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Regulation of the synthesis of pulp degrading enzymes in *Bacillus* isolated from cocoa fermentation

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ABSTRACT

Pectin degrading enzymes are essential for quality of product from cocoa fermentation. Previously, we studied purified pectate lyases (Pel) produced by *Bacillus* strains from fermenting cocoa and characterized the cloned *pel* genes. This study aims to search for biological signals that modulates Pel production and regulators that influence *pel* gene expression. Strains were grown to the end of exponential phase in media containing various carbon sources. Pel enzymes production in *Bacillus* was unaffected by simple sugar content variation up to 2%. Additionally, it appeared that *pel* gene is not under the control of the most common carbon and pectin catabolism regulators *ccpA* and *kdgR*, which could explain the insensitivity of Pel production to carbon source variation. However, a 6-fold decrease in Pel production was observed when bacteria were grown in LB rich medium as opposed to basal mineral medium. Subsequently, bioinformatics analysis of cloned *pel* gene promoter region revealed the presence of DegU binding site. Furthermore, the deletion of *degU* gene dramatically reduces the *pel* gene expression, as revealed by real time quantitative PCR, showing an activation effect of DegU on Pel synthesis in *Bacillus* strains studied. We assumed that, during the latter stage of cocoa fermentation when simple sugars are depleted, production of Pel in *Bacillus* is stimulated by DegU to supply microbial cells with carbon source from polymeric pectic compounds.

1. Introduction

Cocoa fermentation involves numerous microbial species, mainly yeasts and bacteria including *Bacillus*, acetic acid bacteria and lactic acid bacteria (Ardhana and Fleet, 2003; Schwan and Wheals, 2004; Ouattara et al., 2008; Papalexandratou et al., 2011). Microbial activity during cocoa fermentation causes various biochemical reactions, which influence greatly the quality of fermented and dried cocoa bean and chocolate (Schwan, 1998; Jinap et al., 2003). Although not all the reaction mechanisms are known, it is well established that in an exogenous process, sugars contained in the pulp (outer part of bean) are oxidized mainly into ethanol and acetic acid, which penetrate into the beans. This leads to a lowering of the inner pH and an activation of endogenous enzymes, resulting in cascade reactions responsible for the final quality of the fermented beans and chocolate (Biehl et al., 1993; De Brito et al., 2000; Schwan and Wheals, 2004). Moreover, one of the key reactions during this fermentative process is the degradation of the pectin-rich cocoa pulp (Bhumibhamon and Jinda, 1997; Schwan and Wheals, 2004; Ouattara et al., 2011; Cempaka et al., 2014). In fact, the breakdown of pectin contained in the pulp by pectinolytic enzymes, increases the permeability of the beans, connecting the reactions occurring in the outer part of the bean to those taking place deep inside the beans. The former, which are microbial reactions, trigger the latter, which are downstream reactions of the fermentation process (Schwan and Wheals, 2004). Hence, pectin degrading enzymes appear to have a significant impact on cocoa fermented products (Freire et al., 1990; Bhumibhamon and Jinda, 1997; Schwan and Wheals, 2004; Cempaka et al., 2014).

Since cocoa fermentation remains difficult to control, many studies have been undertaken in order to improve this process (Carr et al., 1979; Passos et al., 1984; Hansen et al., 1998; Hashim et al., 1998; Nielsen et al., 2005; Papalexandratou, 2011; Illeghems et al., 2012). In this context, it is assumed that a higher production of pectinolytic enzymes is one of the key factors in

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achieving precise cocoa fermentation control and hence, high crop value for farmers (Bhumibhamon and Jinda, 1997; Schwan, 1998; Schwan and Wheals, 2004; Ouattara et al., 2011; Cempaka et al., 2014). Several microbial cultures, including pectinolytic strains, have been assayed on the farms to assess their potential as starters (Schwan, 1998; Lefeber et al., 2010, 2011; Papalexandratou, 2011).

We recently reported the purification and characterization of pectate lyase, a class of pectinolytic enzymes produced by three *Bacillus* strains (*B. pumilus* BS22, *B. subtilis* BS66, and *B. fusiformis* BS90) isolated from fermenting cocoa beans (Ouattara et al., 2010, 2011). These enzymes display interesting features as they can degrade a large range of pectin compounds, including highly methylated pectins (Ouattara et al., 2010). The inclusion of these strains in the microbial cocktail, with the aim to improve cocoa fermentation is under evaluation. *Bacillus* are reported to be present throughout the fermentation process with high count (10^8 UFC/g of pulp) and generally a peak occurs at 3–4 days of fermentation (Ardhana and Fleet, 2003; Ouattara et al., 2008). However, a thorough understanding of the degradation of cocoa pulp compounds by these bacterial strains is a prerequisite for their efficient manipulation as potential starters in fermentation conditions.

Pectin catabolism involves two mains steps in *Bacillus*. The first step, which takes place outside the bacterium, consists in the degradation of the long polymer of pectin into unsaturated oligo-, tri- and di-galacturonates by extracellular pectinolytic enzymes, mainly pectate lyases (Ouattara et al., 2010). For the second step, the resulting unsaturated digalacturonates, enter into the bacterium and undergo further degradation by intracellular enzymes (see Fig. 1). The genetic organization of the intracellular pathway of unsaturated galacturonate degradation is relatively well established. It is mainly composed of two operons, *kduID* and *kdgRKAT* (Pujic et al., 1998; Lin and Shaw, 2007), the expression of which is induced by galacturonate and repressed by glucose. Furthermore, it has been shown that induction by galacturonate is mediated by KdgR, the specific repressor of galacturonate utilization (Pujic et al., 1998), and that catabolic repression induced by glucose is exerted via CcpA (Carbon catabolite protein A), a master regulator of the general *Bacillus* catabolite-repression system (Pujic et al., 1998; Stulke and Hillen, 2000; Lin and Shaw, 2007). In contrast, very little is known about the control of Pel synthesis, responsible for the extracellular degradation of pectin into unsaturated products. Here we report the impact of growth medium composition and various easily metabolizable sugars on the production of Pel and identify some regulators controlling *pel* gene transcription in *Bacillus* strains isolated from fermenting cocoa beans.

2. Material and methods

2.1. Bacterial strains and culture conditions

Bacillus subtilis BS66 and *Bacillus pumilus* BS22, used in this study, had been previously isolated from fermenting cocoa beans (Ouattara et al., 2008) and characterized (Ouattara et al., 2011). We also used as control the reference strain *B. subtilis* 168 provided by (Pujic et al., 1998). These strains were grown, under shaking (150 rpm), on Luria Broth (LB) (Sambrook et al., 1989) or minimal salt medium containing 0.28% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.2% KH_2PO_4, 0.08% sodium citrate, 0.05% yeast extracts and 0.01% $MgSO_4$. Carbon sources were added at concentrations of 0.5% and polygalacturonate (PGA) was added to a final concentration of 0.4%. *Escherichia coli* and *Bacillus* cells harbouring plasmids were grown on LB supplemented with the required antibiotic: chloramphenicol and ampicillin were used at 5 μg/mL and 100 μg/mL, respectively.

2.2. Competent cell preparation and transformation procedure

Bacillus strains were made competent by a two step method using different growth media notably T base, SpC and SpC II. T base liquid medium was composed of 0.2% $(NH4)_2SO_4$, 1.83% $K_2HPO_4 \cdot 3H_2O$, 0.6% KH_2PO_4 and 0.1% trisodium citrate$\cdot 2H_2O$. SpC medium was obtained by supplementing T base medium with 0.5% glucose, 0.02% magnesium sulfate, 0.2% yeast extract, 0.02% casamino acids and 0.005% tryptophan. SpC II was also prepared from T base medium with addition of 0.5% glucose, 0.08% magnesium sulfate, 0.1% yeast extract, 0.01% casamino acids, 20 μg/mL tryptophan and 0.5 mM $CaCl_2$.

For the competence procedure, a pre-culture of bacteria was prepared by growing *Bacillus* cells on LB plate medium at 30 °C for 24 h. Cells from this pre-culture were used to inoculate 25 mL of SpC liquid medium contained in a 250 mL Erlenmeyer flask followed by incubation of the culture at 37 °C under shaking at (175 rpm). Bacterial growth was monitored by following the OD_{600}. At the transition stage between the exponential and stationary growth phases, the culture was diluted (1:1) with pre-warmed SpII medium (37 °C), and then incubation was continued for 2 h in the same conditions. Competent cells were pelleted by centrifugation, then concentrated in the supernatant, supplemented with glycerol (20%), and stored at −80 °C in Eppendorf tubes.

To transform *Bacillus* competent cells, the DNA solution containing 0.2 μg of the recombinant plasmid was added to the competent cells and incubated at 37 °C, with agitation at 600 rpm. After 30 min, the mixture (DNA + competent cells) was diluted with pre-warmed (37 °C) LB medium and incubated for another 30 min. The transformants were selected on LB agar plates containing the appropriate antibiotic.

2.3. Construction of recombinant plasmids and Bacillus mutants

To construct *Bacillus* mutants with a knocked-out *kdgR* gene, the *kdgR*-disruptive plasmid pDTKDGRI, obtained from Dr Alexeis Sorokin (INRA, Jouy en Josas, France), was used as a vector. pDTKDGRI was constructed as follows: a 370 bp DNA segment, corresponding to an internal *kdgR* fragment spanning from nucleotide 44 to 414 relative to the ATG start codon, was PCR amplified and cloned into the integrative vector, pDT1 (Pujic et al., 1998), to obtain the recombinant plasmid pDTKDGRI. We used this construct to transform the *Bacillus subtilis* BS66 and *Bacillus pumilus* BS22 competent cells. The partial fragment of the *kdgR* gene allows for integration of the pDTKDGRI constructs into the bacterial chromosome by homologous recombination and this provokes the disruption of the *kdgR* gene. We further selected *kdgR* defective mutants of both *Bacillus* strains on LB plates medium containing 5 μg/mL of chloramphenicol. The genotype of selected strains was further confirmed by PCR analysis.

To construct *Bacillus* mutants containing a knocked-out *ccpA* gene, we constructed a CcpA-disruptive plasmid called pDTCCPA. For this purpose, a 500 bp DNA segment corresponding to an internal *ccpA* fragment, spanning from nucleotide 61 to 558 relative to the ATG start codon, was PCR amplified in the presence of genomic DNA from *Bacillus* strains and the primers Ccpa_F243 (5′-CGGAATTCCGTGTCGTGAACGGCAACC-3′) and CcpA_R735 (5′-GCCAAGCTTCCGGAAACGAACGCGATGTCTGT), in the conditions described by Ouattara et al. (2011). The underlined sequences are the restriction sites of *Eco*RI and *Hind*III, respectively. The amplified fragment was further digested by *Eco*RI and *Hind*III restriction enzymes and then cloned between the *Eco*RI and *Hind*III sites of the plasmid pDT1 to give the plasmid pDTCCPA, a *ccpA*-disruptive plamid. The construct was verified by sequencing and then used to transform *Bacillus subtilis* BS 66 and *Bacillus pumilus* BS22