

The Periplasmic Cyclodextrin Binding Protein CymE from *Klebsiella oxytoca* and Its Role in Maltodextrin and Cyclodextrin Transport

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***Klebsiella oxytoca* M5a1 has the capacity to transport and to metabolize α -, β - and γ -cyclodextrins. Cyclodextrin transport is mediated by the products of the *cymE*, *cymF*, *cymG*, *cymD*, and *cymA* genes, which are functionally homologous to the *malE*, *malF*, *malG*, *malK*, and *lamB* gene products of *Escherichia coli*. CymE, which is the periplasmic binding protein, has been overproduced and purified. By substrate-induced fluorescence quenching, the binding of ligands was analyzed. CymE bound α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin, with dissociation constants (K_d) of 0.02, 0.14 and 0.30 μ M, respectively, and linear maltoheptaose, with a K_d of 70 μ M. In transport experiments, α -cyclodextrin was taken up by the *cym* system of *K. oxytoca* three to five times less efficiently than maltohexaose by the *E. coli* maltose system. Besides α -cyclodextrin, maltohexaose was also taken up by the *K. oxytoca cym* system, but because of the inability of maltodextrins to induce the *cym* system, growth of *E. coli mal* mutants on linear maltodextrin was not observed when the cells harbored only the *cym* uptake system. Strains which gained this capacity by mutation could easily be selected, however.**

Cyclodextrins (CDs) are α -1,4-glycosidically linked, cyclic maltooligosaccharides. The main forms are α -, β -, and γ -CDs, which have 6, 7, and 8 glucose residues, respectively (5, 24). Their three-dimensional structure is torus shaped, with a hydrophilic outside ring and a interior hydrophobic cavity. Dependent on the size of the hydrophobic cavity of the respective CD, inclusion complexes with a variety of guest molecules can be formed, which is the basis of broad applications in industry (5, 24). CDs are formed enzymically from starch by CD-glucanotransferases (CGTases) a subgroup of the α -amylase class of enzymes (27, 35). One of the producers of CGTases is *Klebsiella oxytoca* M5a1 (1).

K. oxytoca can utilize starch as the sole carbon and energy source via two metabolic routes. The first one involves the extracellular degradation into linear maltodextrins by hydrolysis of the α -1,6-glycosidic bonds via the cell surface-associated pullulanase (1, 32) and the subsequent cleavage of the α -1,4-glycosidic linkages by the disproportionation activity of the extracellular α -CGTase (1). *Escherichia coli* (11, 15, 16, 36) and *Klebsiella pneumoniae* (7, 44) can then take up these linear maltodextrins (maltose up to maltoheptaose) via a binding protein-dependent ABC transporter (12) consisting of maltoporin (LamB), the maltodextrin-binding protein (MalE), the cytoplasmic membrane proteins (MalF and MalG), and the ATP-binding protein (MalK). Intracellularly, the linear maltodextrins are degraded into glucose and glucose-1-phosphate by the enzymes amylomaltase (MalQ) (31), the maltodextrin phosphorylase (MalP) (43), and maltodextrin glucosidase (MalZ) (30).

In the second pathway, present only in *K. oxytoca*, starch is converted extracellularly into CDs by the cyclization activity of

the α -CGTase, forming first and predominately α -CD, which later on is transformed into the thermodynamically favored β -CD (3, 35). The growth of *K. oxytoca* with CD as its sole carbon and energy source (2) is based on the uptake of the α - and β -CD via the *cym* system (17). Intracellularly, CDs are linearized by a cyclodextrinase (CymH) into linear maltooligosaccharides (14) which enter the maltose degradation pathway (17).

The genes responsible for CD metabolism were localized at the 5' side of the gene coding for the α -CGTase (6, 17). Sequence analysis demonstrated the homology of the gene products of *cymE*, *cymF*, *cymG*, and *cymD* to MalE, MalF, MalG, and MalK, respectively (17). In addition, a functional homology could be shown between the gene product of *cymA* and the maltoporin LamB (29). A mutational analysis showed that *cymE*, *cymF*, *cymD*, and *cymA* were essential for growth at the expense of CDs (17, 29).

The existence of a specific transport system for CDs, which are rigid molecules of considerable size (ranging from 1.37 nm for α -CD to 1.69 nm for γ -CD [outer diameter]), was unexpected. Since their transfer through the outer membrane and the cytoplasmic membrane may involve novel mechanisms, we have initiated the biochemical analysis. Here we report on the biochemical characterization of CymE as a periplasmic CD binding protein as well as the kinetic analysis of the *cym* transport system.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and growth conditions. The bacterial strains and the plasmids used in this study are listed in Table 1, together with their source or derivation.

The rich medium used was Luria-Bertani (LB) medium (26). *mal* and *cym* mutants were discriminated on MacConkey agar plates enriched with 0.5% (wt/vol) maltose or α -CD. The minimal medium employed was salt solution P supplemented with 12 mM ammonium sulfate (18). If not indicated otherwise, particular carbon sources were added at concentrations of 0.2 or 0.5%. The concentrations of antibiotics added and growth conditions were described previously (17).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
<i>Klebsiella oxytoca</i> derivatives		
M5a1	Wild type	1
CYME	Like M5a1, $\Delta(cymE)$	This work
<i>Escherichia coli</i> K-12 derivatives		
JM109	F' (<i>traD36 proAB⁺ lacI^q ZΔM15</i>) <i>recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44</i> $\Delta(lac-proAB)$ λ^-	46
MC4100	F ⁻ , $\Delta(argF-lac)$ U169 <i>araD139 ptsF25 deoC1 relA1 ffbB5301 rpsL150</i> λ^-	13
GM1	Like MC4100, <i>malE::Tn10</i> (Kan ^r)	17
JV2000	Like MC4100, <i>malS::Tn10</i> (Tet ^r) <i>phoA8 rbsR</i>	W. Boos
GM15	Like GM1, <i>malS::Tn10</i> (Tet ^r)	This work
<i>Escherichia coli</i> B derivatives; BL21 (DE-3)		
	F ⁻ <i>hsdS gal</i> λ (DE-3)	41
Plasmids		
pT7-H ₆ -TRXFUS	Ap ^r <i>H₆-trxA</i>	22
pCYME2 (pT7-H ₆ -TRXFUS derivative)	Ap ^r <i>H₆-trxA 'cymE</i>	This work
pSU2719	Cm ^r <i>lacZα</i>	25
pGM200 (pSU2719 derivative)	Cm ^r <i>cymA-J</i>	17
pCYM Δ E (pSU2719 derivative)	Cm ^r <i>cymA-D</i> $\Delta(cymE)$ <i>cymF-J</i>	17

Standard genetic procedures. Standard genetic procedures were adopted from Miller (26) and Sambrook et al. (33). Enzymes for recombinant DNA techniques were used according to the recommendations of the manufacturer.

Immunoblotting analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23) and subjected to immunoblotting analysis (34) with rat anti-CymH monoclonal antibodies at a dilution of 1:10,000 as described previously (14).

Construction of a *K. oxytoca cymE*-deletion mutant. The wild-type *K. oxytoca* strain was transformed with the plasmid pCYM Δ E carrying an in-frame deletion in *cymE* (17). The transformants were grown overnight in LB medium without antibiotics, and the resulting cultures were diluted to cell densities of 1×10^3 to 2×10^3 cells/ml and plated on MacConkey plates supplemented with 0.5% α -CD. Colorless colonies (about 1%) were purified and tested for the inability to grow with α -CD as the sole carbon and energy source as well as for their sensitivity to the antibiotic marker of the plasmid used. The presence of the deletion was verified by PCR employing oligonucleotides priming up- and downstream of the deletion (MC7, 5'-CCCATTTCCGGAATAGAT-3'; MC15, 5'-CAGCCAGGTAAGATTAC-3'). Finally, the strains were tested for complementation of the *cym* phenotype by transformation with a plasmid carrying a wild-type copy of the gene.

Construction of plasmid pCYME2. Plasmid pCYME2 is a derivative of the plasmid pT7-H₆-TRXFUS (22). It carries a translational fusion of the gene coding for a His-tagged thioredoxin, followed by an enterokinase cleavage site and the *cymE* gene lacking its signal sequence coding region (bp 1 to 60). For construction, the '*cymE*' part was amplified via PCR with the oligonucleotide primers CYME6 (5'-GCTTGGGGAGAGAGTATT-3') and CYME7 (5'-TTTCCCGTGCACAATAACATAGTTACTCT-3'). The resulting fragment was phosphorylated and cleaved with *Sal*I. The pT7-H₆-TRXFUS vector part was restricted with *Kpn*I, treated with Klenow fragment, subsequently cleaved by *Sal*I, and dephosphorylated. Finally, both fragments were ligated. All fusion joints and the entire '*cymE*' gene region were verified by sequencing.

Overproduction of the His-tag-thioredoxin-CymE fusion protein. For overproduction and purification of the His-tag-thioredoxin-CymE fusion protein, strain BL21 (DE-3) was transformed with plasmid pCYME2. Cells were grown aerobically at 37°C in 200 ml of LB medium and inoculated 1:100 with an overnight culture. At an A_{600} of 1.0, cells were induced with isopropyl- β -D-thiogalactopyranoside (IPTG; 0.5 mM final concentration), harvested 2 h later by centrifugation, and stored at -80°C.

Purification of CymE. The purification procedure was conducted at 4°C. The protein concentration was assayed during purification according to the method of Whitaker and Granum (45). The CymE content of the fractions was analyzed by SDS-PAGE (23) following the distribution of the 55-kDa band of the His-tag-thioredoxin-CymE fusion protein. After the enterokinase treatment, the appearance of the 39-kDa band of the mature form of CymE was monitored.

Cells were suspended in a 20-fold volume of buffer A (50 mM sodium phosphate [pH 7.4], 500 mM NaCl, 1 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride and broken by three consecutive passages through a French pressure cell at 16,000 lb/in². The extract was clarified by centrifugation for 10 min at 30,000 \times g, resulting in the S30 fraction.

The S30 fraction was applied to a Zn²⁺-chelating Sepharose FF column (1-ml bed volume) that had been equilibrated in buffer A. After loading, the column was rinsed with buffer A and developed with a 30-ml linear gradient ranging from 10 to 200 mM imidazol. The flow rate was 0.5 ml/min. Fractions containing the His-tag-thioredoxin-CymE fusion protein were pooled and dialyzed against buffer B (20 mM Tris-HCl [pH 7.6], 0.5 mM EDTA).

For the cleavage of the fusion protein, the dialysate was incubated with enterokinase (Boehringer Mannheim GmbH) for 6 h at 37°C at a ratio between fusion protein and enterokinase of 1:20 (wt/wt). To separate the mature form of CymE from the His-tag-thioredoxin part of the fusion protein and from the noncleaved fusion protein and minor impurities, ion-exchange chromatography through a Mono Q-HR 5/5 column (1-ml bed volume) was performed. The column was equilibrated with buffer B; after the sample was loaded, the column was washed with 5 ml of buffer B, developed with a 20-ml linear gradient reaching from 0 to 1 M KCl, and finally washed with 5 ml (each) of buffer B and buffer C (buffer B plus 1 M KCl). The flow rate was 0.5 ml/min. Fractions containing the mature form of CymE were pooled and dialyzed against buffer B. The solution was frozen in liquid nitrogen and stored at -80°C.

Fluorescence spectroscopy. Fluorescence spectroscopy was performed to measure the binding of substrates by the purified CymE (42). Fluorescence measurements were carried out in an LS50B Luminescence spectrometer (Perkin-Elmer, Norwalk, Conn.) with excitation and emission slits between 5 and 10 nm and at an excitation wavelength of 280 nm. The emission spectrum was monitored between 300 and 400 nm. Changes in the fluorescence intensity as a function of ligand concentrations were recorded at an emission wavelength of 355 nm. All measurements were performed at room temperature in 1 ml of 50 mM Tris-HCl (pH 7.6), which also served as a reference. Protein concentrations ranged from 0.8 to 8 μ g/ml, and the substrates were added in small volumes. Changes in the intensity of fluorescence emission due to dilutions were corrected with a control containing the same concentration of protein and receiving buffer instead of substrate. For determination of the respective K_d values, a series of substrate concentrations were examined for the degree of quenching of fluorescence. The results reported are the mean values of three consecutive measurements for 5 min each. Maximal quenching was set at 1. The substrate concentration resulting in half-maximal quenching was defined as the K_d and was derived from Lineweaver-Burk plots.

Chromatographic techniques. Linear and cyclic maltooligosaccharides were analyzed qualitatively by thin-layer chromatography (TLC) as described previously (14).

Transport assays. In order to measure transport, the bacteria were first adapted in a preculture to the carbon source employed. For the characterization of CD uptake, 0.5% α -CD or a mixture of 1% succinate plus 0.1% α -CD was used as the carbon source, whereas for studying the maltose system, 0.2% maltose was used as the carbon source. After inoculation of the main culture, cells were grown to the late exponential growth phase, harvested by centrifugation, washed three times with minimal medium without a carbon source, resuspended in the same medium, and adjusted in terms of their cell density. For transport measurements, samples of 3 or 6 ml of this stock solution were adjusted to 25°C. To start the uptake reaction, a small volume of the labeled substrate was

added (time zero). At different time intervals, samples of 500 or 1,000 μ l were withdrawn, filtrated through Schleicher & Schuell (Dassel) NC45 membrane filters (0.45- μ m pore size), washed with 10 ml of minimal medium and dried. Their radioactivity was determined in a TRI-CARB 2100 TR liquid scintillation analyzer (Packard, Dreieich, Germany) with the Ultima Gold scintillation cocktail (Packard). The rate of uptake was taken from the linear portion of the resulting curve (initial rate of uptake).

For the V_{\max} and K_m determination of α -CD and maltohexaose uptake, [α - 14 C]CD (1,890 mCi/mmol) and [14 C]maltohexaose (2 mCi/mmol) were synthesized as described by Pajatsch et al. (28) [α - 14 C]CD of low specific radioactivity (2 mCi/mmol) obtained from Wacker Chemicals (Munich, Germany) was also used. K_m and V_{\max} values were deduced from Lineweaver-Burk plots. Competition of [α - 14 C]CD uptake by unlabeled cyclodextrins or linear maltodextrins was measured with a saturating concentration (20 μ M) of the [α - 14 C]CD of the specific radioactivity (2 mCi/mmol) mixed with the indicated molar excess of unlabeled compounds.

To observe the metabolism of the α -CD taken up by the cells, chromatographic analysis of the intracellular low-molecular-weight compounds was performed. To this end, cells were incubated with a sufficient amount of the [α - 14 C]CD (2 mCi/mmol) to ensure maximal uptake rates over the entire incubation time. At the times indicated, samples were harvested by filtration, washed, frozen immediately in liquid nitrogen, and stored at -80°C . The cells were broken by resuspension in 1 ml of a solution containing 2.5% SDS and 1% chloroform. The solution was clarified by centrifugation, and the supernatant was desalted by the mixed bed ion-exchange material Serdolit MB-1 (Serva, Heidelberg, Germany). Finally, the solution was lyophilized, dissolved in a small volume of water, and separated by TLC. The TLC plate was then autoradiographed and developed.

Special chemicals. The unlabeled α -, β -, and γ -CDs, as well as the [α - 14 C]CD of low specific radioactivity (2 mCi/mmol), were gifts from Wacker Chemicals. [α - 14 C]CD of high specific radioactivity (1,890 mCi/mmol) and [14 C]maltohexaose (2 mCi/mmol) were synthesized as described previously (28). Nonradioactively labeled linear maltodextrins were purchased from Sigma Chemicals (Deisenhofen, Germany). [35 S]dATP used for sequencing was delivered by NEN/DuPont (Dreieich, Germany). Oligonucleotide primers were synthesized by MWG (Ebersberg, Germany). Molecular biological reagents were from Boehringer Mannheim GmbH, Pharmacia (Freiburg, Germany), or New England Biolabs (Schwalbach, Germany).

RESULTS AND DISCUSSION

Overproduction and purification of CymE. A fragment containing the part of the *cymE* gene corresponding to its mature (leaderless) form (17) plus 22 bp downstream of the coding region was amplified via PCR and cloned into the vector pT7-H6-TRXFUS to generate a translational fusion with the gene coding for a His-tagged thioredoxin, which at its C-terminus contains the cleavage site for enterokinase. Considerable attempts to overproduce native CymE from other vector systems and under many growth conditions only led to insoluble protein. For overproduction of the CymE fusion protein, the resulting plasmid, pCYME2, was transformed into *E. coli* BL21 (DE-3), and the expression was induced in LB medium at 37°C by the addition of IPTG. SDS-PAGE of a crude cellular extract showed that a protein of the expected molecular mass was overproduced (Fig. 1, lane 1), the major part in soluble form (data not shown). The purification involved the preparation of a $30,000 \times g$ supernatant, affinity chromatography on a Zn^{2+} -chelating Sepharose FF column, cleavage of the His-tag-thioredoxin-CymE fusion protein by enterokinase, and ion-exchange chromatography through a Mono-Q-HR5/5 column. A symmetrical peak consisting solely of the mature form of CymE was eluted from the final column (data not shown). Figure 1 demonstrates the course of purification as analyzed by SDS-PAGE of the pooled fractions from each step. From 200 ml of culture, 1 mg of purified CymE was obtained.

Specificity and affinity of substrate binding by CymE. The CymE protein contains six tryptophan residues, which, when excited at a wavelength of 280 nm in the absence of ligand, induce a fluorescence emission spectrum with a maximum of 338 nm (Fig. 2). The addition of 0.1 mM substrate such as α -CD (Fig. 2A) decreased the fluorescence intensity and caused a shift of the emission spectrum to shorter wavelengths,

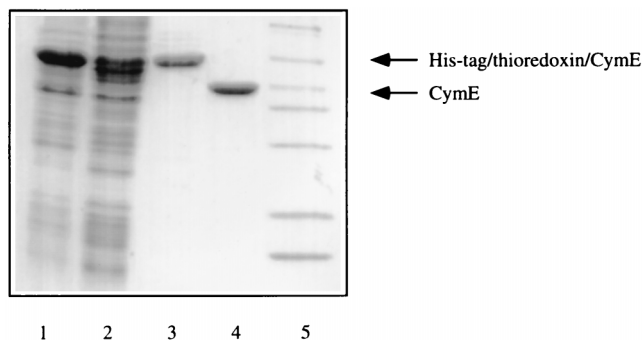


FIG. 1. Course of purification of the mature form of CymE followed by SDS-PAGE of the respective fractions. Lanes: 1, crude extract; 2, S30 fraction; 3, eluate of the Zn^{2+} -chelating Sepharose FF column; 4, eluate of the Mono-Q column; 5, molecular mass standard (97, 85, 55, 39, 33, 27, 19, and 14 kDa). Proteins were stained with Coomassie brilliant blue.

in the case of α -CD by about 4 nm. Addition of 0.1 mM β - and γ -CD and of 0.1 mM maltodextrins also quenched fluorescence, with a shift of the emission peak to shorter wavelengths, although this shift becomes less prominent with increasing size of the CDs or decreasing chain length of the maltodextrins, respectively (data not shown). Addition of maltose (Fig. 2B) was without effect. Since the substrate-induced quenching was maximal at 355 nm (Fig. 2A), this wavelength was used in further experiments.

The substrate specificity of CymE was analyzed by measuring the effect of addition of 0.1 mM cyclic and linear maltodextrins and of several mono- and disaccharides (Fig. 3). The mono- and disaccharides, including maltose, only marginally quenched fluorescence, which may be due to maltodextrin impurities. Linear maltodextrins of a chain length of 3 glucose units and longer, however, had a significant effect. The CDs displayed the highest degree of quenching.

The K_d values of CymE for the three CDs and for maltoheptaose were then determined by titrating the protein with a series of concentrations of the ligands and measuring the effect on fluorescence. As measured by fluorescence quenching, the affinity of CymE for the different sugars decreased with increasing size of the CDs as follows. The K_d values for α -CD, β -CD, and γ -CD were 0.02, 0.14, and 0.30 μM , respectively. The affinity for maltoheptaose (K_d , 70 μM) was much lower than those for the CDs.

CymE dependence of transport of CDs and maltodextrins into *K. oxytoca* and recombinant *E. coli* cells. To gain information on whether the binding affinity of CymE determines the in

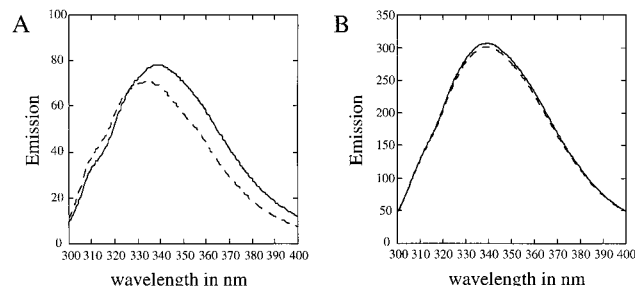


FIG. 2. Fluorescence quenching of CymE induced by α -CD (A) and maltose (B). Excitation was at 280 nm. The emission spectrum of CymE (A, 0.8 $\mu\text{g/ml}$; B, 4 $\mu\text{g/ml}$) before (solid lines) and after (dotted lines) the addition of 0.1 mM substrate is shown.

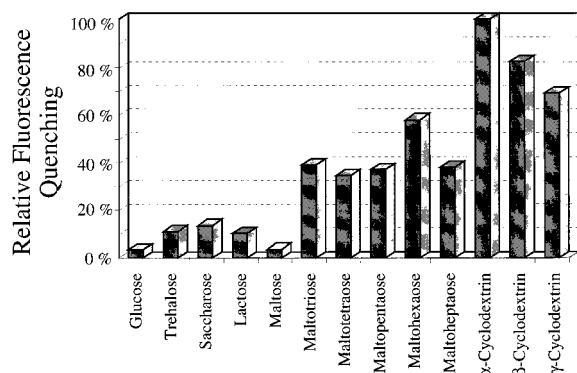


FIG. 3. Decrease in fluorescence of CymE (4 $\mu\text{g/ml}$) induced by different substrates at a concentration of 0.1 mM. The value exhibited by α -CD is set at 100%.

vivo affinity for cellular uptake, transport experiments with radioactively labeled α -CD and maltohexaose were conducted at different substrate concentrations. Several strains were used: *K. oxytoca* (wild type and a *cymE* mutant), *E. coli* GM15/pGM200 harboring a plasmid that encodes the *cym* genes except that for the cyclodextrin glucanotransferase (*cgt*), and *E. coli* GM15/pCYM Δ E with a deletion in *cymE* of pGM200 (both strains lack the *E. coli* maltose transport system because of a chromosomal mutation in *malE*). *E. coli* MC4100 served as control strain for the determination of the uptake of maltohexaose by the *mal* system. All strains, with the exception of MC4100, were grown under conditions used to induce the *cym* system. Table 2 gives the apparent K_m and V_{max} values for α -CD and maltohexaose. The results allow the following conclusions. (i) Under V_{max} conditions, the recombinant *E. coli* strain transports the two substrates at nearly identical rates. (ii) The apparent K_m for α -CD transport in *K. oxytoca* as well as in the recombinant *E. coli* strain is higher than the K_d of the binding protein (1 versus 0.02 μM). (iii) In the recombinant *E. coli* strain, the K_m for α -CD is nearly 10-fold higher than that for maltohexaose. Also, transport of maltohexaose via the *E. coli mal* system is more efficient than that via the *K. oxytoca cym* system.

Since only the initial rate of uptake was used, the kinetic constants of Table 2 reflect the properties of the uptake system and are not influenced by subsequent metabolism or by a possible feedback inhibition due to the accumulating substrate. We also monitored the intracellular fate of radioactive α -CD

TABLE 2. Kinetics of *cym* system-dependent uptake of α -CD and maltohexaose

<i>K. oxytoca</i> or <i>E. coli</i> strain ^a	V_{max} (nmol \cdot min ⁻¹) ^b		K_m (μM)	
	α -CD	Maltohexaose	α -CD	Maltohexaose
Wild type	1.0	ND ^c	0.7–1.0	ND
CYME	— ^d	ND	ND	ND
GM15/pGM200	1.5	1.2	0.7	6.5
GM15/pCYM Δ E	—	—	ND	ND
MC4100	ND	4.3	ND	0.33

^a *K. oxytoca* wild-type and CYME and *E. coli* GM15 strains were grown with a mixture of 1% succinate plus 0.1% α -CD as a carbon source, whereas for *E. coli* MC4100, the carbon source used was 0.2% maltose.

^b Values are for 10^9 cells.

^c ND, not determined.

^d —, from no uptake at all to about 3% of the V_{max} of the respective *cym*⁺ strain.

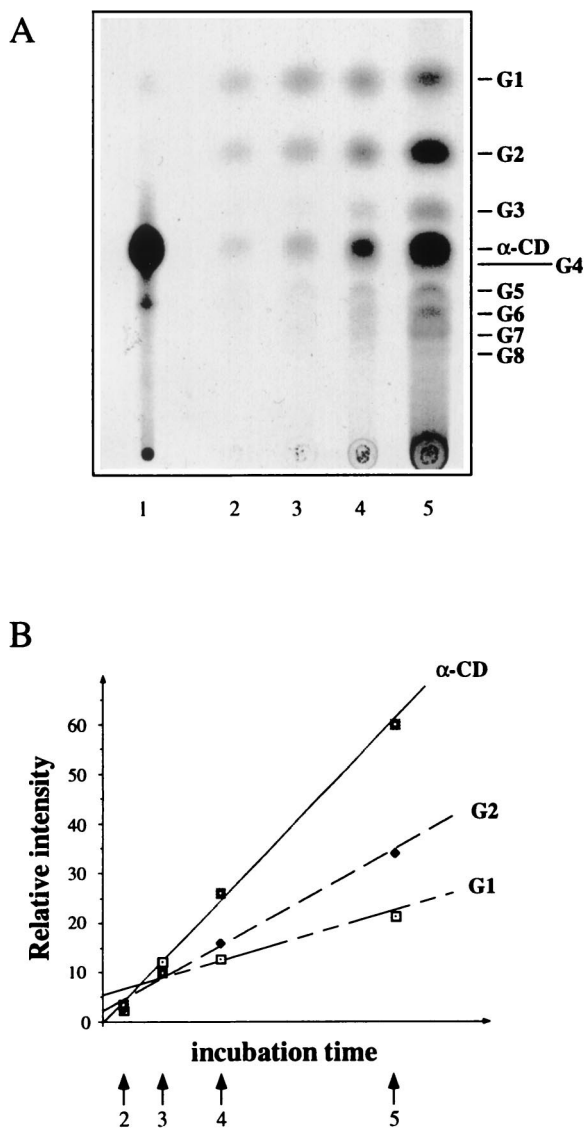


FIG. 4. TLC analysis of the intracellular compounds of the *K. oxytoca* wild type during the uptake of [α -¹⁴C]CD. (A) Scanned autoradiograph. Lanes: 1, authentic [α -¹⁴C]CD applied as standard; 2 to 5, accumulated radioactivity after 20 s, 1 min, 2 min, and 5 min of incubation with [α -¹⁴C]CD, respectively. (B) Comparison of increases in spot intensities for α -CD, G2, and G1. Arrows correspond to the respective lanes of the autoradiograph. G1 and G2 to G8 denote glucose and maltose to maltooctaose, respectively. The spot for α -CD may be contaminated by a minor amount of G4.

during the course of an uptake experiment with the *K. oxytoca* wild type. Figure 4 shows that α -CD is accumulated more rapidly than it is hydrolyzed, which indicates that it is taken up in an unaltered form.

Finally, we have carried out α -CD uptake competition experiments in the absence and presence of competing substrates (Fig. 5). Competition decreased with increasing size of CDs and with decreasing chain length of linear maltooligosaccharides; maltose, again, was without any effect. This pattern roughly reflects the binding affinities of CymE towards these substrates.

Can the *cym* system support growth at the expense of maltooligosaccharides? The results described above have shown that CymE binds maltooligosaccharides, that maltooligosac-

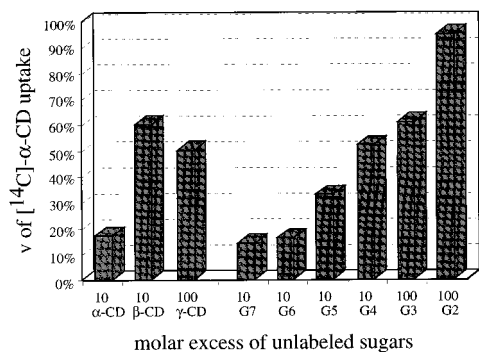


FIG. 5. Competition of the uptake of [^{14}C]CD (20 μM) by the addition of unlabeled substrates. The % v (V_{max}) values are relative to the rate of uptake of [^{14}C]CD. G2 and G3 to G7 denote maltose and maltotriose to maltoheptaose, respectively. The competing substrates were added at the indicated fold concentration.

charides can be transported in a CymE-dependent fashion, and that they compete with the uptake of α -CD. Therefore, one would assume that the *cym* system would allow the cells to grow on these linear maltooligosaccharides. This assumption was tested with strain GM15/pGM200 (Fig. 6A). It grows well in α -CD-containing minimal medium, with a mean doubling time of about 2 h. When subcultured into medium with linear maltodextrins from maltotetraose to maltoheptaose, growth

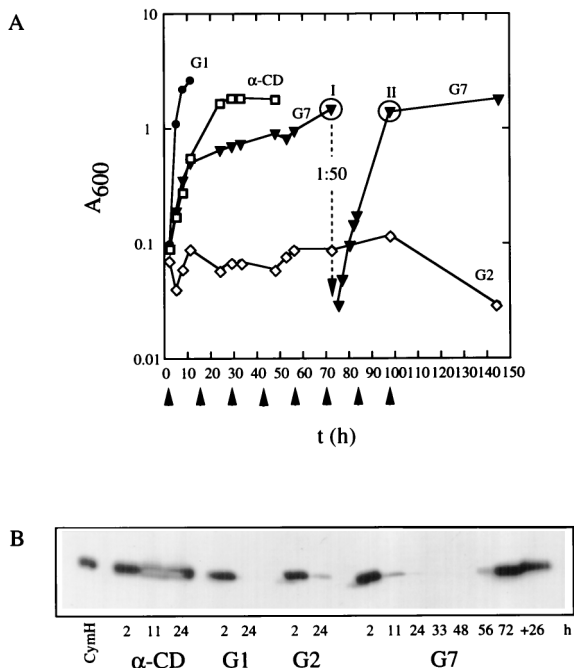


FIG. 6. (A) *cym* system-dependent growth of strain GM15/pGM200 with maltoheptaose (G7) as the carbon source. The preculture was grown with α -CD as the carbon source. Before being subcultured, the cells were harvested, washed, and resuspended in minimal medium. I and II indicate the time points at which samples were withdrawn from the culture to retrieve single colonies which consecutively were characterized. (B) Immunoblotting analysis of the expression of the *cym* system in strain GM15/pGM200 during growth with maltoheptaose (G7) as the carbon source. At the time points indicated, samples of the respective cultures were withdrawn, and a crude extract of the harvested cells was used for immunoblotting with an anti-CymH monoclonal antibody (14). G1 and G2 denote glucose and maltose, respectively. The amount of purified CymH (14) used was 40 ng.

continued with doubling times at least for maltohexaose and maltoheptaose comparable to those with α -CD, but decreased thereafter and finally stopped. However, after a considerable lag, growth resumed and a resubculture grew without a lag. For maltohexaose and maltoheptaose, this lag phase lasted at least 36 h, and it was even longer on maltopentaose and maltotetraose. No growth took place at all on maltose and maltotriose (not shown). Growth on maltodextrins from maltotetraose to maltoheptaose was completely blocked when a mutation was present in *cymE* (not shown).

To test whether the resumption of growth is the consequence of a selection of mutants, we have taken samples at the two time points indicated in Fig. 6. Only 20% of the colonies retrieved from the culture at time I were able to grow on α -CD and on maltoheptaose without a lag phase. This proportion increased to 80% at time 2.

Figure 6B shows that the ability to grow on α -CD correlates precisely with the expression pattern of *cymH*, an enzyme essential for CD metabolism (14, 17). The results of the experiment therefore show that the *cym* system can be used for the uptake of linear maltodextrins, but linear maltodextrins are unable to induce *cym* gene expression. Mutations, however, can be selected which lead to expression. The underlying mutations will be characterized in the future.

In conclusion, the results from previous work—that *cym* genes code for products of a transport system specific for CDs—have been established now by the *in vitro* analysis of CymE, the periplasmic binding protein, as well as by transport studies. Meanwhile, the gene product of *cymA* also has been identified as the functional homolog of the maltoporin LamB (29). CymE binds CDs, especially α -CD, with high affinity. It also binds linear maltooligosaccharides with a chain length of 3 and higher. However, binding appears to be productive and competent for transport only for α -CD and β -CD and for the longer linear maltooligosaccharides. The metabolism of γ -CD requires the activity of CGTase (17), which allows the conclusion that γ -CD has to be converted into α - or β -CD or linearized (3, 4, 29) by this enzyme to be transported. The fact that γ -CD does not support growth (17) could reside in the fact that its complex with CymE does not acquire the conformation required for docking to the membrane CymFG partner (37, 39, 40).

A similar situation has been described for MalE. MalE binds α -CD and β -CD with high affinity (K_d values of 4 and 1.8 μM , respectively) (38), but in the form of an unproductive complex (17, 19–21, 37, 38).

The K_d of CymE for α -CD binding is about 30-fold lower than the K_m in the uptake reaction. Since it appears that the outer membrane does not limit the diffusion (10), the uptake process must be limited at some other step, most probably at the interaction of the substrate-loaded CymE with the membrane complex. Possibly, the amount of CymE is not large enough to ensure equality of K_d with K_m (8, 9).

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REFERENCES

- Bender, H. 1977. Cyclodextrin-Glucanotransferase von *Klebsiella pneumoniae*. 1. Synthese, Reinigung und Eigenschaften des Enzyms von *Klebsiella pneumoniae* M5a1. Arch. Microbiol. **111**:271–282.
- Bender, H. 1977. Cyclodextrin-Glucanotransferase von *Klebsiella pneumoniae*. 2. Bedeutung des Enzyms für den Metabolismus der Cyclodextrine

- bei *Klebsiella pneumoniae* M5a1. Arch. Microbiol. **113**:49–66.
3. **Bender, H.** 1980. Kinetische Untersuchungen der durch die Cyclodextrin-Glycosyltransferase katalysierten (1→4)- α -D-Glucopyranosyltransferreaktionen, insbesondere der Zyklisierungsreaktion, mit (1→4)- α -D-Glucopyranosylketten (durchschnittlicher Polymerisationsgrad von 16) als Substrat. Carbohydr. Res. **78**:133–145.
 4. **Bender, H.** 1981. Enzymology of the cyclodextrins, p. 77–88. In Proceedings of the First International Symposium on Cyclodextrins. D. Reichel Publishing Company, Dordrecht, The Netherlands.
 5. **Bender, H.** 1986. Production, characterization, and application of cyclodextrins. Adv. Biotechnol. Proc. **6**:31–71.
 6. **Binder, F., O. Huber, and A. Böck.** 1986. Cyclodextrin-glycosyltransferase from *Klebsiella pneumoniae* M5a1: cloning, nucleotide sequence and expression. Gene **47**:269–277.
 7. **Bloch, M.-A., and O. Raibaud.** 1986. Comparison of the *malA* regions of *Escherichia coli* and *Klebsiella pneumoniae*. J. Bacteriol. **168**:1220–1227.
 8. **Bohl, E., H. A. Shuman, and W. Boos.** 1995. Mathematical treatment of the kinetics of binding protein-dependent transport systems reveals that both the substrate loaded and unloaded binding proteins interact with the membrane components. J. Theor. Biol. **172**:83–94.
 9. **Bohl, E., and W. Boos.** 1997. Quantitative analysis of binding protein-mediated ABC transport systems. J. Theor. Biol. **186**:65–74.
 10. **Bohl, E., M. Pajatsch, and W. Boos.** Unpublished data.
 11. **Boos, W., R. Peist, K. Decker, and E. Zdych.** 1996. The maltose system, p. 201–229. In E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Company, Georgetown, Tex.
 12. **Boos, W., and J. M. Lucht.** 1996. Periplasmic binding protein-dependent ABC transporters, p. 1175–1209. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: Cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
 13. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
 14. **Feederle, R., M. Pajatsch, E. Kremmer, and A. Böck.** 1996. Metabolism of cyclodextrins by *Klebsiella oxytoca* M5a1: purification and characterisation of a cytoplasmically located cyclodextrinase. Arch. Microbiol. **165**:206–212.
 15. **Ferenci, T.** 1980. The recognition of maltodextrins by *Escherichia coli*. Eur. J. Biochem. **108**:631–636.
 16. **Ferenci, T., M. Muir, K.-S. Lee, and D. Maris.** 1986. Substrate specificity of the *Escherichia coli* maltodextrin transport system and its component proteins. Biochim. Biophys. Acta **860**:44–50.
 17. **Fiedler, G., M. Pajatsch, and A. Böck.** 1996. Genetics of a novel starch utilisation pathway in *Klebsiella oxytoca*. J. Mol. Biol. **256**:279–291.
 18. **Fraenkel, D. G., and F. C. Neidhardt.** 1961. Use of chloramphenicol to study control of RNA synthesis in bacteria. Biochim. Biophys. Acta **53**:96–100.
 19. **Hall, J. A., K. Gehring, and H. Nikaido.** 1997. Two modes of ligand binding in maltose-binding protein of *Escherichia coli*: correlation with the structure of ligands and the structure of binding protein. J. Biol. Chem. **272**:17605–17609.
 20. **Hall, J. A., T. E. Thorgeirsson, J. Liu, Y.-K. Shin, and H. Nikaido.** 1997. Two modes of ligand binding in maltose-binding protein of *Escherichia coli*: electron paramagnetic resonance study of ligand-induced global conformational changes by site-directed spin labeling. J. Biol. Chem. **272**:17610–17614.
 21. **Hall, J. A., A. K. Ganesan, J. Chen, and H. Nikaido.** 1997. Two modes of ligand binding in maltose-binding protein of *Escherichia coli*: functional significance in active transport. J. Biol. Chem. **272**:17615–17622.
 22. **Kromayer, M., R. Wilting, P. Tormay, and A. Böck.** 1996. Domain structure of the prokaryotic selenocysteine-specific elongation factor SelB. J. Mol. Biol. **262**:413–420.
 23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227**:680–685.
 24. **Loftsson, T., and M. E. Brewster.** 1996. Pharmaceutical applications of cyclodextrins. I. Drug solubilisation and stabilization. J. Pharm. Sci. **85**:1017–1025.
 25. **Martinez, E., B. Bartolomé, and F. de la Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZa* reporter gene of pUC8/9 and pUC18/19 plasmids. Gene **68**:159–162.
 26. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 27. **Nakamura, A., K. Haga, and K. Yamane.** 1993. Three histidine residues in the active center of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011: effects of the replacement on pH dependence and transition-state stabilisation. Biochemistry **32**:6624–6631.
 28. **Pajatsch, M., A. Böck, and W. Boos.** Enzymatic preparation of radioactively labeled linear maltodextrins and cyclodextrins of high specific activity from [¹⁴C]maltose using amyloamylase, cyclodextrin glucanotransferase and cyclodextrinase. Carbohydr. Res., in press.
 29. **Pajatsch, M., G. Fiedler, and A. Böck.** Unpublished data.
 30. **Peist, R., C. Schneider-Fresenius, and W. Boos.** 1996. The MalT-dependent and *malZ*-encoded maltodextrin glucosidase of *Escherichia coli* can be converted into a dextrinyltransferase by a single mutation. J. Biol. Chem. **271**:10681–10689.
 31. **Pugsley, A. P., and C. Dubreuil.** 1988. Molecular characterisation of *malQ*, the structural gene for the *Escherichia coli* enzyme amyloamylase. Mol. Microbiol. **2**:473–479.
 32. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57**:50–108.
 33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 34. **Schmid, G., and A. Böck.** 1984. Immunoblotting analysis of ribosomal proteins from archaeobacteria. Syst. Appl. Microbiol. **5**:1–10.
 35. **Schmid, G.** 1996. Enzymology of cyclodextrins, p. 615–626. In J. L. Atwood, J. E. D. Davies, D. D. MacNicol, and F. Vögtle (ed.), Comprehensive supramolecular chemistry, vol. 3. Cyclodextrins. Elsevier Science, Ltd., Oxford, United Kingdom.
 36. **Schwartz, M.** 1987. The Maltose regulon, p. 1482–1502. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
 37. **Sharff, A. J., L. E. Rodseth, J. C. Spurlino, and F. A. Quijcho.** 1992. Crystallographic evidence of a large ligand-induced hinge-twist motion between the two domains of the maltodextrin binding protein involved in active transport and chemotaxis. Biochemistry **31**:10657–10663.
 38. **Sharff, A. J., L. E. Rodseth, and F. A. Quijcho.** 1993. Refined 1.8 Å structure reveals the mode of binding of β -cyclodextrin to the maltodextrin binding protein. Biochemistry **32**:10553–10559.
 39. **Shilton, B. H., H. A. Shuman, and S. L. Mowbray.** 1996. Crystal structures and solution conformations of a dominant-negative mutant of *Escherichia coli* maltose-binding protein. J. Mol. Biol. **264**:364–376.
 40. **Spurlino, J. C., G.-Y. Lu, and F. A. Quijcho.** 1991. The 2.3-Å resolution structure of the maltose- or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis. J. Biol. Chem. **266**:5202–5219.
 41. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. J. Mol. Biol. **189**:113–130.
 42. **Szmelcman, S., M. Schwartz, T. J. Silhavy, and W. Boos.** 1976. Maltose transport in *Escherichia coli* K12. A comparison of transport kinetics in wild-type and λ -resistant mutants with the dissociation constants of the maltose binding protein as measured by fluorescence quenching. Eur. J. Biochem. **65**:13–19.
 43. **Watson, K. A., R. Schinzel, D. Palm, and L. N. Johnson.** 1997. The structure of *Escherichia coli* maltodextrin phosphorylase provides an explanation for the activity without control in this basic archetype of a phosphorylase. EMBO J. **16**:1–14.
 44. **Werts, C., A. Charbit, S. Bachellier, and M. Hofnung.** 1992. DNA sequence analysis of the *lamB* gene from *Klebsiella pneumoniae*: implications for the topology and the pore functions in maltoporin. Mol. Gen. Genet. **233**:372–378.
 45. **Whitaker, J. R., and P. E. Granum.** 1980. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal. Biochem. **109**:156–159.
 46. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **133**:103–119.