Maltoporins and maltose-binding proteins of *Yersinia enterocolitica*

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Two components of the *Yersinia enterocolitica* maltose transport system, maltoporin (OmpM) and an osmotically shockable periplasmic maltose-binding protein (MBP) were identified. The synthesis of OmpM (apparent M_r 43000) and transport of maltose into cells of *Y. enterocolitica* were induced by maltose and maltodextrins. A mutant lacking OmpM was drastically impaired in maltose transport, independent of induction by maltose. The MBP of *Y. enterocolitica* (apparent M_r 40000) was found in the osmotic shock fluid. Its synthesis was induced by maltose. Moreover, rabbit antibodies raised against the MBP of *E. coli* cross-reacted with the analogous protein from *Y. enterocolitica*. The MBP of *Y. enterocolitica* restored the maltose transport activities in $\Delta malE$ mutant cells of *E. coli*.

Introduction

The outer membrane (OM) of enteric bacteria contains pore proteins (porins) that mediate the diffusion of small hydrophilic molecules (Nakae, 1976; Nikaido & Vaara, 1985). Most porins, such as OmpF and OmpC from *Escherichia coli* K12, exhibit low specificity for solutes and act as general diffusion pathways (Nikaido & Vaara, 1985). Other porins are induced in the OM if the bacteria are grown under special conditions. Very few substrate-specific porins have been described so far: among them is LamB, a maltoporin which facilitates the diffusion of maltose and low- M_r maltodextrins (three to seven glucose residues) (Szmecman & Hofnung, 1975; Schwartz, 1987).

In Gram-negative bacteria, the transport of maltose and maltodextrins requires at least four other proteins. One of them is maltose-binding protein (MBP), which is located in the periplasmic space. The MBP of *E. coli* (the product of the *malE* gene) binds maltose and higher $\alpha(1 \rightarrow 4)$ -glucose polymers (Kellerman & Szmecman, 1974). This interaction is necessary for translocation of maltose or maltodextrins through the periplasmic space (for details see the reviews by Hengge & Boos, 1983; Schwartz, 1987).

Yersinia enterocolitica is an enteric bacterium frequently involved in human gastroenteritis (Brenner, 1984). This species is phylogenetically rather distant from *E. coli*, to which other genera such as *Klebsiella*,

Enterobacter and *Citrobacter* are more closely related (Brenner, 1984; Ahmad *et al.*, 1990; Hirvas *et al.*, 1991).

We have identified in the OM of *Y. enterocolitica* 'major' proteins that are physicochemically and functionally similar to OmpA, OmpF and OmpC of *E. coli* (Brzostek & Hrebenda, 1988; Brzostek & Nichols, 1990). The OmpC of *Y. enterocolitica* has been isolated and characterized. In the native form, this protein probably exists as a trimer of identical monomers, which have an apparent M_r of 36000. It behaves as a general diffusion pore with diameter of 1.0 nm. The pore activity exhibits cation selectivity and the biosynthesis of the protein is increased at high osmolarity. These properties characterize the protein as a porin, analogous to OmpC of *E. coli*. Polyclonal antibodies against the pure *Y. enterocolitica* protein cross-react with the trimeric form of OmpC and OmpF from *E. coli* (Brzostek *et al.*, 1989).

In this paper, we describe two other *Y. enterocolitica* proteins, the inducible major OM protein OmpM, analogous to the maltoporin LamB of *E. coli*, and *Y. enterocolitica* maltose-binding protein. In a reconstitution experiment this MBP restored maltose transport in cells of *E. coli* $\Delta malE$.

Methods

Organisms and growth conditions. Strains of *Y. enterocolitica* and *E. coli* are listed in Table 1. Bacteria were grown in minimal medium A (MMA; Miller, 1972) containing glycerol (0.2%, v/v) or glycerol (0.2%, v/v) and maltose (0.2%, w/v) at 28 °C or 37 °C. Maltose (Sigma) was used as inducer for the maltose transport system. Casamino acids (Difco) (0.2%, w/v) were added when cells were grown at 37 °C.

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Abbreviations: MBP, maltose-binding protein; MMA, minimal medium A; OM, outer membrane.

Table 1. *Bacterial strains*

Strain	Known genotype or phenotype	Source or reference
<i>Y. enterocolitica</i>		
Ye5	OmpC ⁺ OmpF ⁺ OmpM ⁺	Brzostek & Hrebenda (1988)
KB1001	OmpC ⁺ OmpF ⁻ OmpM ⁺	
KB1004	OmpC ⁺ OmpF ⁺ OmpM ⁻ isolated after EMS mutagenization of Ye5	
<i>E. coli</i>		
MM134	<i>malT^c-1 Δmal B112 leuB6 his-4 tonA31 tsx78 rpsL136 thi-1 ara14 milI xylI</i>	Brass & Manson (1984)
MC4100	<i>araD139 Δ(argF-lac)U139 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR</i>	Casadaban (1976)
MD15	<i>araD139 Δ(argF-lac)U139 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR malT^c ΔmalE444</i>	W. Boos, University of Konstanz, Germany

Isolation of OmpM⁻ mutants. Ye5 cells were mutagenized with ethyl methanesulphonate (EMS) according to Carlton & Brown (1981). After mutagenesis, the bacteria were plated on maltotriose (5×10^{-4} M) MMA medium. Thirty tiny colonies (<0.01% of total number of colonies) were picked and purified. Each colony was replicated on maltotetraose (5×10^{-4} M) and maltose MMA plates. After 72 h of growth, clones that were unable to grow on maltotetraose (nine clones) were selected and cultured in MMA with glycerol plus maltose. They were used for the isolation of OM proteins. The OM proteins from each clone were separated by electrophoresis (SDS-PAGE). Six Dex⁻ Mal⁺ clones were OmpM⁻. The remaining three were Dex⁻ Mal⁺ OmpM⁺, and in further studies were disregarded.

Isolation of outer membrane proteins. OM proteins were isolated according to a modification of the method of Henning *et al.* (1978). Overnight cultures of the strains were diluted (1:100) in minimal medium A (MMA), grown to the late exponential phase and centrifuged (6000 g for 10 min at 2 °C). The pellet was resuspended in water and sonicated for 75 s (five 15 s cycles with 30 s pauses). Unbroken cells were removed by centrifugation (6000 g for 5 min). The supernatant was centrifuged (12000 g for 10 min at 2 °C), the pellet was washed twice with water and finally resuspended in sample buffer prior to electrophoresis (Laemmli, 1970). Samples (30 µl) containing 50 µg protein were incubated for 5 min in a boiling water bath and applied to the gel.

Preparation of periplasmic proteins. Periplasmic proteins were prepared from exponential phase cells cultured in MMA.

The cold osmotic shock procedure described by Heppel (1971) was used. In some cases, the periplasmic proteins were released by treating the cells with chloroform (Ames *et al.*, 1984). The cold shock fluids were dialysed overnight against 30 mM-Tris/HCl buffer, pH 7.3 and then concentrated with the use of Aquacide (Calbiochem).

SDS-PAGE. Electrophoresis was done in 12% (w/v) SDS-polyacrylamide gels according to Lugtenberg *et al.* (1975). The gels contained acrylamide and bis-acrylamide in the ratio 30:0.8 (w/w) and were stained with Coomassie brilliant blue (Laemmli, 1970).

Western blotting. Western blotting was done with nitrocellulose sheets (Millipore) according to the procedure of Towbin *et al.* (1979), modified as described previously (Brzostek *et al.*, 1989). Bound rabbit

antibodies against *E. coli* MBP were detected by peroxidase-coupled goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories) using *o*-dianisidine (3,3'-dimethoxybenzidine) (Sigma) and H₂O₂.

Maltose-binding activity of shock fluids. [¹⁴C]Maltose [5 µl; specific activity 540 mCi mmol⁻¹ (20 GBq mmol⁻¹)] was added to cold shock fluid and incubated for 5 min at room temperature. For protein precipitation, 1 ml of a saturated solution of ammonium sulphate was used. The samples were filtered (Millipore, 0.45 µm), washed with 5 ml (NH₄)₂SO₄ solution and dried. Radioactivity was determined in a Beckman liquid scintillation counter.

Transport of [¹⁴C]maltose. Maltose uptake assays were done as described by Szmecman & Hofnung (1975). Bacteria were grown in MMA with maltose or glycerol at 28 °C. Cells from the exponential phase of growth were washed twice in MMA (without maltose) and resuspended in 1 ml of MMA to identical optical densities (OD₅₇₈ = 0.1). At time zero, [¹⁴C]maltose [specific activity 540 mCi mmol⁻¹ (20 GBq mmol⁻¹); final concentration 7.0×10^{-2} µM] was added. Samples (200 µl) were removed at 20, 40, 60, 80 and 160 s, immediately filtered through membrane filters (Millipore, 0.45 µm) and washed with 10 ml of MMA. The radioactivity of the dry filters was determined in a toluene-based scintillation fluid.

Reconstitution of maltose transport in *E. coli*. The procedure described by Brass *et al.* (1983) was used. Strains MD15 (*ΔmalB malT^c*) and MM134 (*ΔmalB malT^c*) were grown in MMA plus glycerol and appropriate supplements. Exponentially growing cells (3 ml; 2×10^9 cells ml⁻¹; OD₅₇₈ = 1) were harvested and washed with 5 ml ice-cold 50 mM-Tris/HCl buffer, pH 7.2. The cells were then resuspended in 1 ml of the same buffer containing 300 mM-CaCl₂ (0 °C). After further centrifugation, the cells were suspended in 20 µl Tris/HCl-CaCl₂ buffer (0 °C) and transferred to a new tube containing 20 µl of *Y. enterocolitica* chloroform shock fluid (final concentration of protein about 10 mg ml⁻¹). The sample was shaken for 30 min at 0 °C and the cells were then washed with 1 ml 0.9% (w/v) NaCl at room temperature. For determination of the initial rate of maltose uptake, cells treated as above were suspended at room temperature in 1 ml of MMA containing glycerol. [¹⁴C]Maltose [specific activity 540 mCi mmol⁻¹ (20 GBq mmol⁻¹); final concentration 5×10^{-2} µM] was added.

Results

Identification of maltoporins in OM of *Y. enterocolitica*

Y. enterocolitica cells grown in minimal medium with maltose synthesized an additional OM protein (OmpM) with an apparent *M_r* of 43000 (Fig. 1, lane 6). The synthesis of this protein was also induced by maltodextrins (data not shown). It was not visible on the gel after SDS-PAGE of OM proteins isolated from the culture growing in MMA with glycerol or glucose (Fig. 1, lane 5). The OmpM appeared in the OM independently of culture temperature and Ca²⁺ concentration in the medium (data not shown).

EMS-induced Dex⁻ OmpM⁻ mutants were isolated. They grew in MMA medium with maltose as a carbon source but not in MMA containing maltotetraose. The polypeptide patterns of OM proteins isolated from one of these mutants (KB1004) are shown in Fig. 1 (lanes 7 and 8). Fig. 1 (lanes 3 and 4) indicates the level of OM proteins of mutant strain KB1001 (OmpF⁻ OmpC⁺ OmpM⁺) (Brzostek *et al.*, 1989).

Fig. 1. SDS-PAGE of major OM proteins of *Y. enterocolitica* Ye5 (wild-type) and mutants. Bacteria were grown at 28 °C in MMA containing glycerol (lanes 3, 5 and 7) or glycerol and maltose (lanes 2, 4, 6 and 8). A 50 µg sample of each protein was loaded on to the gel. Lane 2, *E. coli* K12, MC4100 (control, the arrowhead indicates the position of LamB); lanes 3 and 4, *Y. enterocolitica* KB1001; lanes 5 and 6, Ye5; lanes 7 and 8, *Y. enterocolitica* KB1004; lane 1, M_r markers (MW-SDS-70L; Sigma). The apparent M_r values of *E. coli* LamB and *Y. enterocolitica* OmpC are 50000 and 36000 respectively (Lugtenberg, 1981; Brzostek *et al.*, 1989).

Fig. 2. Initial rate of [^{14}C]maltose transport. Bacteria were grown in MMA with glycerol (uninduced cells) or glycerol and maltose (induced cells). At time zero, [^{14}C]maltose was added (final concentration 7×10^{-2} µM). Samples were withdrawn at different times and filtered through membrane filters. The radioactivity of the dried filters was determined. ○, Ye5, uninduced cells; ●, Ye5, induced cells; □, KB1001, uninduced cells; ■, KB1001, induced cells; △, Ye5, after osmotic shock treatment; ⊕, KB1004, uninduced and induced cells. Data shown are means of duplicate experiments.

[^{14}C]Maltose uptake

The initial rate of maltose transport was determined for *Y. enterocolitica* Ye5 (wild-type) and two different

Table 2. Maltose-binding activities of crude shock fluids

All strains were grown at 28 °C in MMA medium with glycerol as a carbon source. The experiment was repeated twice with essentially the same results.

Strain	$10^{-3} \times [^{14}\text{C}]$ Maltose bound [c.p.m. (mg protein) $^{-1}$]
<i>E. coli</i>	
MC4100*	40.00
MD15 ($\Delta malE$)*	0.66
<i>Y. enterocolitica</i>	
Ye5*	54.00
Ye5	1.00
KB1004*	45.10
KB1004	1.40
KB1001*	58.00
KB1001	30.50

*Maltose was added to the medium.

mutants (KB1001 and KB1004). It was measured at a low concentration of maltose (7×10^{-2} µM). The results are shown in Fig. 2. The rate of maltose transport into Ye5 cells increased about 10-fold when the cells were previously induced by maltose. The residual maltose uptake of uninduced cells represented only 6% of the wild-type activity of the induced cells. The mutant lacking OmpM (KB1004) was drastically impaired in transport, independent of maltose induction. This suggests that OmpM of *Y. enterocolitica* may function as a maltoporin.

The rate of maltose transport into uninduced KB1001 (OmpF $^-$ OmpC $^+$ OmpM $^+$) cells was considerably higher

Fig. 3. SDS-PAGE analysis and Western blotting of osmotic shock fluid proteins from *Y. enterocolitica* with anti-*E. coli* MBP antibodies. (a) Gel stained with Coomassie blue; (b) immunoblot showing reactions of antibodies with proteins in the osmotic shock fluid. Bacteria growing in MMA with glycerol (lanes 2, 4 and 6) or glycerol plus maltose (lanes 3, 5 and 7) were subjected to an osmotic shock procedure. A 50 μ l portion of each shock fluid sample was loaded on to the gel. Lanes 2 and 3, shock fluids from *E. coli* K12, MC4100; lanes 4 and 5, *Y. enterocolitica* Ye5; lanes 6 and 7, *Y. enterocolitica* KB1001; lane 1, marker proteins (mixture 4, Serva).

than into uninduced cells of Ye5. It increased after induction of KB1001 cells by maltose.

Maltose-binding proteins

Transport of maltose into induced cells of Ye5 was strongly inhibited after osmotic shock (Fig. 2). We compared the maltose-binding activities of crude shock fluids released from induced and uninduced cells of *E. coli* and *Y. enterocolitica*. The results are summarized in Table 2. In all cases, the same experimental conditions and only one concentration of maltose (2.5 μ M final) were used. The amount of radioactivity bound to the shock

proteins released from induced cells of Ye5 was about 50 times higher than for analogous proteins from uninduced cells. It was comparable to the radioactivity of the shock protein from induced cells of *E. coli* (MC4100) used as a control. The *E. coli* mutant MD15 does not produce maltose-binding proteins. The radioactivity of shock proteins from induced cells of MD15 (1.5% of the binding activities of MC4100) probably represented nonspecific binding of the maltose to the osmotic shock proteins. The maltose-binding activities of crude shock fluids released from induced or uninduced KB1001 cells were higher than the activity of analogous samples obtained from Ye5.

Fig. 4. Reconstitution of active transport in whole cells of *E. coli* $\Delta malE$ with shock fluid from chloroform-treated cells. Cells from exponentially growing cultures of strains MD15 ($\Delta malE malT^+$) and MM134 ($\Delta malB malT^+$) were subjected to the reconstitution procedure. After washing the cells with 0.9% NaCl, maltose uptake was measured. Δ , MD15, without protein in the reconstitution mixture; ∇ , MD15 plus chloroform shock fluid of uninduced cells (10 mg protein ml⁻¹); $\circ$, MD15 plus chloroform shock fluid of induced cells; $\square$, MM134 plus chloroform shock fluid of induced cells. The results are given as the amount of maltose taken up by 1.5×10^8 cells. Results shown are from a single experiment, but repetition gave consistent results.

The electrophoretic (SDS-PAGE) patterns of the periplasmic osmotic shock proteins from different origins are shown in Fig. 3(a). Rabbit antibodies against MBP of *E. coli* were used to identify the *Y. enterocolitica* maltose-binding proteins by immunoblotting (Fig. 3b). The antibodies against *E. coli* MBP recognized the MBP in osmotic shock fluids from *E. coli* (Fig. 3b, lanes 2 and 3) and cross-reacted with analogous proteins from *Y. enterocolitica* (Fig. 3b, lanes 4 and 5). The apparent M_r of the *Y. enterocolitica* MBP was 40 000. The synthesis of this protein, in all strains tested (Ye5 and KB1001) was induced by maltose (Fig. 3b, lanes, 5 and 7).

Three main conclusions result from these experiments: (i) *Y. enterocolitica* produces periplasmic maltose-binding proteins; (ii) the synthesis of these proteins is induced by maltose; (iii) the MBP of *E. coli* is immunologically similar to an analogous protein of *Y. enterocolitica*.

Reconstitution of maltose transport

For the reconstitution of maltose transport in *E. coli* (strain MD15; $\Delta malE malT^+$), osmotic shock fluid from uninduced or maltose-induced Ye5 cells was used. The shock proteins were not lyophilized before the experiment. Strain MD15 had virtually no maltose transport activity, but such activity could be reconstituted in the same cells after pretreatment with MBP from Ye5 in Tris buffer plus Ca²⁺ (Fig. 4). MBP from *Y. enterocolitica* did not restore maltose transport in the *E. coli* control

strain MM134. MM134 is a deletion mutant in *malB* ($\Delta malB malT^+$; missing all *malB* genes).

Discussion

A maltose-inducible OM protein similar to the LamB protein has been identified in most enterobacterial species (Palva & Westerman, 1979; Pick & Wöber, 1979), including *Yersinia enterocolitica*, *Y. intermedia*, *Y. fredericksonii* and *Y. kristensenii* (Bielecki & Hrebenda, 1988).

In this study we have provided the following evidence to suggest that protein OmpM of *Y. enterocolitica* behaves as a maltoporin. (i) Synthesis of OmpM and transport of [¹⁴C]maltose (measured at a low external concentration) were induced by maltose or maltodextrins (maltotetraose was tested). (ii) A mutant of *Y. enterocolitica* defective in production of OmpM (KB1004) was drastically impaired in transport independently of induction by maltose. (iii) In contrast to Ye5 (wild-type), the cells of KB1004 did not grow in MMA containing maltotetraose as a carbon source but showed growth, albeit poor, in the same medium supplemented with maltose. Similar results have been obtained for the maltoporin LamB of *E. coli* (Wandersman *et al.*, 1979), and were explained by assuming that, in the absence of the LamB protein, maltose (M_r 342) can diffuse through the general porins, whereas maltotetraose (M_r 666) is too bulky to do so. (iv) Both LamB and OmpM are murein-associated (Bielecki & Hrebenda, 1988). The following differences between LamB and OmpM were observed: (i) the polypeptide M_r of LamB monomer is 47 392 (Schwartz, 1987) whereas the apparent M_r of OmpM is only 43 000 (see Fig. 1); (ii) the maltose-induced cells of *Y. enterocolitica* are resistant to bacteriophage λ .

Except in the case of most Mal⁺ strains of *Shigella* and one strain of *Erwinia herbicola*, the maltose-inducible OM proteins in the family *Enterobacteriaceae* fail to interact with bacteriophage λ (Schwartz, 1987).

Recently, we found that an OmpF⁻ OmpC⁺ mutant of *Y. enterocolitica* (KB1001) overproduces OmpC and simultaneously synthesizes a small amount of OmpM protein (Brzostek *et al.*, 1989). However, when the cells were induced by maltose the level of OmpM in the OM increased drastically, whereas the amount of OmpC decreased. A similar relationship between OmpC and LamB was described by Diedrich & Fralick (1982). In *E. coli*, this process is partially regulated at the level of transcription.

Two groups of results suggest that the transport of maltose in *Y. enterocolitica* involves an osmotically shockable, i.e. periplasmic, MBP: (i) osmotic shock treatment of maltose-induced cells of Ye5 led to a

reduction in maltose transport in whole cells; (ii) the [^{14}C]maltose bound to the shock proteins released from induced cells of Ye5 was 50 times higher than for analogous proteins from uninduced cells.

The MBP of *Y. enterocolitica* was identified in osmotic shock fluid from Ye5. This protein exhibits an apparent M_r of 40000 (the polypeptide M_r of *E. coli* MBP is 40661; Schwartz, 1987). Its synthesis was induced by maltose, and antibodies against the pure MBP of *E. coli* cross-reacted with the *Y. enterocolitica* MBP. The maltose-binding activity of shock fluids showed that mutant KB1001 synthesizes MBP constitutively. This was not investigated further.

In reconstitution experiments, we demonstrated that cells of a non-polar *malE* deletion strain of *E. coli* (MD15), which lacks periplasmic MBP but constitutively expresses the remaining *malB* genes, were partially restored for maltose transport by the import of *Y. enterocolitica* MBP into the periplasm. This means that MBP of *Y. enterocolitica* can substitute for *E. coli* MBP, and probably is able to interact with the *E. coli* inner membrane MalF, G and K protein complex (Shuman, 1982; Treptow & Shuman, 1985). MBP and OmpM of *Y. enterocolitica* like some of its other proteins e.g. OmpA, OmpH, the murein lipoproteins and the porins, are probably structurally highly conserved within members of the *Enterobacteriaceae* (Hofstra *et al.*, 1980; Palva, 1983; Venegas *et al.*, 1988; Hirvas *et al.*, 1991).

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