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*, 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*, 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 ΔmalE 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* maltodextrins (three to seven glucose residues) (Szmelcman & 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 α(1→4)-glucose polymers (Kellerman & Szmelcman, 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* 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* ΔmalE.

**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.
Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Known genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ye5</td>
<td>OmpC^- OmpF^- OmpM^-</td>
<td>Brzostek &amp; Hrebenda (1988)</td>
</tr>
<tr>
<td>KB1001</td>
<td>OmpC^- OmpF^- OmpM^-</td>
<td>This study</td>
</tr>
<tr>
<td>KB1004</td>
<td>OmpC^- OmpM^- isolated after EMS mutagenization of Ye5</td>
<td></td>
</tr>
<tr>
<td>MM134</td>
<td>maltT^-1 Amlmal B112 leuB6 his-4 tonA31 tex78 rpsL136 thi-1 ara14 mtl1 xytl</td>
<td>Brass &amp; Manson (1984)</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139 A(argF-lac)U139 rpsL150 relA1 deoC1 ptsF25 fjbB3301 rbsR</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>MD15</td>
<td>araD139 A(argF-lac)U139 rpsL150 relA1 deoC1 ptsF25 fjbB3301 rbsR maltT^- AmlmalE444</td>
<td>W. Boos, University of Konstanz, Germany</td>
</tr>
</tbody>
</table>

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^-.

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 (12 000 rpm for 5 min). 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 (12 000 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., 1983) was used. Strains MD15 (Amlmal E444 maltT^-) and MM134 (AmlmalB maltT^-) were grown in MMA plus glycerol and appropriate supplements. Exponentially growing cells (3 ml; 2 × 10^8 cells ml^-1; OD_578 = 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-CaCl_2 (0°C). After further centrifugation, the cells were suspended in 20 μl Tris/HCl-CaCl_2 buffer (0°C) and transferred to a new tube containing 20 μl of Y. enterocolitica chloroform shock fluid (final concentration of 50 μl 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. [3H]Maltose [specific activity 540 mCi mmol^-1 (20 GBq mmol^-1)]; final concentration 5 × 10^-2 M] 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_0 of 43 000 (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 Ca^2+ 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).
Maltose transport in *Yersinia enterocolitica*

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, markers (MW-SDS-70L; Sigma). The apparent M, values of *E. coli* LamB and *Y. enterocolitica* OmpC are 50000 and 36000 respectively (Lugtenberg, 1981; Brzostek et al., 1989).

![SDS-PAGE Image]

Fig. 2. Initial rate of [14C]maltose transport. Bacteria were grown in MMA with glycerol (uninduced cells) or glycerol and maltose (induced cells). At time zero, [14C]maltose was added (final concentration 7 x 10⁻³ 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.

![Graph of Maltose Transport Rate]

[14C]Maltose uptake

The initial rate of maltose transport was determined for *Y. enterocolitica* Ye5 (wild-type) and two different mutants (KB1001 and KB1004). It was measured at a low concentration of maltose (7 x 10⁻³ 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.

Table 2. Maltose-binding activities of crude shock fluids

<table>
<thead>
<tr>
<th>Strain</th>
<th>10⁻³ x [14C]Maltose bound [c.p.m. (mg protein)⁻¹]</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>MC4100*</td>
<td>40-00</td>
</tr>
<tr>
<td>MD15 (ΔmalE)*</td>
<td>0-66</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td></td>
</tr>
<tr>
<td>Ye5*</td>
<td>54-00</td>
</tr>
<tr>
<td>Ye5</td>
<td>1-00</td>
</tr>
<tr>
<td>KB1004*</td>
<td>45-10</td>
</tr>
<tr>
<td>KB1004</td>
<td>1-40</td>
</tr>
<tr>
<td>KB1001*</td>
<td>58-00</td>
</tr>
<tr>
<td>KB1001</td>
<td>30-50</td>
</tr>
</tbody>
</table>

*Maltose was added to the medium.*
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 μM 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.
Maltose transport in Yersinia enterocolitica

Reconstitution of maltose transport

For the reconstitution of maltose transport in E. coli (strain MD15; ΔmalE 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 (ΔmalB 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. frederiksenii 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, in the absence of the LamB protein, maltose (M₄, 342) can diffuse through the general porins, whereas maltotetraose (M₆, 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 Mₐ of LamB monomer is 47392 (Schwartz, 1987) whereas the apparent Mₐ of OmpM is only 43000 (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

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Fig. 4. Reconstitution of active transport in whole cells of E. coli ΔmalE with shock fluid from chloroform-treated cells. Cells from exponentially growing cultures of strains MD15 (ΔmalE malT') and MM134 (ΔmalB 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; V, MD15 plus chloroform shock fluid of uninduced cells (10 mg protein ml⁻¹); O, MD15 plus chloroform shock fluid of induced cells; □, MM134 plus chloroform shock fluid of induced cells. The results are given as the amount of maltose taken up by 1·5 x 10⁶ 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 Mr of the Y. enterocolitica MBP was 40000. 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.
reduction in maltose transport in whole cells; (ii) the 14C-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 Mr of 40000 (the polypeptide Mr 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).

We are grateful to Professor W. Boos for the gift of E. coli strains and rabbit antibodies against MBP.

References


