Analysis of *Yersinia enterocolitica* invasin expression *in vitro* and *in vivo* using a novel *luxCDABE* reporter system

Janja Trček,¹ Thilo M. Fuchs² and Konrad Trülzsch¹

¹Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität, München, Germany
²Zentralinstitut für Ernährung- und Lebensmittelforschung, Abteilung Mikrobiologie, Technische Universität München, Germany

A novel *luxCDABE* plasmid for the analysis of promoter elements by site-specific integration into the genome of *Yersinia enterocolitica* was constructed. The versatility of this reporter system was demonstrated by comparing the activity of the *inv* promoter in the *Y. enterocolitica* high-pathogenic serotype O : 8 (strain WA-314) with that of the low pathogenic serotype O : 9 (strain Y127). The luciferase activity of a transcriptional fusion between the *inv* promoter of serotype O : 8 and *luxCDABE* was about fourfold lower than the activity of the respective O : 9 promoter. This correlated with lower invasin production by *Y. enterocolitica* serotype O : 8 compared with serotypes O : 9, O : 3 and O : 5,27. However, *Y. enterocolitica* of serotype O : 8 revealed higher invasiveness than serotype O : 9. When both invasins were expressed *in trans* at similar levels in the *Y. enterocolitica* O : 8 Δinv background strain, cell invasion assays showed a slightly higher invasiveness of the strain producing Inv(O : 8) than the strain producing Inv(O : 9). We provide experimental evidence that this might be due to a higher binding capacity of Inv(O : 8) for cells expressing β1 integrins compared with Inv(O : 9). The *Y. enterocolitica* O : 8 strain harbouring the Pinv(Inv(O : 8)) : *luxCDABE* fusion was then successfully used to follow *inv* expression in a mouse infection model. These experiments showed for the first time that the *inv* promoter is active in infected living mice, especially in Peyer’s patches of the ileum, the caecal lymph follicle, and the lymph nodes, liver and spleen. The production of invasin in the spleen was demonstrated by Western blot analysis. In conclusion, the presented reporter system enables stable genomic integration of the *luxCDABE* operon into the chromosome of *Yersinia*, facilitates *in vitro* quantification of promoter activities under different bacterial growth conditions, and enables detection of promoter activities in a mouse model.

**INTRODUCTION**

*Yersinia enterocolitica* is an enteropathogenic Gram-negative bacterium, which, after *Salmonella enterica* and *Campylobacter jejuni*, is the third most common cause of foodborne gastroenteritis in Europe (Bottone, 1997). It is able to quickly adapt its metabolism to different ecological niches and temperatures (Bresolin *et al.*, 2006; Heermann & Fuchs, 2008). *Y. enterocolitica* is a psychrotrophic bacterium that proliferates at temperatures as low as 0 °C. This characteristic is of major concern for public health authorities, since *Y. enterocolitica* can grow in food products even during cooling. After oral uptake, yersiniae invade Peyer’s patches of the ileum by entering through specialized epithelial cells called M cells. This is made possible by the interaction of the *Yersinia* invasin with β1 integrins (Isberg & Leong, 1990), which are expressed on the luminal side of M cells but not enterocytes (Clark *et al.*, 1998). After translocation across the mucosal barrier, yersiniae subsequently disseminate to the lymph nodes, spleen and liver, where they form monoclonal micro-abscesses (Oellerich *et al.*, 2007).

Since the entire genome sequence of *Y. enterocolitica* O : 8 is publicly available (Thomson *et al.*, 2006), studies on gene expression and regulation under different environmental conditions are the most straightforward approach to learn more about the adaptation strategies of this versatile
bacterium. For this purpose, different reporters, such as GFP, β-galactosidase (LacZ), firefly luciferase (Luc), bacterial luciferase (LuxAB) and alkaline phosphatase (PhoA), have been established. The drawback of the GFP reporter is that it is very stable and thus its expression responds only slowly to environmental changes. It can also be toxic to cells when expressed at high levels (Greer & Szalay, 2002; Rang et al., 2003). Although GFP variants with lower half-lives have been constructed, the enzyme turnover of these GFP variants, which still show an acceptable level of fluorescence, is generally longer than that of luciferase (Andersen et al., 1998; Ignowski & Schaffer, 2004; Kohlmeier et al., 2007). The other reporters, LacZ, Luc, LuxAB and PhoA, require the addition of substrate to measure enzyme activity. In contrast, luxCDABE encodes not only the luciferase (LuxAB) but also the enzymes involved in substrate synthesis (LuxCDE). Enzymes encoded by the luxCDABE operon of Photobacterium luminescens (applied in this work) are stable at \(37^\circ\)C and above (Meighen, 1993). In contrast to the fluorescence of GFP, the bioluminescence of LuxCDABE requires metabolically active bacteria. The method of measuring luciferase activity is non-invasive and thus can be used to follow the kinetics of bioluminescence during different bacterial growth stages and for imaging bacteria in live mice (Loessner et al., 2007). In Yersinia spp., two other approaches have been used for identification of bacterial genes that are expressed during animal infection: in vivo expression technology (IVET) and signature-tagged mutagenesis (STM) (Darwin & Miller, 1999; Young & Miller, 1997). Recently, a luxCDABE-expressing strain of Yersinia pseudotuberculosis was applied to study the infection process in a mouse model (Isaksson et al., 2009). The benefits of the luxCDABE reporter prompted us to develop a novel plasmid that allows the chromosomal insertion of luxCDABE behind a promoter of choice and thus the analysis of a single-copy promoter activity in yersiniae.

We used this system to study in vitro and in vivo expression of inv by Y. enterocolitica. Invasin is known to be predominantly expressed at low temperature (\(23^\circ\)C) and early stationary phase, conditions which are prevalent in stored foods (Isberg et al., 1988; Pepe et al., 1994). Regulation of inv expression has been extensively studied, and the four proteins RovA, H-NS, YmoA and OmpR have been identified as playing an important role. RovA is a transcriptional activator that positively regulates inv expression, H-NS is a histone-like protein that competes with RovA for overlapping binding sites on the inv promoter, and YmoA forms a transcriptional repression complex with H-NS on the inv promoter (Ellison & Miller, 2006; Tran et al., 2005). The recently described OmpR protein binds to the −15 to −35 promoter region and negatively regulates inv expression (Brozstek et al., 2007). We have shown previously that invasin is not produced by yersiniae washed from the small intestine of mice 2 and 5 days after infection (Oellerich et al., 2007). Invasin, however, has been shown to be produced at \(37^\circ\)C at low pH (5.5) in vitro and has been demonstrated by immunoblotting in mouse Peyer’s patches 2 days after infection (Pepe et al., 1994). It was therefore of great interest to study the in vivo expression of inv in the mouse model.

### METHODS

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are described in Table 1. An inv deletion mutant WA-C(pYYv::cat) \(\Delta\)inv was constructed by Red recombination (Datsenko & Wanner, 2000) using a pair of 71 bp primers (5’-cgcattgttaaagcgataagatggaagagagagaggctggctgg-3’; 5’-ggtcagcctgaagctggctggctggctggctggctgg-3’). The 3’ end of the primers (underlined) amplified a spectinomycin cassette from the \(\Omega\) plasmid (Prenkti & Krisch, 1984). The primers were flanked by a 50 bp homology arm and were designed so that the entire coding region of inv and its 335 bp upstream region were replaced by the antibiotic-resistance cassette. The luxCDABE operon under the arabinose-inducible araBAD promoter (pBAD) was integrated downstream of glms in Y. enterocolitica WA-C, Y. enterocolitica WA-C \(\Delta\)inv and Y. enterocolitica Y127-C by triparental mating. Escherichia coli S17.1 \(\Delta\)pir strains bearing plasmids pHL289 and pUX-BF13 were used for conjugational transfer. The inv gene with its 334 bp upstream region and 328 bp downstream region was cloned into the HindIII site of pACYC184 using primers InvFw (5’-gccgaagcttaagatggaagagagagaggctggctgg-3’) and InvRev (5’-gccgaagcttaagatggaagagagagaggctggctgg-3’) (introduced restriction sites in italic). The plasmids were checked by PCR analysis and DNA sequencing. Some strains were cured of the pYY plasmid by overnight growth in brain heart infusion (BHI) medium with 5 mM EGTA. The colonies were spread on agar and the presence of the pYY plasmid was checked by PCR using primers specific for \(\text{pY}^\circ\). Yersinia and E. coli were routinely grown in Luria–Bertani (LB) broth at 27 and \(37^\circ\)C, respectively. Chloramphenicol (20 \(\mu\)g mL\(^{-1}\)), nalidixic acid (60 \(\mu\)g mL\(^{-1}\)) and kanamycin (50 \(\mu\)g mL\(^{-1}\)) were used as selective antibiotics. E. coli DH5\(\alpha\) (Hanahan, 1983) was used as the primary host in cloning experiments: E. coli S17.1 \(\Delta\)pir (Simon et al., 1983) was used as donor for conjugation.

**Construction of a luxCDABE integration plasmid.** A transposon vector, pUT mini-Tn5 luxCDABE Km2 (Winson et al., 1998), was reconstructed by cutting out the Xhol/PvuI fragment (1607 bp), encoding a transposase, a kanamycin-resistance cassette and a part of the \(\beta\)-lactamase gene. At the same site, a 989 bp fragment encoding a chloramphenicol-resistance gene (660 bp) with its own putative promoter region (245 bp) and putative terminator region (84 bp) was introduced, resulting in plasmid pTHluxCDABE-Cm. The cat gene was ampliﬁed from pKRG (Bresolin et al., 2006) using primers Cmfw (5’-gagcgccgagcgaagagagagag-3’) and Cmrev (5’-ggcgccgagcgaagagagag-3’) (introduced restriction sites in italic type). DNA manipulation, plasmid isolation and the transformation of \(E.\) coli were performed according to standard procedures (Sambrook et al., 1989).

**Construction of promoter–luxCDABE fusions and integration into the chromosome.** The predicted inv promoters (P\(_{\text{inv}}\)) of Y. enterocolitica serotype O:8 (strain WA-314) and Y. enterocolitica serotype O:9 (strain Y127) were PCR-amplified with primers PinvFW (5’-cgacctgctagcagctgctgaagagagagag-3’) and PinRev (5’-gggtcctagctggctggctggctggctggctgg-3’) (introduced restriction sites in italic type) using Pfu DNA polymerase (Fermentas), which exhibits proofreading activity, and cloned into the SacI/KpnI site of pTIluxCDABE-Cm, resulting in a P\(_{\text{inv}}\)-luxCDABE transcriptional fusion. The isolation of chromosomal DNA from Y. enterocolitica has been described previously (Bresolin et al., 2008). The same combination of primers was also applied to PCR amplification and sequencing of putative promoters from the other strains of Y. enterocolitica. The promoter region of the construct was used as the target of a single cross-over with the homologous genome sequence. This was achieved by conjugating the donor strain E. coli S17.1 \(\Delta\)pir, bearing an appropriate construct of
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description and relevant features</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F⁻ deoR endA1 gyrA96 hsdR17(rK mC^') relA1 supE44 λthi-1 Δ(lacZYA-argFV169)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>S1.71 'pir</td>
<td>Sm^R recA thi pro hsdR^- hsdM^- RP4::-Tc::Mu::Km Tn7 'pir lysogen</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F^-ompT gal [dcm] [lon] hsdS^- (rK mC^-) λ ϕ prophage carrying the T7 RNA polymerase gene</td>
<td>Studier et al. (1990)</td>
</tr>
<tr>
<td><strong>Y. enterocolitica strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WA-314</td>
<td>Serotype O: 8 pYV^+</td>
<td>Heesemann &amp; Laufs (1983)</td>
</tr>
<tr>
<td>8081</td>
<td>Serotype O: 8 pYV^+</td>
<td>V. Miller*</td>
</tr>
<tr>
<td>900-36/90</td>
<td>Serotype O: 8 pYV^+</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>Y286</td>
<td>Serotype O: 9 pYV^-</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>W22703</td>
<td>Serotype O: 9 pYV^-</td>
<td>Cornels &amp; Colson (1975)</td>
</tr>
<tr>
<td>Y127</td>
<td>Serotype O: 9 pYV^-</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>Y738</td>
<td>Serotype O: 9 pYV^-</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>H469/87</td>
<td>Serotype O: 3 pYV+</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>Y746</td>
<td>Serotype O: 3 pYV+</td>
<td>Max von Pettenkofer Institute</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUT mini-Tn5 luxCDABE Km2</td>
<td>Suicide plasmid, ori R6K, mini-Tn5, promoterless luxCDABE, mob^+ (RP4), Ap^R Km^R</td>
<td>Winson et al. (1998)</td>
</tr>
<tr>
<td>pTluxCDABE-Cm</td>
<td>Suicide plasmid, ori R6K, promoterless luxCDABE, mob^+ (RP4), Cm^R</td>
<td>This study</td>
</tr>
<tr>
<td>pTluxCDABE-Cm-Pinv(O:8)</td>
<td>pTluxCDABE-Cm carrying 519 bp inv upstream region from WA-314 in front of luxCDABE</td>
<td>This study</td>
</tr>
<tr>
<td>pTluxCDABE-Cm-Pinv(O:9)</td>
<td>pTluxCDABE-Cm carrying 519 bp inv upstream region from Y127 in front of luxCDABE</td>
<td>This study</td>
</tr>
<tr>
<td>pKRG9</td>
<td>Suicide plasmid, ori R6K, mob^+ (RP4), Cm^R</td>
<td>Bresolin et al. (2008)</td>
</tr>
<tr>
<td>pHL289</td>
<td>Suicide plasmid, ori R6K, luxCDABE under P_MAP promoter, Amp^R Km^R</td>
<td>Loesner et al. (2007)</td>
</tr>
<tr>
<td>pUX-BF13</td>
<td>Suicide plasmid, ori R6K, transposase Tn7, Amp^R</td>
<td>Bao et al. (1991)</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Low-copy vector, ori P15A, Cm^R Tc^R</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pACYC-Inv(O:8)</td>
<td>pACYC184 carrying inv of WA-314, Cm^R</td>
<td>This study</td>
</tr>
<tr>
<td>pACYC-Inv(O:9)</td>
<td>pACYC184 carrying inv of Y127, Cm^R</td>
<td>This study</td>
</tr>
<tr>
<td>pGEX-4T3</td>
<td>GST gene fusion expression vector</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pGEX-4T3-Inv397(O:8)</td>
<td>Translational fusion between gst and inv397 from WA-314</td>
<td>Wiedemann et al. (2001)</td>
</tr>
<tr>
<td>pGEX-4T3-Inv397(O:9)</td>
<td>Translational fusion between gst and inv397 from W22703</td>
<td>This study</td>
</tr>
</tbody>
</table>

*The University of North Carolina, NC, USA.

pTluxCDABE-Cm-Pinv, and the recipient strain of Y. enterocolitica on LB agar plates for 6–12 h. To prevent the loss of the virulence plasmid pYV from yersiniae, the plates were incubated at 27 °C. The exconjugants were selected on LB agar plates with chloramphenicol (20 μg ml^-1) and nalidixic acid (60 μg ml^-1) to select against E. coli. Since Y. enterocolitica Y127 does not possess any known antibiotic-resistance markers, the exconjugants were selected on the Yersinia-specific agar CIN (Oxoid) supplemented with chloramphenicol.
(20 μg ml⁻¹). The appropriate site of luxCDABE insertion was checked by PCR analysis and DNA sequencing.

**In vitro measurement of luminescence.** To measure luciferase activity, each exconjugant was grown in 5 ml LB medium in glass tubes at 27 or 37 °C overnight. This was then used as the inoculum (1:100 dilution) for 100 ml LB medium with chloramphenicol in 500 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (180 r.p.m.). Every 90 min, 200 μl was removed to measure luciferase activity in white 96-well plates using a luminometer (MicroLumat Plus LB 96V, Berthold Technologies). The signal was measured for 2 s and recorded as relative light units (RLU). The bacterial growth was measured as OD₆₀₀.

**Analysis of Inv production.** The correlation between promoter activity and Inv production was determined by immunoblotting. The strains were grown overnight at 27 °C, diluted 1:20 and grown to OD₆₀₀ 1.0. The protein concentration was determined by the Bio-Rad Protein Assay based on the method of Bradford (1976). Equal amounts of protein were loaded into each lane of an 11 % SDS-PAGE gel and blotted onto a nitrocellulose membrane. The membrane was blocked in PBS containing 3 % BSA for 1 h at room temperature, and incubated with polyclonal anti-Inv antibodies (dilution 1 : 5 000) in PBS containing 0.5 % Tween (PBS-T) for 1 h. After washing with PBS-T, the membrane was incubated in the presence of horseradish peroxidase-conjugated anti-rabbit IgG antibodies (dilution 1 : 10 000) in PBS-T for 1 h. Detection was carried out using the ECL system (GE Healthcare). As a control, different serotypes of *Y. enterocolitica* were also incubated with polyclonal antibody raised against heat-killed *Y. enterocolitica* (Heesemann & Laufs, 1983).

**Surface detection of Inv by flow cytometry.** For detection of invasin production on the surface of whole-cell *Y. enterocolitica*, immunostaining in combination with flow cytometric analysis was performed. Approximately 5 × 10⁸ bacteria were prepared as described above and blocked in PBS containing 2.5 % goat serum for 30 min at 4 °C. Then the polyclonal anti-Inv antibodies (dilution 1 : 5000) were added and incubated for 1 h at 4 °C. The cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit antibodies (Invitrogen, dilution 1 : 500) in PBS containing 2.5 % goat serum for 1 h at 4 °C. After washing with PBS, the bacteria were fixed in PBS containing 4 % paraformaldehyde for 15 min at 4 °C, washed twice with PBS containing 2 mM EDTA and 0.5 % BSA, and kept in the same buffer in the dark at 4 °C until analysis. Data were collected for 10 000 events using a FACS Canto II flow cytometer (Becton Dickinson) equipped with 488 nm laser excitation. Fluorescence was detected at 530 nm.

**Infection of cell cultures.** To compare the efficiency of bacterial internalization mediated by Inv(O : 8) and Inv(O : 9), 3 × 10⁴ cells per well of HEP-2, GE-11 β1 (Gimond *et al.*, 1999) and GE-11 β1-null cell lines (Gimond *et al.*, 1999) were seeded in sterile, white, 96-well plates 24 h before infection. The HEP-2 cells were grown in RPMI 1640 medium (Invitrogen), and GE11 β1 and GE11 cells in DMEM high-glucose medium (Invitrogen), all supplemented with 10 % heat-inactivated fetal calf serum (FCS) and incubated in a humidified 5 % CO₂ atmosphere at 37 °C. The cells were infected with *Y. enterocolitica* WA-C *inv* *attTn7*::luxCDABE expressing inv(O : 8) or inv(O : 9) from pACYC184. The infection was performed at m.o.i. 50 for 2 h, after which the extracellular bacteria were washed away. The eukaryotic cells were then additionally incubated in medium with gentamicin (100 μg ml⁻¹) to kill extracellular bacteria and L-arabinose (0.2 %) to induce luxCDABE. The intracellular bacteria were measured 2 h after addition of gentamicin by the intensity of the Lux signal, as described by Flintet *et al.* (2008). The same procedure was used to compare invasiveness between *Y. enterocolitica* WA-C *attTn7*::luxCDABE and Y127-C *attTn7*::luxCDABE *Y. enterocolitica* WA-C Δinv *attTn7*::luxCDABE was used as a negative control. A standard curve correlating c.f.u. and luminescence was made after infection of HEP-2 with WA-C *attTn7*::luxCDABE at different m.o.i.

**Production of recombinant Inv397.** The recombinant Inv397(O : 9) encompassing the C-terminal end, consisting of domains D1, D3, D4 and D5 from *Y. enterocolitica* W22703 (serotype O : 9), was produced as described elsewhere for Inv397(O : 8) from WA-314 (Wiedemann *et al.*, 2001). Briefly, a PCR fragment, inv397(O : 9), was amplified with primers Inv397fw (5’-acgtgatattcctaccagccagataa-3’) and Inv397Rev (5’-gcgcttgatattgacttcggcc-3’) (introduced restriction sites in italic type) and cloned into pGEX-4T3. Inv397(O : 9) was overexpressed in *E. coli* BL21 (DE3) grown at 37 °C after induction with 1 mM IPTG. The bacterial cells were harvested and disrupted with a French press. Glutathione S-transferase (GST)-Inv397(O : 9) was purified from the supernatant using Glutathione Sepharose (Pharmacia Biotech) and Superdex 200 gel filtration.

**Binding of invasin to β1 integrins.** The GST fusion protein (0.1 mg), 50 μl 1 M NaHCO₃ and 0.25 mg Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen) suspended in 20 μl DMSO was mixed in a total volume of 500 μl PBS and incubated for 1 h in the dark at room temperature by mixing on a shaker. The non-attached dye was removed from the solution by dialysing against PBS at 4 °C. The labelled protein was stored at −20 °C until further use. The GE11 and GE11 β1 cells were enzymatically detached using trypsin EDTA, harvested and suspended in FACS buffer (27 mM Tris, pH 6.4, 2.4 mM KCl, 137 mM NaCl). The cells and proteins were incubated for 1 h at 4 °C with 1 mM MnCl₂. Approximately 5 × 10⁶ cells suspended in 200 μl FACS buffer were mixed with 2 μg Alexa Fluor 488-labelled GST–Inv397(O : 8) or GST–Inv397(O : 9) and incubated at 4 °C for 1 h. The cells were centrifuged at 1100 r.p.m. for 10 min and washed three times with FACS buffer. Binding was detected by flow cytometry.

**In vivo monitoring of inv expression and Inv production.** Infection in a mouse model was carried out in 6–8-week-old female BALB/c mice. The luminescent yersiniae were grown in LB medium at 27 °C with shaking to the late exponential phase, washed twice and suspended in LB medium containing 15 % (v/v) glycerol. The bacteria were stored at −80 °C and c.f.u. were determined by plating serial dilutions. Five mice were orally infected with 10⁶ c.f.u. and three intravenously in the lateral tail vein with 10⁶ c.f.u. of *Y. enterocolitica* WA-C(pYV::cat) P₃ inv(O : 8) :: luxCDABE. inv expression was followed daily for 4 days using the IVIS Lumina System (Xenogen). Before imaging, mice were anaesthetized with isoflurane using the Xenogen XGI-8 gas anaesthesia system. After live imaging, mice were sacrificed by CO₂ asphyxiation, and the entire intestinal tract was removed along with the liver, spleen, and mesenteric and cervical lymph nodes and subjected to analysis with the IVIS Lumina system. To analyse invasin production, spleens from mice infected with *Y. enterocolitica* WA-C(pYV::cat) as well as with *Y. enterocolitica* WA-C(pYV::cat) Δinv were collected 5 days after oral infection as described above. The spleens were homogenized, and equal amounts of sample were subjected to Western blotting and immunostaining with anti-Inv antibodies, as described above.

**Statistical analysis.** Experiments were repeated at least three times. Quantitative data were statistically evaluated using the non-parametric Mann–Whitney test by using GraphPad Prism software. Differences were considered significant at P<0.05.

**RESULTS AND DISCUSSION**

**Validation of pITluxCDABE-Cm by measuring inv promoter activity.** The chromosomal integration of the transcriptional fusion between the inv promoter and luxCDABE resulted in a
merodiploid strain, with the \( P_{\text{inv}} \)-\( \text{luxCDABE} \) fusion being followed by the rest of the suicide plasmid \( pJT\text{luxCDABE-Cm} \) and a wild-type copy of the \( \text{inv} \) gene. To study the effects of temperature and growth phase, yersiniae of serotypes O:8 and O:9 were grown at 27 and 37 °C for a period of 51 h (Fig. 1). These experiments showed that the luminescence of yersiniae harbouring the transcriptional fusions responds well to different temperatures (about fourfold lower at 37 °C than at 27 °C). This is in accordance with the published data, which show that \( \text{inv} \) expression in \( Y. \text{enterocolitica} \) is fourfold lower at 37 °C than at 23 °C (Pepe et al., 1994). During the stationary phase, the luminescence signal finally decreased (Fig. 1a). The effect of growth phase on \( \text{inv} \) expression, as

![Graphs showing luciferase activity](https://example.com/graph.png)

**Fig. 1.** Effect of temperature (a) and growth phase (b) on luciferase activity exhibited by \( P_{\text{inv(O:8)}} \)::\( \text{luxCDABE} \) present in \( Y. \text{enterocolitica} \) WA-C(pYV::\( \text{kan} \)) and \( P_{\text{inv(O:9)}} \)::\( \text{luxCDABE} \) present in \( Y. \text{enterocolitica} \) Y127. Filled triangles and squares represent luminescence at 27 and 37 °C, respectively. Open triangles and squares represent the optical density of bacterial cultures grown at 27 and 37 °C, respectively. The exponential (log) phase, late exponential (late log) phase and stationary (station.) phases are denoted for bacteria taken from the cultures at OD600 0.15, 1.0 and 2.0, respectively. All data represent the mean ± SD from three independent experiments.
determined by the luxCDABE reporter, is presented in Fig. 1(b). These data confirmed earlier findings (Pepe et al., 1994) that inv expression at 27 °C reaches its maximum in the stationary phase. Interestingly, serotype O : 9, which in contrast to serotype O : 8 shows no growth retardation at 37 °C, expressed inv more strongly in the exponential phase than in the stationary phase at 37 °C. To show that luciferase activity reflects differences in the promoter region of inv, P_{inv(O:8)}::luxCDABE was also integrated into the chromosome of Y. enterocolitica O : 9. The activity of the P_{inv(O:8)}::luxCDABE transcriptional fusion in the background of the O : 9 serotype was identical to that of the same fusion in Y. enterocolitica O : 8, suggesting that no specific regulation factor in Y. enterocolitica O : 8 is responsible for differences in inv expression between serotype O : 8 and O : 9.

Analysis of invasin production by immunoblotting and FACS analysis

To determine whether differences in inv expression demonstrated by luminescence measurements could be confirmed by immunostaining, yersiniae of serotypes O : 8, O : 9, O : 5,27 and O : 3, as well as an inv mutant, were grown in vitro and subjected to immunoblotting and FACS analysis using polyclonal anti-Inv antibodies. These experiments confirmed stronger invasin production in Y. enterocolitica serotype O : 9 compared with serotype O : 8, as estimated by densitometry (Fig. 2a). The serotypes O : 5,27 and O : 3 also produced more invasin than serotype O : 8 (Fig. 2a). Analysis of additional strains of the same serotype confirmed this observation, suggesting that the amount of invasin produced is not strain-
serotype-specific. To investigate whether there is a difference in the amount of surface-exposed invasin among different serotypes of \textit{Y. enterocolitica}, flow cytometric analysis of whole-cell bacteria was performed. This analysis showed about fourfold stronger invasin production on the surface of serotype O:9 compared with serotype O:8 (Fig. 2b). Additionally, only about 50\% of serotype O:8 cells were fluorescent in contrast to about 95\% of serotype O:9 cells. Invasin production as well as the number of fluorescent yersiniae of serotype O:5,27 were higher than for serotype O:8 but lower than for serotype O:9 (Fig. 2b). The observation that only 50\% of O:8 serotype cells have invasin on the surface might indicate specific regulation of \textit{inv} expression on the single-cell level. Lower production of Inv(O:8) per bacterium and fewer \textit{inv}-expressing yersiniae might also contribute to the clonal invasion phenomenon of \textit{Y. enterocolitica} O:8 recently described by us (Oellerich et al., 2007).

### Analysis of \textit{inv} promoter sequences

In order to determine whether the observed differences in \textit{inv} expression between the tested \textit{Yersinia} serotypes were

![Sequence comparison of RovA and H-NS binding sites (Ellison & Miller, 2006) in \textit{inv} upstream regions among different serotypes of \textit{Y. enterocolitica}. Alignment was performed with CLUSTAL \_ X (Thompson et al., 1997). The RovA binding sites are marked with light-grey shading and the H-NS binding sites with dark-grey shading. Nucleotides typical of serotype O:8 in the RovA and H-NS binding sites that might affect the efficiency of regulator binding are boxed. The "35\/-10 region and the transcriptional start (Pepe et al., 1994) are shown in bold and italic type, respectively. Asterisks indicate identical nucleotides.](https://example.com/sequence.png)
due to differences in the nucleotide sequence of their respective promoters, we compared the inv upstream regions of different Y. enterocolitica serotypes (Fig. 3). Close to the translational start, a typical deletion of three nucleotides was detected in serotype O:8 that was not observed in the sequences of the analysed O:9, O:3 and O:5,27 serotypes of Y. enterocolitica (data not shown). Further analysis of the promoter regions revealed nucleotides typical of serotype O:8 in the RovA and H-NS binding sites, which might affect the efficiency of regulator binding to DNA targets (Fig. 3). The RNAxDraw program (Matzura & Wennborg, 1996) was used to fold the untranslated mRNA to identify potential folds that might affect translation. We were, however, unable to identify any folds that were typical of Y. enterocolitica serotype O:8 strains.

Comparison of invasiveness between Y. enterocolitica serotypes O:8 and O:9

Since Y. enterocolitica serotype O:8 produced less invasin than serotype O:9, we wondered whether this correlated with a reduced invasion capacity for HEp-2 cells. Therefore, cell invasion assays were performed using arabinose-inducible luminescent yersiniae, as described in Methods. These experiments revealed that WA-C (serotype O:8) was more invasive than strain Y127-C (serotype O:9) (Fig. 4a). This was surprising, since WA-C produced less invasin than Y127-C. This might be due to differences in binding capacity to β1 integrins or to other serotype-specific differences between the two strains. To exclude such differences, both inv(O:8) and inv(O:9) were cloned and expressed in the WA-C Δinv attTn7::luxCDAE mutant background. Immunoblot analysis revealed that inv(O:8) and inv(O:9) expressed under their own promoters from pACYC184 produced equal amounts of Inv (data not shown). Gentamicin protection assays performed with WA-C Δinv attTn7::luxCDAE pACYC-Inv(O:8) and WA-C Δinv attTn7::luxCDAE pACYC-Inv(O:9) showed that Inv(O:8) mediated slightly higher invasiveness compared with Inv(O:9) in the same background strain (Fig. 4b). This observation suggests that the higher invasiveness of Y. enterocolitica O:8 is probably attributable to a 13 amino acid difference between the inv-coding regions of Y. enterocolitica WA-314 (serotype O:8) (accession no. FN594891) and Y. enterocolitica W1024 (serotype O:9) (accession no. Z48169). To find out whether these amino acid differences result in different binding properties of the two invasins to β1 integrins, we performed binding assays.

**Fig. 4.** Comparison of invasiveness between Y. enterocolitica serotype O:8 and serotype O:9 (a) and between Y. enterocolitica WA-C Δinv pACYC184-Inv(O:8) and WA-C Δinv pACYC184-Inv(O:9) (b) using the indicated cell lines. A gentamicin protection assay was performed with luminescent yersiniae. (c) Flow cytometric analysis of eukaryotic cells after binding between the indicated cell line and fluorochrome-conjugated recombinant protein GST–Inv397(O:8) or GST–Inv397(O:9). An asterisk indicates a significant difference (P<0.05). Histograms are presented in Supplementary Fig. S1. (d) Correlation between c.f.u. and luminescence (RLU).
between labelled invasins and epithelial cells with and without β1 integrins. As shown in Fig. 4(c), the results of these experiments indicated a slightly higher binding capacity of Inv(O : 8) for β1 integrins in comparison with Inv(O : 9). This could explain the higher invasiveness mediated by Inv(O : 8) compared with Inv(O : 9).

Fig. 5. In vivo inv expression of Y. enterocolitica. BALB/c mice were infected orally with $1 \times 10^9$ c.f.u. (a) and intravenously with $1 \times 10^4$ c.f.u. (b) of Y. enterocolitica WA-C(pYV::kan) harbouring $P_{inv(O : 8)}::luxCDABE$ in their chromosome. The standard curve (c) was made from measurements of luminescence and c.f.u. in Peyer’s patches. The luminescence of live mice was followed for 4 days using the IVIS Lumina System (Xenogen). Extracted organs were visualized 3 days (a) and 4 days (b) post-infection. The examined organs are marked as L (liver), S (spleen), LN (lymph nodes), C (caecum), LU (lung), LI (large intestine), SI (small intestine) and PP (Peyer’s patches).
Application of the luxCDABE system in a mouse infection model

To determine whether the luxCDABE system is suitable for in vivo inv expression studies, we infected BALB/c mice with WA-C(pYV::kan) P_{inv(O:8)}::luxCDABE orally as described in Methods. Only 1 day after infection, luminescent Peyer’s patches of the gut were observed. From days 1 to 3, bacterial luminescence of infected Peyer’s patches increased strongly, demonstrating for the first time inv expression in living mice (Fig. 5a). Bacteria that were washed from the small intestinal lumen did not show any significant luminescence, confirming our previous data showing that Inv is not produced by yersiniae 2 and 5 days post-infection as analysed by Western blotting (Oellerich et al., 2007). Furthermore, this shows that there is no significant non-specific inv expression in the mouse model. Three days post-infection, the small intestine, large intestine, cervical lymph nodes, spleen and liver were removed for a more detailed analysis of P_{inv} activity. At this time point, multiple Peyer’s patches were strongly luminescent along with the caecal lymph follicle and cervical lymph nodes (Fig. 5a). No luminescence was detected in the liver or spleen due to the expected low colonization at this time point. In order to achieve strong systemic colonization, three mice were infected intravenously with WA-C(pYV::kan) P_{inv(O:8)}::luxCDABE. On days 3 and 4 post-infection, the luminescence of living mice increased strongly (Fig. 5b). Extraction of organs revealed that this was due to the luminescence of the spleen, liver and lungs as well as the lymphoid tissue of the entire gastrointestinal tract. Culturing yersiniae from different organs demonstrated 99% stability of the luciferase construct for at least 4 days in the mouse model. These experiments show for the first time that inv is expressed not only in lymphoid tissue of the gut but also in the spleen, liver and lungs of live mice. It is thus conceivable that invasin plays an important role in pathogenesis in these organs besides mediating invasion (Arencibia et al., 1997; Ennis et al., 1993; Grassl et al., 2003; Lundgren et al., 1996; Schulte et al., 2000). With the aim of confirming invasin production in the mouse infection model, we analysed spleens from mice infected with Y. enterocolitica WA-C(pYV::cat) and Y. enterocolitica WA-C(pYV::cat) Δinv by Western blot analysis. As shown in Fig. 6, specific bands were confirmed in the spleens of mice infected with wild-type Y. enterocolitica WA-C(pYV::cat) but not in spleens of mice infected with the inv deletion mutant. The detected invasin was shorter (~85 kDa) than full-length Inv, which might be the result of proteolytic degradation of Inv, as noted before (Pepe et al., 1994).

Since many genome sequences of yersiniae have been determined and annotated, the presented reporter system will facilitate the analysis of promoter activities and of the expression of virulence factors under different growth conditions, thus supporting the search for functional coding sequences in yersiniae and the study of the in vivo expression of Yersinia virulence factors in mice.

**ACKNOWLEDGEMENTS**

We would like to thank Holger Loessner, Helmholtz Centre for Infection Research, for plasmids pHL289 and pUX-BF13, Ingo Autenrieth, University Hospital Tübingen, for polyclonal anti-Inv antibodies, Hicham Buoabe and Virginie Nägele for help with the flow cytometry, and Kathrin Biersch for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft (DFG) grant TR 740/2-1. J.T. was partially supported by the German Academic Exchange Service in the programme Modern Applications of Biotechnology.

**REFERENCES**


enterocolitica O:8 to pathogenicity in the mouse infection model. Infect Immun 72, 5227–5234.


Edition by: H. Hilbi