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

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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 $\beta 1$ integrins compared with Inv(O:9). The *Y. enterocolitica* O:8 strain harbouring the $P_{inv(O:8)}::luxCDABE$ 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.

Received 16 January 2010

Revised 8 June 2010

Accepted 11 June 2010

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 $\beta 1$ 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

Abbreviations: GST, glutathione S-transferase; RLU, relative light units.

The GenBank/EMBL/DDBJ accession numbers for the construct pJT*luxCDABE*-Cm and for *inv* from *Y. enterocolitica* WA-314 are FN594890 and FN594891, respectively.

A supplementary figure, showing the results of FACS analysis of eukaryotic cells after binding of fluorochrome-conjugated recombinant proteins, is available with the online version of this paper.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description and relevant features	Source or reference
<i>E. coli</i> strains		
DH5 α	F [−] <i>deoR endA1 gyrA96 hsdR17(r_K[−] m_K⁺) recA1 relA1 supE44 λthi-1 Δ(<i>lacZYA-argFV169</i>)</i>	Hanahan (1983)
S17.1 λ pir	Sm ^R <i>recA thi pro hsdR[−] hsdM⁺</i> RP4::2-Tc::Mu::Km Tn7 λ pir lysogen	Simon <i>et al.</i> (1983)
BL21(DE3)	F [−] <i>ompT gal [dcm] [lon] hsdS_B (r_B[−] m_B[−])</i> , λ prophage carrying the T7 RNA polymerase gene	Studier <i>et al.</i> (1990)
<i>Y. enterocolitica</i> strains		
WA-314	Serotype O:8 pYV ⁺	Heesemann & Laufs (1983)
WA-C	WA-314 pYV [−]	Heesemann & Laufs (1983)
8081	Serotype O:8 pYV ⁺	V. Miller*
900-36/90	Serotype O:8 pYV ⁺	Bresolin <i>et al.</i> (2008)
Y286	Serotype O:8 pYV ⁺	Bresolin <i>et al.</i> (2008)
W22703	Serotype O:9 pYV [−]	Cornelis & Colson (1975)
Y-96-P	Serotype O:9 pYV ⁺	Heesemann (1987)
Y127	Serotype O:9 pYV ⁺	Bresolin <i>et al.</i> (2008)
Y738	Serotype O:9 pYV ⁺	Bresolin <i>et al.</i> (2008)
H469/87	Serotype O:3 pYV ⁺	Bresolin <i>et al.</i> (2008)
Y746	Serotype O:3 pYV ⁺	Max von Pettenkofer Institute
MvP	Serotype O:5,27 pYV ⁺	Max von Pettenkofer Institute
SZ1249/04	Serotype O:5,27 pYV ⁺	Bresolin <i>et al.</i> (2008)
WA-C(pYV::kan)	WA-C harbouring kanamycin-resistance cassette in pYV	Oellerich <i>et al.</i> (2007)
WA-C(pYV::cat)	WA-C harbouring chloramphenicol-resistance cassette in pYV	Trülsch <i>et al.</i> (2004)
WA-C(pYV::cat) Δ inv	<i>inv</i> replaced with spectinomycin-resistance cassette	This study
WA-C attTn7::luxCDABE	WA-C with chromosomally integrated <i>luxCDABE</i> downstream of <i>glmS</i>	This study
WA-C Δ inv attTn7::luxCDABE	WA-C Δ inv with chromosomally integrated <i>luxCDABE</i> downstream of <i>glmS</i>	This study
Y127-C attTn7::luxCDABE	Y127 with chromosomally integrated <i>luxCDABE</i> downstream of <i>glmS</i> , pYV [−]	This study
WA-C(pYV::kan)	WA-C(pYV::kan) with chromosomally integrated	This study
P _{inv} (O:8)::luxCDABE	P _{inv} (O:8)- <i>luxCDABE</i> fusion	
Y127 P _{inv} (O:9)::luxCDABE	Y127 with chromosomally integrated P _{inv} (O:9)- <i>luxCDABE</i> fusion	This study
Y127 P _{inv} (O:8)::luxCDABE	Y127 with chromosomally integrated P _{inv} (O:8)- <i>luxCDABE</i> fusion	This study
Plasmids		
pUT mini-Tn5 <i>luxCDABE</i> Km2	Suicide plasmid, <i>ori</i> R6K, mini-Tn5, promoterless <i>luxCDABE</i> , <i>mob</i> ⁺ (RP4), Ap ^R Km ^R	Winson <i>et al.</i> (1998)
pJT <i>luxCDABE</i> -Cm	Suicide plasmid, <i>ori</i> R6K, promoterless <i>luxCDABE</i> , <i>mob</i> ⁺ (RP4), Cm ^R	This study
pJT <i>luxCDABE</i> -Cm-P _{inv} (O:8)	pJT <i>luxCDABE</i> -Cm carrying 519 bp <i>inv</i> upstream region from WA-314 in front of <i>luxCDABE</i>	This study
pJT <i>luxCDABE</i> -Cm-P _{inv} (O:9)	pJT <i>luxCDABE</i> -Cm carrying 519 bp <i>inv</i> upstream region from Y127 in front of <i>luxCDABE</i>	This study
pKRG9	Suicide plasmid, <i>ori</i> R6K, <i>mob</i> ⁺ (RP4), Cm ^R	Bresolin <i>et al.</i> (2008)
pHL289	Suicide plasmid, <i>ori</i> R6K, <i>luxCDABE</i> under P _{BAD} promoter, Amp ^R Km ^R	Loessner <i>et al.</i> (2007)
pUX-BF13	Suicide plasmid, <i>ori</i> R6K, transposase Tn7, Amp ^R	Bao <i>et al.</i> (1991)
pACYC184	Low-copy vector, <i>ori</i> P15A, Cm ^R Tc ^R	New England Biolabs
pACYC-Inv(O:8)	pACYC184 carrying <i>inv</i> of WA-314, Cm ^R	This study
pACYC-Inv(O:9)	pACYC184 carrying <i>inv</i> of Y127, Cm ^R	This study
pGEX-4T3	GST gene fusion expression vector	GE Healthcare
pGEX-4T3-Inv397(O:8)	Translational fusion between <i>gst</i> and <i>inv</i> 397 from WA-314	Wiedemann <i>et al.</i> (2001)
pGEX-4T3-Inv397(O:9)	Translational fusion between <i>gst</i> and <i>inv</i> 397 from W22703	This study

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pJT*luxCDABE*-Cm-P_{inv}, 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 1:5000) 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 × 10⁶ bacteria were prepared as described above and blocked in PBS containing 2.5 % goat antiserum 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 antiserum 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 Δ*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 Flentie *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 Δ*inv 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 a 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'-acgtgaattctaccagtcacgaagataa-3') and Inv397Rev (5'-gccgctcgagctattgaggtccgcac-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 were 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 enzymically 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 × 10⁵ 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 10⁹ c.f.u. and three intravenously in the lateral tail vein with 10⁴ c.f.u. of *Y. enterocolitica* WA-C(pYV::kan) P_{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) Δ*inv* 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 pJT*luxCDABE*-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_{inv} - $luxCDABE$ fusion being followed by the rest of the suicide plasmid pJTLuxCDABE-Cm and a wild-type copy of the *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 *inv* expression in *Y. 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 *inv* expression, as

Fig. 1. Effect of temperature (a) and growth phase (b) on luciferase activity exhibited by $P_{inv(O:8)}::luxCDABE$ present in *Y. enterocolitica* WA-C(pYV::kan) and $P_{inv(O:9)}::luxCDABE$ present in *Y. 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 $\pm$ 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- but

Fig. 2. Production of invasin in different serotypes of *Y. enterocolitica* as determined by immunoblot analysis (a). In each lane of the Western blot, 30 µg protein of the following *Y. enterocolitica* strains was loaded: 1, WA-314 (serotype O:8); 2, 8081 (serotype O:8); 3, W22703 (serotype O:9); 4, SZ1249/04 (serotype O:5,27); 5, H469/87 (serotype O:3); 6, Y127 (serotype O:9); 7, Y746 (serotype O:3); 8, Y-96-P (serotype O:9); 9, WA-C(pYV::cat) Δinv . (b) Production of invasin on the surface of whole-cell *Y. enterocolitica* of different serotypes as determined by immunostaining in combination with flow cytometry. The mean fluorescence of bacteria and the percentage of fluorescent yersiniae for selected serotypes are presented. Histograms of fluorescent bacteria after detection by flow cytometry are also shown for different serotypes. The vertical lines indicate the gating used to determine the number of FITC-positive cells.

serotype-specific. To investigate whether there is a difference in the amount of surface-exposed invasins among different serotypes of *Y. enterocolitica*, flow cytometric analysis of whole-cell bacteria was performed. This analysis showed about fourfold stronger invasins 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 invasins on the surface might indicate specific regulation of *inv* expression on the single-cell level. Lower production of Inv(O:8) per bacterium and fewer *inv*-expressing yersiniae might also contribute to the clonal invasion phenomenon of *Y. enterocolitica* O:8 recently described by us (Oellerich *et al.*, 2007).

Analysis of *inv* promoter sequences

In order to determine whether the observed differences in *inv* expression between the tested *Yersinia* serotypes were

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Y.e. WA-314 (O:8)      AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AACTATTTTGATA--ATAATAATA--
Y.e. 8081 (O:8)       TGCGCAGAGAGTCTATTTTATGAGACGAATGTA-AACTATTTGATA--ATAATAATA--
Y.e. 900-36/90 (O:8)  AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AACTATTTTGATA--ATAATAATA--
Y.e. Y286 (O:8)       AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AACTATTTTGATA--ATAATAATA--
Y.e. 22703 (O:9)      AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. Y127 (O:9)       AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. Y738 (O:9)       AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. Y96P (O:9)       AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. MvP (O:5,27)     AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. SZ1249/04 (O:5,27) AATGCAGAGAGTCTATTTTATGAGACGAATGTA-AGCTATTTTGATA--ATAATAACA--
Y.e. Y746 (O:3)       ACGCTCCTTCTCTGAGTTTAAACACTCACATTTAGGCACCTTGTATGCCGTAAAGTTGG
                        * * * * *
Y.e. WA-314 (O:8)      -----TATCACAATATATATA--CATGCTAAATATAACCTGACAATTAAATTAACAAGC
Y.e. 8081 (O:8)       -----TATCACAATATATATATATCTGCTAAATATAACCTGACAATTAAATTAACAAGC
Y.e. 900-36/90 (O:8)  -----TATCACAATATATATATATCTGCTAAATATAACCTGACAATTAAATTAACAAGC
Y.e. Y286 (O:8)       -----TATCACAATATATATATATCTGCTAAATATAACCTGACAATTAAATTAACAAGC
Y.e. 22703 (O:9)      -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. Y127 (O:9)       -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. Y738 (O:9)       -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. Y96P (O:9)       -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. MvP (O:5,27)     -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. SZ1249/04 (O:5,27) -----TATCAC--CATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
Y.e. Y746 (O:3)       ACGCTCCATTTTATTATATATATTCAGGCTAAATATAACCTGACAATTAAATTAATAGCAAGC
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Y.e. WA-314 (O:8)      TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. 8081 (O:8)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. 900-36/90 (O:8)  TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. Y286 (O:8)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. 22703 (O:9)      TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. Y127 (O:9)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. Y738 (O:9)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. Y96P (O:9)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. MvP (O:5,27)     TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. SZ1249/04 (O:5,27) TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
Y.e. Y746 (O:3)       TAATATTACCATGATGAATTTTTTTT-GCATTTCATTGTCATTGCTGTTATTTTTTAATT
                        *****
Y.e. WA-314 (O:8)      TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. 8081 (O:8)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. MvP (O:5,27)     TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. 900-36/90 (O:8)  TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. Y286 (O:8)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. SZ1249/04 (O:5,27) TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. 22703 (O:9)      TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. Y127 (O:9)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. Y738 (O:9)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. Y96P (O:9)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
Y.e. Y746 (O:3)       TTTTAATTTTATTTTTT-GTAAGTTCTGCTATTCTATGTT----TAGTGTTTGCGAGAGA
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Fig. 3. Sequence comparison of RovA and H-NS binding sites (Ellison & Miller, 2006) in *inv* upstream regions among different serotypes of *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.

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 RNAdraw 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 $\beta 1$ 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::luxCDABE* 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::luxCDABE* pACYC-Inv(O:8) and WA-C Δinv *attTn7::luxCDABE* 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 $\beta 1$ 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).

Fig. 5. *In vivo inv* expression of *Y. enterocolitica*. BALB/c mice were infected orally with 1×10^9 c.f.u. (a) and intravenously with 1×10^4 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).

between labelled invasins and epithelial cells with and without $\beta 1$ integrins. As shown in Fig. 4(c), the results of these experiments indicated a slightly higher binding

capacity of Inv(O:8) for $\beta 1$ integrins in comparison with Inv(O:9). This could explain the higher invasiveness mediated by Inv(O:8) compared with Inv(O:9).

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 invasins play 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 invasins 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 invasins were 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.

Fig. 6. Invasin production in spleens of mice infected with *Y. enterocolitica* WA-314, as detected by immunoblotting with polyclonal anti-Inv antibodies.

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 Berschl 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.

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Edited by: H. Hilbi