Role of CcpA in Polyhydroxybutyrate Biosynthesis in a Newly Isolated *Bacillus* sp. MA3.3

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Key Words

CcpA • Polyhydroxybutyrate • Catabolite repression • Nitrogen metabolism • Xylose • *Bacillus*

Abstract

The *ccpA* gene was inactivated in the polyhydroxybutyrate (PHB)-producing strain *Bacillus* sp. MA3.3 in order to reduce glucose catabolite repression over pentoses and develop improved bacterial strains for the production of PHB from lignocellulosic hydrolysates. Mutant *Bacillus* sp. MSL7 Δ CcpA are unable to grow on glucose and ammonia as sole carbon and nitrogen sources, respectively. Supplementation of glutamate as the nitrogen source or the substitution of the carbon source by xylose allowed the mutant to partially recover its growth performance. RT-PCR showed that CcpA stimulates the expression of the operon (*gltAB*), responsible for ammonia assimilation via glutamate in *Bacillus* sp. MA3.3. Moreover, it was demonstrated that the supplementation of xylose or glutamate was capable of stimulating *gltAB* operon expression independently of CcpA. In PHB production experiments in mineral media, it has been observed that the glucose catabolite repression over the pentoses was partially released in MSL7. Although the carbohydrate consumption is faster in the *ccpA* mutant, the biomass and PHB bio-

synthesis are lower, even with supplementation of glutamate. This is attributed to an increase of acetyl-CoA flux towards the tricarboxylic acid cycle observed in the mutant.

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Introduction

Polyhydroxybutyrate (PHB) is produced by many bacteria and archaea in response to various environmental conditions. The accumulation of PHB increases in some bacteria when growth is limited by a nutrient other than carbon, such as nitrogen [McCool and Cannon, 2001]. PHB structural properties are similar to those of polypropylene, with the advantage of being biodegradable, biocompatible and produced from renewable carbon sources [Harding et al., 2007].

Gram-negative bacteria, such as *Escherichia coli*, contain outer membrane lipopolysaccharide endotoxins, which may contaminate PHB during the extraction process and may induce undesirable immunogenic reactions [Chen and Wu, 2005; Lee et al., 1995]. For that reason,

W. Hillen has since passed away.

Gram-positive microorganisms are more suitable to produce PHB for medical applications [Valappil et al., 2008]. In a previous screening for PHB-producing bacteria using inexpensive substrates, a mangrove soil-isolated *Bacillus* sp. MA3.3, closely related to *B. megaterium*, was isolated and reached promising results on PHB accumulation [Lopes et al., 2009]. However, when it grows in sugars usually found in lignocellulosic hydrolysates, glucose exerts a strong catabolite repression over xylose and arabinose.

In Gram-positive bacteria, the metabolism of different carbon sources is coordinated by CcpA. Glucose metabolism activates CcpA that binds to catabolite-responsive elements (*cre*) which usually overlap promoters of catabolite-repressed genes. For example, the xylose catabolism *xylABE* operon in *B. megaterium* has three *cre* sites that can respond to different signals in different intensities [Gösseringer et al., 1997]. However, CcpA mediates both repression and activation of genes. Among the genes repressed by CcpA are those encoding enzymes required for the utilization of secondary carbon sources, as well as genes of the tricarboxylic acid (TCA) cycle. The genes activated by CcpA include those required for overflow metabolism, glycolysis and the biosynthesis of certain amino acids [Fujita, 2009].

A *Bacillus* sp. MSL7 Δ CcpA, carrying a *ccpA* chromosomal deletion, was constructed to reduce glucose catabolite repression over pentoses, thus allowing the improvement of PHB production from lignocellulosic hydrolysates.

Results and Discussion

Construction and Characterization of the CcpA Mutant

Using primers P2C and P4C, we characterized the gene *ccpA* of *Bacillus* sp. MA3.3 and its flanking region (fig. 1, lane 2). Those regions showed high similarity with the orthologous regions of *B. subtilis* 168 (online suppl. table 1, www.karger.com/doi/10.1159/000324502). Sprehe et al. [2007] investigated the carbon catabolite repression in *B. subtilis* 168 and constructed pWH338 to obtain a chromosomal deletion of *ccpA* by substituting *ccpA* with the kanamycin resistance gene *aphA3*. Due to the high similarity found between MA3.3 and *B. subtilis* 168, the previous construction made by Sprehe et al. [2007] was used to delete *ccpA* in *Bacillus* sp. MA3.3. After protoplast transformation of *Bacillus* sp. MA3.3 with pWH338, the kanamycin-resistant *Bacillus* sp. MSL7 Δ CcpA was selected. Using

Fig. 1. PCR analysis of gene substitution in *Bacillus* sp. MSL7 (Δ CcpA). The gene *ccpA* of *B. megaterium* MA3.3 was amplified using primers P2C and P4C (1,200 bp; lane 2). The same primers were used to confirm that *ccpA* was substituted by the kanamycin resistance gene *aphA3* (1,000 bp; lane 3). 100-bp peqGOLD reference marker (Peqlab; lane 1).

Fig. 2. Western blot analysis with specific anti-CcpA antibodies *Bacillus* sp. MSL7 Δ CcpA. SDS-PAGE gel was loaded with the same concentration of crude protein extract of *Bacillus* sp. MA3.3 (lane 2) and *Bacillus* sp. MSL7 (lane 3), and subjected to Western blot with anti-CcpA. Purified CcpA was used as a control (lane 1).

the aforementioned primers, P2C and P4C, the PCR analysis of MSL7 demonstrated that *ccpA* was substituted by the kanamycin resistance gene *aphA3* (fig. 1, lane 3). The gene substitution resulted in a shorter DNA amplicon in comparison to the wild-type amplicon (fig. 1).

We analyzed the CcpA synthesis in the wild-type *Bacillus* sp. MA3.3 and in the recombinant MSL7 Δ CcpA by Western blot assays with specific anti-CcpA antibodies. The translation of CcpA in *Bacillus* sp. MA3.3 was confirmed (fig. 2, lane 2), while no translation product was detected in the recombinant strain (fig. 2, lane 3). A purified CcpA from *B. subtilis* 168 was used as the experiment control (fig. 2, lane 1). Therefore, both PCR and Western

Fig. 3. Growth experiments with *Bacillus* sp. MA3.3 and *Bacillus* sp. MSL7 Δ CcpA in M9 medium or M9 supplemented with glutamate.

blot analysis confirmed the deletion of *ccpA* in *Bacillus* sp. MSL7 (Δ CcpA).

Growth Experiments of *Bacillus* sp. MSL7 Δ CcpA

To evaluate the growth capability of the mutant *Bacillus* sp. MSL7 Δ CcpA, experiments were performed with M9 supplemented with carbohydrates (fig. 3). The mutant was unable to grow on glucose and presented reduced growth on xylose or mixtures of carbohydrates. Moreover, the supplementation of glutamate allowed the mutant to partially recover its growth performance.

The involvement of CcpA on glutamate biosynthesis in *B. subtilis* has been described previously [Belitsky et al., 2000; Faires et al., 1999; Wacker et al., 2003]. Ammonia is assimilated via glutamate, which is exclusively synthesized by the glutamine synthetase and glutamate synthase (encoded by the *gltAB* operon). Glutamine synthetase catalyses the formation of glutamine from glutamate and ammonium, and glutamate synthase converts 2-oxoglutarate and glutamine to two molecules of glutamate.

One of these molecules of glutamate can be recycled to glutamine, while the second molecule is now available for anabolism [Belitsky et al., 2000; Faires et al., 1999]. CcpA is needed to induce the *gltAB* operon when supplemented with glucose and ammonium as a carbon and nitrogen source, respectively [Belitsky et al., 2000; Faires et al., 1999; Wacker et al., 2003].

RT-PCR was used to investigate how CcpA controls the expression of *gltAB* operon in the *Bacillus* sp. MA3.3 and its recombinant. Analyses were performed using the RNA extraction of a culture of the wild-type strain in M9 supplemented with glucose and xylose (fig. 4, lanes 2 and 3, respectively), RNA from *Bacillus* sp. MSL7 Δ CcpA cultivated in M9 with glucose and xylose (fig. 4, lanes 4 and 5, respectively), and, finally, RNA from *Bacillus* sp. MSL7 Δ CcpA cultivated in M9 supplemented with glutamate and glucose and xylose (fig. 4, lanes 6 and 7, respectively). As described previously for *B. subtilis*, the *ccpA* mutant was incapable of inducing a *gltAB* operon when supplemented with glucose and ammonium as the carbon and

Table 1. PHB production by *Bacillus* sp. MA3.3 and *Bacillus* sp. MSL7 in mineral minimal medium supplemented with glucose (G); xylose (X); glucose and xylose (G+X); and glucose, xylose and arabinose (G+X+A)

Sugar	MA3.3 (wild type)				MSL7 (Δ CcpA mutant)			
	CDW, g·l ⁻¹	X _R , g·l ⁻¹	X _{PHB} , g·l ⁻¹	%PHB	CDW, g·l ⁻¹	X _R , g·l ⁻¹	X _{PHB} , g·l ⁻¹	%PHB
<i>Mineral media</i>								
G	5.76 ± 0.35	2.18	3.58	62.15 ± 2.23	0.73 ± 0.23	0.73	0.00	0.00 ± 0
X	5.23 ± 0.29	2.02	3.21	61.36 ± 1.78	2.05 ± 0.43	1.60	0.45	21.84 ± 1.15
G+X	3.86 ± 0.34	2.38	1.47	38.16 ± 3.12	2.24 ± 0.18	1.62	0.62	27.82 ± 4.26
G+X+A	3.99 ± 0.42	2.40	1.59	39.89 ± 2.76	1.92 ± 0.45	1.45	0.47	24.32 ± 2.12
<i>Mineral media supplemented with glutamate</i>								
G	6.33 ± 0.21	2.50	3.83	60.43 ± 2.23	2.65 ± 0.42	1.77	0.88	33.27 ± 2.01
X	5.54 ± 0.18	2.15	3.39	64.25 ± 1.92	3.16 ± 0.52	1.51	1.65	52.18 ± 1.21
G+X	4.23 ± 0.31	2.51	1.72	37.52 ± 3.14	3.64 ± 0.12	1.09	1.09	44.69 ± 2.12
G+X+A	4.25 ± 0.29	2.51	1.74	40.74 ± 2.63	3.61 ± 0.21	1.18	1.18	45.27 ± 2.78

Cell dry weight (CDW), residual biomass (X_R), PHB concentration (X_{PHB}), and PHB content from cell dry weight (%PHB) are presented.

Fig. 4. RT-PCR of *gltB* (107 bp) expression in *Bacillus* sp. MA3 wild type and *Bacillus* sp. MSL7 (Δ CcpA) cultivated in M9. Lane 1: 100-bp DNA ladder. Lanes 2 and 3: wild-type strain cultivated in glucose and xylose, respectively. Lanes 4 and 5: Δ CcpA mutant cultivated in glucose and xylose, respectively. Lanes 6 and 7: M9 was supplemented with glutamate and the Δ CcpA mutant was cultivated in glucose and xylose, respectively.

nitrogen source, respectively (fig. 4, lane 4). However, the substitution of the carbon source by xylose promotes *gltAB* expression and growth independently of CcpA (fig. 4, lane 5). Similar results were verified by Wacker et al. [2003] in experiments with *B. subtilis* with glycerol and glucitol. While glucose and fructose were unable to induce the expression of *gltAB* when CcpA was absent, glycerol and glucitol induced *gltAB* expression. Thus, it is another example of non-PTS carbon sources inducing the expression of the *gltAB* operon and allowing growth even in *ccpA* mutants.

In a further analysis of ammonia consumption in the presence of glutamate, it was confirmed that glutamate

not only stimulates the expression of *gltAB* (fig. 4, lane 6), but also promotes ammonia uptake in mineral medium containing glucose, probably by triggering the consumption of carbohydrates (fig. 5). Therefore, the data presented here show that glutamate could promote *gltAB* expression allowing the utilization of glucose by *ccpA* mutants of *Bacillus* sp. MA3.3. However, the mechanism of *gltAB* induction by CcpA is still not clear. Wacker et al. [2003] proposed that CcpA promotes the glycolysis overflow and subsequently *gltAB* induction.

PHB Production Experiments

PHB production experiments with mineral media with higher sugar concentrations (15 g·l⁻¹) were performed to observe the impact of *ccpA* deletion on growth and PHB biosynthesis (table 1). The total cell dry weight is expressed separately in two parameters: biomass formation (X_R) and PHB production (X_{PHB}).

In experiments with ammonium as the only nitrogen source, the mutant presented lower values of cell dry weight and PHB content (%PHB) in comparison with the wild type. In experiments using mineral media supplemented with glutamate, the wild type reached higher values of total biomass. As observed in growth experiments, glutamate allowed the *ccpA* mutant cells to partially overcome the growth defect, especially in glucose. As demonstrated by the growth experiments, glutamate can stimulate carbohydrate and ammonia assimilation.

The glucose catabolite repression over the pentoses was partially released in MSL7 (fig. 5). Even though the

Fig. 5. Ammonia consumption of *Bacillus* sp. MA3.3 wild type and *Bacillus* sp. MSL7 (Δ CcpA) in M9 medium supplemented with glucose (▲) or M9 supplemented with glucose and glutamate (■).

carbohydrate consumption is faster in the *ccpA* mutant, the biomass and PHB biosynthesis are lower. Therefore, the next question leads to the flux changes in central carbon metabolism in the mutant cell.

In the PHB biosynthetic pathway of *Ralstonia eutropha*, two molecules of acetyl coenzyme A (acetyl-CoA) are condensed by β -ketothiolase (PhaA), followed by a stereo-specific reduction catalyzed by an acetoacetyl-CoA reductase (PhaB) to produce the monomer *R*-(3)-hydroxybutyryl-CoA, which is polymerized by PHA synthase (PhaC). These three *pha* genes are encoded by the *phaCAB* operon. In *B. megaterium*, the *phaA* gene is not located in the same operon, and other genes associated to PHB synthesis are clustered with *phaC* [McCool and Cannon, 1999]. PHB synthesis is regulated at the enzymatic level by the intracellular concentration of acetyl-CoA and free CoASH [Haywood et al., 1988; Lee et al., 1995]. Therefore, citrate synthase is an important control point based on the availability of CoASH [Henderson and Jones, 1997], which regulates the activity of 3-ketothiolase [Park and Lee, 1996].

In *B. subtilis*, CcpA directly represses the operon *citZ-icd-mdh*. This operon is formed by the genes *citZ* (citrate synthase), *icd* (isocitrate dehydrogenase) and *mdh* (malate dehydrogenase) encoding the first enzymes of the TCA cycle [Kim et al., 2002; Lulko et al., 2007; Sonenshein, 2007]. To evaluate if the deletion of CcpA caused the higher flux of acetyl-CoA, measurements of CO₂ production were performed during the PHB production experiments. MSL7 produced 0.23 ± 0.01 g of CO₂ per

Fig. 6. PHB production by *Bacillus* sp. MA3.3 (solid line) and *Bacillus* sp. MSL7 (dashed line) in mineral media supplemented with glutamate and sugar mixtures: glucose (◆), arabinose (+), xylose (■), cell dry weight (CDW; ▲), PHB content from cell dry weight (%PHB; ×).

Fig. 7. CO₂ evolution in by MA3.3 (solid line) and MSL7 (dashed line) in mineral media supplemented with glutamate and sugar mixtures: cell dry weight (CDW; ▲), and CO₂ production (○). The average from at least two independent experiments for each test, with the standard deviation lower than 0.03 for CO₂ evolution.

gram of biomass, about 30% more than the wild type which produced 0.18 ± 0.01 g · l⁻¹. The increase on CO₂ production rate in MSL7 indicates a higher flux through the TCA cycle, which could explain the lower biomass and PHB biosynthesis observed in the *ccpA* mutant. Those results suggest that a similar regulation mechanism of PHB biosynthesis observed in *B. subtilis* is presented in *Bacillus* sp. MA3.3, based on the control of *citZ-icd-mdh* by CcpA. Additionally, the higher flux through the TCA cycle also results in a higher concentration of

free CoASH, also considered inhibitory to PHB biosynthesis [Henderson and Jones, 1997].

Even in mineral media supplemented with glutamate, the *ccpA* mutant growth was lower than the wild-type growth (table 1). The literature suggests that this can be solved with the addition of methionine and branched-chain amino acids to the growth medium. The biosynthesis of those compounds also depends on a functional CcpA in *B. subtilis* [Ludwig et al., 2002]. On the other hand, the addition of a complex nitrogen source may make the medium more expensive, hampering the advantages of using inexpensive lignocellulosic hydrolysates.

In conclusion, the *ccpA* mutant of *Bacillus* sp. MA3.3 showed a reduction of catabolite repression and, consequently, consumed sugar mixtures faster than the wild type. However, deregulation of genes necessary for the growth on mineral minimal media, including those required for ammonia consumption, was also observed. Moreover, since CcpA plays an important role in coordinating the carbon central metabolism in *Bacillus* sp. MA3.3, its deletion caused a higher flux to the TCA cycle, resulting in lower yields of biomass and PHB.

The efficient utilization of lignocellulosic hydrolysates relies on different cellular phenotypes. High PHB productivity depends on a strain with reduced catabolite repression and resistance to the inhibitor compounds present in the hydrolysate. In contrast, a high PHB yield is dependent on the fluxes of the central carbon metabolism. Recently, a procedure to improve complex phenotypes was described, one which is based on random mutagenesis of global regulators, inducing global perturbations of the transcriptome, and selection of phenotypic improvements [Alper and Stephanopoulos, 2007]. Therefore, instead of knocking out *ccpA* as is described here, *ccpA* could be subjected to random mutagenesis and further selection of the desired cellular phenotype.

Experimental Procedures

Bacterial Strains and Growth Conditions

Bacillus sp. MSL7 Δ CcpA (Kan^r) is a derivative of the soil-isolated *Bacillus* sp. MA3.3 [Lopes et al., 2009], and both were cultivated in nutrient broth at 30°C and 200 rev·min⁻¹. M9 medium [Sambrook et al., 1989] containing single sugar (2 g·l⁻¹) or sugar mixtures (1 g·l⁻¹ of each) was used in growth experiments. Mineral minimal medium [Rocha et al., 2008] supplemented with glucose (15 g·l⁻¹); xylose (15 g·l⁻¹); glucose and xylose (7.5 g·l⁻¹ of each); or glucose, xylose and arabinose (5 g·l⁻¹ of each) was used for the PHB production experiments. For further experiments, M9 and mineral media were supplemented with sodium glutamate (2 g·l⁻¹).

Construction and Characterization of the CcpA Mutant

The gene *ccpA* and its flanking region of *Bacillus* sp. MA3.3 were amplified using primers P2C: 5'-TACGTAAATACAAGA-AATGA-3' and P4C: 5'-CCG TGTCTGAACCTTGG-3' (1 min of denaturation at 95°C, 1 min of annealing at 59°C, and 3 min of extension at 72°C for a total of 30 cycles.). This sequence was compared with the orthologous region of *B. subtilis* 168 using the software DIALIGN [Morgenstern et al., 1996]. Protoplast transformation of *Bacillus* sp. MA3.3 [Rygus and Hillen, 1992] with pWH338 [Sprehe et al., 2007] was performed. In pWH338, the *ccpA* gene of *B. subtilis* 168 was substituted by the kanamycin resistance gene *aphA3* [Sprehe et al., 2007]. For that reason, pWH338 was used to promote *ccpA* deletion by homologous recombination in *Bacillus* sp. MA3.3. After protoplast regeneration, the cells were plated on M9 minimal medium containing glucose (0.2%) and kanamycin (80 mg/l). The confirmation of *ccpA* deletion was carried out by PCR analysis of the CcpA region and Western blot immunodetection using anti-CcpA rabbit antibodies as described previously [Küster et al., 1996; Sambrook et al., 1989].

Reverse Transcription PCR

Total RNA extraction was performed using Trizol (Gibco) and DNase treatment (Promega) as described by the manufacturers. RNA integrity was tested by electrophoresis in agarose gels using the phosphate buffer method [Farrel, 2005]. RNA quantification was performed by measuring absorbance at 260 nm using NanoDrop 1000 (Thermo Scientific). RNA samples were subjected to PCR with primers for the operon encoding for 16S rRNA to detect possible contamination with genomic DNA. cDNA was always synthesized with 5 µg of RNA sample and the SuperScriptTM III First-Strand Synthesis SuperMix kit (Invitrogen) with random hexamers. cDNA was used as a template for PCR using specific primers for glutamate synthase, *gltB* (5'-acggttggtgtcaaaagcca and 5'-accttggtgccaggaagctg). The PCR program was conducted using a denaturation step of 3 min at 94°C, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min, with a final extension step at 72°C for 10 min.

Analytical Methods

Bacterial growth was evaluated spectrophotometrically (OD₆₀₀ nm). Cell dry weight was determined gravimetrically as described by Silva et al. [2004], carbohydrates were determined by HPLC [Silva et al., 2004], and PHB amount was determined by gas chromatography of hydroxyalkanoate esters [Gomez et al., 1996]. CO₂ was entrapped using barium hydroxide (0.025 N) and quantified by titration with HCl (0.05 M) using phenolphthalein (0.04%) as a pH indicator. The following equation was used to calculate the CO₂ evolution of the culture: CO₂ (mg) = (Volume HCl¹ - Volume HCl²), with Volume HCl¹ representing the volume of HCl used in the sample and Volume HCl² representing the volume used in the blank reaction.

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