMC as substrate (Table 2). The RBcel11 activity was strongly inhibited by all the tested alcohols, resulting in an ~10% residual activity. Moreover, EDTA also inhibited the hydrolytic enzyme activity to an extent of 40 to 3% remaining activity in the presence of 1 mM to 50 mM of EDTA, respectively. Triton X-100, PEG6000, NaCl, $CaCl_2$ and $MgCl_2$ seemed to have very little influence on the CMC hydrolysis by RBcel1.

RBcel1 kinetic parameters and substrate specificity

The kinetic parameters for CMC hydrolysis were determined after substrate hydrolysis (from 0.09% to 2.7% in a 20 mM polybuffer, pH 7.0) for 25 min using dinitro-salicylic acid assay (Figure 4a). The

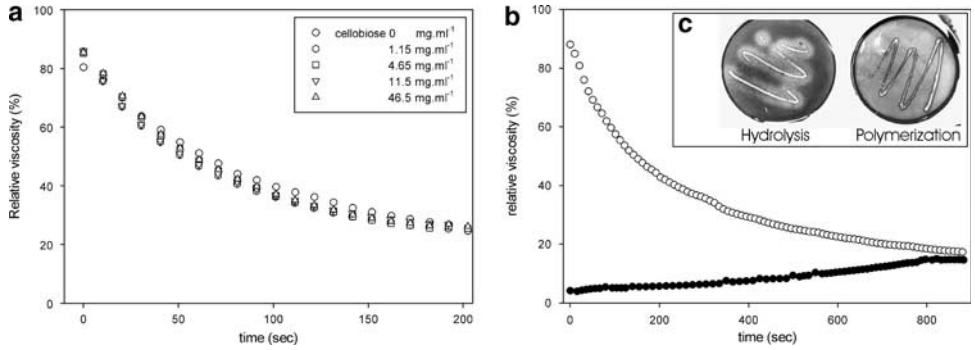

Figure 5 (**a**) Normalized viscometric assay for CMC hydrolysis (2.4%) by RBcel1 in presence of increasing amounts of cellobiose. (**b**) Viscometric assay for CMC hydrolysis (2.7%; ○) and cellobiose polymerization (0.1M; ●) by RBcel1. (**c**) Congo-red staining of CMC (hydrolysis) or cellobiose (polymerization) containing LBA plates incubated with RBcel1 expressing cells.

deduced kinetic values were apparent parameters because saturation was not achieved even when high substrate concentrations were used. The apparent *Km* and k_{cat} values for CMC were $11 \pm 2\,mg\,ml^{-1}$ and $22 \pm 2\,s^{-1}$, respectively. The kinetic efficiency (k_{cat}/Km) was calculated as $2 \pm 0.5\,ml\,mg^{-1}\,s^{-1}$. In addition, as far as the reaction products profile obtained by TLC analysis is concerned, it seemed that CMC hydrolysis mainly produced cellobiose (G_2) and cellotriose (G_3), but not glucose (G_1) (Figure 4b), and this supports an endotype of action of the RBcel1 enzyme, which was confirmed by a viscometric assay of CMC hydrolysis, leading to a quick decay of viscosity (Figure 5).

Substrate specificity was investigated using various polysaccharides. Tests were carried out in standard conditions in which the CMC substrate (1%) was substituted by microcrystalline cellulose (Avicel, β-1,4 glucan), chitin (β-1,4 N-acetyl-glucosamine), starch (α-1,4 glucan), birch wood xylan (β-1,4 xylan) or 4 mM p-nitrophenyl-cellobioside ($pNPC_2$). No significant hydrolysis was observed after 2 h at 37 or 55 °C (data not shown).

Viscometric assay

To elucidate the inability to reach substrate saturation during the hydrolysis of CMC, an inhibition by the product was hypothesized. During hydrolytic reaction, the production of an increasing amount of a potential inhibitor (cellobiose) should progressively decrease the rate of the reaction. As no synthetic substrate was degraded by RBcel1, the decrease in the viscosity of a CMC-containing solution in the presence of RBcel1 and the degree of increase in cellobiose were investigated. It is known that viscometric methods are more rapid and sensitive than dinitro-salicylic acid assay in the initial phase of the reaction (Almin and Eriksson, 1967), and that a quick decay of viscometry is related to the action of an endo-hydrolysis. Such a decrease in the relative viscosity of an RBcel1-containing CMC solution was observed, whereas large amounts of oligosaccharides were released as shown by the TLC. However, in the tested range of cellobiose concentrations (0–46 $mg\,ml^{-1}$), no significant changes in the rate of initial viscosity decrease was observed, suggesting that cellobiose was not an RBcel1 inhibitor at the tested concentrations (Figure 5a).

Endoglucanases catalyze the hydrolysis of CMC by decreasing the energy activation barrier, allowing water molecules to react with and cleave O-glycosidic bonds. The reaction is limited to an equilibrium between the concentration of CMC and carboxymethyl-cellobiose. Cellobiose polymerization produces cellulose, which is not soluble and precipitates in the reaction mix. In these conditions, the equilibrium is displaced toward the formation of the polymer. However, no enzymatic evidence for the existence of such an equilibrium is available. Nevertheless, the deletion of an endo-glucanase-encoding gene (*bcsZ*) from the BCS operon in cellulose-producing bacteria results in their inability to produce polymeric materials (Standal *et al.*, 1994; Matthysse *et al.*, 1995). Under this perspective, it was noteworthy that when RBcel1 was incubated in the presence of cellobiose alone, a significant increase in viscosity was observed (Figure 5b). As increasing concentrations of oligosaccharide would only slightly increase relative viscometry, the significant increase in viscosity when RBcel1 was incubated with cellobiose was considered as the result of the production of cello-oligosaccharides of higher molecular weight or cellulose.

To test the production of a polymeric material by RBcel1, *E. coli* 39.5 was incubated on cellobiose or CMC-containing agar media. After 2 days of incubation at 37 °C and Congo red staining, a clear CMC-hydrolysis halo and a dark polysaccharide zone were observed on CMC and cellobiose-containing media, respectively (Figure 5c). This indicates that RBcel1 has both hydrolyzing and polymerizing activities (Recouvreux *et al.*, 2008).

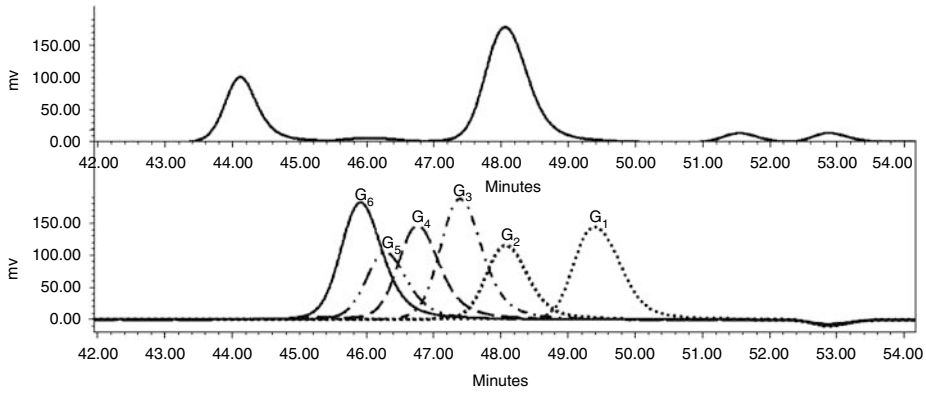

Figure 6 Figure 6 GPC elution profile of 10 kDa-filtered cellobiose polymerization product (upper) revealing the unconsumed cellobiose, cellohexose and high molecular weight polymer compared to standard elution profile (lower).

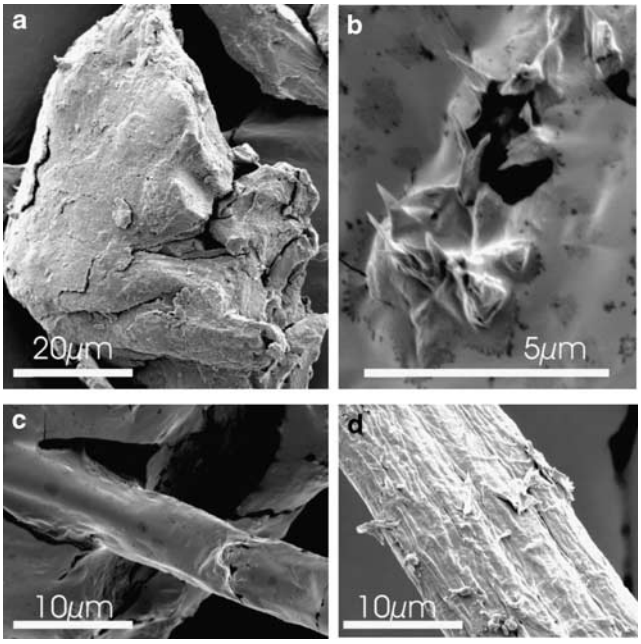

Figure 7 SEM analysis of cellobiose polymerization products (**a**, **b**, **c**) and cellulose (**d**) revealing the presence of fibrous material related to cellulose.

Analysis of the polymerization product by gel permeation chromatography and scanning electron microscopy

Polymerization products were recovered from a viscometric assay, filtered to remove the protein and analyzed by GPC. The elution profile (Figure 6) showed the presence of different forms of polymeric material. Peaks corresponding to residual cellobiose, cellohexose and high-molecular-weight polysaccharides, compared with standard curve, were detected. On the basis of the calibration curve obtained using commercial cello-oligosaccharides, the HMW polysaccharide was estimated to be $\sim$7 kDa, which corresponds to $\sim$45 polymerized glucose units. Such an HMW polysaccharide was finally observed by SEM analysis carried out on a crude polymerization product. The presence of an amorphous product (Figure 7a), probably composed of the remaining cellobiose and small oligosaccharides, covered by small picks (Figure 7b) and forming scattered fibers (Figure 7c), was highlighted. Non-fibrous material was regarded as the remains of cellobiose and low-molecular-weight cello-oligosaccharides, whereas high molecular weight polysaccharides were related to fibrous material. These fibers exhibited a homogeneous diameter ($\sim$10 μm), which is consistent with the crystalline cellulose structure (Figure 7d). Moreover, a comparison of SEM pictures obtained from crystalline or *in vitro*-produced cellulose shows the non-reticulated shape of this synthetic polymer.

Discussion

Here, a new metagenomics study of an Antarctic soil sample is reported. The constructed library was screened for cellulolytic activity and allowed the isolation of one clone, named *E. coli* 39.5, which produced the RBcel1 cellulase.

RBcel1 enzymatic analysis shows ambivalent features of both CMC degradation to cellobiose, and cellobiose polymerization to cellulose. Owing to the very low molar enthalpy value for β-1–4 glycosidic bonds hydrolysis, $\pm$580 cal mol^{-1} (Tewari and Goldberg, 1989; Lonhienne *et al*., 2001), and because enzymatic catalysis decreases the energy activation barrier, the reaction process in both hydrolysis and synthesis direction will occur. The initial reactant concentrations will direct the reaction to reach a kinetic equilibrium characterized by concomitant hydrolysis and synthesis of CMC. With regard to RBcel1, the existence of such a kinetic equilibrium could explain the observed decrease in the CMC-hydrolysis rate measured during the reaction. Moreover, as generally suspected for such an enzymatic comportment, no inhibition by the product (cellobiose) was detected during CMC hydroysis.

Many enzymes are known to perform both polymerization and degradation of polymers such as cellulose. In fact, endoglucanases are routinely used

for so-called reverse hydrolysis or hydrolase-catalyzed transglycosylation in low water concentration reactions (Wang, 2008). Xyloglucan endotransglycosylases (EC 2.4.1.207) catalyze both the cleavage and religation of polysaccharides and are enzymes related to the production of cellulose in plants (Steele *et al.*, 2001). But interestingly, in some bacteria that synthesize cellulose, the last step of the process is catalyzed by an endoglucanase from the GH family 8, which transfers a soluble cellulose precursor from the lipid II to the earlier existing cellulose (Standal *et al.*, 1994; Matthysse *et al.*, 1995; Romling, 2002). Soluble, chemically modified cellulose, such as CMC, is routinely used for the enzymatic characterization of endoglucanases. But, as described here, purified cellulases are generally unable to catalyze crystalline cellulose degradation, which is generally carried out by large protein complexes referred to as cellulosomes.

The RBcel1 sequence analysis highlights the enzyme classification. RBcel1 is a new subtype of endoglucanase from the GH family 5, related to different groups of enzymes from phytopathogenic bacteria (*Xanthomonas, Ralstonia*), from symbiotic bacteria involved in the lignocellulose digestion (*Bacteroides*) or from enzyme derived from plant root-associated organisms (*Gluconoacetobacter*).

Comparing RBcel1, Pst_2494 from *P. stutzeri* and 1GZJ from *T. aurantiacus*, sequences alignment shows RBcel1 adaptation to cold temperature and highlights the lack of W_{174} (also absent from Pst_2494) involved in cellulose binding during the hydrolysis of CMC and which should have a significant effect on the reaction kinetics.

Complete insert sequence analysis shows the phylogenetic linkage of the microorganisms to *Pseudomonodaceae*-like bacteria by synteny conservation. Little is known about the cellulose metabolism of these bacteria, although biofilm-associated cellulose was earlier detected for several *Pseudomonas* species, including *P. stutzeri* (Ude *et al.*, 2006). After searching for genes from the BCS operon, all the required BCS-associated genes were located on the *P. stutzeri* genome (from locus Pst_278–280), but no endoglucanase related to the GH family 8-encoding gene (*bcsZ*) was detected. The ability of *P. stutzeri* to produce cellulose then requires the presence of a nonassociated BCS operon endoglucanase, as described for several groups of cellulose-producing bacteria, in which the *bcsZ* gene is known to be located outside the BCS operon (Standal *et al.*, 1994; Romling, 2002).

Thus, on the basis of the sequence homology of the 39.5 insert to the *P. stutzeri* genome and that of the RBcel1 homology to the Pst_2494 locus, the physiological role of these two proteins is supposed to be identical and is suspected to be the last step of a cellulose production process. Although no evidence is available, this reaction was suspected to be catalyzed by enzymes referred to as endoglucanases, performing, in physiological conditions, a polymerization reaction (Delmer, 1999; Romling, 2002). Herein, we present the first explicit enzymatic proof that endoglucanases catalyze the polymerization of cellulosic material. Moreover, whereas all the endoglucanases required for bacterial cellulose biosynthesis are from the GH8 family, RBest1 and Pst_2494 belong to the GH5 family.

The analysis by GPC and SEM of the so-produced polymer, showed the production of high-molecular-weight polysaccharides of un-reticulated shape, which were regarded as cellulose.

This work highlights the involvement of cellulases in bacterial cellulose production. Although this production remains negligible compared with plant production, its environmental impact is highly importance. Indeed, the cellulose production by bacteria was associated to plant root and *Rhizobium* interaction (Smit *et al.*, 1992), to biofilm formation (Branda *et al.*, 2005; Da Re and Ghigo, 2006) and to plant infection by phytopathogens (Matthysse and McMahan, 1998; Rodríguez-Navarro *et al.*, 2007). Our finding gives new insights into a better understanding of these processes that affect both micro and macro ecosystems.

Hence, the possibility of isolating new genes and proteins from extreme environments is described herein. However, the characterization of the RBcel1 enzyme makes evident the fact that activity-driven screening is dedicated to a specific phenotype (CMC hydrolysis). Moreover, attempts to understand the physiological role of isolated enzymes lead to the conclusion that new proteins are not always just what we were searching for at the beginning.

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