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Presence of a functional flagellar cluster Flag-2 and low-temperature expression of flagellar genes in *Yersinia enterocolitica* W22703

Geraldine Bresolin, Janja Trček, Siegfried Scherer and Thilo M. Fuchs

Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL), Abteilung Mikrobiologie, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany

Correspondence
Thilo M. Fuchs
thilo.fuchs@wzw.tum.de

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Twelve *Yersinia enterocolitica* mutants carrying *luxCDABE*-transposon insertions in motility and chemotaxis genes were isolated on the basis of strong low-temperature induction. Two transposons were located within an 11.2 kb enteric flagellar cluster 2 (Flag-2) of *Y. enterocolitica* biotype 2, serotype O : 9 strain W22703. The Flag-2 gene cluster is absent from the corresponding genomic location of the sequenced strain *Y. enterocolitica* biotype 1B, serotype O : 8 strain 8081. Evidence for the functionality of the O : 9 Flag-2 genes, probably located within the plasticity zone of the genome, is provided by swarming assays. PCR analysis of 49 strains revealed the presence of Flag-2 genes in biotypes 2–5, but not in biotypes 1A or 1B. Bioluminescence, measured between 6 and 37 °C, showed that the expression of all genes located in Flag-2 and in the known flagellar cluster, Flag-1, was highest at approximately 20 °C, and that expression of two Flag-2 genes is FlhC-dependent. In a motility assay, a non-motile and a hyper-motile phenotype resulted from knockout mutations of the Flag-1 genes *fliS1* and *fliT*, respectively. Complemented strains validated these results, confirming the regulatory role of FliT.

INTRODUCTION

Yersinia enterocolitica belongs to the family *Enterobacteriaceae* and is a psychrotolerant, peritrichously flagellated human pathogen that causes a range of gastrointestinal diseases (Bottone, 1999). This Gram-negative, rod-shaped micro-organism is widely distributed in nature and able to survive for extended periods in terrestrial and aquatic environments at ambient temperature. The bacterium has been isolated from numerous species including flies (Fukushima *et al.*, 1979; Rahuma *et al.*, 2005). Swine have been identified as a major source of human infection (Bottone, 1997; Fredriksson-Ahomaa *et al.*, 2006). A multiphasic life cycle, which comprises a free-living phase, a potential insect-associated phase and a host-dependent phase, may be characteristic of biotypes 2–4 of this pathogen (Bresolin *et al.*, 2006b).

In *Y. enterocolitica*, low temperature is a key environmental factor for the expression of several genes important for survival outside mammalian hosts (Bresolin *et al.*, 2006a; Kapatral *et al.*, 2004) as well as for the coordinate expression and assembly of the flagellar structure (Kapatral & Minnich, 1995; Kapatral *et al.*, 2004; Rohde *et al.*, 1994). So far, all functional flagellar genes of *Y. enterocolitica* have been found to be located in the common flagellar cluster 1 (Flag-1), which is also required for full virulence (Young *et al.*, 2000). A second, but inactive, flagellar cluster, termed Flag-2, has been found in around one-fifth of *Escherichia coli* strains tested and also in *Yersinia pestis* and *Yersinia pseudotuberculosis*, but not in *Y. enterocolitica* 8081 (Ren *et al.*, 2005). The distribution of Flag-2 in the *Y. enterocolitica* biotypes has not been investigated.

Except for some distinct differences, the flagellar hierarchy in *Y. enterocolitica* resembles that of *E. coli* and *Salmonella* spp. (Horne & Pruss, 2006), in which the regulation and function of flagellar genes are well understood. Their genomes contain more than 50 flagellar genes, which can be categorized into three promoter classes. These correspond to the temporal requirements for flagellar gene products during the morphological development of the flagellum, which consists of a membrane-spanning hook–basal-body and an external structure (Aldridge & Hughes, 2002; Chilcott & Hughes, 2000). The regulation of flagellar gene expression is coordinated by the *flhDC* master operon, which is transcribed in a temperature-independent manner from a class 1 promoter (Kapatral *et al.*, 2004). FlhD and FlhC act as transcriptional activators for class 2 promoters including the transcriptional regulators FliA (σ^{28}) and FlgM (anti-σ^{28}) and genes required for the

Abbreviation: RLU, relative light units.

The GenBank/EMBL/DDBJ accession number for the Flag-2 sequence of *Yersinia enterocolitica* is AM600695.

A supplementary table listing the primers used is available with the online version of this paper.

formation of the hook–basal-body. Most genes necessary for late flagellar morphogenesis, motor rotation and chemotactic signalling are transcribed from class 3 promoters. Rohde *et al.* (1994) reported 23 °C as being the temperature for maximal flagellar gene expression. However, little is known about the induction and transcription of flagellar genes in *Y. enterocolitica* at temperatures below 25 °C, and in what way their expression differs from that in related species.

In this study, screening of a *luxCDABE*-reporter mutant library of *Y. enterocolitica* W22703 at low temperature identified transposon insertions within 12 genes involved in motility and chemotaxis. Sequence analysis of two mutants revealed a strain-specific flagellar region, which is homologous to the flagellar cluster Flag-2 of *Y. pestis* and *Y. pseudotuberculosis*. FlhC-dependent expression of Flag-2 genes is demonstrated, and evidence for their functionality is provided. Furthermore, we show that Flag-1 and Flag-2 genes are maximally expressed at approximately 20 °C, indicating that enhanced motility is part of the psychrotolerant life style of *Y. enterocolitica*, which includes survival and growth at environmental temperatures.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Tables 1 and 3. All cultures were grown in LB broth (10 g tryptone l^{-1}, 5 g yeast extract l^{-1} and 5 g NaCl l^{-1}) or on LB agar (LB broth supplemented with 1.5 %, w/v, agar). *E. coli* was grown at 37 °C and *Y. enterocolitica* at the temperatures indicated below. If appropriate, the media were supplemented with the following antibiotics: 20 μg chloramphenicol ml^{-1}, 50 μg kanamycin ml^{-1}, 20 μg nalidixic acid ml^{-1} or 18 μg tetracycline ml^{-1} (for *E. coli*) and 12 μg tetracycline ml^{-1} (for *Y. enterocolitica*).

General molecular techniques. DNA manipulation was performed according to standard procedures (Sambrook & Russell, 2001). To isolate chromosomal DNA, 1.5 ml of bacterial culture was centrifuged, and the sediment was resuspended in 400 μl lysis buffer (100 mM Tris, pH 8.0, 5 mM EDTA, 200 mM NaCl). After incubation for 15 min on ice, 10 μl 10 % SDS and 5 μl proteinase K (10 mg ml^{-1}) were added, and the sample was incubated at 55 °C overnight. The chromosomal DNA was then precipitated with 500 μl 2-propanol, washed in ethanol, dried and dissolved in 500 μl TE buffer (10 mM Tris/HCl, 1 mM Na_2EDTA, pH 7.4) containing 1 μl RNase (10 mg ml^{-1}). Transposon mutagenesis using pUT mini-Tn*5* *luxCDABE* Km2 and mating with *E. coli* S17.1 λ*pir* as the donor strain has been described elsewhere (Bresolin *et al.*, 2006a). PCRs were carried out with *Taq* polymerase (Fermentas) using the following programme: one cycle at 95 °C for 2 min; 30 cycles at 95 °C for 10 s, at the appropriate annealing temperature for 30 s, and at 72 °C for 45–180 s depending on the expected fragment length; one cycle at 72 °C for 10 min. All primers are listed in Supplementary Table S1, available with the online version of this paper. Chromosomal DNA (100 ng μl^{-1}) was used as a template for PCR amplification.

Inverse PCR and DNA sequencing. Identification and characterization of transposon insertion sites was performed by inverse PCR (Bresolin *et al.*, 2006a). Briefly, 400 ng chromosomal DNA of each transposon mutant was completely digested with *Cla*I, *Hin*dIII or *Ssp*I (Fermentas), enzymes were heat-inactivated and fragments were treated with T4 DNA ligase (Invitrogen) to allow self-ligation, resulting in circular molecules. Subsequent inverse PCR (Ochman *et al.*, 1990) was performed using transposon-specific primers derived from the O-end or the I-end of mini-Tn*5* (Table S1). The PCR fragments obtained were sequenced by MWG-Biotech with primers hybridizing to a 100 bp transposon region near the O-end or the I-end.

Sequencing of strain-specific DNA was performed by inverse PCR (as described above) using the restriction enzymes *Hae*III (USB), *Hha*I, *Hin*dIII, *Hpa*I, *Msp*I, *Mun*I, *Rsa*I, *Ssp*I and *Vsp*I (Fermentas) and primers listed in Table S1. Sequencing was performed by 4base lab.

Bioinformatics. Mapping of mini-Tn*5* *luxCDABE* insertions was performed with the *Y. enterocolitica* BLAST Server from the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/y_enterocolitica). Sequences without similarities to the sequence of *Y. enterocolitica* 8081 (accession no. AM286415/AM286416) were classified as specific for strain W22703, used in this study. Sequence assembly was done with Vector NTI Advance (Invitrogen). The resulting sequence was annotated using the NCBI ORF-Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Homology searches of the single ORFs were performed with BLASTP from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Promoter sequences located upstream of the identified genes were deduced using a promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). The accession number of the *Y. pseudotuberculosis* genome sequence is NC_006155.

Measuring expression profiles using a *luxCDABE* reporter. Bioluminescence measurements were performed in white 96-well plates with clear bottoms (Matrix Technologies). Transposon mutants were grown overnight at 30 °C with shaking (500 r.p.m.) in deep-well microtitre plates filled with 800 μl selective LB broth, and then 1 : 4000 diluted in 200 μl LB broth with kanamycin. For investigation of the expression profiles, the plates were incubated at 6, 10, 15, 20, 25, 30 and 37 °C. Bioluminescence (at 490 nm) and OD_{405} of all plates were measured in parallel using a Wallac VICTOR^2 1420 multilabel counter (Perkin Elmer Life Sciences). Bioluminescence was recorded as relative light units (RLU), and background activity of 0.15 RLU was subtracted. Cells were measured in the late exponential phase (OD_{405} ~0.8–0.9). To allow a direct comparison of results obtained at all temperatures applied, RLU were related to the growth of cells (RLU/OD).

Complementation of W22703-*fliS1*::Tn*5lux* and W22703-*fliT*::Tn*5lux*. The complete coding sequence of *fliS1* (YE2525) and 411 nucleotides of its upstream sequence, as well as the coding sequence of *fliT* (YE2526) and 207 nucleotides located upstream, were amplified at an annealing temperature of 50 °C and with an elongation time of 2 min using the oligonucleotides HAPF1/fliTR1 and fliSF1/ybcMR1 (Table S1), respectively. Each fragment was digested with *Eco*RI and cloned separately into the *Eco*RI site of pACYC184, resulting in the recombinant plasmids pACYC184/*fliS1* and pACYC184/*fliT*. Cloning was performed in *E. coli* DH5αMCR and constructs were confirmed by PCR and restriction analysis. The direction of gene transcription corresponds to that of the disrupted plasmid gene encoding chloramphenicol acetyltransferase. Plasmid constructs were transformed by electroporation into the respective *Y. enterocolitica* mutant strains.

Construction of insertional knockout mutants. Knockout mutants of the putative flagellin gene *flaA* (*orf9*), the putative regulatory gene (*orf10*) and the hook-length-control gene *fliK* (*orf5*) of the new flagellar region Flag-2 as well as the flagellar regulatory gene *flhDC* were generated by plasmid insertion via homologous recombination. Short intragenic fragments of the target genes were

Table 1. Strains and plasmids used in this study

Numbers in parentheses indicate the Tn*5* insertion site with respect to the gene.

Strain/Plasmid	Relevant characteristics	Reference or source
Strains		
E. coli S17.1 *λpir*	*λpir* lysogen of S17.1 (Tp^r Sm^r *thi pro hsdR*$^-$*M*$^+$ *recA* RP4::2-Tc::Mu-Km::Tn*7*)	Simon *et al.* (1983)
E. coli DH5αMCR	*F*$^-$ *mcrA* Δ*(mcr-hsdRMS-mcrBC)* ϕ*80lacZ*Δ*M15* Δ*(lacZYA–argF)U169 recA-1 endA-1 supE-44 thi-1 gyrA-96 relA-1*	Invitrogen
Y. enterocolitica W22703	Biotype 2, serotype O:9, Nal^r, Res$^-$, Mod$^+$, pYV$^-$	Cornelis & Colson (1975)
Y. enterocolitica 8081	Biotype 1B, serotype O:8, Nal^r	V. Miller, St Louis, USA
W22703-*fliB*(434)::Tn*5lux*	W22703 with *luxCDABE* reporter inserted into YE2520 (Flag-1) encoding flagellin N-methylase	Bresolin *et al.* (2006a)
W22703-*fleC*(963)::Tn*5lux*	As above, into YE2521 (Flag-1) encoding flagellin	Bresolin *et al.* (2006a)
W22703-*fliS1*(9)::Tn*5lux*	As above, intoYE2525 (Flag-1) encoding a putative flagellin-specific chaperon	Bresolin *et al.* (2006a)
W22703-*fliT*(119)::Tn*5lux*	As above, into YE2526 (Flag-1) encoding a putative regulatory protein	Bresolin *et al.* (2006a)
W22703-*ybcM*(30)::Tn*5lux*	As above, into YE2527 (Flag-1) encoding a putative AraC type regulatory protein	Bresolin *et al.* (2006a)
W22703-*fliF*(1027)::Tn*5lux*	As above, into YE2537 (Flag-1) encoding a flagellar basal body M-ring protein	Bresolin *et al.* (2006a)
W22703-2575(797)::Tn*5lux*	As above, into YE2575 encoding a methyl-accepting chemotaxis protein	Bresolin *et al.* (2006a)
W22703-*cheA*(1437)::Tn*5lux*	As above, into YE2577 encoding chemotaxis protein CheA	Bresolin *et al.* (2006a)
W22703-YE2673::Tn*5lux*	W22703 with *luxCDABE* reporter inserted 68 bp downstream of YE2673 encoding a putative chemotaxis signal transduction protein	Bresolin *et al.* (2006a)
W22703-YE2848(285)::Tn*5lux*	W22703 with *luxCDABE* reporter inserted into YE2848 encoding a putative chemotaxis methyl-accepting transducer	Bresolin *et al.* (2006a)
W22703-*fliD2*::Tn*5lux*	As above, into *fliD* (Flag-2) encoding a flagellar hook-associated protein; four independent insertions at nucleotide positions 311, 458, 507, and 1156	This study
W22703-*fliS2*(238)::Tn*5lux*	As above, into *fliS* (Flag-2)	This study
W22703-*flaA*(214)::pKRG9	W22703 with disrupted putative flagellin gene (Flag-2)	This study
W22703-*orf10*(171)::pKRG9	W22703 with disrupted putative regulatory gene (Flag-2)	This study
W22703-*fliK*(181)::pKRG9	W22703 with disrupted hook-length control gene (Flag-2)	This study
W22703-*fliD2*::Tn*5lux-flhC*::pKRG9	W22703 with double knockout of *fliD2* and *flhC*	This study
W22703-*fliS2*::Tn*5lux-flhC*::pKRG9	W22703 with double knockout of *fliS2* and *flhC*	This study
W22703-*fleC*::Tn*5lux-flhC*::pKRG9	W22703 with double knockout of *fleC* and *flhC*	This study
W22703-*fliT*::Tn*5lux-flhC*::pKRG9	W22703 with double knockout of *fliT* and *flhC*	This study
W22703-YE2848::Tn*5lux-flaA*::pKRG9	W22703 with double knockout of YE2848 and *flhC*	This study
W22703-YE2673::Tn*5lux-flaA*::pKRG9	W22703 with double knockout of YE2673 and *flhC*	This study
W22703-*fleC*::Tn*5lux-flaA*::pKRG9	W22703 with double knockout of *fleC* and *flaA*	This study
Plasmids		
pUT mini-Tn*5 luxCDABE* Km2	Suicide vector, *ori* R6K, mini-Tn*5* Km2 *luxCDABE* transposon, *mob*$^+$ (RP4), Ap^r Km^r	Winson *et al.* (1998)
pACYC184	Cloning vector, P15A origin, Cm^r Tc^r	Chang & Cohen (1978)
pACYC184/*fliS1*	pACYC184 with an 876 bp *Eco*RI fragment carrying *fliS1* (YE2525) and 411 bp of its upstream region	This study
pACYC184/*fliT*	pACYC184 with an 891 bp *Eco*RI fragment carrying *fliT* (YE2526) and 207 bp of its upstream region	This study
pKRG9	Derivative of suicide vector pGP704; *ori* R6K, *mob*$^+$ (RP4), Cm^r Ap^s	Creatogen
pKRG-*flaA*′	pKRG9 with a 774 bp intragenic fragment of *flaA* (Flag-2)	This study
pKRG-*orf10*′	pKRG9 with a 747 bp intragenic fragment of a putative regulatory gene (Flag-2)	This study
pKRG-*fliK*′	pKRG9 with a 389 bp intragenic fragment of *fliK* (Flag-2)	This study
pKRG-*flhDC*′	pKRG9 with a 463 bp intragenic fragment of *flhDC*	This study

amplified from *Y. enterocolitica* chromosomal DNA using primers listed in Table S1. Fragments were digested with *Xba*I and *Sac*I (Fermentas) and ligated into the *Xba*I/*Sac*I-restricted suicide plasmid pKRG9. The recombinant plasmids were transformed into *E. coli* S17.1 λ*pir* by electroporation and transferred into *Y. enterocolitica* W22703 via plate mating. For this purpose, five colonies of donor strain and approximately 20 colonies of recipient strain were mixed on an LB agar plate and incubated for 6 h at 30 °C. The complete lawn was scraped off the plate and resuspended in 1 ml LB medium. Serial dilutions were prepared and conjugants were selected on plates containing nalidixic acid and chloramphenicol. To exclude illegitimate recombination, the correct insertion of the recombinant plasmid was confirmed by PCR using a gene-specific primer or a cloning primer, and a plasmid-derived primer (Table S1).

Motility assay. Motility was tested by assessing swimming phenotypes on motility agar (LB medium containing 0.3 % agar without antibiotics). Streak cultures of *Y. enterocolitica* wild-type, mutant and complementing strains were prepared on LB agar plates containing the appropriate antibiotics and incubated overnight at 30 °C. From these plates single colonies were stabbed onto motility agar plates and incubated initially for 2 h at 37 °C to start the assay with non-motile bacteria. The plates were subsequently incubated at 15 °C (44 h), 20 °C (21 h), 25 °C (21 h) and 30 °C (42 h).

RESULTS

Y. enterocolitica W22703 carries a flagellar region homologous to Flag-2

We recently reported the identification of *luxCDABE* insertions into the genome of *Y. enterocolitica* strain W22703 that resulted in increased light emission at 10 °C as compared to 30 °C (Bresolin *et al.*, 2006a). Among mutants with strongly elevated light emission, we identified 21 clones harbouring transposon insertions in 12 different genes associated with motility and chemotaxis (Table 1; Fig. 1a), six of which are part of the Flag-1 cluster. Interestingly, five insertion sites were located within a different genome region of W22703 showing significant homologies to flagellar genes of *Y. pestis* (Fig. 1b) and *Y. pseudotuberculosis*. The sequences of these five transposon mutants were assembled into a 2367 bp fragment that was extended by inverse PCR to a total of 11 526 bp. BLASTN analysis revealed that 290 bp at the very 5′-end of this fragment were identical to the sequence of YE3610 within the *Y. enterocolitica* 8081 genome sequence. No further sequence identities were observed, indicating that the 11.2 kb region is absent in strain 8081. YE3610 is located within the 199 kb plasticity zone of *Y. enterocolitica* 8081, which is the largest region of species-specific genomic variation found within this species (Thomson *et al.*, 2006). Further sequence analysis resulted in the annotation of 12 ORFs (Fig. 1b) with identities of deduced amino acid sequences to proteins from other pathogenic *Yersinia* species and from *E. coli* of between 31 % and 94 % (Table 2). All the ORFs were highly similar to the Flag-2 genes of *Y. pestis* CO92 (Parkhill *et al.*, 2001) and *Y. pseudotuberculosis* IP32953 (YPTB3329–YPTB3322) as well as to the Flag-2 flagellar cluster from *E. coli* 042 (Ren *et al.*, 2005).

Distribution of Flag-2 in different biotypes of *Y. enterocolitica*

Y. enterocolitica strains W22703 and 8081 belong to biotypes 2 and 1B, respectively. To gain further information on the distribution of the Flag-2 gene cluster in the six biotypes of *Y. enterocolitica*, we subjected a total of 49 strains to PCR analysis using six oligonucleotides that amplify fragments I–VI of the Flag-2 gene cluster as depicted in Fig. 1(b). The strains included W22703 and 8081 as a positive- and negative control, respectively, and 47 strains that had been isolated on five continents from humans and animals (Table 3). Distinct PCR fragments were obtained when chromosomal DNA of biotypes 2–5 served as a template. In each of these reactions, the PCR product showed the expected length of 1554 bp (I), 1834 bp (II), 1529 bp (III), 1817 bp (IV), 2171 bp (V), and 1517 bp (VI). In contrast, none of the six primer pairs produced amplified fragments when genomic DNA of biotype 1A or 1B was used, with the exception of fragment III, representing *flaA*, which was amplified from genomic DNA of strains 213-36/89 and 214-36/84 (Table 3). The presence of Flag-1 in each strain was confirmed by the amplification of *fliT* using primers fliSF1 and ybcMR1. No PCR amplification was observed for two strains, 237 and SZ4501/97, and the latter strain was found to be less motile than the wild-type strain in the swarming assay (results not shown).

Expression of motility and chemotaxis genes is maximal at approximately 20 °C

Most experiments addressing temperature-dependent transcription of virulence or flagellar genes of *Y. enterocolitica* are performed within a relatively narrow temperature range (24–37 °C). We therefore decided to investigate the transcriptional response of all mutants with insertions into chemotaxis and flagellar genes located in Flag-1 and Flag-2 at 6, 10, 15, 20, 25, 30 and 37 °C at an OD_{405} between 0.8 and 0.9. Very low light emission was observed for all mutants at 37 °C, with bioluminescence signals only slightly above background level (Fig. 2a, b). This finding is in accordance with the non-motile phenotype of *Y. enterocolitica* cells at human body temperature. The reporter activity of all mutants measured increased with decreasing temperature with a maximum at 20 °C, with the exception of W22703-YE2575::Tn*5lux* (Fig. 2b) and W22703-*fleC*::Tn*5lux* (Fig. 2a), which had maximum activity at 25 °C. The highest expression was observed for *fleC*, which encodes a flagellin (Fig. 2a). Mutants W22703-*fliD2*::Tn*5lux* and W22703-*fliS2*::Tn*5lux* showed another temperature optimum at 30 °C (Fig. 2c), suggesting a putative role of the Flag-2 genes in virulence towards mammals. The temperature-dependent activity of enzymes involved in bioluminescence was also taken into account by using a correction factor derived from our own experimental data and the Arrhenius prediction (Bresolin *et al.*, 2006a). This approach gave an optimal temperature of

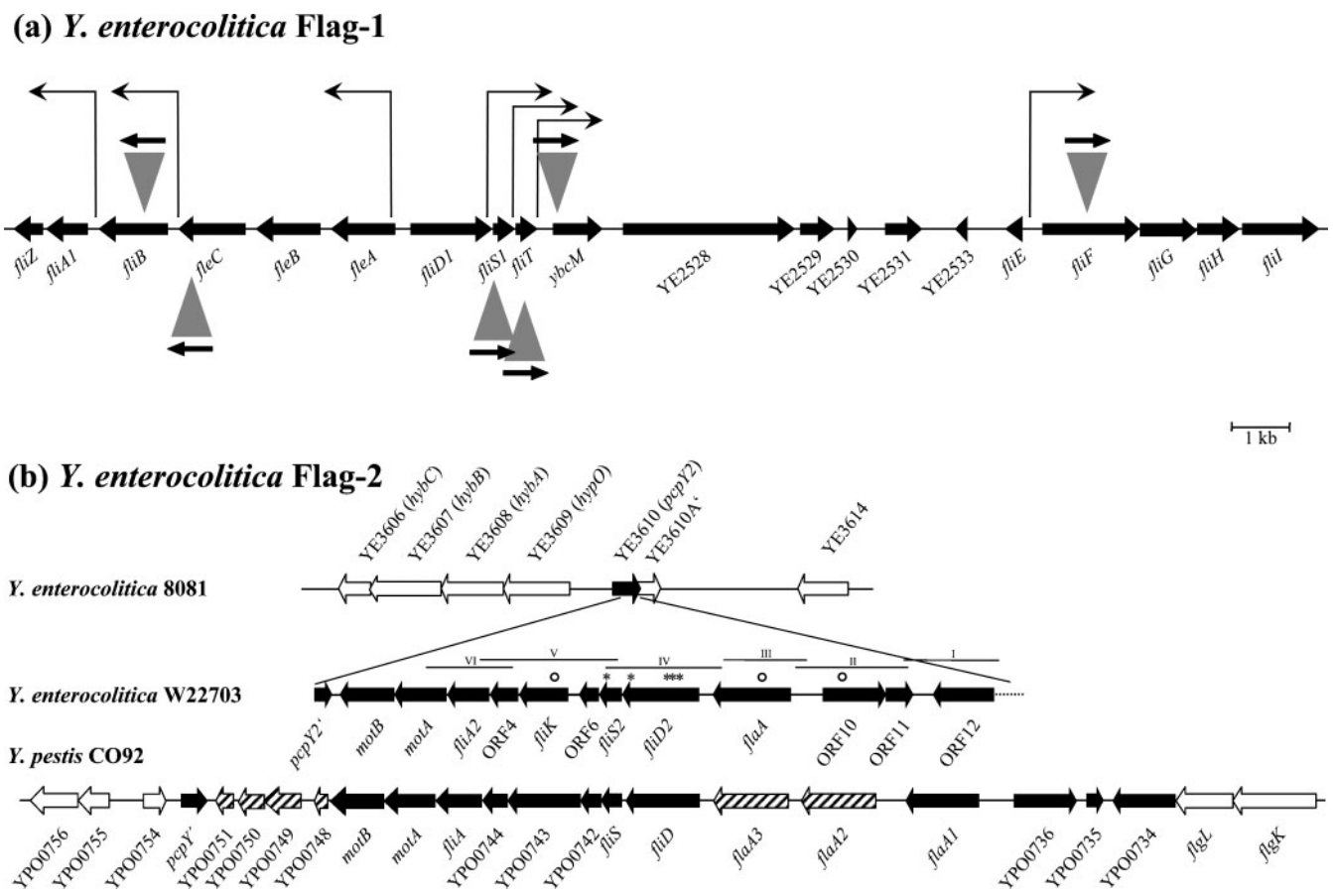

Fig. 1. (a) Partial map of the *Y. enterocolitica* 8081 flagellar cluster Flag-1. Gene organization and annotation was adapted from that used by the Sanger Institute, and gene nomenclature from Horne & Pruss (2006). Positions and orientations of ORFs are indicated by thick black arrows, those of predicted promoters by thin bent arrows. Chromosomal mini-Tn*5 luxCDABE* insertions into the genome of W22703, localized by sequence analysis, are indicated by triangles. Arrows at the base of each triangle indicate the orientation of the *luxCDABE* operon. (b) Part of the flagellar cluster Flag-2 identified in strain W22703. The region was annotated using the NCBI ORF-Finder and compared to the genomes of *Y. enterocolitica* 8081 and *Y. pestis* CO92 (accession no. AL590842). Homologous genes are indicated by black arrows; additional genes within Flag-2 of *Y. pestis* without a homologue within the known Flag-2 sequence of strain W22703 are indicated by hatched arrows. Circles indicate three site-directed insertional mutants; asterisks indicate five transposon insertions described in Table 1. Fragments I–VI refer to the PCR analysis of Flag-2 distribution (Table 3).

15–20 °C for the expression of all genes investigated (data not shown).

Evidence for functional Flag-2 genes

Two genes of Flag-2, *fliD2* and *fliS2*, also showed maximal expression at 20 °C (Fig. 2c). To investigate their regulatory dependence on the flagellar activator FlhC, *flhC* was mutated by insertional knockout in strains W22703-*fliD2*::Tn*5lux*, W22703-*fliS2*::Tn*5lux*, W22703-*fliT*::Tn*5lux* and W22703-*fleC*::Tn*5lux*. The latter two served as control strains. The luciferase activity was then measured at 6–37 °C. The results obtained demonstrate that not only *fliT* or *fleC*, but also the Flag-2 genes *fliS2* and *fliD2* are under the control of the flagellar master operon at lower temperatures (Fig. 2c).

Four transposons had inserted into an ORF encoding a putative flagellar hook-associated protein HAP (*fliD2*), and the fifth transposon insertion was located in the neighbouring downstream gene, termed *fliS2*, which encodes a flagellin-specific chaperone FliS2 (Fig. 1b). To test the functionality of Flag-2 genes in strain W22703, we generated three insertional mutants with knockouts of the putative flagellar subunit gene *flaA* (*orf9*), a predicted regulatory gene (*orf10*) and *fliK* (*orf5*) which is similar to flagellar hook-length-control genes. Their *in vitro* growth at both 15 and 30 °C was identical to that of the wild-type strain. The swimming phenotypes of the mutants were investigated in motility assays at different temperatures. We observed an increase of motility at temperatures below 30 °C (Fig. 3a). No effect on motility was observed when *flaA* was mutagenized in strains with a Tn*5lux* insertion

Table 2. Nomenclature of ORFs of the new flagellar region in *Y. enterocolitica* W22703 as derived from identity and similarity of predicted proteins with and to sequences in the SWISS-PROT and GenBank databases

Gene	aa*	MM†	Homologous protein/putative function	Identity/similarity (%)‡
orf1	302	33.5	Flagellar motor protein MotB	88/94^{Yp}; 88/93^{Yptb}
orf2	288	31.7	Flagellar motor protein MotA	88/94^{Yptb}; 87/94^{Yp}
orf3	231	26.6	Sigma factor FliA2	86/94^{Yptb}; 85/94^{Yp}
orf4	155	17.7	Putative flagellar biogenesis protein	73/81^{Yptb}; 71/81^{Yp}
orf5	316	33.8	Flagellar hook-length control protein FliK	50/63^{Yp}; 50/62^{Yptb}
orf6	109	12.5	Hypothetical protein	38/62^{Yp}; 38/60^{Yptb}
orf7	127	14.1	Flagellin-specific chaperone FliS2	58/75^{Yptb}; 58/75^{Yp}
orf8	422	45.7	Putative flagellar-hook-associated protein FliD2	33/60^{Yptb}; 32/60^{Yp}
orf9	430	44.1	Putative flagellin FlaA	32/47^{Yptb}; 31/47^{Yp}
orf10	351	39.8	Putative regulatory protein, DNA-binding winged-HTH domains	51/64^{Yptb}; 50/64^{Yp}
orf11	153	17.2	Hypothetical protein	57/71^{Yp}; 56/71^{Yptb}
orf12	336	37.3	Hypothetical protein	45/63^{Yptb}; 44/63^{Yp}

*aa, length (amino acids) of product.
†MM, predicted molecular mass (kDa) of product.
‡*Yp*, *Y. pestis*; *Yptb*, *Y. pseudotuberculosis*.

into *cheA*, *fleC*, *fliB*, *fliD2*, *fliS2*, *ybcM*, YE2575 or YE2848 (data not shown).

The *fliT* mutant reveals a hyper-motile phenotype in *Y. enterocolitica*

All strains but one (W22703-YE2673::Tn*5lux*) were knockout mutants of *Y. enterocolitica* flagellar or chemotaxis genes. This allowed us to directly investigate the biological function of mutated Flag-1 genes in a motility assay. The *in vitro* growth properties of all mutants measured at 15 and 30 °C did not differ from that of strains W22703 and 8081. As expected, the knockout mutant of FliF, forming the flagellar MS ring, W22703-*fliF*::Tn*5lux*, and the two chemotaxis mutants W22703-*cheA*::Tn*5lux* and W22703-2575::Tn*5lux* exhibited a non-motile phenotype at all temperatures tested (data not shown). W22703-*fliT*::Tn*5lux* showed a hyper-motile phenotype at all temperatures examined, and W22703-*fliS1*::Tn*5lux* a non-motile phenotype below 30 °C (Fig. 3b, c). FliS1 probably acts as a cytosolic chaperone, and FliT is involved in the transcriptional regulation of flagellar genes (Auvray *et al.*, 2001; Kutsukake *et al.*, 1999). The motility phenotypes of the remaining transposon mutants, as well as of a *fleC*/*flaA* double mutant, did not differ from the wild-type phenotype under the tested conditions (data not shown).

To confirm the results obtained for the *fliT* and *fliS1* mutants, the wild-type strain and the mutants W22703-*fliS1*::Tn*5lux* and W22703-*fliT*::Tn*5lux* were transformed with the constructs pACYC184/*fliS1* and pACYC184/*fliT*, respectively. Each fragment cloned carried the putative native promoter sequence (Table 1). In swarming assays, the hyper-motility of W22703-*fliT*::Tn*5lux* was drastically reduced in the presence of pACYC184/*fliT* (Fig. 3b). A similar non-motile phenotype resulted from *fliT* over-expression in the wild-type, indicating that FliT acts as repressor of flagellar synthesis in *Y. enterocolitica*. Motility of the non-motile mutant W22703-*fliS1*::Tn*5lux* was successfully restored with the plasmid pACYC184/*fliS1* (Fig. 3c).

DISCUSSION

Here, we report, to our knowledge for the first time, the presence of the flagellar cluster Flag-2 in a *Y. enterocolitica* genome. We observed a conserved synteny with respect to the genomes of *Y. pestis* CO92 and *Y. pseudotuberculosis* IP32953. In strain W22730, Flag-2 is probably part of the plasticity zone, a key locus for high pathogenicity. Interestingly, remnants of Flag-2 are still present in strain 8081, indicating that a 100 kb deletion contributes to the high genomic variability of *Y. enterocolitica* strains (Thomson *et al.*, 2006). By PCR analysis of 49 strains representing six biotypes, we show that the newly described flagellar region Flag-2 in *Y. enterocolitica* is specific to strains from the low-pathogenic biotypes 2–5 and is absent from the high-pathogenic biotype 1B strains and from the biotype 1A strains tested. No pseudogenes were identified among the 12 ORFs annotated. The temperature-and *flhDC*-dependent expression of *fliD2* and *fliS2* were demonstrated in this study, as well as the functionality of at least three other Flag-2 genes, *fliK*, *flaA* and *orf10*. Their products are predicted to be involved in flagellar hook-length control and in regulation, activities that might explain the slightly hyper-motile phenotype of the respective mutants. Due to the absence of *flaA* in two *Y. enterocolitica* strains, as well as the wild-type-like motility

Table 3. *Y. enterocolitica* strains investigated for Flag-2 genes

ND, Not defined.

Biotype	Serotype	Strain	Geographical origin	Biological origin
1A	ND	MZ0124*	ND	Concentrate of whey
1A	O : 41,43	SZ634/04†	Germany	Human
1A	O : 5	Y755‡	France	Pony
1A	O : 5	H79/83†	Germany	Human
1A	O : 5	H1527/93†	Germany	Human
1A	O : 41,43	SZ593/04†	Germany	Baby food
1A	O : 41,43	SZ554/04†	Germany	Food
1A	O : 4,33	SZ1167/04†	Germany	Human
1A	O : 10	SZ671/04†	Germany	Human
1A	O : 5	1527-36/93†	Germany	Human
1A	O : 5	SZ662/97†	Germany	Human
1A	O : 14	SZ4331/97†	Germany	Human
1A	O : 15	SZ4501/97†	Germany	Human
1A	O : 41,43	767-36/91†	Germany	Human
1A	O : 48	SZ4643/93†	Germany	Human
1A	O : 19,8	88/50‖	Germany	Water
1A	O : 5	5/29807‖	Germany	Swine
1A	O : 50	54/1‖	Germany	Water
1A	O : 6,30	55/2‖	Germany	Liquid manure
1A	O : 7,8	53/30444‖	Germany	Swine
1B	O : 8	SZ375/04†	Germany	Human
1B	O : 8	WA-314‡	USA	Human
1B	O : 13	Y293§	ND	ND
1B	O : 8	SZ506/04†	Germany	Human
1B	O : 8	Y286§	USA	ND
1B	O : 8	SZ5108/01†	Germany	Human
1B	O : 8	900-36/90†	Germany	Human
1B	O : 8	19-36/92†	Germany	Human
1B	O : 21	209-36/84†	Germany	Human
1B	O : 21	213-36/89†	Germany	Human
1B	O : 21	214-36/84†	Germany	Human
2	O : 9	H692/94†	Germany	ND
2	O : 9	H621/87†	Germany	Human
2	O : 5,27	SZ1249/04†	Germany	Human
2	O : 9	Y738‡	France	Human
2	O : 9	Y127§	ND	ND
2/3	O : 5,27	237‡	USA	ND
3	O : 5,27	H230/89†	Germany	Human
3	O : 1	NCTC 10460¶	Denmark	Chinchilla
3	O : 3	Y746‡	Japan	Human
4	O : 3	Y747‡	Sweden	Human
4	O : 3	Y755‡	South Africa	Human
4	O : 3	Y769‡	New Zealand	Human
4	O : 3	SZ425/04†	Germany	Pig tongue
4	O : 3	SZ687/04†	Germany	Dog faeces
5	O : 2a,2b,3	Y772‡	France	Hare
5	O : 2a,2b,3	Y773‡	France	Hare

*Authors' collection.
†Institut für Hygiene und Umwelt, Hamburg, Germany.
‡Max von Pettenkofer-Institut, München, Germany.
§Institut für Mikrobiologie der Bundeswehr, München, Germany.
‖Robert-Koch-Institut, Berlin, Germany.
¶National Collection of Type Cultures, London, UK.

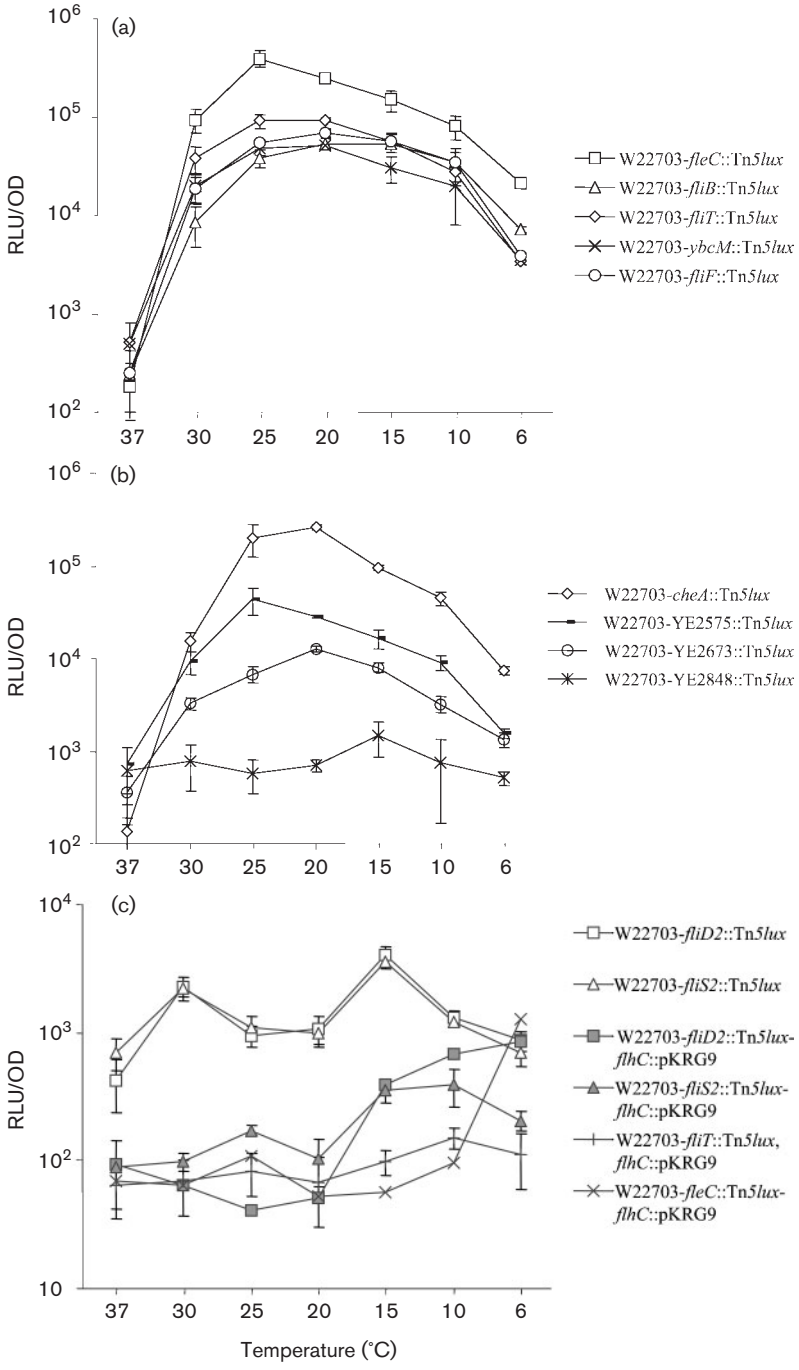

Fig. 2. Temperature-dependent expression of *Y. enterocolitica* W22703 genes involved in chemotaxis and motility. Overnight cultures were diluted 1 : 4000. RLU values were divided by OD_{405} values measured within a range of 0.8–0.9. For each strain and temperature, the figure shows the mean ± SD of 3–16 independent measurements. (a) Five mutants with insertions of the luciferase reporter into Flag-1 genes show similar expression profiles, with maximal bioluminescence at approximately 20 °C. (b) Significantly weaker transcription is observed for the four genes involved in chemotaxis. (c) Temperature-dependent expression of the two Flag-2 genes *fliD2* and *fliS2*. Upon *flhC* knockout, expression of both *fliD2* and *fliS2* is significantly reduced. As a control, the drastic decrease in *fliT* and *fleC* expression (compare with panel a) upon *flhC* knockout is also shown.

of several double mutants in a *flaA*$^{-}$ background, the role of this additional flagellar subunit gene remains unclear. The distribution pattern of Flag-2 is similar to that of other genome domains, such as the insecticidal pathogenicity island *tc*-PAI^{Ye} (Bresolin *et al.*, 2006b), indicating an evolutionary split of biotypes 1A and 1B from biotypes 2–5. We suggest that biotypes 2–5, but not 1A or 1B, use insects as transmission vectors, and that Flag-2 genes contribute to the fitness of pathogenic *Y. enterocolitica* outside their mammalian hosts, possibly by increasing the chance of encountering an invertebrate host.

We also tested the phenotype of three Flag-1 mutants. The non-motility of the *fliF* mutant is congruent with the finding that inactivation of *fliF* in *Caulobacter crescentus* (Grunenfelder *et al.*, 2003) and *Listeria monocytogenes* (Bigot *et al.*, 2005) abolishes flagella production and consequently bacterial motility. The increased motility of the *fliT* mutant indirectly confirms the finding that a *Salmonella enterica* sv. Typhimurium *fliT*::*kan* mutant, in which flagellar structure and filament length were indistinguishable from those of wild-type flagella, produces twice as many flagella as the wild-type strain. However, no

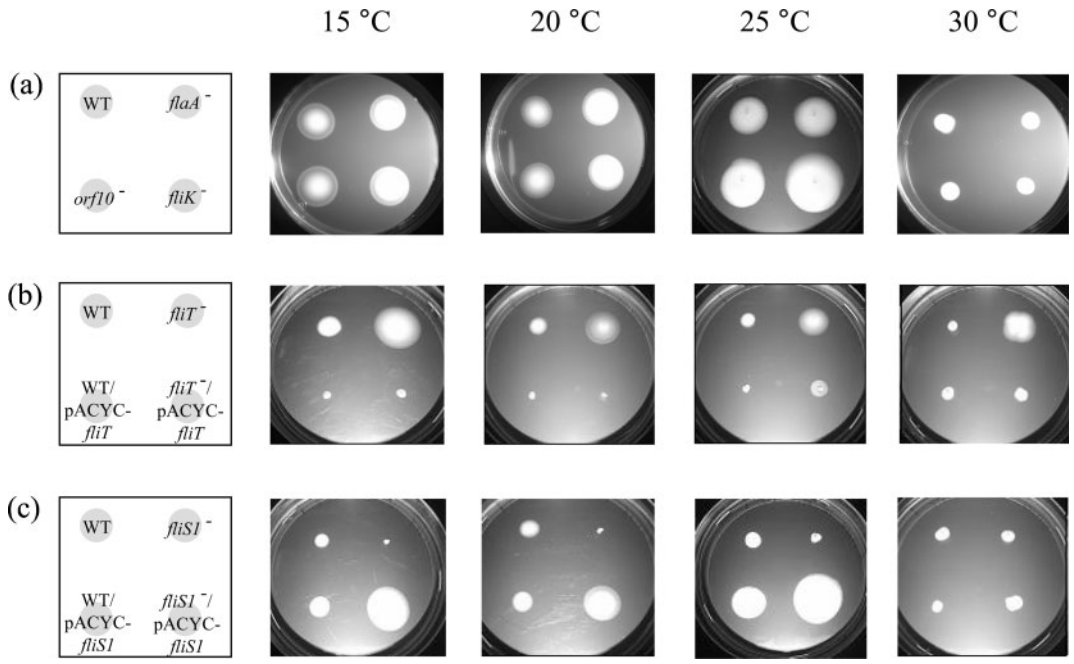

Fig. 3. Motility tests. W22703 wild-type strain (WT) and mutant strains were stabbed onto motility agar plates and monitored at 15, 20, 25 and 30 °C. Each experiment was repeated at least six times; representative data are shown. (a) Flag-2 mutants with inactivated *orf10*, *fliK* or *flaA* showed a significant increase in motility at lower temperature. (b) The W22703-*fliT*::Tn*5lux* mutant, which lacks a putative repressor of class 2 flagellar operons, is hyper-motile. (c) An insertional knockout of W22703-*fliS1*::Tn*5lux*, which putatively encodes a cytoplasmic chaperone, results in a non-motile phenotype. The two knockout mutations were complemented with pACYC184/*fliS1* and pACYC184/*fliT*, respectively, thus restoring the wild-type phenotype.

corresponding phenotype of *S. enterica* sv. Typhimurium with increased motility could be identified (Yokoseki *et al.*, 1995, 1996). The hyper-motile phenotype of the *fliT* mutant of *Y. enterocolitica* is in line with the assumption that FliT acts as a negative regulator of the transcription of flagellar genes (Kutsukake *et al.*, 1999). This function is confirmed by our observation that even the wild-type strain showed a decreased motility as a result of *fliT* overexpression (Fig. 3b). In contrast, *Y. enterocolitica* motility was abolished in a *fliS1* mutant. The motility of the *fliS1* strain, but not of the wild-type, after FliS1 overexpression was significantly higher than that of the wild-type alone (Fig. 3c). This effect might be explained by a coupled transcription of *fliS1* and *fliT*, so that strain W22703-*fliS1*::Tn*5lux*/pACYC-*fliS1* genotypically resembles the hyper-motile *fliT* mutant. Flagellar filaments produced by a *fliS* mutant in *S. enterica* sv. Typhimurium were much shorter than those produced by the wild-type strain, indicating that FliS is a cytosolic chaperone involved in controlling flagellin polymerization and preventing FliC degradation (Auvray *et al.*, 2001; Ozin *et al.*, 2003). Motility of a *Salmonella fliS* mutant was clearly impaired but not completely abolished (Yokoseki *et al.*, 1995).

A causal connection between motility and virulence has been demonstrated in several studies. At 37 °C, *Y. enterocolitica* cells are non-motile, autoagglutinate and express and secrete Yops. The invasion capabilitiy of this bacterium has been shown to be affected by motility, suggesting that motility is required for migrating to and contacting host cells (Young *et al.*, 2000). The *fliA* gene, encoding the alternative σ factor of the flagellar system, is highly induced at 25 °C and repressed at 37 °C (Kapatral *et al.*, 2004). FliA probably indirectly inhibits the expression at 25 °C of seven genes that are encoded by the pYV virulence plasmid, indicating that FliA plays a role in the inverse temperature regulation of flagellar and virulence genes (Horne & Pruss, 2006). This observation is in line with the finding that a non-motile *flhDC* mutant of *Y. enterocolitica* also secretes larger amounts of Yops, encoded by the pYV plasmid, than the wild-type bacteria (Bleves *et al.*, 2002). Evidence has also been found for a temperature-dependent synthesis antagonism between type III secretion in *Y. enterocolitica*, which is necessary for the survival of the bacterium in the mammalian host, and its flagellar assembly system (Bleves *et al.*, 2002). The flagellar export apparatus functions as a secretion system for the virulence-associated phospholipase YplA (Young *et al.*, 1999). Microarray data on *S. enterica* sv. Typhimurium motility have only recently revealed several FlhDC-controlled virulence genes, among them the virulence operon *srfABC*, thus demonstrating that the association

between motility and virulence is a phenomenon not restricted to *Y. enterocolitica* (Frye *et al.*, 2006; Wang *et al.*, 2004). Recently, it has been shown that in the insect pathogen *Xenorhabdus nematophila* the expression of a novel haemolysin, which is required for full virulence of *X. nematophila* against insects, is also regulated by the flagellar master-operon (Cowles & Goodrich-Blair, 2005). Interestingly, within the scope of identifying genes that are upregulated during prolonged growth of *Y. enterocolitica* at low temperatures, e.g. during proliferation in insects, we identified a *srfA* homologue and a haemolysin secretion gene (Bresolin *et al.*, 2006a). It might therefore be speculated that temperature-driven non-motility at 37 °C and maximal motility at approximately 20 °C essentially contribute to the control of the *Y. enterocolitica* switch between two pathogenicity phases related to mammals and invertebrates.

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