Osmotic regulation of transport processes in Lactobacillus plantarum
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The Mechanism of Osmotic Activation of the Quaternary Ammonium Compound Transporter (QacT) of \textit{Lactobacillus plantarum}

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ABSTRACT

The accumulation of quaternary ammonium compounds (QAC) in *Lactobacillus plantarum* is mediated via a single transport system with a high affinity for glycine betaine ($K_m^{\text{app}}$ of 18 µM) and other positively charged analogues, and a low affinity for proline ($K_m^{\text{app}}$ of 950 µM). Mutants defective in the uptake of glycine betaine were generated by UV irradiation, and selected on the basis of the resistance towards dehydroproline, a toxic proline analogue. Three independent DHP-resistant mutants showed reduced glycine betaine uptake rates and accumulation levels, but behaved similar to the wild type in terms of direct enzymatic activation of uptake by high osmolality conditions. Kinetic analysis of glycine betaine uptake and efflux in the wild type and mutant cells is consistent with one uptake system for quaternary ammonium compounds in *L. plantarum* and separate system(s) for their excretion. The mechanism of osmotic activation of the quaternary ammonium compound transport system (QacT) was studied. It was observed that the uptake rates were inhibited by the presence of internal substrate. Upon raising the medium osmolality, the QacT system was rapidly activated (increase in maximal velocity) through an diminished inhibition by *trans* substrate as well as an effect that is independent of intracellular substrate. We also studied the effects of the cationic amphipath chlorpromazine, which inserts into the cytoplasmic membrane, and thereby influences the uptake and efflux of glycine betaine. The results provide further evidence for the notion that the rapid efflux of glycine betaine upon osmotic downshock is mediated by a channel protein that is responding to membrane stretch or tension. The activation of QacT upon osmotic upshock seems to be effected by a turgor-related parameter other than membrane stretch or tension.

INTRODUCTION

Bacteria protect themselves against high external osmolality by the uptake or synthesis of a limited number of so-called compatible solutes. The predominant compatible solute in many organisms is glycine betaine, which usually is accumulated through an osmoregulated uptake system. Analogues of glycine betaine have been found in several bacteria, and many glycine betaine uptake systems facilitate their uptake as well. The osmotic regulation of the transport systems may occur at the genetic or enzymatic level or both, and these aspects have been studied in most detail in enteric bacteria. In *Escherichia coli* glycine betaine (and proline)
are taken up via a low affinity secondary transport protein (ProP), and a high affinity ATP-binding cassette transport system (ProU) (Csonka, 1989). Both ProP- and ProU-mediated uptake are stimulated (at the protein level) by an increase in external osmolality, although the mechanisms of osmosensing are most likely different (Poolman and Glaasker, 1998; Milner et al., 1987; Koo et al., 1991; Faatz et al., 1988). Homologues of ProU have been identified in the Gram-positive bacterium Bacillus subtilis (Kappes et al., 1996; Kempf and Bremer, 1995), whereas a homologue of ProP is present in Erwinia chrysanthemi (Gouesbet et al., 1996). Important structural information regarding the nature of the osmosensing domain has recently been obtained for the BetP protein of Corynebacterium glutamicum, a member of the third family of osmoregulated uptake systems for glycine betaine and proline (Peter et al., 1998). There is clear evidence that the carboxyl-terminal region (55 amino acids) has a central role in osmosensing.

Glycine betaine is the major compatible solute in the cytoplasm of Lactobacillus plantarum grown in high-salt chemically defined media containing glycine betaine. L. plantarum is unable to synthesize or metabolize glycine betaine, and the final accumulation levels of glycine betaine are thus solely determined by the relative rates of uptake and efflux (Glaasker et al., 1996a). Previous studies have indicated that osmotic regulation of glycine betaine uptake occurred mainly at the protein level, whereas changes in protein expression were relatively small as compared to systems such as ProU (Lucht and Bremer, 1994). However, it could not be excluded that more than one system effected the uptake, whereas efflux of glycine betaine upon osmotic downshock seemed to be mediated by more than one efflux system (Glaasker et al., 1996b). In this study mutants defective in glycine betaine uptake were generated and characterized to elucidate the contribution of the transport systems to the overall flux of glycine betaine. We also describe the substrate specificity and the kinetics of the glycine betaine uptake system under high and low osmolality conditions, as well as the effect of a cationic amphipath on the uptake and efflux activities in L. plantarum.

MATERIALS AND METHODS

Bacterial strains, culture conditions and media. Lactobacillus plantarum ATCC 14917 was grown at 30°C in a chemically defined medium (CDM) or modified-CDM (without proline) at pH 6.7, containing 0.5% (w/v) glucose as described previously (Glaasker et al., 1996a). High osmolality media were obtained by adding 0.8 M KCl to the standard CDM.

Isolation of mutants defective in glycine betaine uptake. A 3 ml aliquot of exponentially
growing cells (A\text{660} of 0.2-0.6) in low osmolality CDM, was dispersed over a petri-dish (diameter 9 cm), and irradiated for 1 minute with Ultra Violet light of 254 nm at a distance of 24 cm from the petri-dishes. The survival rate was around 1% as estimated from the plating of irradiated and non-irradiated samples on MRS agar plates. The irradiated culture was washed and concentrated in CDM without proline, and, subsequently, plated confluent on CDM agar plates without proline. The toxic proline analogue dehydroproline (DHP; 20 µl of a 100mM solution) was spotted in the middle of the plates (Patchett et al., 1992). After 48 hours of incubation, putative DHP-resistant mutants were picked from the colony free zone around the DHP spots. DHP is a toxic proline analogue that was found to competitively inhibit the uptake of glycine betaine and proline. Transport of leucine and glutamic acid was not affected by DHP (data not shown). Several independently isolated DHP-resistant mutants were able to grow on CDM agar without proline in the presence of 1 mM of DHP. The protein patterns of three mutants (DHP\text{R}-38.1, -38.2, -38.3) were analyzed on a Comassie-stained SDS-polyacrylamide gel, and were found to be similar to each other. The patterns of the mutants differed from that of the wild type in four protein bands with apparent molecular masses of 120, 90, 75, and 31 kDa (data not shown). These protein bands are missing, or at least significantly reduced in the three mutants.

**Transport assays.** Uptake assays, and enzymatic synthesis of \textsuperscript{14}C-glycine betaine from [N-methyl-\textsuperscript{14}C] choline (40-60 mCi/mmol), and uptake of \textsuperscript{14}C-proline (260 mCi/mmol) were performed as described previously (Glaasker et al., 1996a). Briefly, the cells were washed and resuspended in 50 mM potassium phosphate, pH 6.5 plus 0.8 M KCl or 50 mM potassium phosphate, pH 6.5. The latter buffer system was used to release most of the compatible solutes that were accumulated during the growth under high-osmolality conditions. The data of the kinetic experiments were fitted with the Michaelis-Menten equation, from which the apparent affinity constant (K\textsubscript{m,app}) and maximal rate of uptake (V\textsubscript{max}) were calculated.

**Miscellaneous.** Protein was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as a standard. Total protein extracts of wild type and DHP-resistant mutants of \textit{L. plantarum} were subjected to SDS-polyacrylamide (10% w/v) gelelectrophoresis after lysis of the cells by sonication. The osmolalities of media and buffers were measured by freezing-point depression with an Osmomat 030 (Gonotec, Berlin, Germany). Growth experiments were performed in sterile low protein-binding microplates. Plate wells containing 200 µl of culture were sealed by adding 75 µl of sterile silicone oil (1.03 g/ml), and growth rates were determined from A\text{620} increases using a Multiscan MCC/340 MKII (Flow Laboratories, Lugano, Switzerland). For the calculation of intracellular concentrations a value of 3 µl/mg of protein for the specific internal volume was used.

**RESULTS AND DISCUSSION**

**Substrate specificity of the glycine betaine uptake system.** In several Gram-positive and Gram-negative bacteria, proline and glycine betaine are taken up via the same system despite their difference in molecular structure (Milner et al., 1987; Pourkomalian and Booth, 1994; Molenaar et al., 1993). In \textit{L. plantarum}, a 100-fold excess of unlabelled proline did not affect glycine betaine uptake, whereas proline transport was completely inhibited by a 100-fold excess of unlabelled glycine.
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Betaine (Table I). Experiments with other substrate-analogues showed that glycine betaine uptake was strongly inhibited by tetra-methyl-ammonium and even by di-methyl-sulphonium-propionic acid, i.e. compounds with a positive charge at the nitrogen or sulphur atom. The substrate analogue carnitine abolished the uptake to the same extent as glycine betaine itself. Substrate analogues without a positive charge, like di-methyl-glycine, tri-methyl-amine, and 4-amino-butyric acid, had no significant inhibitory effect on the glycine betaine uptake rates. In fact, some of these compatible solutes stimulated the glycine betaine uptake. Since the analogues were added at a concentration of 125 mM, the increased uptake most likely reflects osmotic activation of the glycine betaine uptake system (see below), which may even have masked small inhibitory effects.

The tetra-methyl-ammonium compounds, as well as the compounds without a positive charge, inhibited the uptake of proline completely. These results are consistent with a single uptake system, with a high affinity for glycine betaine (and other compounds with a positive charge at the nitrogen or sulphur atom) and a low affinity for proline (and other analogues without a positive charge). The transport system was termed QacT (Quarternary ammonium compound Transporter). To substantiate this conclusion we determined the kinetic parameters for glycine betaine and proline uptake under various osmotic conditions.

**Kinetic analysis of glycine betaine and proline uptake.** With cells grown in CDM and uptake assayed at low osmolality, the uptake of glycine betaine was monophasic with an apparent $K_m$ of 18 µM and a $V_{max}$ of 27 nmol/min per mg of protein (Table II; line 1). Proline uptake was also monophasic under these conditions with an apparent $K_m$ of 950 µM and $V_{max}$ of 21 nmol/min per mg of protein (Table II; line 7). The uptake of glycine betaine and proline was also studied in cells cultured at high osmolality, but the kinetic parameters were not significantly different. The apparent $K_m$ for glycine betaine increased to 33 µM and the $V_{max}$ to 105 nmol/min per mg of protein, when high osmolality assay media were used (Table II; line 1). Similar changes were found for the uptake of proline, the apparent $K_m$ and $V_{max}$ for proline increased to 1500 µM and 150 nmol/min per mg of protein at high assay osmolality (Table II; line 7). These results indicate that the increased rate of uptake upon an osmotic upshift mainly involves an increased $V_{max}$ as a result of activation of QacT. Since the effects of culture and assay conditions on $K_m$ and $V_{max}$ are similar for glycine betaine and proline uptake, and the uptake is monophasic under all conditions tested, the data are best-explained by uptake via a single system.