Yersinia enterocolitica YopQ: strain-dependent cytosolic accumulation and post-translational secretion

Janja Trček,†‡ Gottfried Wilharm,† Christoph A. Jacobi and Jürgen Heesemann

Author for correspondence: Jürgen Heesemann. Tel: +49 89 5160 5200. Fax: +49 89 5160 5202. e-mail: heesemann@m3401.mpk.med.uni-muenchen.de

Max von Pettenkofer-Institut, Pettenkoferstr. 9a, D-80336 Munich, Germany

INTRODUCTION

Pathogenic Yersinia species use a plasmid (pYV)-encoded type III secretion system to evade the host primary immune defences. The system enables yersiniae to deliver different effector proteins (YopE, YopH, YopM, YopO/YpkA, YopP/YopJ, YopT) into susceptible eukaryotic host cells (Cornelis et al., 1998). The secretion signal of Yops studied thus far was located within the first 10–17 codons of the respective gene (Anderson & Schneewind, 1997, 1999; Schesser et al., 1996; Sory et al., 1995). For some of the Yops, such as YopE and YopN, a second, chaperone-dependent secretion signal has been proposed (Cheng et al., 1997).

Anderson & Schneewind (1997) reported that several frameshift mutations, which completely altered the peptide sequence of the first 15 codons of the yopE and yopN genes, did not prevent secretion. This finding led to the assumption that the secretion signal is at the mRNA level. It was further suggested that an mRNA element signals transport of Yops by coupling translation and secretion (Anderson & Schneewind, 1999). Recently, Lloyd et al. (2001b) performed a systematic mutational analysis within the N-terminal sequence of YopE. Some of the mutations, changing the amino acid sequence, had significant effects on the secretion of YopE. Therefore, they concluded that secretion of YopE is directed by the N terminus and not by the 5′ end of yopE mRNA.

YopQ of Yersinia enterocolitica (YopK of Yersinia pseudotuberculosis) is a secreted protein (ca 18 kDa) that is required for virulence (Holmström et al., 1995; Mulder et al., 1989). YopK was shown to be located in association with bacteria infecting cultured cells and it has been suggested that YopK controls translocation of Yops by regulating the size of the translocation pore (Holmström et al., 1997). No chaperone has been described for YopQ/K. Based on the results performed with Y. enterocolitica strain W22703, Anderson & Schneewind (1999) predicted a co-transla-

Keywords: YopQ-GFP accumulation, yopQ/yopK sequences, mRNA secondary structures
tional mechanism of YopQ secretion. They proposed that an mRNA secretion signal at the 5' end is necessary and sufficient for secretion of YopQ. In accordance with their prediction they could not detect YopQ intracellularly. Moreover, they demonstrated lack of post-translational secretion of YopQ (Anderson & Schneewind, 1999). In contrast, it has been shown that YopK of Y. pseudotuberculosis is accumulated intracellularly (Holmström et al., 1995), indicating that the mRNA signal hypothesis cannot be applied to YopQ/K of all pYV-carrying Yersinia species.

In this study YopQ production and secretion was investigated in different serotypes of Y. enterocolitica. Cytosolic accumulation and secretion of YopQ was analysed by immunoblotting. yopQ translational fusions with gfp, encoding the green fluorescent protein (Jacobi et al., 1998), were constructed to study cytosolic accumulation of the YopQ-GFP hybrid protein in Yersinia under various culture conditions. The intensity of fluorescence was measured by flow cytometry. By infecting HeLa cells we tried to localize YopQ-GFP by confocal laser scanning microscopy.

**METHODS**

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria–Bertani (LB) medium (Davis et al., 1980) at 37 °C (Escherichia coli) or 28 °C (Yersinia). Chloramphenicol (Cm, 20 µg ml⁻¹), naldixic acid (60 µg ml⁻¹), carbenicillin (250 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) were used as selective antibiotics. E. coli DH-5α (Hanahan, 1983) was used as the primary host organism in cloning experiments. Recombinant plasmids were introduced into Y. enterocolitica by electroporation. E. coli S17-1pir (Simon et al., 1983) was used as donor for conjugation. For induction of the yop regulon, overnight cultures of yersinia were diluted (1:40) in brain–heart infusion (BHI; Difco) and grown for 2 h at 37 °C. The medium was supplemented with 15 mM MgCl₂, 0.2% glucose and 5 mM EGTA (EGTA-BHI) to induce secretion, and with 5 mM CaCl₂ (Ca-BHI) to repress secretion (cultivation for 2 h at 37 °C; Heesemann et al., 1984).

For inhibition of protein synthesis Cm (100 µg ml⁻¹) was added to the medium (Lloyd et al., 2001b). For inhibition experiments after Yop induction, bacteria were cultured in BHI for 2 h at 37 °C, then medium was supplemented to obtain EGTA-BHI and incubated for 20 min at 37 °C. Subsequently, Cm (100 µg ml⁻¹) was added and incubation was continued for another 20 min; then the cells were centrifuged, washed and further incubated for 1.5 h under Yop-inducing conditions in the presence of Cm. Inhibition of protein synthesis prior to induction of Yop secretion was performed as follows. Strains were grown in Ca-BHI for 2 h at 37 °C, then Cm (100 µg ml⁻¹) was added and cultures were incubated for 20 min at 37 °C. Bacteria were pelleted, resuspended in EGTA-BHI (plus 100 µg Cm ml⁻¹) and grown for another 20 min before harvesting the cells.

**Nucleic acid manipulation.** Plasmid DNA preparation, restriction enzyme digests, ligations and transformations were performed as described by Sambrook et al. (1989). Plasmid DNA was sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the ABI Prism 377XL DNA Sequencer (Applied Biosystems).

Plasmids pJTYopQO8-GFP3 and pJTYopQO9-GFP3 were constructed by cloning yopQ from Y. enterocolitica WA-314, serotype O:8 (Heesemann, 1987), and Y. enterocolitica MRS 40, serotype O:9 (Sory et al., 1995), respectively. The yopQ PCR-amplified fragments were ligated into the HindIII/BamHI restriction sites of plasmid pCJ-G3 (Jacobi et al., 1998). The yopQ gene, including 391 bp upstream of the translational start but without stop codon, was PCR-amplified using the forward primer 5'-GGGATCCCCATATGCTATCATGCTGTTTACG-3' and reverse primer 5'-ACGCAAGCTTGTCGCTGGTCAGAGATCG-3', both preceded by appropriate restriction sites (underlined). yopQO8-gfp3 and yopQO9-gfp3 were cointegrated into virulence plasmids pYV08 and pYV09, respectively, by homologous recombination. Fragments yopQO8-gfp3 and yopQO9-gfp3 were also inserted into the SalI/XbaI restriction sites of suicide plasmid pKAS32 and transformed into E. coli S17-1pir (Skorupski & Taylor, 1996). After conjugation and single cross-over, suicide plasmids pKASYopQO8-GFP3 and pKASYopQO9-GFP3 were co-integrated into the virulence plasmids of Y. enterocolitica WA-314 and Y. enterocolitica MRS 40, respectively. The correct co-integration was confirmed by sequencing.

**Flow cytometric measurement.** A Coulter Epics flow cytometer equipped with an argon 488 nm laser was used. Bacteria were diluted and detected by side scatter as described by Russo-Marie et al. (1993) and Jacobi et al. (1998). The scale was logarithmic, and fluorescence data and scatter data were collected for 10000 events.

**Protein analyses.** Yersinia were harvested by centrifugation (12000 g, 10 min) and the culture supernatant was collected. Released proteins were precipitated with trichloroacetic acid and prepared as described previously (Heesemann et al., 1986). To eliminate cell-surface-associated Yops, pelleted cells were resuspended in PBS containing 600 µg proteinase K ml⁻¹. After incubation for 15 min at 30 °C, PMSF was added to 5 mM and cells were pelleted and dissolved in SDS sample buffer (see below) supplemented with 5 mM PMSF. Samples from supernatant and whole bacteria were dissolved in a modified SDS sample buffer consisting of 0.1 M MgCl₂, 4% SDS, 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue and 100 mM Tris/HCl, pH 9. MgCl₂ was added to SDS sample buffer to precipitate DNA of whole-cell lysates (Chen & Christen, 1997). Tris/HCl, pH 9, instead of pH 6.8, was used to eliminate hydrolysis of aspartyl-prolyl peptide bonds (Cannon-Carlson & Tang, 1997). The volume of samples was adjusted in accordance to the OD₅₆₀ values of bacterial cultures. Electrophoresis in 12% polyacrylamide gels in the presence of SDS was performed as described by Laemmli (1970). Proteins were transferred to a nitrocellulose membrane by electroblotting (Towbin et al., 1979). The membrane sheets were blocked with 0.5% Tween 20 (Sigma–Aldrich) and 5% bovine serum albumin (Sigma–Aldrich) in PBS. Immunostaining of YopQ/K, YopE and GFP was performed with rabbit anti-glutathione S-transferase (GST)-YopQ polyclonal antibodies (dilution 1:10000), anti-YopE polyclonal antibodies (dilution 1:5000), or anti-GFP polyclonal antibodies (dilution 1:3000) and peroxidase-conjugated secondary anti-rabbit antibodies (dilution 1:5000). Detection was carried out using the ECL Western Blotting System (Amersham Pharmacia Biotech). For immunostaining of YadA the mAb 8D1 (Roggenkamp et al., 1995) was used. Subsequently, secondary binding of alkaline-phosphatase-conjugated anti-mouse IgG was detected with BCIP/NBT substrate (Sigma).

**Preparation of polyclonal antibodies.** Polyclonal antibodies against fusion protein GST-YopQ were generated by immuni-
zation of a rabbit. The yopQ gene was amplified by PCR and cloned in-frame downstream of gst in the vector pGEX-4T-3 (Amersham Pharmacia Biotech). The fusion protein could not be successfully expressed in E. coli; therefore wild-type Y. enterocolitica was used as host organism. Protein was purified by GST purification modules according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Microscopic examination.** In the HeLa cell infection model the recombinant Yersiniae with GFP constructs were visualized by using an Aristoplan epifluorescence and confocal laser scanning microscope (Leica TCS 4D) (CLSM) as described by Jacobi et al. (1998).

**Computer data analysis.** The sequences were aligned by using the CLUSTAL X 1.8 program (Thompson et al., 1994) and folded by using the RNAdraw program (Matzura & Wennborg, 1996); in both cases using the default settings.

### RESULTS AND DISCUSSION

**Intracellular detection of YopQ in Y. enterocolitica of different serotypes**

In a first approach we examined the production of YopQ in five strains of Y. enterocolitica serotypes O:3, O:8 and O:9 and in Y. pseudotuberculosis YPIII(pBI1). Yersiniae were induced for Yop-secretion in EGTA-BHI broth at 37 °C. In parallel, secretion was blocked by the addition of calcium (Ca-BHI). Irrespective of whether the cells were induced or blocked for secretion, we could detect YopQ in total cell lysates by immunoblot analysis (Fig. 1, left panel). Similar results were obtained when cells were treated with proteinase K prior to dissolving in SDS sample buffer (Fig. 1, right panel). The efficiency of proteinase K treatment was validated by detection of cell-surface-exposed Yersinia adhesin YadA (Fig. 1). The mAb used was raised against YadA of serotype O:8 and cross-reacts with YadA of other Y. enterocolitica serotypes. Accordingly, signals for the other serotypes were weaker. The only signal detected after proteinase K treatment was a degradation product of the full-length YadA in the case of Y. enterocolitica MRS 40 (Fig. 1, right panel). Thus, the detected YopQ signals can be attributed to their intracellular accumulation. Strikingly, significantly stronger YopQ protein bands were always detectable in strain WA-314, serotype O:8, in comparison to strains of serotypes O:3 and O:9. In accordance with Holmström et al. (1995) we were also able to detect YopK in the cytosol of Y.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>endA1 bsdR17 (r_m2) supE44 thi-1 recA1 gyrA relA1Δ(lacZYA-argF)U169 (880lacZAM15)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>S17-134pir</td>
<td>pir' tri', Spec'</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Y. enterocolitica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WA-314</td>
<td>Clinical isolate, harbouring pYVO8, serotype O:8, Nal'</td>
<td>Heesemann (1987)</td>
</tr>
<tr>
<td>MRS 40</td>
<td>Serotype O:9, Nal'</td>
<td>Sory et al. (1995); Sarker et al. (1998)</td>
</tr>
<tr>
<td>Y-96-P</td>
<td>Clinical isolate, serotype O:9</td>
<td>Heesemann (1987)</td>
</tr>
<tr>
<td>Y-108-P</td>
<td>Clinical isolate, serotype O:3</td>
<td>Heesemann (1987)</td>
</tr>
<tr>
<td>Y-03 122/00</td>
<td>Clinical isolate, serotype O:3</td>
<td>R. Gierczynski, National Institute of Hygiene, Warsaw, Poland</td>
</tr>
<tr>
<td>WA-C</td>
<td>Plasmid-less derivative of strain WA-314, spontaneous Nal'</td>
<td>Heesemann (1987)</td>
</tr>
<tr>
<td>WA-C (pYV-515)</td>
<td>lcrD::Tn5; Tn5 insertional inactivation of lcrD, Km'</td>
<td>Ruckdeschel et al. (1996)</td>
</tr>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YPIII(pBI1) YPIII</td>
<td>Wild-type</td>
<td>Bölín et al. (1982)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCJ-G3</td>
<td>pACYC184 carrying gfp3</td>
<td>Jacobi et al. (1998)</td>
</tr>
<tr>
<td>pJTYopQO8-GFP3</td>
<td>pCJ-G3 carrying yopQ and yopQ upstream region from Y. enterocolitica WA-314</td>
<td>This study</td>
</tr>
<tr>
<td>pJTYopQO9-GFP3</td>
<td>pCJ-G3 carrying yopQ and yopQ upstream region from Y. enterocolitica MRS 40</td>
<td>This study</td>
</tr>
<tr>
<td>pKASYopQO8-GFP3</td>
<td>pKAS32 carrying translational fusion between yopQO8 and gfp3</td>
<td>This study</td>
</tr>
<tr>
<td>pKASYopQO9-GFP3</td>
<td>pKAS32 carrying translational fusion between yopQO9 and gfp3</td>
<td>This study</td>
</tr>
<tr>
<td>pYVO8::pKASYopQO8-GFP3</td>
<td>pYVO8 (WA-314) with co-integrated pKASYopQO8-GFP3</td>
<td>This study</td>
</tr>
<tr>
<td>pYVO9::pKASYopQO9-GFP3</td>
<td>pYVO9 (MRS 40) with co-integrated pKASYopQO9-GFP3</td>
<td>This study</td>
</tr>
<tr>
<td>pGEX-4T3</td>
<td>GST gene fusion vector, Ap'</td>
<td>Amersham Pharmacia Biotech</td>
</tr>
</tbody>
</table>

---

**Comparison of YopQ and YopK production**

We were not able to detect YopK in the cytosol of Y. enterocolitica strains. The mAb used was raised against YadA of serotype O:8 and cross-reacts with YadA of other Y. enterocolitica serotypes. According to the results presented in Table 1, signals for the other serotypes were weaker. The only signal detected after proteinase K treatment was a degradation product of the full-length YadA in the case of Y. enterocolitica MRS 40 (Fig. 1, right panel). Thus, the detected YopQ/K signals can be attributed to cell-surface-associated secreted YopQ/K, but can be attributed to their intracellular accumulation. Strikingly, significantly stronger YopQ protein bands were always detectable in strain WA-314, serotype O:8, in comparison to strains of serotypes O:3 and O:9. In accordance with Holmström et al. (1995) we were also able to detect YopK in the cytosol of Y. enterocolitica.
pseudotuberculosis. The higher electrophoretic mobility of YopK in comparison to YopQ can be explained by differences in the amino acid composition: two lysine and one glycine residue of YopQ are replaced by glutamic acid residues in YopK.

However, we had to change the conventional Laemmli sample buffer for SDS-PAGE to obtain reproducible results of intracellular YopQ levels. Aspartyl-prolyl peptide bonds, which are present in YopQ and YopK, are prone to hydrolyse during heating in Laemmli sample buffer because of a drop in pH (Cannon-Carlson & Tang, 1997). We found that YopQ signals were enhanced and reproducible when we changed the pH of Laemmli sample buffer from 6.8 to 9.

As a second approach, we studied the production of YopQ-GFP hybrid proteins by Y. enterocolitica WA-314 and Y. enterocolitica MRS 40. Reporter constructs were under the control of their autochthonous YopQ promoter and were inserted into the low-copy-number plasmid pACYC184 (Chang & Cohen, 1978). Both bacteria induced for secretion of Yops, as well as those blocked for secretion, fluoresced. The flow cytometry analysis showed that Y. enterocolitica WA-314 carrying plasmid pJTYPqO8-GFP3 fluoresced more strongly than Y. enterocolitica MRS 40 harbouring pJTYPqO9-GFP3 (Fig. 2b). These results are supported by immunoblot analysis (Fig. 2a). However, bacteria induced for secretion fluoresced more strongly than those in which secretion was blocked, although secretion of the hybrids was detected. This was surprising, since in the case of an open secretion pore, one would expect the majority of the fusion protein to be released from the bacteria. This might be due to impaired secretion of the hybrid protein compared to native YopQ (Fig. 3a). In accordance with this, the secreted YopQ-GFP did not differ significantly among the strains compared (Fig. 2a).

Anderson & Schneewind (1999) have shown that overproduction of YopQ caused its intracellular accumulation, implying overtitration of some regulatory component. To exclude the copy number effect of the introduced plasmids in the analysed strains, the yopQ-gfp constructs were co-integrated into the virulence plasmid of Y. enterocolitica WA-314 and Y. enterocolitica MRS 40, respectively. As presented in Fig. 2, co-integration of the fusions reduced fluorescence compared to expression of yopQ-gfp from pACYC184. However, Y. enterocolitica WA-314 again fluoresced more strongly than Y. enterocolitica MRS 40.

Thus, all these results contradict the co-translational model of YopQ secretion which predicts no YopQ accumulation in the cytosol of yersiniae (Anderson & Schneewind, 1999). One reason for the discrepancy might be that Anderson & Schneewind established the model for YopQ secretion based on the characteristics of one single strain of Y. enterocolitica and its mutants, that is Y. enterocolitica W22703, serotype O:9 (Cornelis & Colson, 1975). Our comparative analysis of Y. enterocolitica strains of different serotypes shows that YopQ expression appears to be strain-dependent and that significant amounts of YopQ are not co-translationally secreted. The contradictory results may also be caused by the instability of YopQ during sample preparation for SDS-PAGE.

The specificity and efficiency of plasmid-encoded type III secretion was proven for strain Y. enterocolitica WA-
Expression and secretion of *Y. enterocolitica* YopQ

314(pJTYopQO8-GFP3) and was further used to monitor translocation of YopQ into HeLa cells (Fig. 3). A comparison with the secretion-deficient *lcrD* mutant WA-C(pYV-515) harbouring the same plasmid served as a control for specificity of protein release. Fig. 3(a) additionally reveals that YopQ-GFP detected in whole-cell lysates is not associated with the cell surface. Approximately 3–5% of the intracellular amount of YopQ-GFP was detected in the supernatant (Fig. 3a). However, we could not demonstrate translocation of YopQ-GFP fusion protein into eukaryotic cells (Fig. 3b). This is in line with the results of Holmström *et al.* (1997) who could not find translocated YopK.

**Comparison of the yopQ sequences among the *Y. enterocolitica* strains**

To explain the differences in the level of *yopQ* expression among the *Y. enterocolitica* strains, we sequenced the 5′ end as well as the upstream region of the *yopQ* genes from *Y. enterocolitica* WA-314, serotype O:8, and *Y. enterocolitica* Y-108-P, serotype O:3. We compared the sequences to each other and to the homologous sequences of *Y. enterocolitica* serotype O:9 (accession no. NC 002120), *Y. pseudotuberculosis* (U18804) and *Y. pestis* (NC_001882). Downstream of the transcriptional start site of *yopQ*, which was determined by Anderson & Schneewind (1999), an obvious difference exists between the sequences. The sequence of *Y. enterocolitica* O:8, 5 nt after the transcriptional start, shows a deletion of 8 nt compared to the homologous sequences of *Y. enterocolitica* serotype O:9, *Y. enterocolitica* serotype O:3 and *Y. pseudotuberculosis*. We speculate that this difference might be responsible for a modulation of transcription efficiency. To test this hypothesis, we isolated total RNA from different *Yersinia* serotypes and performed semiquantitative RT-PCR. However, we could not detect significant differences in the intensity of the RT-PCR signals.

---

**Fig. 2.** (a) Western blot of cell lysates (above) and supernatant (below) from *Y. enterocolitica* serotype O:8 (WA-314) harbouring plasmids pJTYopQO8-GFP3 or pYVO8::pKASYopQO8-GFP3, and from *Y. enterocolitica* serotype O:9 (MRS 40) harbouring plasmids pJTYopQO9-GFP3 or pYVO9::pKASYopQO9-GFP3. Detection was performed with rabbit anti-GFP polyclonal antibodies. Five times more supernatant than cell pellet was loaded. (b) Corresponding intensity of fluorescence (flow cytometer) of diverse *Yersinia* strains. ■, 27 °C; □, 37 °C+CaCl2; ▶, 37 °C–CaCl2. Error bars indicate SD (three independent analyses).
among the analysed serotypes of yersiniae (data not shown).

Anderson & Schneewind (1997) had predicted a stem–loop in the secretion signal of YopE and YopN, with the start codon and a possible Shine–Dalgarno ribosome-binding site being base-paired. They suggested that such an RNA structure inhibits a successful translation of yopQ by preventing ribosome loading (Anderson & Schneewind, 1997). By folding yopQ mRNA sequences, we searched for such structures in the putative YopQ secretion signal. Three different mRNA secondary structures were predicted (Fig. 4). Since the nucleotide sequences of YopQ from the Y. enterocolitica serotype O:9 and Y. enterocolitica serotype O:3 are the same, they could form the same predicted mRNA secondary structure. In that structure (Fig. 4a) the first two nucleotides of the start codon as well as the upstream sequence with the putative ribosome-binding site are base-paired in a stem. The situation is different in the predicted mRNA secondary structure of yopQ from serotype O:8, where the start codon as well as the putative ribosome-binding site are not base-paired (Fig. 4b). The predicted yopQ mRNA secondary structures of Y. pseudotuberculosis and Y. pestis are the same (Fig. 4c). In that structure the first two nucleotides of the start codon are not base-paired, whereas in contrast to the O:8 sequence, the third nucleotide is part of a duplex structure. The ribosome-binding site is not base-paired in Y. pseudotuberculosis and Y. enterocolitica O:8.

The observed nucleotide differences in yopQ mRNA sequences thus influence the predicted yopQ mRNA secondary structures. There is a particularly significant difference between the predicted structures of serotypes O:9 and O:8. As suggested by Anderson & Schneewind (1999), the mRNA base-paired duplex at the Shine–Dalgarno region and at the start codon might hinder the ribosomes from binding to the ribosome-binding site. The differences between serotypes O:8 and O:9 in their predicted yopQ mRNA structures and YopQ expression levels support this suggestion of Anderson & Schneewind (1999). We conclude that these differences in yopQ mRNA secondary structures might have an influence on...
the rate of translational initiation and therefore on the quantity of intracellular YopQ in Y. enterocolitica. However, the structural differences shown in Fig. 4 do not support the notion of an mRNA secretion signal for different serotypes.

**Post-translational secretion of YopQ**

Based on the detection of cytosolic YopQ we addressed the question of post-translational secretion of this cytosolic pool. We inhibited protein synthesis by adding Cm after induction of secretion. To ensure a complete inhibition of protein synthesis, antibiotic treatment lasted for 20 min. Supernatant was subsequently removed and the cells were washed. Then they were further incubated in fresh medium under inducing conditions in the presence of antibiotics. Finally, supernatants were collected to analyse post-translationally secreted proteins. As shown in Fig. 5, YopQ of Y. enterocolitica serotypes O:8 and O:9 was still secreted after blocking translation, indicating possible post-translational secretion. Intracellularly, YopQ was not detectable any more after inhibition of protein synthesis, proving the efficiency of inhibition of YopQ biosynthesis and indicating that the cells were bleed out by the procedure. This cannot be explained by the half-life of YopQ alone, since the secretion-deficient control strain WA-C(pYV-515) still harbours YopQ after the procedure (Fig. 5). This is additional evidence that the intracellular YopQ pool is secretion-competent. For YopE a post-translational secretion has been demonstrated (Lloyd et al., 2001b). Here, analysis of supernatants for YopE secretion shows comparable results to those found for YopQ (Fig. 5).

To analyse whether YopQ synthesized prior to Yop induction (Ca²⁺ depletion) is competent for secretion we inhibited protein synthesis 20 min before induction (Lloyd et al., 2001b) and collected supernatants after induction and ongoing inhibition of protein synthesis. As shown in Fig. 6 YopQ/K and YopE are both secreted independently from protein synthesis in this experimental approach. In a similar experiment Lloyd et al. (2001b) demonstrated a chaperone-assisted rapid secretion of YopE independently from protein synthesis. We mimicked their approach and found that not only YopE but also YopQ was secreted within seconds after induction in a first burst (data not shown). Finally, we analysed the effect of Cm on Yop secretion in a time-course experiment (Fig. 6b). We could demonstrate that after Cm treatment the secretion of YopE is decreased more rapidly than secretion of YopQ. This can only be explained by different half-lives of the secretion-competent fraction of the respective Yop. The half-life of YopQ (under overexpressing conditions) has been determined by Anderson & Schneewind (1999) to be approximately 30 min. The decrease of YopQ secretion described here is in line with their observation.

In summary, the experiments shown in Figs 5 and 6 do not indicate any differences in the secretion mechanism of the two Yops.

These findings are in contradiction to the work presented by Anderson & Schneewind (1999), who could
not demonstrate post-translational secretion. However, their experimental approach is not comparable as they used a tac promoter for induction of yopQ. This overproduction has been shown by the same authors to influence the regulation of YopQ secretion. It might also be that YopQ overproduction leads to overtitration of an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has recently been discussed by Lloyd erones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ. The role of chaperones in creating a hierarchy of Yop secretion has an unknown chaperone for YopQ.

In conclusion our comparative analysis of different serotypes of Y. enterocolitica demonstrates (i) strain-dependent YopQ production and accumulation in the cytosol that might be due to differences in folding structure of the 5’ end of the corresponding mRNA, and (ii) that a significant portion of YopQ is post-translationally secreted.

ACKNOWLEDGEMENTS

We thank Verena Lehmann for excellent technical assistance and Elke Wilharm and Konrad Trülsch for critical comments on this manuscript. We also thank the referees for valuable suggestions. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 594, Teilprojekt B6). J.T. was partially supported by a fellowship from the DAAD (Deutscher Akademischer Austauschdienst).

Fig. 6. Western blot showing secretion of YopQ/K and YopE after inhibition of protein synthesis prior to induction of Yop secretion. (a) Strains were grown in Ca-BHI for 2 h at 37 °C, then Cm was added (100 µg ml⁻¹) and cultures were incubated for 20 min at 37 °C. Bacteria were pelleted, resuspended in EGTA-BHI (plus 100 µg Cm ml⁻¹) and grown for another 20 min before harvesting the cells. An aliquot (50 µl) of culture was loaded for analysis of pellet fractions and 500 µl culture supernatant was loaded; detection was with polyclonal antisera raised against YopQ and YopE, respectively. The secretion-deficient strain WA-C(pYV-S15) served as a control for specificity of protein release. (b) Time-course of the secretion of YopQ and YopE after Cm treatment and subsequent Yop induction. Strain WA-314 was grown in Ca-BHI for 2 h at 37 °C, then Cm was added (100 µg ml⁻¹). At the time points indicated aliquots of the culture were pelleted, washed in BHI (plus Cm), resuspended in EGTA-BHI (plus 100 µg Cm ml⁻¹) to induce Yop secretion and grown for another 20 min at 37 °C before supernatants were prepared for SDS-PAGE and subsequent Western blotting. The control, lane C, shows the amount of Yops secreted within 20 min in the absence of Cm (an aliquot of the culture taken immediately before Cm treatment).

REFERENCES


Expression and secretion of Y. enterocolitica YopQ


Received 20 August 2001; revised 3 January 2002; accepted 15 January 2002.