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Genomic, proteomic and biochemical analysis of the chitinolytic machinery of *Serratia marcescens* BJL200

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ABSTRACT

The chitinolytic machinery of *Serratia marcescens* BJL200 has been studied in detail over the last couple of decades, however, the proteome secreted by this Gram-negative bacterium during growth on chitin has not been studied in depth. In addition, the genome of this most studied chitinolytic *Serratia* strain has until now, not been sequenced. We report a draft genome sequence for *S. marcescens* BJL200. Using label-free quantification (LFQ) proteomics and a recently developed plate-method for assessing secretomes during growth on solid substrates, we find that, as expected, the chitin-active enzymes (ChiA, B, C, and CBP21) are produced in high amounts when the bacterium grows on chitin. Other proteins produced in high amounts after bacterial growth on chitin provide interesting targets for further exploration of the proteins involved in degradation of chitin-rich biomasses. The genome encodes a fourth chitinase (ChiD), which is produced in low amounts during growth on chitin. Studies of chitin degradation with mixtures of recombinantly produced chitin-degrading enzymes showed that ChiD does not contribute to the overall efficiency of the process. ChiD is capable of converting *N,N′*-diacetyl chitobiose to *N*-acetyl glucosamine, but is less efficient than another enzyme produced for this purpose, the Chitobiase. Thus, the role of ChiD in chitin degradation, if any, remains unclear.

1. Introduction

Chitin, found in crustaceans, insects, and fungal cell walls, is often considered as the second most abundant biopolymer in nature, after cellulose. The shells of crustaceans consist, in addition to chitin, of calcium carbonate and protein. The extraction of chitin from these resources is today done by use of harsh, environmentally unfriendly chemicals, and further processing of the chitin, e.g. to make chitosan, also involves such chemicals [1,2]. It is therefore desirable to make this process more environmental friendly, for instance by use of enzymes. Many microorganisms are known to degrade chitin and by studying the proteins secreted by an organism growing on chitin, a better understanding of the enzymatic degradation of chitin-rich biomasses can be obtained.

The chitinolytic machinery of *Serratia marcescens* BJL200, a Gram-negative soil bacterium, is well studied [3–5]. It is already known that, during growth on chitin, this bacterium produces three chitinases (ChiA, ChiB, ChiC), belonging to glycosyl hydrolase (GH) family 18, a lytic polysaccharide monooxygenase (LPMO), belonging to auxiliary activity (AA) family 10, named CBP21, and a β-hexosaminidase (Chitobiase), belonging to the GH20 family [6–11]. The latter enzyme is located in the periplasm and converts *N,N′*-diacetyl chitobiose, the primary product of the chitinases, into *N*-acetyl glucosamine for further utilization by the cell [12]. ChiA, ChiB and ChiC are known to be secreted by *S. marcescens*, but ChiB and ChiC do not harbor a conventional sec-signal peptide [6,10,13]. Both ChiB and ChiC are known to be located in the periplasm before export to the extracellular space [6,13], possibly through a Type 2 secretion system commonly found in Gram-negative bacteria [14,15]. However, Hamilton et al. [13] showed that *S. marcescens* Db10/Db11 lacks a typical Type 2 secretion system, and demonstrated that a holin-like protein (ChiW) and an endopeptidase (ChiX) are essential for secretion of chitinolytic enzymes. In addition, a LysR-type transcription regulator (ChiR) is essential for production of the chitinolytic machinery of *S. marcescens* 2170 [16]. The *chiR*, *chiW* and *chiX* genes are located in the same region as the *chiB* and *cbp21* genes on the chromosome of *S. marcescens* Db10/Db11 [13].

The genome of *S. marcescens* BJL200 likely encodes at least one more GH18 chitinase (ChiD), since this enzyme is encoded in the genomes of other members of the *Serratia* genus [17,18]. Chitinase D from *Serratia proteamaculans* (*Sp*ChiD) has been characterized in detail and displays both hydrolytic and transglycolytic activities [17,19–21], whereas, similar properties have recently been described for a chitinase from *S. marcescens* GPS5 [18]. However, a quantitative characterization of ChiD activity has not been described and little is known about how the activity of ChiD relates to the activity of the other chitinolytic enzymes. To obtain more insight into chitin degradation by *S. marcescens*,

Abbreviations: CAZymes, carbohydrate active enzymes; GH, glycosyl hydrolase; LPMO, lytic polysaccharide monooxygenase; LFQ, label free quantification.

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and potentially discover additional proteins involved in chitin conversion, we have carried out a quantitative proteomics study of the secretome of *S. marcescens* BJL200 during growth on chitin. Identified proteins were mapped on the draft genome sequence of the bacterium which was determined as part of this work. The genome indeed contained a *chiD* gene, and the proteomic data showed that the ChiD protein is much less abundant during growth on chitin compared to the other chitinases and CBP21. We have therefore performed an in-depth characterization of ChiD to investigate its possible role in chitin degradation by *S. marcescens*.

2. Materials and methods

2.1. DNA extraction

DNA extraction was done essentially as described by Rosewarne et al. [22], with minor modifications. Quantification of DNA was done using the Qubit™ fluorometer and the Quant-iT™ dsDNA BR Assay Kit (Invitrogen, CA, USA), before sequencing.

2.2. Genome sequencing and annotations

Illumina MiSeq sequencing was performed at the Norwegian Sequencing Centre (Oslo, Norway), using TruSeq sample preparation and a 2 × 300 paired-end sequencing kit (Illumina, CA, USA). Low quality base calls (Phred quality score < 20) and short sequences (length < 20 bp) were trimmed using Sickle PE [23], and the sequences were *de novo* assembled using IDBA UD [24]. Paired-end reads files were merged prior to assembly using the fq2fa program implemented the IDBA package. Annotation of the contigs was done using Rapid Annotation Subsystem Technology version 2.0 (RAST, http://rast.nmpdr.org/) [25–27]. The raw sequencing data has been uploaded to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/SRA/) under accession number SRP076778. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MSEC00000000. The version described in this paper is version MSEC01000000.

The subcellular location of proteins was predicted using the LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP/) [28], and the PRED-TAT software (http://www.compgen.org/tools/PRED-TAT/) [29]. Proteins annotated as cytosolic (CYT) by the LipoP server were further analyzed using the SecretomeP 2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/) [31] to detect possible non-classical secretion. We considered proteins with a SecretomeP score of 0.5 or higher as secreted in a non-classical (NC) fashion. Annotation of carbohydrate active enzymes (CAZymes) according to the CAZy database (http://www.cazy.org/Citing-CAZy.html) [11] was done using dbCAN (http://csbl.bmb.uga.edu/dbCAN/index.php) [30] with hidden Markov models version 3.0.

2.3. Proteomics

S. marcescens BJL200 was grown on agarose (1%) plates with 1% wt/vol α-chitin (extracted from *Pandalus borealis*, Seagarden, Husøyvegen 278, Karmsund Fiskerihavn, 4262 Avaldsnes, Norway), 1% wt/vol β-chitin (extracted form squid pen, Batch 20140101, France Chitin, Chemin de Porte Claire, F-84100 Orange, France), or 0.2% wt/vol glucose (VWR International) as sole carbon source in M9 minimal medium. The M9 minimal medium was supplemented with 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$. The plates were prepared essentially as described by Tuveng et al. [31], using a sterile Grade QM-A Quartz Filter, circle, 47 mm (GE Healthcare Life Sciences, Oslo, Norway). The plates comprise two layers of identically composed solid medium; the filter is located between the two layers and separates cells (growing on the top of the plate) from the bottom of the plate, where protein samples were collected [32]. Glass petri dishes with a diameter of 80 mm were used and the incubation temperature for bacterial growth was 30 °C.

Secretomes from cells grown on plates were collected at different time points using biological triplicates for each time point. Samples were prepared as described by Bengtsson et al. [32] with the exception that trypsinated samples were dried under vacuum (Concentrator plus, Eppendorf, Denmark) to concentrate the samples, and dissolved in 0.1% (vol/vol) trifluoro acetic acid (TFA), before purification of peptides using ZipTip C18 pipette tips (Merck Millipore, Cork, Ireland). To produce samples for analysis of intracellular proteins, we collected cells by scraping them off the plates and suspending them in lysis buffer (50 mM Tris, 0.1% triton X-100, 200 mM NaCl, 1 mM DTT, pH 7.1). Glass beads (acid washed, ≤106 μm, Sigma, Oslo, Norway) were then added and the cells were disrupted using a FastPrep-24 (MP Biomedicals, CA, USA) for 3 × 1 min, followed by centrifugation. Proteins in the supernatant were precipitated by adding trichloro acetic acid (TCA) to a final concentration of 16% (vol/vol). After collecting the precipitated proteins by centrifugation at 15,000 × *g*, the proteins were dissolved in SDS-buffer and loaded onto a SDS-PAGE gel for standard electrophoretic separation. In-gel digestion was performed essentially as described by Shevchenko et al. [33] and peptides were purified using ZipTips. All peptide samples were dried under vacuum, dissolved in 10 μl 2% (vol/vol) acetonitrile (ACN), 0.1% (vol/vol) TFA, and analyzed by LC-MS/MS.

2.4. Mass spectrometry

Mass spectrometry analysis of peptides was done essentially as described by Tuveng et al. [31]. In brief, peptides were analyzed using two technical replicates for each biological replicate or one technical replicate for the cell lysis samples. The system used was a nanoHPLC-MS/MS system consisting of a Dionex Ultimate 3000 RSLCnano (Thermo Scientific, Bremen, Germany) connected to a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a nano-electrospray ion source. Samples were loaded onto a trap column (Acclaim PepMap100, C_{18}, 5 μm, 100 Å, 300 μm i.d. × 5 mm, Thermo Scientific, Bremen, Germany) and back-flushed onto an analytical column (Acclaim PepMap RSLC C_{18}, 2 μm, 100 Å, 75 μm i.d., Thermo Scientific, Bremen, Germany). In order to isolate and fragment the 10 most intense peptide precursor ions at any given time throughout the chromatographic elution, the mass spectrometer was operated in data-dependent mode to switch automatically between orbitrap-MS and higher-energy collisional dissociation (HCD) orbitrap-MS/MS acquisition. The selected precursor ions were then excluded for repeated fragmentation for 20 s. The resolution was set to $R = 70{,}000$ and $R = 35{,}000$ for MS and MS/MS, respectively. For optimal acquisition of MS/MS spectra, automatic gain control (AGC) target values were set to 50,000 charges and a maximum time of 128 milliseconds.

2.5. Data analysis

MS raw files were analyzed using MaxQuant version 1.4.1.2 [34,35], and proteins were identified and quantified using the MaxLFQ algorithm [36]. Technical replicates, i.e. HPLC re-injections, were combined during MaxQuant analysis. The data were searched against a custom database of the predicted proteome of *S. marcescens* BJL200 (5202 protein sequences), supplemented with common contaminants such as keratins, trypsin and BSA. In addition, reversed sequences of all protein entries were concatenated in order to estimate the false discovery rate (FDR). The tolerance levels for matching to the database were 6 ppm for MS, 20 ppm for MS/MS. Trypsin was used as digestion enzyme, and two missed cleavages were allowed. N-terminal acetylation, oxidation of methionine, conversion of glutamine to pyro glutamic acid, and deamidation of asparagine and glutamine were set as variable modifications (carbamidomethylation of cysteine residues was set as fixed modification in analysis of cell lysis samples). The 'match between runs' feature of MaxQuant was enabled with default parameters, in order to transfer identifications between samples based on accurate mass and