The level of Yop proteins secreted by *Yersinia enterocolitica* is changed in maltose mutants

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Abstract

Enteropathogenic *Yersinia enterocolitica* strains express a set of plasmid-encoded proteins called Yops, involved in pathogenicity. We studied the influence of the maltose system on the production of Yop proteins and found that the level of Yop proteins of *Y. enterocolitica* O:9 was reduced in the presence of maltose. Transposon insertion mutants impaired with the maltose transport activity showed a decreased level in the production of Yop proteins. The transcription of the *yopH* gene for YopH phosphatase in these maltose mutants was unchanged and revealed a maltose mutation impaired in the secretion of Yop proteins instead of their expression. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Yop protein; Maltose system; *Yersinia enterocolitica*

1. Introduction

*Yersinia enterocolitica* is a facultative intracellular pathogen associated with a broad range of gastrointestinal syndromes in patients. The ability of *Y. enterocolitica* and other pathogenic *Yersinia* species to resist the non-specific defense of the host, i.e. phagocytosis by macrophages or killing by complement, depends on the presence of a 70-kb pYV plasmid [1]. Contact with eukaryotic cells or growth conditions in a low calcium concentration and temperature of 37°C induce the secretion of 12 different plasmid-encoded proteins, called Yops, that are important pathogenicity determinants [2]. The functions of individual Yops are now extensively studied. The YopH and YopE proteins are key elements in the process that allows the pathogen to proliferate in lymphoid tissue [3,4]. YopH from *Y. enterocolitica* is a phosphotyrosine phosphatase (PTPase) that acts on tyrosine-phosphorylated proteins of macrophages, which contributes to the inhibition of bacterial uptake [5]. Although the molecular target of YopH is unknown, it has been suggested that these eukaryotic proteins are involved in signal transduction.

The production of Yops is coordinately regulated by the ‘Ca<sup>2+</sup> dependence region’, a 20-kb DNA fragment of the plasmid pYV required for regulation, secretion and polarized translocation of Yop proteins [6,7]. Transcription of the *yop* genes is activated by VirF regulator which is in turn modulated by a chromosomal encoded histone-like protein called YmoA. The secretion of Yops by *Yersinia* species does not involve the cleavage of a classical signal sequence but involves a III type machinery [8]. Accordingly, the production of Yop proteins is modulated by various environmental parameters. Besides the effects of Ca<sup>2+</sup> and temperature, other nutritional and physical parameters of the environment, such as exogenous nucleotides and osmolarity, are known to influence *yop* genes expression [9,10].

It was recently found that maltose affects the production and secretion of virulence factors of *Vibrio cholerae* [11]. It is likely that other still unidentified regulons (maltose regulon) of *Y. enterocolitica* may assist in the fine-tuning of virulence genes required for successful infection. We have previously identified two components of the *Y. enterocolitica* maltose transport system, maltoporin (OmpM) and maltose binding proteins (MBPs), analogous to LamB and MBP of *Escherichia coli*, respectively [12,13]. The aim of this work was to determine whether the maltose system could affect the production and secretion of Yop proteins, virulence factors of *Y. enterocolitica*.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

*Y. enterocolitica* strain Ye9 serotype O:9 was from the collection of the State Institute of Hygiene, Warsaw, Poland. The pYV plasmid of that strain is indistinguishable by restriction analysis from the pYV plasmid described for other strains of serotype O:9 [14]. *Y. enterocolitica* strains were grown at 25°C in brain-heart (BHI) infusion medium or Luria broth (LB) with antibiotics, where appropriate. To study the effect of maltose, minimal medium A [15] supplemented with casamino acids (0.5%), glycerol (0.2%) and maltose (0.2%) was used. Construction of the Ye9 maltose mutants is described below. The *Y. enterocolitica* Ye9, rifampicin resistant strain was isolated [15]. *E. coli* SM10 λpir [16] was used as the mating donor to mobilize pUT mini-Tn5xyIE [17] into *Y. enterocolitica* strain Ye9. To promote the expression of plasmid-encoded proteins (Yops), all *Y. enterocolitica* strains were cultivated at 37°C in the absence of Ca²⁺ ions in the medium (the growth medium was supplemented with 20 mM sodium oxalate and 20 mM MgCl₂; MOX version).

2.2. Induction and analysis of secreted Yop proteins

Strains were grown in BHI medium at 25°C for 18 h. These were used to inoculate BHI-MOX medium to an optical density (OD₆₀₀) of 0.1. The cultures were incubated at 25°C to an OD₆₀₀ of 0.3 and then transferred to 37°C. After 2.5 h of vigorous incubation, the bacterial cells were removed by centrifugation. Proteins in the supernatant were precipitated by the addition of trichloroacetic acid (TCA; final concentration 5%) and incubation on ice for 2 h. After centrifugation (13 000 g for 30 min at 4°C), the pellets were washed with ice-cold acetone. Yop proteins were resuspended in the same volume of sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels.

2.3. Assay of YopH phosphatase (PTPase) activity

The YopH PTPase activity was measured according to the method described by Persson et al. [4] in the supernatant of a culture of *Y. enterocolitica* strains growing under conditions that promote the expression of Yop proteins. The PTPase assay was performed at 37°C in a volume of 200 μl containing 25 mM *p*-nitrophenyl phosphate (p-NPP, Sigma) as substrate, 40 mM 2-(N-morpholino)-ethanesulfonic acid pH 5.0 buffer and 1.6 mM dithiothreitol. The addition of supernatant (the source of enzyme) initiated the reaction that was stopped by 0.1 M NaOH (200 μl). Spectrophotometric measurements at 410 nm of the amount of released *p*-nitrophenol were carried out on a Pharmacia Biotech, Ultrospec 2000 spectrophotometer. The total YopH phosphatase activity was calculated as total PTP units per bacterial cell density monitored at 578 nm.

2.4. Western blotting

The Yops were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Sigma). Western blot was done according to the procedure of Towbin et al. [18].

2.5. Transport of [¹⁴C]maltose

Maltose uptake assays were performed as described previously [12].

2.6. DNA manipulations

Chromosomal and plasmid DNA isolation, restriction endonuclease digestion, and agarose electrophoresis were performed according to standard methods [19]. The enzymes used for DNA manipulations were purchased from Roche Molecular Biochemicals.

2.7. Construction of *Y. enterocolitica* maltose mutants

pUT with mini-Tn5xyIE transferred from donor strain *E. coli* SM10 λpir into *Y. enterocolitica* Rif⁺ strains by mobilization with a filter mating technique. Donor and recipient strains grown in LB medium to an OD₆₀₀ of 0.4 were mixed in 2 ml of 10 mM MgSO₄ and immobilized on a membrane filter (0.45 μm). The filters were incubated for 18 h at room temperature on the surface of LB plates. The mating mixture was then suspended in RF solution and plated out on a selective medium: LB with kanamycin (95 μg ml⁻¹) and rifampicin (50 μg ml⁻¹). The selection of maltose mutants was carried out on McConkey agar plates with 1% maltose [15] and the appropriate antibiotics.

2.8. Characterization of transposon insertions

Mini-Tn5xyIE insertions in maltose mutants were confirmed by polymerase chain reaction (PCR). For this purpose, PCRs for each mutants were performed for 30 cycles at 58°C with primers corresponding to the kanamycin resistance gene (the upstream primer K1 (5’-CTCATCGAG-CATCAAATGAAAC-3’) and the downstream primer K2 (5’-CTCTGATGTTACATTGACAAG-3’)). The Km⁸ gene product was analyzed by agarose gel. To determine the site of insertion, chromosomal DNA from the mutant strains was digested with EcoRI that cuts at one end of the transposon and leaves the Km⁸ determinant intact. Southern blot analysis was performed using a nucleic acid labeling and detection kit (Boehringer Mannheim). The Km⁸
determinant from the pUT mini-Tn5xylE plasmid was used as a probe.

2.9. RNA extraction and Northern blot analysis

RNA was extracted after 120 min of induction of Yop proteins at 37°C in BHI-MOX medium using a Qiagen kit—following the manufacturer’s procedure. RNA electrophoresis in 1% agarose gels and Northern blot on nylon membranes (Sigma) were performed as described by Sambrook et al. [19]. DNA–RNA hybridizations were performed with a DNA probe. The yopH probe was synthesized by PCR. A 441-bp fragment of yopH gene was amplified by PCR with the chromosomal DNA of strain Ye9 used as a template. The upstream primer P1 (5’-CAGTGGTGCCAGAGAGTCA-3’) consisting of nucleotides from 459 to 478 and the downstream primer P2 (5’-TCTGGGCCATAAGGAGAAACAGT-3’) complementary to the nucleotides from 877 to 900 were used [20]. A fragment of yopH gene was labeled with digoxigenin by the random prime kit (Boehringer Mannheim).

3. Results and discussion

The results presented here provide the first step towards establishing the role of maltose and the maltose system in the expression and secretion of Yop proteins of Y. enterocolitica O:9. Detailed studies were performed on YopH protein, which exhibited a tyrosine phosphatase activity and was released in abundant amounts into the growth medium.

In response to cultivation at 37°C in the absence of Ca2+ ions, strain Ye9 synthesizes and secretes a series of plasmid-encoded Yop proteins into the growth medium. Fig. 1 (lane 2) shows a Coomassie-stained SDS–PAGE gel of the more abundant Yops released by Y. enterocolitica strain Ye9. From 51 kDa to the bottom of the gel are: YopH, YopM, V antigen, YopD, B, T proteins, usually comigrated as one band, YopN, YopP and YopE. The above proteins correspond well with the Yops described earlier by Cornelis et al. [6] and recently for Y. enterocolitica O:8 by Aepfelbacher et al. [2]. To analyze the effect of maltose on the expression of Yop proteins, Y. enterocolitica strain Ye9 was incubated under conditions allowing Yops expression and secretion (37°C; MMA medium with 0.2% glycerol, MOX version). At the beginning of incubation, maltose was added to a final concentration of 0.2% to one of the cultures. After 3 h of induction, the YopH PTPase activity was determined in samples of supernatant, then the secreted proteins were precipitated with TCA and SDS–PAGE was performed. Analysis of the Yop protein profiles revealed differences in the amount of Yops obtained for different induction conditions (with or without maltose). The results show that, upon exposure to maltose, the level of Yop proteins is reduced (Fig. 1, lane 3). These results were confirmed in repeated experiments provided with special attention to the same amount of cells used in the induction of Yops. Maltose has been shown in V. cholerae to inhibit the production of virulence factors secreted into the extracellular milieu (mannose-sensitive hemagglutinin, soluble hemagglutinin-protease and cholera toxin and in contrast, maltose induced synthesis of toxin-coregulated pilus associated with the bacterial cell surface) [11].

YopH protein is an essential virulence determinant of pathogenic strains of Y. enterocolitica. In this study, YopH protein was released by the Ye9 strain after induction at 37°C in Ca2+-deprived medium and identified on polyacrylamide gels at a position corresponding to the apparent molecular mass of about 51 kDa (Fig. 1). It has been established that YopH is a protein tyrosine phosphatase related to eukaryotic PTPases and exhibits the highest specific activity among all PTPases so far described [5,21]. This PTPase activity can be monitored using the chromogenic substrate p-NPP [4]. The PTPase activity of YopH protein was measured in the supernatants of cultures of Ye9 strain growing in MMA medium with and without maltose. The PTPase activity was about five times greater in the culture medium without maltose than in the medium supplemented with this sugar (data not shown). These results confirmed the reduced level of YopH protein obtained from SDS–PAGE analysis.
To test whether genes of the maltose system have an effect on the secretion and production of Yop proteins, we isolated maltose mutants by transposon mutagenesis (see Section 2). Maltose mutants of *Y. enterocolitica* Ye9 were isolated from a transposition pool of 20 independent mating procedures. Eight transposon mutants were selected as Mal strains on McConkey agar plates supplemented with maltose. Then, we attempted to determine if these mutants are impaired in maltose transport. To clarify this, [14C]maltose uptake was investigated. The initial rate of [14C]maltose transport was determined for *Y. enterocolitica* Ye9 strain and Mal transposon mutants (Fig. 2). Two mutants, from all tested, were impaired in maltose transport compared with the wild-type strain. The maltose uptake of mutants AG18 and AG20 represented only 20% of the wild-type activity. The effect of maltose mutations on Yops production (secretion) was further investigated. Yop proteins from all Mal strains were isolated and separated by PAGE. A comparison of the Yops profile of strain Ye9 with Mal mutants indicated that two mutants, AG18 and AG20, from the maltose mutant pool exhibited a reduced level of Yop proteins. An electrophrogram of Yops released into the growth medium (BHI, MOX version) by the wild-type strain Ye9 and maltose mutants AG18 and AG20 is presented in Fig. 3A. Yops prepared from both mutants exhibited only traces of those expressed by the Ye9 strain. The presence of YopH protein in the culture supernatants of these maltose mutants and the wild-type strain was studied by immunoblotting, using anti-YopH antibodies (Fig. 3B). Both maltose mutants secreted the YopH protein in minute amounts compared with the Ye9 strain. The immunological reaction paralleled those obtained from SDS-PAGE analysis.

An important step in the study of maltose mutants was to confirm that the obtained phenotypes are caused by integration of mini-Tn5xyle transposons. This was verified by PCR using primers K1 and K2 for the kanamycin resistance gene (Fig. 4A). Amplified products of both mu-
tants corresponded well with the size of Km R determinants of the transposon delivered by plasmid pUT mini-Tn5xyE. Southern hybridization analysis of mutants AG18 and AG20, using the Km R gene as a probe, revealed that they have single insertions in the same loci (Fig. 4B). To find out whether a reduced level of Yop proteins in maltose mutants AG18 and AG20 is the result of differences in the transcription of yop genes, Northern blot analysis was performed. Total RNA was extracted from induced cells of the wild-type strain Ye9 and both mutant strains, and analyzed by Northern blot hybridization with a yopH probe (Fig. 5). As expected, in the absence of Ca2+ ions in the medium and at a temperature of 37°C for induction, the transcription of YopH in strain Ye9 was evident. The specific yopH probe also hybridized with mRNA isolated from mutant strains growing under induction conditions (Fig. 5, lanes 2 and 3). These results suggest that phenotypic changes in the profile of Yop proteins in maltose mutants are probably not due to the reduction of yop transcription. Rather, the maltose system may directly interact with the proteins required for the secretion of Yop proteins. The intact maltose transport system seems to be crucial for translocation of the virulence factor of V. cholerae [11]. The results of our studies may reflect the alteration in secretion of virulence factors of the enteropathogenic Y. enterocolitica strains in response to differences of environmental niches of the human organism. Maltose, the most available sugar in the intestinal tract, could be an important signal in the regulation of the expression of Yop proteins. Yops are not involved in the first stages of infections like other virulence factors (adhesion and invasion proteins), so they would simply be repressed. Moreover, the role of maltose in the function of the export pathways of Yops in Y. enterocolitica is quite possible because the significant homology of YscC, the protein involved in the specific secretion of Yops, with maltose-regulated PulD of Klebsiella pneumoniae, is known [22]. PulD is the protein involved in the secretion of pullulanase whose expression is induced by growth in the presence of this sugar and it is positively regulated by MalT protein [23]. Our preliminary studies on the maltose induction of LamB and MBP proteins of Y. enterocolitica indicate that the MalT regulator exists in Y. enterocolitica [12]. The explanation for the role of the maltose system in the secretion of Yop proteins needs further experimental analysis.

Fig. 4. Genetic identification of transposon insertion. A: Agarose gel electrophoresis showing the PCR-amplified gene from maltose mutants encoding a kanamycin resistance gene of mini-Tn5xyE transposon. Lanes: 1, DNA molecular mass marker X (Roche); 2, strain Ye9; 3, mutant AG18; 4, mutant AG20. B: Southern blot of transposon mutants of Y. enterocolitica. Chromosomal DNA was prepared from AG18 (lane 1) and AG20 (lane 2) maltose mutants and from the wild-type Y. enterocolitica Ye9 as a negative control (lane 3). All DNA samples were digested with EcoRI. The PCR-amplified Km resistance gene was used as a probe.

Fig. 5. Northern blot analysis of yopH transcription. Whole cell RNA was prepared from Y. enterocolitica Ye9 (lanes 1) and maltose mutants AG18 (lane 2) and AG20 (lane 3) induced at 37°C for 2 h. The yopH mRNA was identified with oligonucleotide yopH probe labeled by a random prime with digoxigenin. The arrows point to the two yopH transcripts [24].
References


