

# Cross Communication Between Components of Carbon Catabolite Repression of *Lactobacillus casei* and *Bacillus megaterium*

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## Abstract

In low-GC Gram-positive bacteria, carbon catabolite repression (CCR) is exerted by transcriptional regulation through a protein complex consisting of catabolite control protein CcpA and serine phosphorylated phosphocarrier protein HPr (HPr-ser-P). We investigated the interaction between these components of *Lactobacillus casei* and *Bacillus megaterium*. CcpA of *L. casei* could not complement a *B. megaterium* *ccpA* mutant strain, whereas it was found to be functional in *Bacillus subtilis*. To explore the nature of the non-complementing phenotype, we overproduced and purified CcpA and HPr of *L. casei* for *in vitro* analyses. Electrophoretic mobility shift assays revealed a failure in CCR signal transduction at the level of protein-protein interaction between *L. casei* CcpA and *B. megaterium* HPr-ser-P, while binding of CcpA to the *B. megaterium* target site was intact. We established a method based on surface plasmon resonance that allowed a quantitative analysis of CcpA/HPr-ser-P interactions. Calculation of the apparent dissociation constants revealed that the interaction of *L. casei* CcpA with *B. megaterium* HPr-ser-P was fivefold weaker than with its own HPr-ser-P suggesting that the reduced affinity was responsible for the non-complementing phenotype.

## Introduction

In low-GC Gram-positive bacteria, carbon catabolite repression (CCR) is exerted by energetically favorable carbon sources via the histidine-containing phosphocarrier protein HPr of the phosphoenolpyruvate-sugar

phosphotransferase system (PTS) and the central regulator catabolite control protein CcpA (for a review see Hueck and Hillen, 1995; Stülke and Hillen, 1999). Upon growth on preferred sources of carbon, HPr is phosphorylated at a regulatory residue serine 46 (HPr-ser-P) by the metabolite-activated ATP-dependent HPr kinase/phosphatase HPrK (Reizer *et al.*, 1998; Kravanja *et al.*, 1999). HPr-ser-P is capable of interacting with CcpA promoting the specific binding to DNA target sequences termed catabolite responsive elements (*cre*), which are located upstream or within catabolic genes and operons (Deutscher *et al.*, 1995; Miwa *et al.*, 2000). This results in a repression of gene expression at the transcriptional level as has been demonstrated for a wide variety of catabolite-controlled systems including the xylose utilization operon *xylAB* of *Bacillus megaterium* (Rygus and Hillen, 1992). CcpA has been identified in bacilli, streptococci, staphylococci, and some industrially relevant lactic acid bacteria, such as *Lactococcus lactis* and *Lactobacillus casei* (Henkin *et al.*, 1991; Egeter and Brückner, 1996; Monedero *et al.*, 1997; Luesink *et al.*, 1998; Simpson and Russell, 1998; Schick *et al.*, 1999; Mahr *et al.*, 2000). CcpA proteins were found to comprise a subfamily within the LacI/GalR family of bacterial repressors with some of the CcpA-specific residues eventually forming a continuous patch on the protein surface, which is thought to represent the binding surface of HPr-ser-P (Jones *et al.*, 1997; Kraus *et al.*, 1998). In addition, a mutational analysis of CcpA of *B. megaterium* identified amino acid positions critical for its function in CCR (Kraus and Hillen, 1997; Kraus *et al.*, 1998; Küster *et al.*, 1999).

To elucidate the function of CcpA in the dairy starter bacterium *L. casei*, we studied complementation of CCR of the *xylAB* system of *B. megaterium* by *L. casei* CcpA *in vivo*. Surprisingly, we found that CcpA of *L. casei* was not able to confer CCR to a *B. megaterium* *ccpA* mutant, although similar heterologous cross complementation had been demonstrated earlier, including *in vivo* complementation of a *B. subtilis* *ccpA* mutant with *L. casei* *ccpA* (Davison *et al.*, 1995; Egeter and Brückner, 1996; Monedero *et al.*, 1997; Schick *et al.*, 1999). In order to find the reason for this non-complementing phenotype, we established *in vitro* systems that allowed to monitor the involved protein-protein and protein-DNA interactions of *L. casei* and *B. megaterium* components. The results suggest that *L. casei* CcpA could not confer CCR in *B. megaterium* due to a reduced affinity to *B. megaterium* HPr-ser-P.

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## Results

### CcpA of *L. casei* Cannot Complement a *B. megaterium* *ccpA* Mutant

For the *in vivo* complementation of a *B. megaterium* *ccpA* mutant with *ccpA* of *L. casei*, we chose plasmid pGCCPA, which carries the *L. casei* *ccpA* gene under the control of the vegetative Gram-positive promoter SPO2 and contains a ribosome binding site and a start codon adapted to *B. subtilis* (Monedero *et al.*, 1997). This construct which was derived from plasmid pGAL9 was shown to partially complement CCR of the gluconate operon *gntRK* of *B. subtilis* GM1225 (*ccpA* mutant) (Monedero *et al.*, 1997). Since the plasmid-encoded erythromycin gene was not functional in *B. megaterium* a neomycin resistance cassette (*neo*) of pWH1509K was cloned in plasmids pGCCPA and pGAL9. This yielded plasmids pWH153 (*ccpA*<sup>Lca</sup>) and pWH152 (control), respectively, which were transformed into *B. megaterium* WH353  $\Delta$ *ccpA*.

To study the ability of *L. casei* CcpA to confer CCR to the *B. megaterium* *ccpA* deletion mutant, we measured  $\beta$ -galactosidase activities of the chromosomal *xylA::lacZ* fusion. The *xylAB* promoter in this strain responds to CCR only, which is due to a deletion of the xylose repressor gene rendering the system expressed in the absence of xylose. As depicted in Figure 1, *B. megaterium* WH353(pWH2040), carrying *ccpA*<sup>Bme</sup>, showed a 5.2 fold reduction of  $\beta$ -galactosidase activity in the presence of glucose, while WH353(pWH1509K; control) was defective in CCR. Enzyme activities of strains WH353(pWH153), carrying *ccpA*<sup>Lca</sup>, and WH353(pWH152; control) were similar under repressing conditions leading to the conclusion that CcpA of *L. casei* was not promoting CCR of the *xyl* operon in *B. megaterium*.

To assure that *ccpA* of *L. casei* was heterologously expressed in the *B. megaterium* *ccpA* mutant,

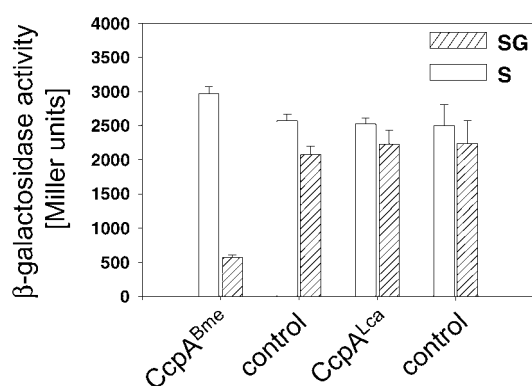


Figure 1. *In vivo* complementation of a *B. megaterium* *ccpA* mutant with the *L. casei* *ccpA* allele. The figure displays  $\beta$ -galactosidase activity measurements obtained for a  $\Delta$ *xylR xylA::lacZ* fusion in *B. megaterium* WH353. Cells were grown in the presence of succinate (S, open bars) for non-repressing growth conditions and in the presence of succinate plus glucose (SG, hatched bars) for glucose-repressing growth conditions. Strains containing plasmids pWH2040 (*CcpA*<sup>Bme</sup>), pWH1509K (control), pWH153 (*CcpA*<sup>Lca</sup>), and pWH152 (control) are indicated.

we performed a Western blot experiment using polyclonal antibodies raised against CcpA of *B. megaterium* (Küster *et al.*, 1996). CcpA could be detected in *L. casei* wild-type extracts, but not in the isogenic *L. casei* *ccpA* mutant (Figure 2, lanes 3 and 4). A signal was also obtained in *B. megaterium* WH353(pWH153, *ccpA*<sup>Lca</sup>) corresponding to the *L. casei* CcpA protein (lane 5). Such a band was not present in extracts of the control strain WH353(pWH152) (lane 6). CcpA of *B. megaterium* was readily detectable in WH353 (pWH2040, *ccpA*<sup>Bme</sup>) cell extract, whereas the control extract WH353(pWH1509K) showed no CcpA signal (lanes 8 and 7). Hence, although *L. casei* CcpA could not complement the *ccpA* deletion mutant of *B. megaterium*, it could be shown to be indeed being expressed in *B. megaterium*.

### Overproduction and Purification of CcpA and HPr of *L. casei*

In order to find an explanation for this non-complementing phenotype we examined the *in vitro* interaction of *L. casei* CcpA with the *B. megaterium* target sequence *cre*<sup>xyl</sup> in the presence and absence of *B. megaterium* or *L. casei* HPr-ser-P. For this purpose overproduction plasmids for CcpA and HPr of *L. casei* were constructed and purification protocols for both proteins were established. CcpA and HPr could be purified to homogeneity following the procedure described in *Materials and Methods* yielding approximately 20 mg and 12 mg pure protein per liter of *Escherichia coli* culture, respectively. Phosphorylation of HPr of *L. casei* at serine 46 was achieved upon incubation of the purified protein with HPr kinase/phosphatase of *B. subtilis* and subsequent separation of proteins by anion exchange chromatography.

### *In vitro* Analysis of the *cre*/CcpA/HPr-ser-P Complexes

The interactions of purified components were examined by electrophoretic mobility shift assays. It could be observed that CcpA of *B. megaterium* formed a

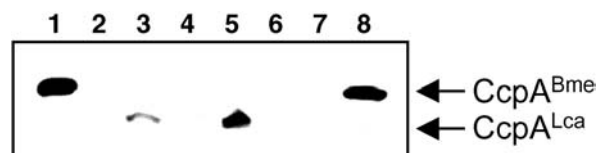


Figure 2. Western blot analysis of expression of *ccpA* of *L. casei* (*CcpA*<sup>Lca</sup>) in *B. megaterium*. A Western blot of a sodium dodecyl sulfate-7.5% polyacrylamide gel is shown after incubation with polyclonal antibodies raised against CcpA of *B. megaterium* (*CcpA*<sup>Bme</sup>). Lane 1, 50 ng of purified CcpA of *B. megaterium*; lane 2, molecular weight marker; lanes 3 and 4, 50  $\mu$ g cell extract of *L. casei* ATCC393 (wild type) and *L. casei* BL71 (*ccpA* mutant), respectively; lane 5, 50  $\mu$ g cell extract of *B. megaterium* WH353(pWH153, *CcpA*<sup>Lca</sup>); lane 6, 50  $\mu$ g cell extract of WH353(pWH152, control); lane 7, 1  $\mu$ g cell extract of WH353(pWH1509K, control); lane 8, 1  $\mu$ g cell extract of WH353(pWH2040, *ccpA*<sup>Bme</sup>). CcpA of *B. megaterium* always migrated slower in denaturing protein gels than CcpA of *L. casei*. It should be noted that detection of *L. casei* CcpA required higher amounts of cell extract due to the lower affinity of the antibody.

complex with  $cre^{xyI}$  only in the presence of HPr-ser-P<sup>Bme</sup> (Figure 3A, lane 3). CcpA of *L. casei* already exhibited some binding to  $cre^{xyI}$  in the absence of HPr-ser-P under these experimental conditions (lane 5). As anticipated, a much more efficient binding of *L. casei* CcpA to  $cre^{xyI}$  was found when *L. casei* HPr-ser-P was added to the reaction, but interestingly not when HPr-ser-P of *B. megaterium* was used (lanes 7 and 6). This observation could be confirmed in a mobility shift assay using *L. casei* CcpA and the homologous  $cre$  sequence of the *L. casei* lac operon ( $cre^{lac}$ ). Again, binding of *L. casei* CcpA to  $cre^{lac}$  was much more efficient in the presence of *L. casei* HPr-ser-P than with HPr-ser-P of *B. megaterium* (Figure 3B, lanes 4 and 3). Furthermore, there was no reciprocity in this interaction defect, since binding of *B. megaterium* CcpA to  $cre^{xyI}$  could be greatly enhanced by the addition of HPr-ser-P of *L. casei* (Figure 3A, lane 4).

#### Determination of Equilibrium Parameters for CcpA/HPr-ser-P Interactions

To quantify the differences in these protein-protein interactions, they were analyzed in real-time using surface plasmon resonance. *L. casei* CcpA was immobilized on a CM5 sensor chip via amino coupling. When equimolar concentrations of HPr-ser-P of *L. casei* or *B. megaterium* were applied at non-saturating CcpA-binding conditions, *L. casei* HPr-ser-P gave a sixfold higher response signal, indicating that its binding ability to *L. casei* CcpA was correspondingly sixfold greater than that of *B. megaterium* HPr-ser-P (Figure 4A). Apparent equilibrium dissociation constants of the complexes of *L. casei* CcpA with either *L. casei*, *B. megaterium*, or *B. subtilis* HPr-ser-P were subsequently elucidated and found to be  $3.9 \times 10^{-6}$  M,  $2.0 \times 10^{-5}$  M, and  $2.0 \times 10^{-5}$  M, respectively, showing that HPr-ser-P<sup>Bme</sup> and his-tagged HPr-ser-P<sup>Bsu</sup> had a very similar affinity for CcpA of *L. casei* (Figure 4B and 4C). This affinity value was 5.1 fold lower than that of *L. casei* CcpA for its own HPr-ser-P, which approximately coincided with the data from response signals obtained before (Figure 4A).

#### Discussion

This study was prompted by the initial observation that *L. casei* CcpA could not mediate CCR in *B. megaterium*, although its synthesis was readily detectable. The finding contrasted with published data, since, until date, functional complementation of CcpA between different Gram-positive bacteria had apparently always been successful (Davison *et al.*, 1995; Egeter and Brückner, 1996; Monedero *et al.*, 1997; Schick *et al.*, 1999). The lack of complementation was considered an interesting ground to set up comparative experiments that would eventually help to understand protein-protein and DNA-protein interactions taking place during the final stages of the CCR signal transduction process.

In the ternary complex  $cre/CcpA/HPr-ser-P$ , CcpA must interact with two partners and, as a consequence, the non-complementing phenotype observed could be due to a failure in an effective  $cre/CcpA$  or  $CcpA/HPr-ser-P$  interaction. Therefore, the binding ability of purified *L. casei* CcpA to  $cre$  sites in the presence and absence of HPr-ser-P proteins was compared by electrophoretic mobility shift assays. *L. casei* CcpA could efficiently recognize  $cre^{xyI, Bme}$  and  $cre^{lac, Lca}$  in the presence of its own HPr-ser-P, but not by the addition of *B. megaterium* HPr-ser-P. This indicated that the lack of complementation was due to a failure in the interaction of *L. casei* CcpA with the endogenous HPr-ser-P.

This experiment further showed that *L. casei* CcpA could bind  $cre$  already without corepressor, which would be a discrepancy to the *in vivo* results. However, this effect was considered unspecific, since *L. casei* CcpA exhibited some binding to the non-specific DNA included in the assay. This was monitored by Coomassie Brilliant Blue-staining of the gel shown in Figure 3A, where a major fraction of *L. casei* CcpA was detectable at the height of the non-specific DNA (data not shown). In contrast, purified CcpA of *B. megaterium* did not show unspecific binding of DNA, indicating that the binding abilities between two CcpAs cannot be directly compared with this method.

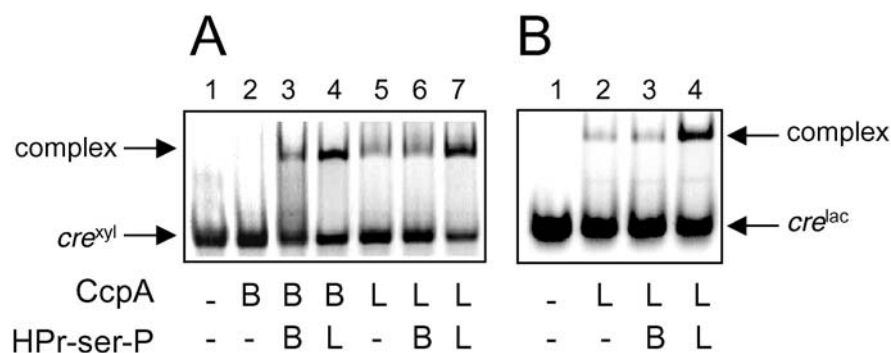


Figure 3. Electrophoretic mobility shift assay using a 48 bp oligonucleotide containing the  $cre$  sequence of the *B. megaterium*  $xyI$  gene (A) and a 26 bp oligonucleotide containing the  $cre$  sequence of the *L. casei* lac operon (B). Purified CcpA and HPr-ser-P of *B. megaterium* [B] and/or *L. casei* [L] were combined with the respective  $cre$  containing oligonucleotides as indicated. An excess of unspecific DNA was added to all reactions.

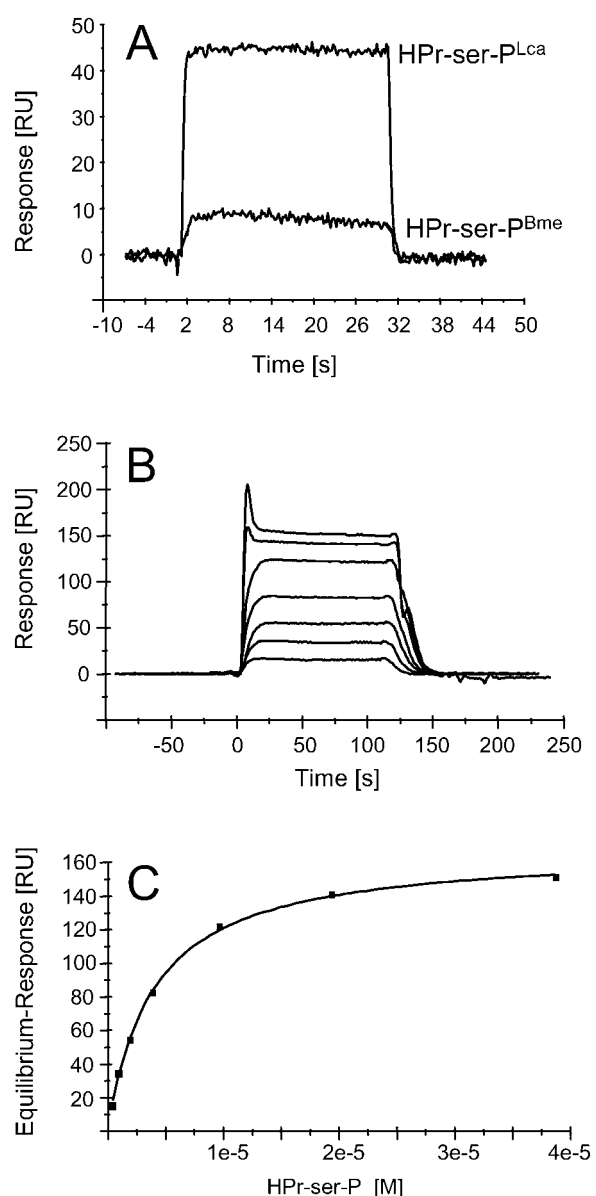


Figure 4. (A) Interaction analysis of the *L. casei* CcpA protein with HPr-ser-P of *L. casei* and *B. megaterium* by surface plasmon resonance. *L. casei* or *B. megaterium* HPr-ser-P were allowed to flow over immobilized *L. casei* CcpA at equimolar concentrations (11  $\mu$ M). (B) Estimation of the apparent equilibrium dissociation constant for the interaction of HPr-ser-P<sup>Lca</sup>/CcpA<sup>Lca</sup>. HPr-ser-P was flowed over CcpA at several concentrations (38.8, 19.4, 9.7, 3.9, 1.9, 1.0 and 0.4  $\mu$ M). (C) The response in equilibrium for each sensorgram was calculated and plotted against HPr-ser-P concentrations to obtain the apparent equilibrium dissociation constant. The same experiment was performed using HPr-ser-P<sup>Bme</sup> or HPr-ser-P<sup>Bsu</sup> (data not shown).

Another result to discuss is that *B. megaterium* CcpA showed a weaker interaction with its own HPr-ser-P compared to its binding to *L. casei* HPr-ser-P. This shows that heterologous cross communication between two components might not always yield reciprocal results.

To determine the affinities between *L. casei* CcpA and different HPr-ser-P species in a more quantitative way, we chose the method of the surface plasmon

resonance to monitor protein-protein interactions in real-time. This sensitive technique allowed to estimate the apparent equilibrium dissociation constants of interactions between these protein pairs, which have up to date not been described for this system (Karlsson and Falt, 1997). Using purified CcpA and HPr-ser-P of *L. casei* the apparent  $K_D$  of the homologous interaction was  $3.9 \times 10^{-6}$  M. This is in good agreement with an apparent  $K_D$  of  $7.0 \pm 2.9 \times 10^{-6}$  M determined by fluorescence titration for the CcpA-homologue PurR of *E. coli* to its effector guanine (Xu *et al.*, 1998). The apparent  $K_D$  value obtained for the interaction of *L. casei* CcpA with *B. megaterium* HPr-ser-P was fivefold lower compared to that of both *L. casei* proteins.

We anticipated that HPr-ser-P of *B. subtilis* would exhibit a higher binding affinity to *L. casei* CcpA since *in vivo* complementation was reported (Monedero *et al.*, 1997). Surprisingly, the  $K_D$  was the same as determined for the pair CcpA<sup>Lca</sup>/HPr-ser-P<sup>Bme</sup>. Obviously, similar heterologous cross communication was sufficient for complementation of CCR in *B. subtilis*, while it did not work in *B. megaterium*. It should be noted that CcpA of *L. casei* complemented only partially (about 40%) CCR of the *B. subtilis* *gnt* target system (Monedero *et al.*, 1997). Thus, other parameters such as protein expression levels, the choice of the reporter system, and the presence of a second HPr-ser-P-like protein (Crh-ser-P) could have accounted for this difference (Galiniere *et al.*, 1997).

As mentioned before, functional interaction between HPr-ser-P and CcpA belonging to different species has been demonstrated, but has not been studied at the molecular level. The here established method to quantitatively determine CcpA/HPr-ser-P interactions may provide a useful tool to investigate CcpA and HPr protein variants in a combinatorial manner.

#### Experimental Procedures

##### Bacterial Strains, Plasmids and Growth Conditions

The strains and plasmids used in this work are listed in Table 1. *L. casei* cells were grown in MRS medium (Difco) at 37°C under static conditions. *E. coli* was grown with shaking at 37°C in Luria-Bertani (LB) medium. *B. megaterium* cells were grown in LB medium and M9 mineral medium supplemented with 25 mM succinate at 28°C with shaking. Glucose at a concentration of 10 mM was added when repressing growth conditions were required. Plating of bacteria was performed on the same media solidified with 1.5% agar. When appropriate, the concentrations of antibiotics used were 100  $\mu$ g/ml of ampicillin or 30  $\mu$ g/ml of kanamycin for *E. coli* and 4  $\mu$ g/ml of neomycin for *B. megaterium*.

##### Construction of *L. casei* ccpA Expression Vectors

A neomycin resistance cassette (*neo*) of pWH1509K was cloned in plasmids pGCCPA and pGAL9 using standard procedures and *E. coli* DH5 $\alpha$  as host (Sambrook *et al.*, 1989). The fragment containing the *neo* cassette was obtained by *Xba*I restriction of pWH1509K and inserted into pGCCPA and pGAL9 digested with the same restriction enzyme yielding plasmids pWH153 and pWH152, respectively.

##### Enzyme Assays

Quantification of  $\beta$ -galactosidase activity in *B. megaterium* cells was carried out as described by Hueck *et al.* (Hueck *et al.*, 1995). Cells were grown in M9 mineral medium supplemented with 25 mM succinate (non-repressing growth conditions) or with 25 mM succinate

Table 1. Strains and plasmids used in this study.

strain/plasmid	genotype/description	reference
<i>B. megaterium</i> WH353	$\Delta ccpA$ $gdh2\phi(xylA1-spoVG-lacZ)$ $\Delta xylR$	Küster <i>et al.</i> , 1999
<i>L. casei</i> BL23	ATCC 373 [pLZ15']	B. Chassy (University of Illinois, Urbana)
<i>L. casei</i> BL71	<i>L. casei</i> BL23 <i>ccpA</i> mutant	Monedero <i>et al.</i> , 1997
<i>E. coli</i> DH5 $\alpha$	general cloning host	Sambrook <i>et al.</i> , 1989
<i>E. coli</i> BL21	protein overproduction strain (DE3, pLysS)	Studier, 1991
pGAL9	<i>erm</i>	Monedero <i>et al.</i> , 1997
pGCCPA	<i>erm</i> , <i>ccpA</i> ( <i>L. casei</i> )	Monedero <i>et al.</i> , 1997
pWH1509K	<i>neo</i>	Rygus and Hillen, 1992
pWH2040	<i>neo</i> , <i>ccpA</i> ( <i>B. megaterium</i> )	Hueck <i>et al.</i> , 1995
pWH152	pGAL9 plus <i>neo</i> from pWH1509K	<i>This work</i>
pWH153	pGCCPA plus <i>neo</i> from pWH1509K	<i>This work</i>
pET3c	<i>bla</i>	Studier <i>et al.</i> , 1990
pET3c- <i>ccpA</i>	pET3c containing <i>ccpA</i> of <i>L. casei</i>	<i>This work</i>
pET3c- <i>ptsH</i>	pET3c containing <i>ptsH</i> of <i>L. casei</i>	<i>This work</i>

plus 10 mM glucose (repressing growth conditions). Experiments were repeated in triplicate.

#### Western Blot Analysis

*B. megaterium* strains derived from WH353( $\Delta ccpA$ ) transformed with plasmid pWH153 carrying *L. casei ccpA* (*ccpA*<sup>Lca</sup>), pWH152 (control), pWH2040 carrying *B. megaterium ccpA* (*ccpA*<sup>Bme</sup>), and pWH1509K (control), were grown in LB medium supplemented with neomycin. *L. casei* strains ATCC393 (wild type) and BL71 (*ccpA* mutant) were grown in MRS medium (Difco). Cells were harvested at an OD<sub>600</sub> of 0.5 by centrifugation and cell extracts were subsequently prepared as described previously (Monedero *et al.*, 1997; Mahr *et al.*, 2000). Proteins of cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Fluorotrans) by electroblotting. CcpA was detected with a rabbit polyclonal antiserum raised against CcpA of *B. megaterium* (Küster *et al.*, 1996). CcpA antibodies on the polyvinylidene difluoride membrane were visualized using the ECL Western blot analysis system (Amersham).

#### Construction of CcpA and HPr Overproduction Vectors

Plasmid pET3c-*ccpA* for the overproduction of *L. casei* CcpA was constructed by amplifying the *ccpA* gene from plasmid pUCCPA (Monedero, unpublished) with oligonucleotides CCPA1 (GGA-GAAAAATCATATGGAAAAGC) and CCPA2 (AAAAGGATCCAT-TATTCGTTG) introducing *NdeI* and *BamHI* restriction sites (underlined), respectively. PCR was performed with *Pfu* DNA polymerase. The restricted amplification product was cloned in the expression vector pET3c digested with the same restriction enzymes yielding pET3c-*ccpA*. Plasmid pET3c-*ptsH* for overproduction of HPr of *L. casei* was obtained by a similar procedure. The *ptsH* gene (encoding HPr) of *L. casei* was amplified from chromosomal DNA with oligonucleotides PTSH1 (CAGATCACATATGGAAAACGCG) and PTSH2 (AAATGTGGATCCATTATTCAGCC). All constructs were verified by DNA sequencing.

#### Protein Overproduction and Purification

For protein purification, plasmids pET3c-*ccpA* and pET3c-*ptsH* were transformed in *E. coli* BL21(DE3) (pLysS) (Studier, 1991). Overexpression of proteins was achieved by isopropyl thio- $\beta$ -D-galactoside (IPTG) induction as described (Parche *et al.*, 1999). For the purification of CcpA, one liter of *E. coli* culture was grown in the presence of 1 mM IPTG for 3 h. Cells were harvested by centrifugation and washed with buffer A (20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol). The pellet was resuspended in 5 ml of buffer A and crude extracts were prepared by sonification at 45 W  $\times$  30 s (Labsonic U, Braun) and subsequent removal of cell debris by centrifugation. The cleared cell extract was used for an anion exchange chromatography (HQ/M 16/100; Poros) using a gradient from 0 to 1 M NaCl. Fractions containing CcpA were pooled, concentrated, and proteins were further separated by gel filtration (Superdex G75; Pharmacia) using a buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, and 200 mM NaCl, giving a homogeneous pure CcpA preparation. Purification of heat-stable HPr was achieved following a similar procedure with the exception that the cleared cell extract was boiled for 10 min and

centrifuged to remove the majority of cell proteins. The supernatant was subjected to anion exchange chromatography as described above yielding fractions containing homogeneous pure HPr.

#### In vitro Phosphorylation of HPr at Serine 46

HPr of *L. casei*, *B. megaterium*, or *B. subtilis* was phosphorylated at serine 46 with HPr kinase/phosphatase from *B. subtilis* expressed from p4813 as previously described (Kraus *et al.*, 1998; Reizer *et al.*, 1998).

#### Electrophoretic Mobility Shift Assays

Two small DNA fragments that contained characterized *cre* sites were used in these experiments. A synthetic 48 bp double-stranded oligonucleotide containing the *cre* sequence of the *B. megaterium xylA* gene (position +105 to +152 of *xylA*) (*cre*<sup>xyl</sup>) (5  $\mu$ M) or a 26 bp double-stranded oligonucleotide comprising the *cre* sequence of the *L. casei lac* operon (position -23 to -48 of *lacT*) (5  $\mu$ M) were mixed with purified CcpA (10  $\mu$ M) of *B. megaterium* or *L. casei* with or without 10  $\mu$ M HPr-ser-P of *B. megaterium* or *L. casei* in a total volume of 10  $\mu$ l. Samples were incubated in the presence of 2  $\mu$ g unspecific DNA (linearized plasmid pWH802) at 37°C in a buffer containing 100 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 10 min before loading on a non-denaturing 5% polyacrylamide gel. Gels were run with a buffer containing 100 mM Tris-HCl (pH 8.8) and 1 mM EDTA at 70 V for 45 min. DNA was visualized by ethidium bromide staining.

#### Surface Plasmon Resonance Experiments

The interaction of *L. casei* CcpA with HPr-ser-P from *L. casei*, *B. megaterium*, or *B. subtilis* was analyzed by surface plasmon resonance with a BiaCore X biosensor (Pharmacia Biosensor AB). CcpA was immobilized on a CM5 sensor chip (Pharmacia Biosensor AB) by amino coupling to 1.5 to 1.7 ng/mm<sup>2</sup> following instructions supplied by the manufacturer. The difference in CcpA binding between *L. casei* and *B. megaterium* HPr-ser-P was determined by subsequently flowing these phosphorylated effectors over the sensor chip in HBS-EP buffer (BiaCore AB) at a flow rate of 100  $\mu$ l/min. To determine equilibrium constants, a set of sensorgrams was collected at a flow rate of 5  $\mu$ l/min using various concentrations of *L. casei* or *B. megaterium* HPr-ser-P or his-tagged HPr-ser-P from *B. subtilis*. Apparent equilibrium dissociation constants of the complexes were estimated by plotting the equilibrium response against HPr-ser-P concentrations and fitted to the model  $R_{eq} = K_A \times C \times R_{max} / (K_A \times C + R_{max})$ , where  $R_{eq}$  is the response in equilibrium,  $K_A$  the apparent association constant (inverse of  $K_D$ ),  $C$  the concentration of HPr-ser-P,  $R_{max}$  the maximal response (when the chip reaches saturation) and  $n$  a sterical impediment correction factor. This fitting allowed the calculation of the apparent  $K_D$  of protein-protein interactions. Sensorgrams were analyzed using the BIAevaluation 3.1 software (BiaCore AB).

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## References

- Davison, S.P., Santangelo, J.D., Reid, S.J., and Woods, D.R. 1995. A *Clostridium acetobutylicum* regulator gene (*regA*) affecting amylase production in *Bacillus subtilis*. *Microbiology* 141: 989–996.
- Deutscher, J., Küster, E., Bergstedt, U., Charrier, V., and Hillen, W. 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. *Mol. Microbiol.* 15: 1049–1053.
- Egeter, O., and Brückner, R. 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. *Mol. Microbiol.* 21: 739–749.
- Galinier, A., Haiech, J., Kilhoffer, M.C., Jaquinod, M., Stülke, J., Deutscher, J., and Martin-Verstraete, I. 1997. The *Bacillus subtilis* *crh* gene encodes a HPr-like protein involved in carbon catabolite repression. *Proc. Natl. Acad. Sci. U.S.A.* 94: 8439–8444.
- Henkin, T.M., Grundy, F.J., Nicholson, W.L., and Chambliss, G.H. 1991. Catabolite repression of  $\alpha$ -amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli* *lacl* and *galR* repressors. *Mol. Microbiol.* 5: 575–584.
- Hueck, C.J., and Hillen, W. 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? *Mol. Microbiol.* 15: 395–401.
- Hueck, C.J., Kraus, A., Schmiedel, D., and Hillen, W. 1995. Cloning, expression and functional analyses of the catabolite control protein CcpA from *Bacillus megaterium*. *Mol. Microbiol.* 16: 855–864.
- Jones, B.E., Dossonnet, V., Küster, E., Hillen, W., Deutscher, J., and Klevit, R.E. 1997. Binding of the catabolite repressor protein CcpA to its DNA target is regulated by phosphorylation of its corepressor HPr. *J. Biol. Chem.* 272: 26530–26535.
- Karlsson, R., and Falt, A. 1997. Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *J. Immunol. Methods* 200: 121–133.
- Kraus, A., and Hillen, W. 1997. Analysis of CcpA mutations defective in carbon catabolite repression in *Bacillus megaterium*. *FEMS Microbiol. Lett.* 153: 221–226.
- Kraus, A., Küster, E., Wagner, A., Hoffmann, K., and Hillen, W. 1998. Identification of a co-repressor binding site in catabolite control protein CcpA. *Mol. Microbiol.* 30: 955–963.
- Kravanja, M., Engelmann, R., Dossonnet, V., Blüggel, M., Meyer, H.E., Frank, R., Galinier, A., Deutscher, J., Schnell, N., and Hengstenberg, W. 1999. The *hprK* gene of *Enterococcus faecalis* encodes a novel bifunctional enzyme: the HPr kinase/phosphatase. *Mol. Microbiol.* 31: 59–66.
- Küster, E., Hilbich, T., Dahl, M.K., and Hillen, W. 1999. Mutations in catabolite control protein CcpA separating growth effects from catabolite repression. *J. Bacteriol.* 181: 4125–4128.
- Küster, E., Luesink, E.J., de Vos, W.M., and Hillen, W. 1996. Immunological crossreactivity to catabolite control protein CcpA from *Bacillus megaterium* is found in many Gram-positive bacteria. *FEMS Microbiol. Lett.* 139: 109–115.
- Luesink, E.J., van Herpen, R.E., Grossiord, B.P., Kuipers, O.P., and de Vos, W.M. 1998. Transcriptional activation of the *glycolytic las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. *Mol. Microbiol.* 30: 789–798.
- Mahr, K., Hillen, W., and Titgemeyer, F. 2000. Carbon catabolite repression in *Lactobacillus pentosus*: analysis of the *ccpA* region. *Appl. Environ. Microbiol.* 66: 277–283.
- Miwa, Y., Nakata, A., Ogiwara, A., Yamamoto, M., and Fujita, Y. 2000. Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucleic Acids Res.* 28: 1206–1210.
- Monedero, V., Gosalbes, M., and Pérez-Martínez, G. 1997. Catabolite repression in *Lactobacillus casei* ATCC 393 is mediated by CcpA. *J. Bacteriol.* 179: 6657–6664.
- Parche, S., Schmid, R., and Titgemeyer, F. 1999. The phosphotransferase system (PTS) of *Streptomyces coelicolor*: identification and biochemical analysis of a histidine phosphocarrier protein HPr encoded by the gene *ptsH*. *Eur. J. Biochem.* 265: 308–317.
- Reizer, J., Hoischen, C., Titgemeyer, F., Rivolta, C., Rabus, R., Stülke, J., Karamata, D., Saier, M.H.J., and Hillen, W. 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. *Mol. Microbiol.* 27: 1157–1169.
- Rygu, T., and Hillen, W. 1992. Catabolite repression of the *xyl* operon in *Bacillus megaterium*. *J. Bacteriol.* 174: 3049–3055.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schick, J., Weber, B., Klein, J.R., and Henrich, B. 1999. PepR1, a CcpA-like transcription regulator of *Lactobacillus delbrückii* ssp. *lactis*. *Microbiology* 145: 3147–3154.
- Simpson, C.L., and Russell, R.R. 1998. Identification of a homolog of CcpA catabolite repressor protein in *Streptococcus mutans*. *Infect. Immun.* 66: 2085–2092.
- Studier, F.W. 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* 219: 37–44.
- Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185: 60–89.
- Stülke, J., and Hillen, W. 1999. Carbon catabolite repression in bacteria. *Curr. Opin. Microbiol.* 2: 195–201.
- Xu, H., Moraitis, M., Reedstrom, R.J., and Matthews, K.S. 1998. Kinetic and thermodynamic studies of purine repressor binding to corepressor and operator DNA. *J. Biol. Chem.* 273: 8958–8964.