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

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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.

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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′-CG<u>GAATTC</u>CGTGTCGTGAACGGCAACC-3′) and CcpA_R735 (5′-GCC<u>AAGCTT</u>CCGGAAACGAACGCGATGTCTGT), 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

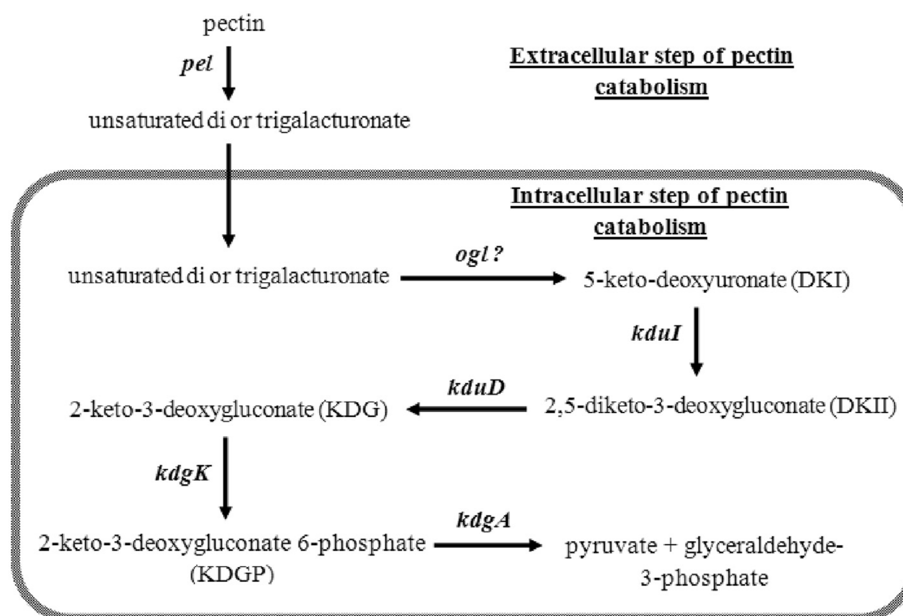

Fig. 1. Possible degradative pathways of pectin in *Bacillus subtilis*. The different steps are catalysed by the products of the genes specified near the corresponding arrow. Biochemical pathways are based on genetic and biochemical studies in Gram-negative bacteria and *Bacillus* (Lin, 1996; Hugouvieux-Cotte-Pattat et al., 1996; Ouattara et al., 2010). Question marks indicate functions whose attribution to *B. subtilis* gene is unclear. pel, pectate lyase; ogl, oligogalacturonate lyase; kdul, 5-keto-4-deoxyuronate isomerase; kduD, 2-keto-3-deoxygluconate oxidoreductase. kdgK, 2-keto-3-deoxygluconate kinase; kdgA, 2-keto-3-deoxygluconate-6-phosphate aldolase.

competent cell, resulting in *ccpA* defective mutants of both *Bacillus* strains. The partial fragment of *ccpA* gene allows for integration of the pDTCCPA constructs into the bacterial chromosome, by homologous recombination and provokes the disruption of the *ccpA* gene. Selection of mutants was performed on LB plates medium containing 5 μg/mL of chloramphenicol. The genotype of the mutant was further confirmed by PCR analysis.

The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material.

2.4. Pectate lyase activity

An overnight pre-culture of *Bacillus* strains was used to inoculate the basal mineral medium described above. Polygalacturonate (PGA) as pectic compound was added to a final concentration of 0.4% (w/v). The culture (25 mL), contained in a 250 mL Erlenmeyer flask, was maintained under agitation at 150 rpm, in a 30 °C water bath until the end of the exponential growth phase. After centrifugation at 6000 × g for 5 min, pectate lyase activity was measured in the cell free supernatant. Pectate lyase activity was determined by the degradation of polygalacturonate to unsaturated products that absorb at 235 nm (Moran et al., 1968). The standard assay mixture consisted of 1 g/L PGA in 0.1 M Tris HCl and 0.1 mM $CaCl_2$, pH 8, in a total volume of 1 mL. Specific activity is expressed as μmol of unsaturated products liberated per min per mg of bacterial dry weight. The molar extinction coefficient of unsaturated oligogalacturonates was assumed to be 5200 (Moran et al., 1968).

2.5. RNA extraction, real time quantitative PCR and pel gene expression quantification

Bacterial strains were grown in different culture conditions, the kinetic growth was monitored by measuring the absorbance (OD_{600}) at regular time intervals. At the end of exponential growth phase, cells were harvested and pelleted by centrifugation at 6000 × g for 5 min then suspended in 0.5 mL of extraction buffer (50 mM sodium acetate pH 5.2; 10 mM EDTA) immediately followed by the addition of 0.5 mL phenol-chloroform solution (pH 5.2) then mechanically broken with glass bead (Sigma, Paris, France) using Fast prep adjusted to speed 6 for 40 s. RNA contained in the solution was extracted according to the method described by Schmitt et al. (1990). All the solutions used for RNA manipulation were prepared with RNase free water (Sigma, Lyon, France) and the materials used was also RNase free. Isolated RNA was quantified basing on its absorption at 260 nm, using ND 2000 Nanodrop spectrophotometer, the quality was checked by visualization on 0.5% agarose gel and stored at −80 °C for further use. RNA extract was subjected to DNase I treatment (Roche), then a PCR was performed with primers used for quantitative PCR to rule out DNA contamination. cDNA were synthesized from 1 μg RNA and 50 ng random hexamers using RevertAid^{M} First strand cDNA synthesis kit (Fermentas, France), following the manufacturer's instructions. Moreover the efficiency of retrotranscrition was evaluated by quantification of cDNA produced from 2.10^5 copies/μL of RNA pAW added to the reaction of reverse transcription (Lautier et al., 2007; Lautier and Nasser, 2007; Wisniewski and Rogowsky, 2004).

To run the qPCR, 1 μL of the reverse transcription reaction was added as template to the Roche SYBR Green mix containing *pel* gene-specific primers and the reference or housekeeping gene-specific primer. The thermal cycling conditions and data analysis were performed using the Lightcycler LC 480 from Roche, as previously described by Hommais et al. (2011).

2.6. Statistical analysis

To search for significative differences between the data obtained from different cultures conditions, an analysis of variation (ANOVA) was performed using two replications of each culture condition (two biological replicates) and three samples from each culture condition (three technical repetitions). The analysis was realized with the software SPSS version 12.0.1 using Duncan's test at a 95% confidence level.

3. Results

Previous studies have shown that Pel produced by *B. subtilis* BS66 and *B. pumilus* B22 display significant differences in their biochemical and enzymatic characteristics (Ouattara et al., 2010). For example, Pel-22, produced by *B. pumilus* had a low specific activity compared with Pel-66, from *B. subtilis*, whereas Pel-22 displayed a higher affinity for the substrate than Pel-66. We, therefore, retained these two enzymes for the regulation studies.

3.1. Sensitivity of pel genes to pectic compounds

To determine whether polygalacturonate, the preferential substrate of Pels, or galacturonate, the degradative product of polygalacturonate, could induce the synthesis of Pel in *B. pumilus* 22 or *B. subtilis* 66, we compared enzyme production in medium containing these two pectic compounds with enzymes production in medium without pectic compounds, containing glucose (basal medium), during the growth of strains to the end of exponential growth phase. In *Bacillus subtilis* BS66 Pel production varied between 3.88 (±0.31) U/mg of bacterial dry weight (BDW) obtained in PGA and 4.43 (±0.15) U/mg of BDW obtained in galacturonate, whiles the variation of enzyme production in *Bacillus pumilus* BS22 was in the range 2.142 (±0.09) U/mg of BDW obtained in basal medium and 2.257 (±0.06) U/mg of BDW obtained in galacturonate. In all case, the highest production was achieved in galacturonate although the increase was slight (less than 10%) comparatively to the basal medium (Fig. 2).

Previous studies have shown that, in *Bacillus subtilis*, galacturonate acts as an activator of genes involved in intracellular catabolism of pectic compounds by relieving the KdgR repression on these genes (Pujic et al., 1998). To investigate whether the *kdgR* gene might be involved in regulation of the Pel-22 and Pel-66 synthesis, *kdgR* disruption mutants were constructed, as described in Materials and Methods section. Fig. 3 shows that enzyme production in the presence of various carbon sources remained at the same level in the mutants, compared to the wild type strains. To assess the results, we used as a control, the strain BSPP1 (Pujic et al., 1998) which is *kdgR* gene defective, derivative from *Bacillus subtilis* 168 strain. Similar results were obtained with BSPP1 and *B. subtilis* 168 strains, in term of Pel production, so no significant variation was observed (Fig. 3). The statistical analysis revealed that the small differences observed in this study are not significant and hence, there is no difference in Pel production between *kdgR* mutants and wild types.

3.2. Sensitivity of Pel-22 and Pel-66 to catabolic repression induced by glucose

We further tested whether the synthesis of Pel-22 and Pel-66 is sensitive to catabolic repression induced by glucose or by another readily metabolizable carbon source (fructose or sucrose). Basal mineral medium or LB medium, containing PGA or GalU, was supplemented with glucose, fructose or sucrose at different concentration up to 2%. Fig. 3 shows that in basal mineral medium, a slight decrease in Pel production (less than 8%) was observed in both *B. subtilis* 66 and *B. pumulis* 22 when glucose, fructose or sucrose were added to the PGA or GalU medium at 2%. Additionally, in basal LB medium that initially contains glucose at 0.5%, no further decrease in Pel production was observed following the addition of others readily metabolizable carbon sources.

Investigations have been undertaken, in regulatory mutant to deepen the results obtained in the parental strains. It has been previously reported that the CcpA protein is a major transcription

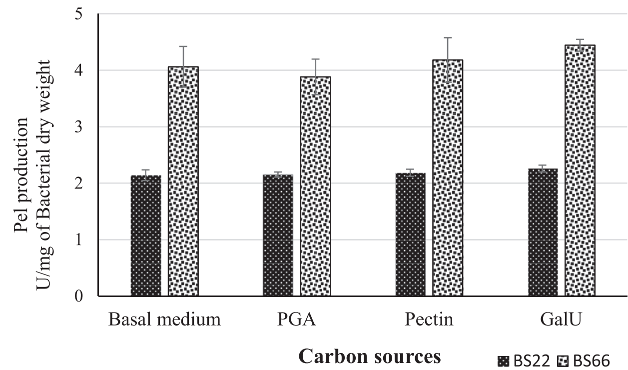

Fig. 2. Pectate lyase production in *Bacillus subtilis* BS66 and *Bacillus pumilus* BS22 in the presence of various pectic compounds. Experiences were duplicated and error bars indicate biological standard deviation.

factor mediating catabolite repression in *B. subtilis* (Stulke and Hillen, 2000). To further test whether the *ccpA* gene might be involved in Pel-22 and Pel-66 production control, *ccpA* disruption mutants were constructed, as described in the Materials and Methods section. Fig. 3 shows that inactivation of the *ccpA* gene has no significant impact on Pel-22 and Pel-66 production, whatever growth conditions are used.

A similar pattern of Pel production was observed with the control strain BM1034, a *ccpA* mutant derived from *B. subtilis* 168 (Lin and Shaw, 2007), and its corresponding wild type strain, confirming the results obtained with *Bacillus* strains BS66 and BS22 wild types and the *ccpA* mutants.

3.3. DegU as a positive regulator of the pel gene from Bacillus subtilis BS66

We next examined whether differences in the composition of growth medium based on factors other than the carbon source may impact the synthesis of Pel-22 and Pel-66. As shown in Fig. 4, the growth of the bacteria in LB nutrient rich medium instead of in the basal mineral medium containing the same concentration of carbon source, resulted in a decrease of about 6-fold in Pel production in both *Bacillus* wild type strains. This result indicates that the synthesis of Pel-22 and Pel-66 is repressed by high nutrient availability and suggests the involvement of unsuspected regulatory mechanisms in the production of these enzymes.

We subsequently, performed a computer analysis in the regulatory regions of the *pel* gene from *Bacillus subtilis* BS66 that we have previously cloned and characterized (Ouattara et al., 2010), using the PRODORIC database (Munch et al., 2005; www.prodoric.tu-bs.de/vfp/vfp_promoter.php), to search for binding sites of the known *B. subtilis* regulators. As expected, no binding sites for CcpA and KdgR were found in the promoter region of *pel66* gene. Rather, a binding site for DegU was identified 225 bp upstream from the ATG codon of *pel* gene with a highly significant score (Fig. 5). To check the possible involvement of DegU in regulation of *pel* gene, mutants' derivative from *Bacillus subtilis* 168 (Msadek et al., 1991, 1995) were essayed for Pel production in different culture conditions. The results show that Pel enzymes production decreases in the *degU* mutant comparatively to the wild type (Fig. 6). Furthermore, real time experiments revealed a strong decreased (60 folds) in the *pel* gene transcript in the *degU* mutant (Fig. 7). These data thus strongly suggest that DegU acts on the accumulation of *pel* gene transcripts in *Bacillus*.

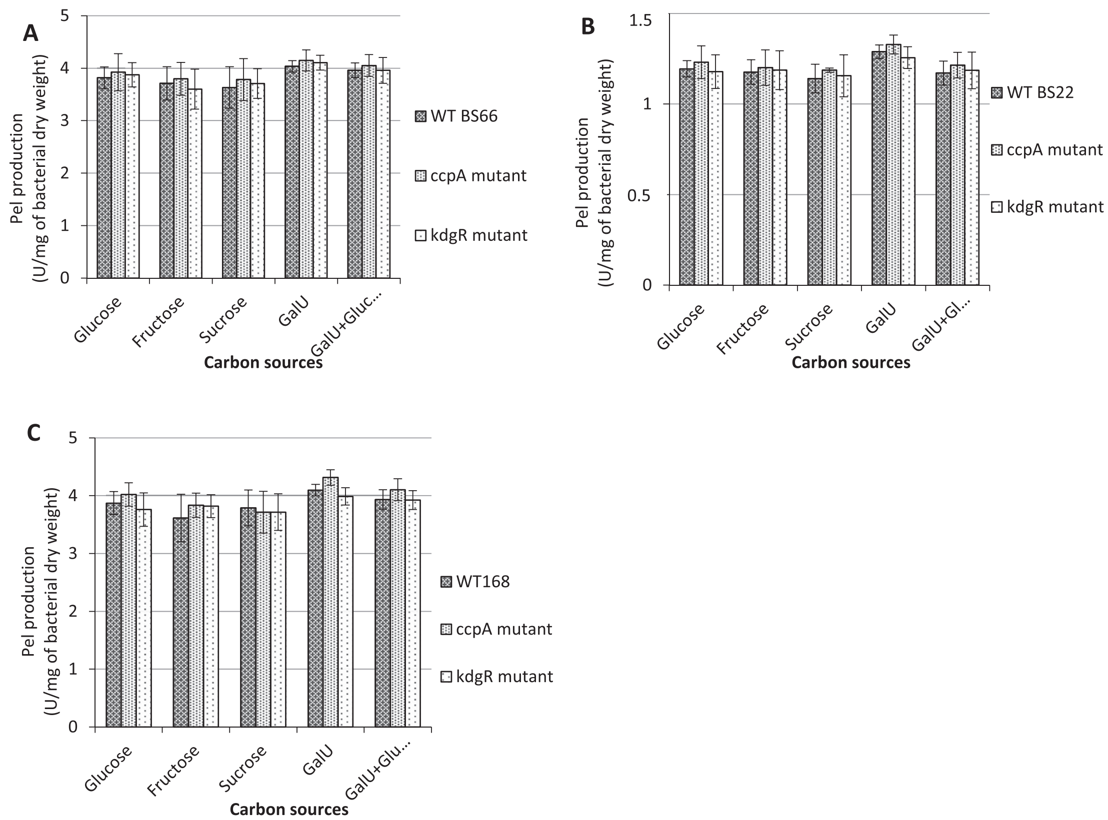

Fig. 3. Pectate lyase production in *Bacillus* wild types and mutants in the presence of various carbon sources. Error bars indicate standard deviation between two biological replications.

4. Discussion

In this work, we studied the regulation of pectate lyase (Pel) production in *Bacillus* involved in cocoa fermentation. The main objective of this study was to identify biological signals and transcriptional regulators that significantly modulate the production of these enzymes and, therefore susceptible to play an important role in the cocoa fermentation process.

The results showed that Pel production in *Bacillus* strains from fermenting cocoa beans do not depend on carbon source since no significant difference was observed in the presence of glucose, fructose or sucrose. On the other hand, we observed that Pel production is not induced by pectic compounds (PGA, pectin) or by the derivative galacturonate. These observations are in concurrence with previous studies reporting that pectinolytic enzymes are not inducible by pectic compounds and constitutively produced in *Bacillus* (Ward and Fogarty, 1974). In contrast, Ouattara et al. (2011) recently reported that Pel production by *Bacillus* strains is weakly induced by PGA and pectin when cells are grown until the late stationary growth phase. In the present study, enzyme production and gene expression in *Bacillus*, were rather analyzed at the end of exponential growth phase, which could explain the differences observed. The stationary growth phase corresponds to nutritional limitation, when many genes are derepressed and the transcripts are generally degraded (Priest, 1977). Hence, the exponential growth phase is more appropriate for assessing the impact of culture conditions on gene expression and production of enzymes involved in pectic compounds metabolism in *Bacillus* (Pujic et al., 1998).

Similarly to *pel* gene, the operon *kdgRKAT* was also reported to be not induced by polymeric pectic compound, such as PGA or pectin (Pujic et al., 1998). The operon *kdgRKAT* constitute with operon *kduID*, the genes coding for enzymes catalyzing the intracellular degradation of pectic compounds (see Fig. 1). However, both operons were reported to be induced, 4-fold, in the presence of galacturonate, the end-product of pectin or PGA degradation (Pujic et al., 1998; Lin and Shaw, 2007), by opposition with *pel* gene which is not induced by this compound.

Very less variation in Pel enzyme production was observed with sugar concentration up to 2%. This insensitivity of the *pel* gene to sugar concentration in *Bacillus* strains studied in this work, is

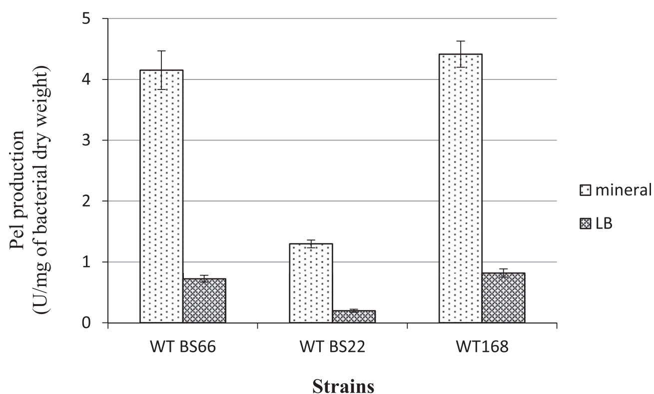

Fig. 4. Comparative level of Pel production in LB and basal mineral medium in wild type *Bacillus* strains. Error bars indicate standard deviation between two biological replications.

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1    CGGCGGGTCGACTCTAGAGGTCGGAGCCAAAGAAACAGGTTTCAAGCACGCGGTGGGAAA 60
61   TATCTTCAATAAAAAAGGAGACGAGCTCCGCAATAAAATTCACGAAATCGGTTTTTCTGA 120
121  AGATGAAGCCGCTCAATTTGAAAAACGCTTAGATGAAGGAAAAGTGCTTCTCTTTGTGAC 180
181  AGATAACGAAAAAGTGAAAGCTTGGGCATAAAGCAAGGAAAAAACCAAAAGGCCAATGTC 240
241  GGCCTTTTGGTTTTTTTGCGGTCTTTGCGGTGGGATTTTGCAGAATGCCGCAATAGGATA 300
301  GCGGAACATTTTCGGTTCTGAATGTCCCTCAATTTGCTATTATATTTTTGTGATAAATTG 360
361  GAATAAAATCTCACAAAATAGAAAATGGGGGTACATAGTGGATG
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Fig. 5. Location of regulon DegU binding sequences (in grey bright and underlined) at nucleotide 225 from ATG codon, in the promoter region of *pel66* gene previously characterized (Ouattara et al., 2010). Regulon motifs were found with the prodoric database (Munch et al., 2005) accessible on www.prodoric.tu-bs.de/vfp/vfp_promoter.php. The DegU binding sequence (ACAAAGAGAAGCACTTTTCCT) was identified on the reverse strand. The complementary sequence on the other strand is presented.

undoubtedly a real advantage for the cocoa fermentation process. In fact, the synthesis of many extracellular degradative enzymes in *Bacillus*, such as amylase (Singh et al., 2008) and cellulase (Kotchoni and Shonukan, 2002), is subjected to catabolic repression. Furthermore, Singh et al. (2008) reported a strong repression of beta galactosidase and xylosidase by glucose, which reduces the production of these enzymes by 48-fold. A repression such as this on Pel production, in *Bacillus* involved in cocoa fermentation should not be relevant since cocoa pulp contains up to 2% glucose. Taken together, the results show that Pel production in potential starter *Bacillus* strains is not sufficiently regulated by carbon sources to be hindered by cocoa pulp in the natural fermentation process, where there is a high content of carbon sources. In this context, the potential of pectinolytic enzymes in these strains could be maximally exploited.

Moreover, this study clearly showed that the most common regulators involved in carbon catabolism, CcpA (Stülke and Hillen, 2000; Deutscher et al., 2002), and the repressor involved in pectin catabolism in bacteria, KdgR, (Nasser et al., 1992; Pujic et al., 1998), were surprisingly found to have no role in *pel* gene expression in *Bacillus* studied since no significant variation in Pel enzyme production was observed in the ccpA and kdgR mutants comparatively to the wild type. This is strongly supported by absence of binding site for these regulators in *pel* gene regulatory regions as revealed by bioinformatics analysis. The lack of control of these regulators on *pel* gene could also explain the insensitivity of this gene to carbon source variation observed in *Bacillus* strains studied.

Unlike *pel* gene, the expression of these two *kduID* and *kdgRKAT* operons were reported to be subjected to 10–100 folds catabolite repression by 2% glucose, and this repression was shown to be mediated by the regulators KdgR and CcpA (Lin and Shaw, 2007). These observations, therefore, suggest that, as regards the pectin catabolism pathway in *Bacillus*, genes encoding enzymes for the intracellular pathway (operons *kduID* and *kdgRKAT*) and genes encoding extracellular enzymes (*pel*) are not regulated in the same way. This conclusion is contrary to previous studies concerning the plant pathogenic bacteria *Dickeya* and *Pectobacterium*, where coordinated regulation between the intracellular and extracellular parts of the pectin catabolism pathway has been reported (Hugouvieux-Cotte-Pattat et al., 1996; Kepseu et al., 2010).

Contrary to CcpA and KdgR regulators, DegU was found to regulate *pel* gene regarding the results obtained in the wild type and its *deg* mutants. In fact, Pel enzyme production and *pel* gene expression were higher in *Bacillus subtilis* wild type 168 than in the defective *degU* mutant.

This suggests that the Deg system acts as a positive regulator, activating the transcription of *pel* gene. DegU is known to be a regulator involved in the development of genetic competence occurring at the end of exponential growth phase whiles its phosphorylated form DegU-P is involved in synthesis of extracellular degradative enzymes (Dahl et al., 1992; Hamoen et al., 2000). DegU is phosphorylated by the DegS kinase protein (Dahl et al., 1991). Previously the mutant *degU32*(Hy), containing high levels of phosphorylated DegU (Dahl et al., 1991) was showed by transcriptomic approach to be an overproducer of degradative extracellular enzymes including PelB (Mader et al., 2002). Overall, these data reveal that the DegS-DegU system activates the synthesis of Pel enzyme.

The DegS-DegU system is involved in the regulation of transition state-specific processes which are often associated with depletion and modification of the growth media nutrient content or changes in the environmental conditions (Kunst et al., 1994; Dartois et al., 1998; Hamoen et al., 2000; Yasumura et al., 2008). It could be assumed that during the latter stage of cocoa bean fermentation (six days fermentation), when simple sugars and others nutrients

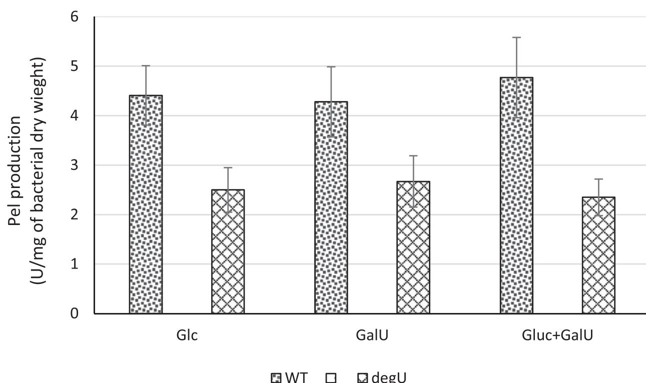

Fig. 6. Enzymes production in *Bacillus* wild type and *deg* mutants grown in different conditions. Error bars indicate standard deviation between two biological replications.

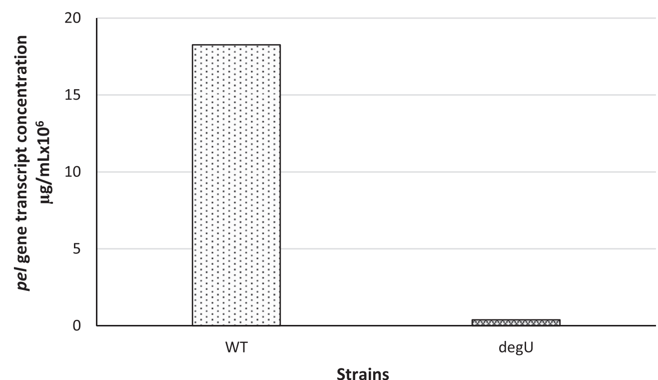

Fig. 7. Quantification of the *pel* gene expression in *Bacillus* wild type and *degU* mutant. Strains were grown in mineral medium containing galacturonate (GalU) + glucose until the end of exponential phase. Carbon sources were added at 2% final concentration. RNA was extracted, retro transcribed and used to quantify the *pel* gene expression in Quantitative PCR assays; *rpsU* gene was used as reference for *pel* gene expression normalization.

are depleted, the production of Pel is stimulated by the DegS-DegU system to supply microbial cells with carbon source from degradation of polymeric pectic compounds contained in the cocoa pulp.

5. Conclusion

This study enriches our knowledges on pectin metabolism regulation in *Bacillus*, notably by providing information on *pel* gene expression. Pel synthesis in *Bacillus* studied was found to be insensitive to sugar content variation due to the lack of control of the two main carbon catabolism regulators such as CcpA and KdgR on *pel* gene. However, *pel* gene expression appear to be regulated by DegU, which may play an important role during cocoa fermentation by activating the yield of this enzyme in nutrient limitation conditions in the latter stage of the fermentative process. The nature of the signal modulating the Deg system remains to be elucidated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.12.004.

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