LcoP, an osmoregulated betaine/ectoine uptake system from *Corynebacterium glutamicum*

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Abstract In *Corynebacterium glutamicum*, four uptake systems for compatible solutes have been characterized so far. DHPE (ΔbetPΔputPΔproPΔectP), a derivative of the *C. glutamicum* type strain ATCC 13032 carrying deletions in the corresponding genes, still showed a low betaine uptake rate of 1.4 nmol/(min mg cdm). Genome analyses revealed the presence of a putative carrier, named low capacity osmoregulated permease (LcoP), which shows similarities to compatible solute transporters of the betaine/carnitine/choline transporter (BCCT)-family. Deletion of *lcoP* in DHPE resulted in betaine and ectoine uptake deficiency. LcoP, a betaine and ectoine permease is regulated at the expression and the activity level by the external osmolality. Addition of local anesthetics modulated the activity of BCCT-family members BetP, EctP, and LcoP in a different manner, indicating a different type of lipid–protein interaction.

Keywords: Betaine/carnitine/choline transporter; Low capacity osmoregulated permease; Activity regulation; Expression regulation; Local anesthetics; *Corynebacterium glutamicum*

1. Introduction

A soil bacterium, like *Corynebacterium glutamicum*, experiences dramatic changes of the external osmolality in its natural habitat. To cope with the deleterious effects of severe osmotic stress, effective adaptation mechanisms are necessary. A widely distributed strategy of eubacteria to overcome hyperosmotic conditions, which lead to a loss of cytoplasmic water, is the accumulation of compatible solutes by biosynthesis and/or uptake [1–4]. Since uptake of compatible solutes is faster and energetically cheaper than de novo synthesis, normally a fast activation of uptake systems for compatible solutes occurs under hyperosmotic conditions [5].

In *C. glutamicum* four secondary carriers for the uptake of compatible solutes have been characterized. PutP, a high affinity proline uptake system, is independent from the external osmolality and seems to be involved in proline utilization for anabolic purposes rather than in osmotic stress adaptation [6]. In contrast, BetP, EctP and ProP are regulated at the level of activity, i.e., they become activated if the external osmolality exceeds a certain threshold level. Interestingly, they possess an identical activity optimum at an osmolality of approx. 1.3 osM [5,7,8]. BetP and EctP belong to the betaine/carnitine/choline transporter (BCCT)-family [5], whereas ProP is a member of the MFS-family of transport proteins [2]. BetP is highly specific for betaine [8], whereas the proline/ectoine permease ProP and the ectoine/betaine/proline permease EctP have a broader substrate spectrum [5]. The transport velocities are ranging between 27 and 110 nmol/(min mg cell dry mass). Thus, *C. glutamicum* seems to be well equipped with uptake systems for compatible solutes allowing an effective adaptation to hyperosmotic stress if betaine, ectoine or proline is provided by the environment. Nevertheless, in DHPE, a derivative of the *C. glutamicum* type strain ATCC 13032, where the genes *betP*, *putP*, *proP*, and *ectP* were stepwise deleted [5], still a low uptake activity of betaine (1.4 nmol/min mg cdm) was detected. In this work, we addressed the question whether low capacity osmoregulated permease (LcoP), encoded in the *C. glutamicum* genome and showing similarities to BetP and EctP, is responsible for the residual betaine uptake activity in DHPE. Furthermore, the physiological role of LcoP and its osmostress-dependent regulation was investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

C. glutamicum type strain ATCC 13032 and its derivatives were grown either in Brain heart infusion (Difco, Detroit, MI, USA) or in CgXII medium with or without 1 M NaCl [9] at 30 °C. The construction of pEKEX2-betP (pGTG) was recently described [10].

2.2. Construction of C. glutamicum deletion strain DHPF

The gene *lcoP* was deleted by the method of allelic replacement, based on the selection of a chromosomal deletion by two recombination events [11]. The flanking regions together with the *lcoP* gene were amplified by PCR by the use of 5′[TTC ATC TGG ATC GTC G] and 5′[GCA CTT CAG ATT G] as primers. The resulting 2.5 kb fragment was ligated into the *Sma*I site of pUC18 leading to pUC18*lcoP*chrom and sequenced for control. An internal *Nru*I/*Nco*I fragment of 360 bp was removed and the plasmid was religated after a Klenow fill-in reaction leading to pUC18Δ*lcoP*chrom. Subsequently, the 2.1 kb fragment Δ*lcoP*chrom was isolated after *Pvu*II restriction and ligated into the *Sma*I site of pK19*mobsacB*. The resulting integration plasmid pK19Δ*lcoP* was transferred into strain DHPE via conjugation [12]. The deletion mutant DHPF (Δ*betP*, Δ*proP*, Δ*putP*, Δ*ectP*, and Δ*lcoP*), resulting from a double chromosomal recombination event of pK19mobsacB plasmid (integration/excision), was identified via PCR.

2.3. Construction of pEKEX2-lcoP and pEKEX2-ectP

For overexpression of *lcoP* and *ectP*, the corresponding genes were amplified via PCR. As template chromosomal DNA of *C. glutamicum* type strain ATCC 13032 was used. For amplification of *lcoP*, the

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primers 5′[ATG GAG AGG ACA CAC ATG] and 5′[TTA ATC CTT TTT CGC GTC C] were used, whereas for *ectP* the primers 5′[GGA TCC ATG AGC TCA AAT ATA G] and 5′[GAA TTC TTA AAT ATC GTA TTC] were chosen. The PCR products were ligated into the *Sma*I site of vector pUC18 and sequenced for control. Subsequently, the two fragments were ligated in the *Bam*HI/*Eco*RI site of the IPTG inducible plasmid pEKEX2 [13] and transformed into DHPF leading to DHPF/pEKEX2-lcoP or DHPF/pEKEX2-ectP, respectively.

2.4. Synthesis of [^{14}C]betaine and transport assays

Synthesis of [^{14}C]betaine and transport assays was described earlier [7,8]. For the transport assays, [^{14}C]betaine at a final concentration of 750 μM was used. The strains were grown overnight in Brain Heart Infusion (BHI) medium (Difco, Detroit, MI, USA) with 600 mM NaCl or, in simple BHI medium, if transcription of the plasmid-encoded genes was induced by the addition of 0.2 mM IPTG. Cells were washed once in 50 mM KP_i (pH 7.5) containing 50 mM NaCl, suspended in the same buffer and energized with 10 mM glucose, before they were subjected to an osmotic upshift. After several time intervals, samples were taken and the activity was quantified by scintillation counting.

2.5. RNA hybridization experiments

The purification of RNA from *C. glutamicum* was performed as described recently [14]. For construction of RNA antisense probes, intragenic *lcoP* fragments of a size of roughly 500 bp was amplified via PCR by the use of ATCC 13032 cells as template. The primers 5′[CCA TGG CTA TCC CCT CC] and 5′[TTA ACT CTT TTT CGC GTC C] were chosen. The PCR-fragment was cloned into the *Sma*I site of pGEM4Z (Promega, Mannheim, Germany). DIG-11dUTP-labeled antisense RNA was obtained by in vitro transcription (1 h, 37 °C) from *Eco*RI-linearized vectors by the use of T7 polymerase. Transcription changes were monitored by RNA hybridization experiments with digoxygenin-labeled antisense RNA probes as described recently [14].

3. Results and discussion

We previously measured that *C. glutamicum* strain DHPE, carrying deletions of *betP*, *putP*, *proP* and *ectP*, has a residual betaine uptake rate of 1.4 nmol/(min mg cdm) [5]. In DHPE carrying the control plasmid pEKEX2, which is routinely used as expression vector for *betP*, *ectP* and *proP*, this activity was found to be increased fourfold due to unknown reasons. Experiments revealed that the betaine uptake activity in DHPE and DHPE/pEKEX2 (Fig. 1) is regulated by the medium osmolality. This observation challenged the former interpretation that the residual low activity might be an unspecific side reaction of an unknown amino acid transporter. In the *C. glutamicum* genome sequence an open reading frame was identified (NCBI No.: NCgl2251), encoding a protein with 630 amino acids designated LcoP. This putative carrier shows high similarity to members of the BCCT-family, e.g., to BetP and EctP from *C. glutamicum* (40% and 32%) and OpuD from *Bacillus subtilis* (36%), which are all regulated at the level of activity in response to the external osmolality [5,8,15]. Similarities between BetP, LcoP and EctP were only found in the membrane part of the proteins, but not in the hydrophilic N- and C-terminal domains (Fig. 2). It was previously shown for BetP that these domains are involved in the activity regulation [10,16].

The *lcoP* gene was deleted in the genome of DHPE. The resulting strain, DHPF, was grown under conditions of high osmolality, in order to induce putative residual uptake systems for compatible solutes. After a hypo-osmotic washing step cells were used for uptake measurements. These revealed that DHPF was unable to take up betaine and ectoine under high osmolality conditions (Fig. 3), but a very low proline uptake

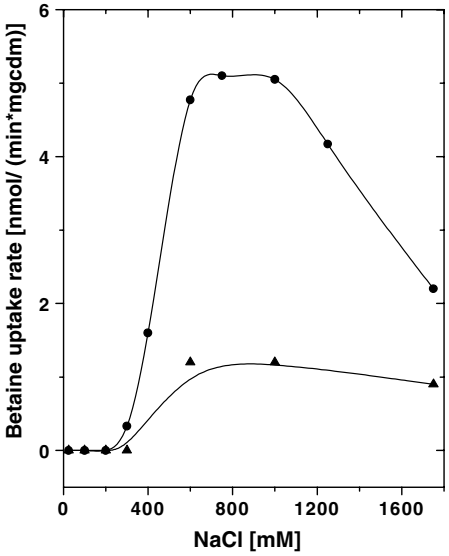

Fig. 1. Betaine uptake rate of *C. glutamicum* DHPE in dependence of the external osmolality. DHPE (triangles) and DHPE/pEKEX2 (circles).

rate of 0.2 nmol/(min mg cdm) was detected which was independent from the external osmolality (data no shown). Therefore, LcoP seems to be the last osmostress-regulated uptake system for compatible solutes in *C. glutamicum*. This assumption is further strengthened by data mining in the *C. glutamicum* genome sequence, which revealed that no other open reading frame encoding a putative compatible solute transporter is present. To characterize the kinetic parameters of the transporter, *lcoP* was cloned into the IPTG-inducible expression vector pEKEX2, and subsequently pEKEX2-lcoP was transformed into DHPF. Only a low proline uptake rate of 0.2 nmol/(min mg cdm) was detectable, which is likely to reflect the residual activity of DHPF, whereas betaine and ectoine uptake was increased up to 8 nmol/(min mg dw). The K_m determinations were performed in 50 mM KP_i buffer (pH 7.5) with 750 mM NaCl (Table 1) and led to values for ectoine which were three times higher than the K_m for betaine (154 μM). Na^+, the co-substrate of the uptake reaction, has a K_m of 36 mM. In this case, osmolality for reaching maximal stimulation was increased by the addition of 1500 mM sorbitol instead of NaCl. Other substrates were not tested, because it is unlikely that LcoP transports other compatible solutes than betaine and ectoine. In growth experiments carried out in wild type cells under conditions of severe osmotic stress, a growth advantage was only found if betaine, ectoine or proline was added to the medium, whereas no effect was detected with carnitin, choline, or glycine [7, unpublished results].

The regulation of transport activity in dependence of external osmolality was investigated either by variation of NaCl or sorbitol (Fig. 3). LcoP was activated by hyperosmotic stress above 600 mosmol/kg. In the case of sorbitol, the maximal transport activity was found at an osmolality of 1300 mosmol/kg (corresponding to 1000 mM sorbitol). This value was shifted to 1600 mosmol/kg if NaCl was used (corresponding to 750 mM NaCl). Thus, LcoP is characterized by an activity maximum in a similar range as detected for BetP, EctP and

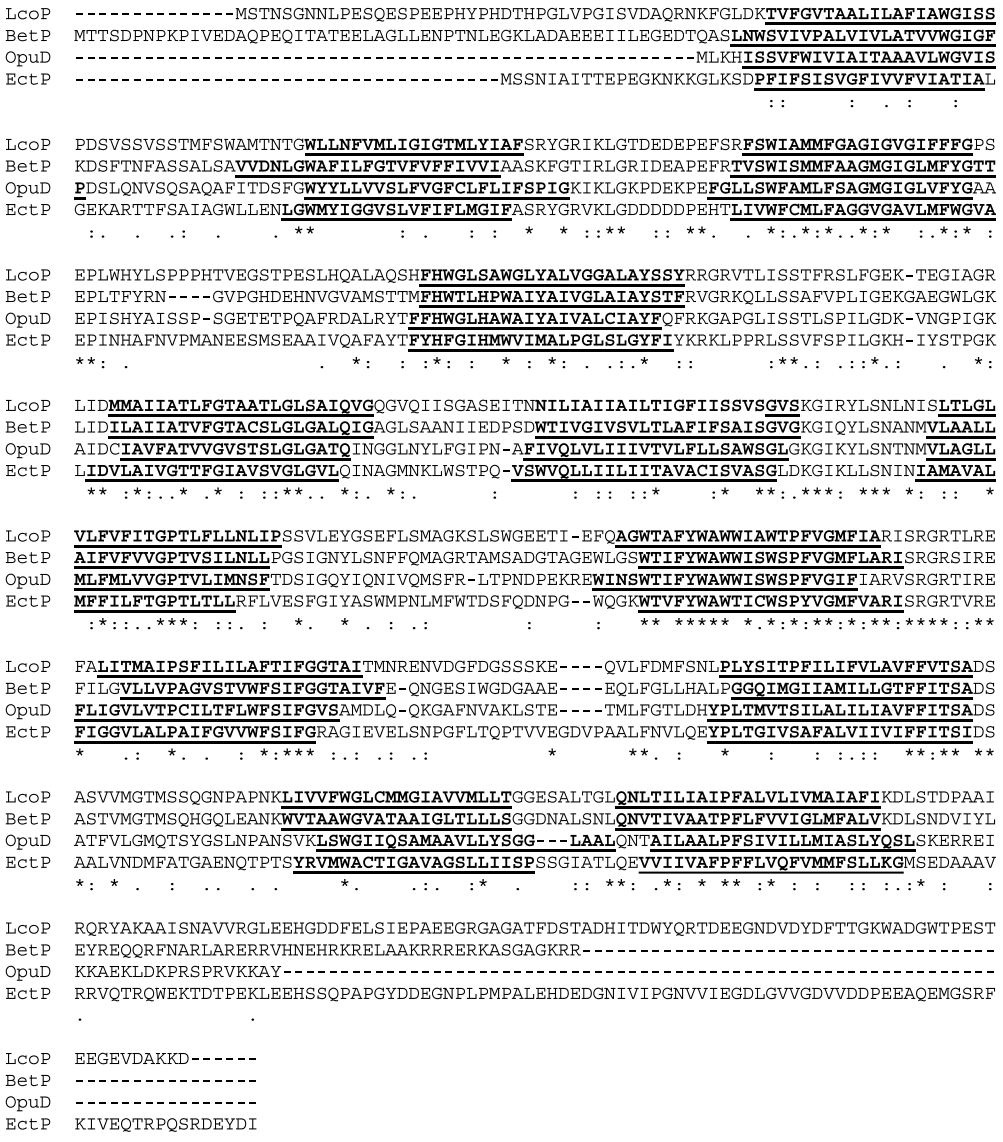

Fig. 2. Sequence alignment of LcoP, EctP [5], and BetP [8] from *C. glutamicum* and of OpuD from *B. subtilis* [15]. Stars indicate identical amino acids, whereas colons and dots represent conserved or semi-conserved substitutions, respectively. The predicted transmembrane segments are shown in underlined bold letters. For the prediction of transmembrane segments, the program TMHMM [http:/www.cbs.dtu.dk/services] was used.

ProP, but in contrast to them it has only a low transport activity [5,7] (Fig. 1).

Local anesthetics, which alter the physical state of the membrane, have been used for characterization of the sensing process of osmoregulated carriers, since changes in the protein/membrane interaction are supposed to be a possible trigger for carrier activation under osmotic stress [10,17,18]. Due to the similar activation regulation of the BCCT-family members [5,7] (Fig. 3) and the fact that the activation threshold of BetP was found to be influenced by tetracaine addition [10,17], we tested whether differently charged local anesthetics [18] also had a similar effect on the activities of BetP, EctP and LcoP. For this purpose, the concentration of tetracaine (positively charged), decane (uncharged) and capric acid (negatively charged) was varied between 0 and 1.3 mM in the presence of 450 mM NaCl. Surprisingly, the local anesthetics had a very different influence on the activity regulation of the BCCT-type carriers (Fig. 4). Whereas BetP activity was increased by about 60% by tetracaine and up to 20% by decane and capric acid, EctP was inhibited by each compound, but most efficiently by tetracaine (by about 70%). Addition of tetracaine resulted in a pronounced inhibition of LcoP, too, but in contrast to EctP decane and capric acid were slightly stimulatory. Up to now, the activation in response to osmotic stress was analyzed in detail only in the case of BetP, revealing that BetP activity is triggered in proteoliposomes solely by increasing internal K^+ (or Rb^+ or Cs^+) concentrations, whereas other cations like Na^+ were not effective [19,20] and that the last 25 amino acids of the C-terminal domain of BetP are involved in K^+-sensing [16]. In addition, there is experimental evidence that BetP activity can be influenced by the composition of the surrounding phospholipid membrane and by tetracaine [17, this work] indicating that the membrane is somehow involved in BetP regulation. Two different results argue for the hypothesis that the osmostress-induced activation mechanisms are, at least partially, different within the BCCT family. On the one hand

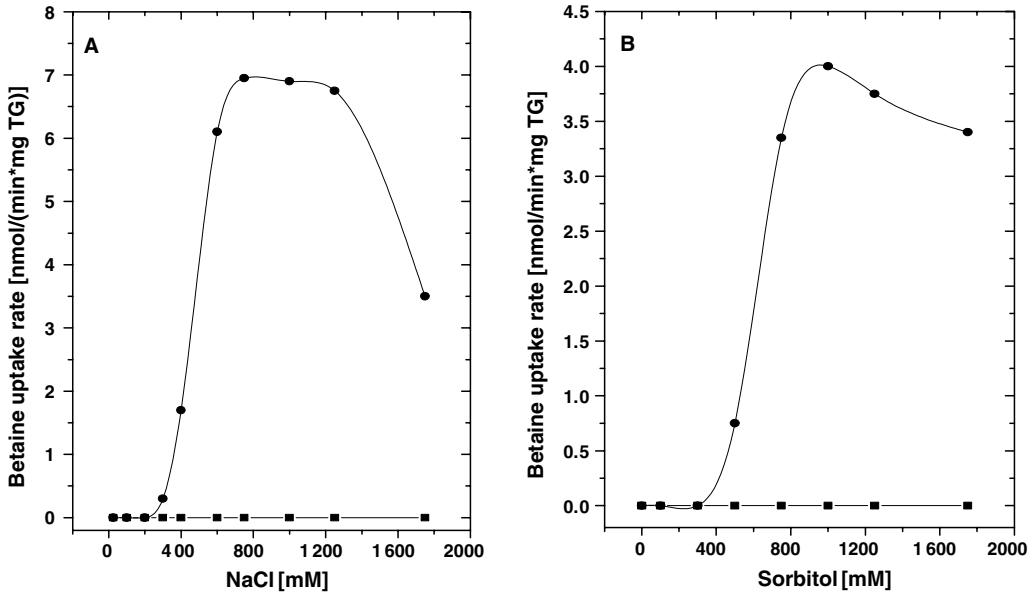

Fig. 3. Activation of betaine uptake by NaCl or sorbitol in *C. glutamicum* strains DHPF/pEKEX2-lcoP and the control strain DHPF/pEKEX2. Variation of the external osmolality by the addition of NaCl (A), or sorbitol (B). DHPF/pEKEX2-lcoP (circles); DHPF/pEKEX2 (squares). The osmolality of the buffer was 0.2 osmol/kg. Osmolality was increased by up to 3.5 osmol/kg by NaCl or by sorbitol by up to 2.2 osmol/kg.

the activities of BetP, EctP and LcoP were differently changed by addition of local anesthetics, indicating that the physical state of the membrane has a different influence on BCCT carrier activity. On the other hand, no conserved residues within the C-terminal domains of BetP, EctP, and LcoP are found (Fig. 2), although in the case of BetP a stretch of 25 amino acids was shown to be important for K^+-sensing, which is the key event for BetP activation [19,20]. Therefore, a common activation mechanism for the BCCT carriers seems to be unlikely.

Besides regulation at the level of activity, we also analyzed whether the *lcoP* gene is induced under hyperosmotic stress. For this purpose exponentially growing cells, pre-adapted to low osmolality medium, were exposed to different hyperosmotic shifts (from 0.3 to 0.9 osmol/kg, from 0.3 to 1.8 osmol/kg, from 0.3 to 2.4 osmol/kg, or from 0.9 to 2.4 osmol/kg). Cells were harvested after the indicated times and the RNA was isolated (Fig. 5). RNA hybridization analyses revealed (Fig. 5) that the expression level of *lcoP* depended on the osmolality of the medium (0.3 versus 0.9 osmol/kg) and on the extent of the hyperosmotic shift. A small change of the osmolality led to a smaller expression change as compared to severe osmotic stress. Furthermore, the time course of the response was influenced, too. *C. glutamicum* cells needed more time for the initiation of the expression response after an extreme upshock from 0.3 to 2.4 osmol/kg in comparison to cells which were already pre-adapted to 0.9 osmol/kg and shifted to 2.4 osmol/kg. Taken together, these results demonstrated that the expression of *lcoP* strictly depends on the external osmolality.

Table 1
Kinetic parameters of LcoP

Kinetic parameters	*C. glutamicum* DHPF/pEKEX2-lcoP
K_m betaine (μM)	154 ± 15
K_m ectoine (μM)	539 ± 41
K_m Na^+ (mM)	36 ± 2
V_{max} betaine (nmol/(min mg dw))	8.5 ± 0.2
V_{max} ectoine (nmol/(min mg dw))	8.6 ± 0.3

The values were derived from three independent measurements.

Although LcoP is efficiently regulated at the level of expression and activity, the rather low transport rates of LcoP in comparison to other osmoregulated transporters of *C. glutamicum* led to the question of the putative physiological relevance of this system. To investigate whether LcoP is sufficient to protect *C. glutamicum* against hyperosmotic conditions, the influence of 10 mM betaine on growth was investigated in ATCC 13032 (wild type), DHPE (ΔbetPΔputPΔproPΔectP), and DHPF (ΔbetPΔputPΔproPΔectPΔlcoP). For this purpose an overnight culture, grown in minimal medium CgXII, was used to inoculate fresh minimal medium containing additionally 1 M NaCl (Fig. 6A). Whereas for wild type and DHPE, addition of 10 mM betaine significantly reduced the lag phase compared to the non-supplemented strains, no beneficial effect of betaine was observed in DHPF. Consequently, LcoP alone is still able to protect DHPE to the same extent as the joint contribution of all osmoregulated uptake systems in wild type cells under the conditions tested. Recently, we showed that under conditions of high osmolality the accumulation of betaine causes a reduction of the cytoplasmic proline pool [21], which was shown to be the major compatible solute in nitrogen-rich medium in the absence of external compatible solutes [14]. A similar influence of betaine on proline accumulation was detected in wild type and DHPE but not in DHPF, thus further proving that this strain is devoid of any betaine uptake system (Fig. 6B). The analysis of DHPF furthermore shows that although being strongly involved in the osmostress response of *C. glutamicum*, these uptake systems are not essential for cell's survival under conditions of severe osmotic stress at

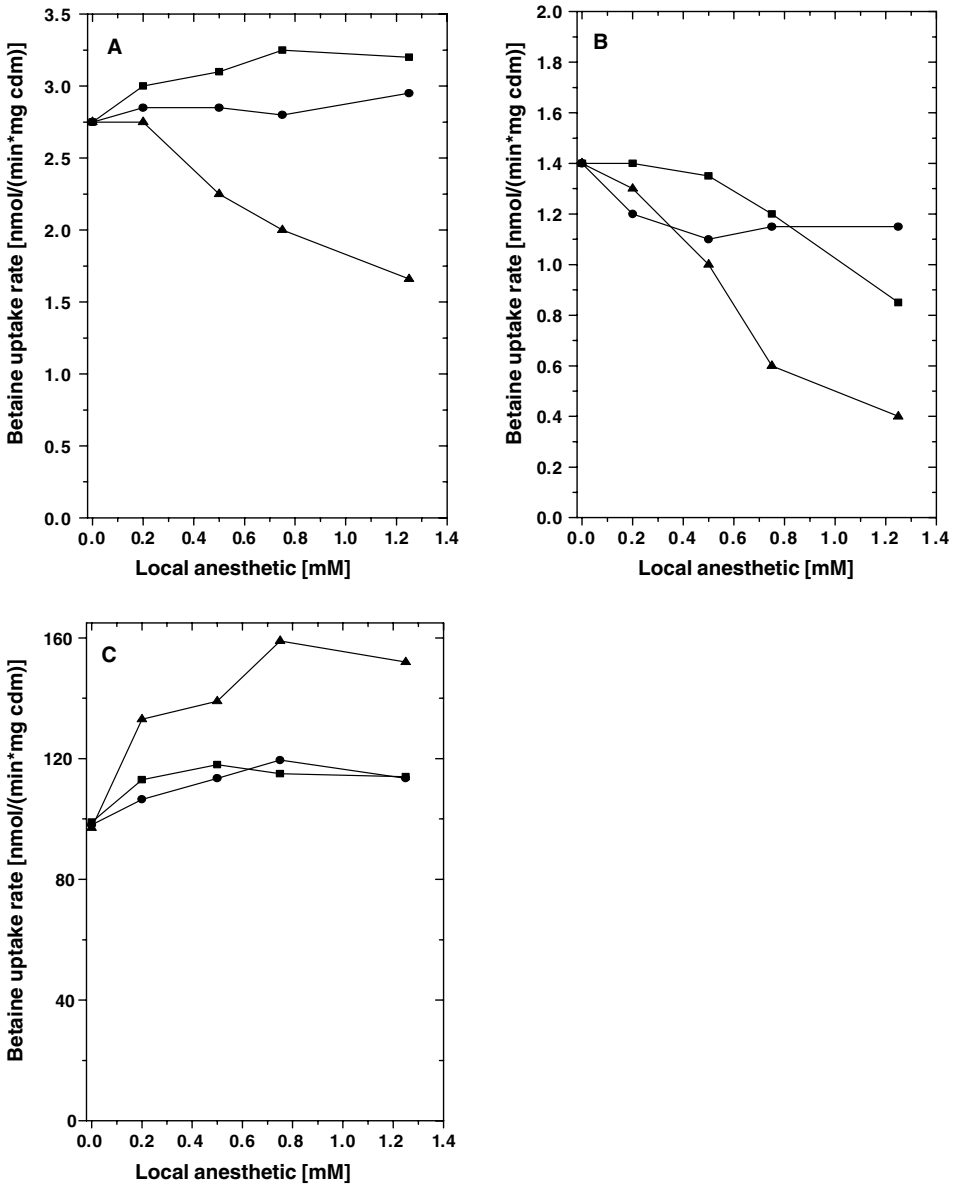

Fig. 4. Activity regulation of LcoP (A), EctP (B), and BetP (C) in dependence of the local anesthetic concentration. The external osmolality was adjusted by addition of 450 mM NaCl. At this concentration, the carriers reached 30% of the fully stimulated transport activity. Tetracaine (triangles), decane (circles), capric acid (squares).

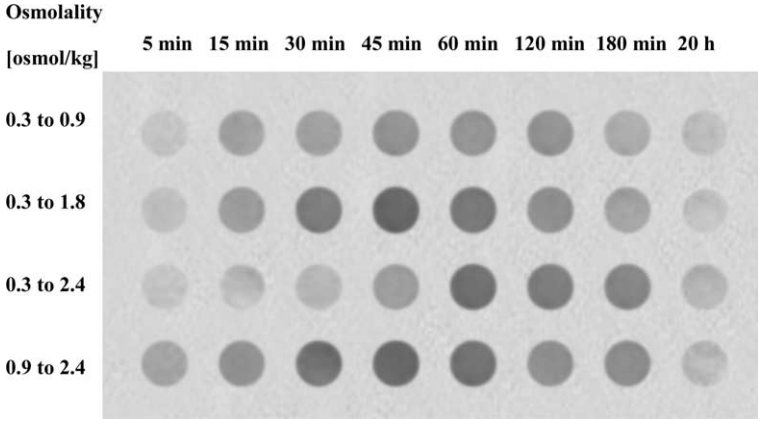

Fig. 5. RNA hybridization experiments: Induction of *lcoP* expression by different osmotic upshifts.

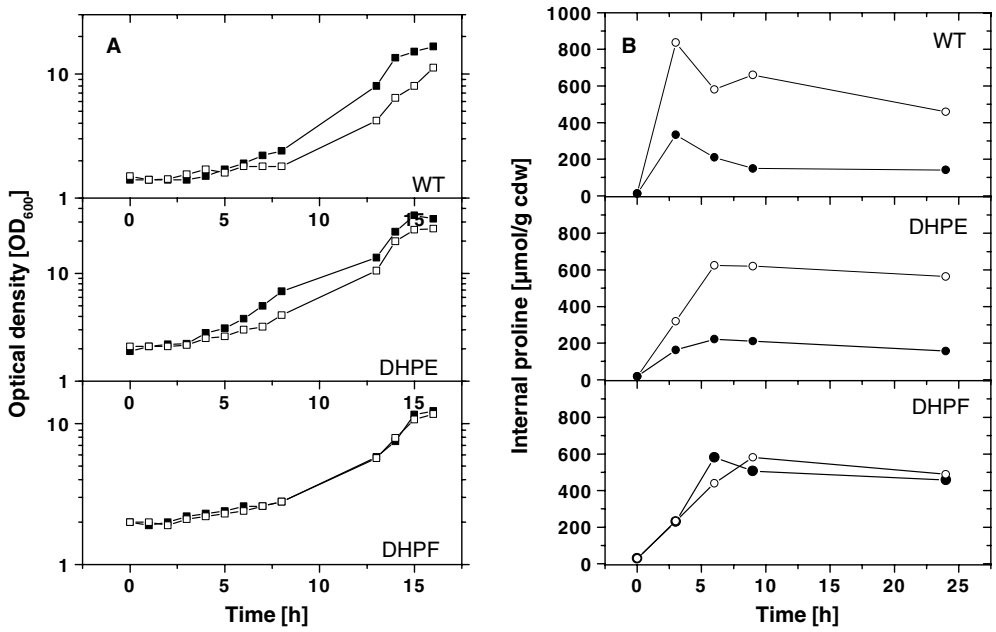

Fig. 6. Growth and internal proline content of several *C. glutamicum* strains in CgXII medium at an external osmolality of 2.4 osmol/kg in the presence (closed symbols) or absence (open symbols) of 10 mM betaine. Growth (A) and proline content (B). Wild type, DHPE (ΔbetPΔputPΔproPΔectP) and DHPF (ΔbetPΔputPΔproPΔectPΔlcoP).

least under the conditions tested. Obviously, the de novo synthesis of proline, glutamine and trehalose [14] in DHPF is sufficient to allow growth even at an external osmolality of 2.4 osmol/kg, although the lag phase after the hyperosmotic shock is prolonged. Nevertheless, the high redundancy of uptake systems and biosynthesis pathways for compatible solutes is a strong indication for the importance of effective protection mechanisms against hyperosmotic stress in *C. glutamicum*. Future work must reveal which individual role the osmoregulated uptake carriers have within the osmotic stress response of *C. glutamicum*. A mixture between constitutive rescue systems and carriers, which are effectively regulated at the level of expression by the prevailing osmotic conditions, would allow a sophisticated stress adaptation. Nevertheless, the function of a system, like LcoP, which has only a rather small uptake rate, is still puzzling but may be understood in view of the fact that growth in the natural habitat, i.e., the soil, is more restricted than under controlled laboratory conditions. Thus, even the small contribution of LcoP within the scenario of carriers for compatible solutes may provide a growth advantage under extreme conditions.

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