Transcriptional activation of the tad type-IVb pilus operon by PypB in *Yersinia enterocolitica*

by

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**ABSTRACT**

Type-IV pili are virulence factors in various bacteria and mediate among other functions the colonization of diverse surfaces. Various subclasses of type-IV pili have been identified, but information on pilus expression, biogenesis, and the associated phenotypes is sparse in the genus *Yersinia*. We recently described the identification of PypB as a transcriptional regulator in *Yersinia enterocolitica*. Here we show that the *pypB* gene is associated with the *tad* locus, a genomic island that is widespread among bacterial and archaeal species. The genetic linkage of *pypB* with the *tad* locus is conserved throughout the *Yersiniae*, but is not found in other bacteria carrying the *tad* locus. We show that the genes of the *tad* locus form an operon in *Y. enterocolitica* that is controlled by PypB and that *pypB* is part of this operon. The *tad* genes encode functions necessary for biogenesis of the Flp subfamily of type-IVb pili initially described in *Aggregatibacter actinomycetemcomitans* to mediate a tight adherence phenotype. In *Y. enterocolitica*, the Flp pilin protein shows some peculiarities in its amino acid sequence that imply similarities as well as differences to typical motifs found in the Flp subtype of type-IVb pili. Flp is expressed and processed after PypB overproduction, resulting in microcolony formation, but not in increased adherence to biotic or abiotic surfaces. Our data describe transcriptional regulation of the *tad* type-IVb pilus operon by PypB in *Y. enterocolitica*, but fail to show most previously described phenotypes associated with this type of pili in other bacteria.

**INTRODUCTION**

Bacteria are able to colonize diverse biotic and abiotic surfaces. The attachment of a pathogenic bacterium to the surface of a eukaryotic cell is an important step for a successful infection and also the prerequisite for subsequent events like internalization, or contact-dependent translocation of effector molecules by type-III secretion systems. For the interaction with surfaces, many bacteria make use of fimbrial or non-fimbrial adhesins (10). Fimbriae or pili are hair-like structures that extrude from the bacterial surface and usually consist of only one structural protein called pilin (29). Pili can be grouped by their morphological characteristics. Recently, however, they have been classified according to their biosynthesis. In Gram-negative bacteria one can distinguish chaperone-usher, curli, and type-IV pili (T4P) (9), with
T4P being the most abundant pili so far described. T4P are characterized as 1-4 µm long, flexible filaments with an average diameter of 5-8 nm. The functions of T4P are quite diverse. Besides mediating adhesion to and, in part, invasion into, eukaryotic cells, they have been shown to play a role in microcolony and biofilm formation, twitching motility on surfaces, and the uptake of DNA by natural transformation. In addition, bacteriophages make use of pili as a receptor. Contact of pili with receptors on eukaryotic cells can result in the induction of signaling cascades. Therefore, it is not surprising that pili are essential virulence factors of many bacteria (6).

According to sequence similarities, T4P can be divided into type-IVa (T4a) and type-IVb (T4b) pili. The N-terminal signal sequence of the structural protein of T4a pili is relatively short and consists of only 5-6 amino acids, while the signal sequence of T4b pilin contains 15-30 aa. Similarly, the mature T4a pilin is relatively short compared to the T4b pilin (ca. 150 aa vs. 190 aa) (6, 29). Interestingly, genes encoding T4a pili are usually scattered throughout the genome, and are widespread throughout the bacterial kingdom. In contrast, distribution of T4b pili is restricted to enteropathogenic bacteria including *Escherichia coli*, *Vibrio cholerae*, and *Salmonella enterica* (6, 29).

Studies in the periodontitis-causing bacterium *Aggregatibacter (Actinobacillus) actinomycetemcomitans* revealed the presence of T4b pili with specific characteristics. These include a long leader peptide and a relatively short mature pilin of 50-80 aa. Due to its unique features, it has been described as the Flp subfamily of T4b pili (17). In *A. actinomycetemcomitans*, the Flp pili mediate unspecific attachment to surfaces and the formation of microcolonies and tenacious biofilms. Further analysis revealed that Flp pili can be found in a wide variety of bacterial and archaeal species encoded by the *tad* (tight adherence) locus that has spread via horizontal gene transfer (15, 31, 37).

The Gram-negative human pathogen *Yersinia enterocolitica* causes a variety of gastrointestinal syndromes. After uptake by the human host via contaminated food or water, *Y. enterocolitica* colonizes the intestine and crosses the epithelial barrier via M cells to reach the underlying lymphoid tissues, where the bacteria multiply extracellularly and in rare cases may spread to deeper tissues to cause a systemic disease (2). Besides *Y. enterocolitica*, two more *Yersinia* species are pathogenic for humans. While *Y. pestis* is transmitted via a fleabite and causes the devastating disease plague, *Y. pseudotuberculosis* is a gastrointestinal pathogen. All three
species have in common a tropism for lymphatic tissues. Extracellular survival and replication in lymphoid tissues is made possible by the virulence plasmid-encoded type III secretion system that secretes anti-phagocytic and anti-inflammatory effector proteins into the host cells (5). Y. enterocolitica uses several adherence and invasion factors to colonize its host, including Inv, Ail, and the virulence plasmid-encoded YadA (8, 14, 25). While pili have been described to mediate surface attachment in many bacterial species, they are not well characterized in Yersinia. Y. enterocolitica possesses Myf fibrillae (similar to pH6 antigen of Y. pestis) that can be found on the bacterial surface by immunogold labelling, but the role for pathogenesis remains ill defined (13). Recent analysis identified a type IV pilus operon in Y. pseudotuberculosis that contributes to pathogenicity in the mouse model of infection. This pil operon is located on a genomic island termed YAPI (Yersinia adhesion pathogenicity island). A homolog of this island was also identified by in silico analysis in Y. enterocolitica, but not Y. pestis (3, 4, 35).

In a previous analysis we identified three regulators termed PypA, PypB, and PypC in Y. enterocolitica that activate transcription of the in vivo expressed hreP gene encoding a protease necessary for full virulence in the mouse model of yersiniosis (38). Analysis of the organization of the genomic locus around pypB revealed that it is located upstream of a putative operon homologous to the tad locus of A. actinomycetemcomitans. In this study we show that pypB is part of the tad operon and characterize transcriptional regulation and expression of Flp pili in Y. enterocolitica.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. For routine growth, all strains were grown in Luria-Bertani (LB) broth or on agar plates at 26°C for Y. enterocolitica or 37°C for E. coli or as otherwise indicated. Antibiotics were used as described previously (38). For the induction of protein expression from the \( P_{BAD} \) promoter, bacteria were grown in the presence of 0.1-0.2 % (w/v) arabinose.

Construction of bacterial strains and plasmids
Primers used for PCR amplification of DNA fragments are listed in Table 2. For the overproduction of PypB under control of the $P_{BAD}$ promoter, we cloned the respective gene in pBAD33. For this purpose, the plasmid pBAD-pypB(38) was digested with XbaI/PstI and the $pypB$ fragment (660 nt) was isolated. Subsequent ligation into XbaI/PstI-digested pBAD33 resulted in plasmid pBAD33-pypB.

For the construction of the *Y. enterocolitica* flp deletion strain (GHY545), we first amplified the flp gene including approximately 500 nt upstream and downstream using the primers JS-flpKpnI.for and JS-flpXbaI.rev and ligated the PCR fragment into the suicide plasmid pEP185.2. The resulting plasmid was then used as a template in an inverse PCR using the primers JS-flpXhol.rev and JS-flpXhol.for, thereby deleting the flp gene. After digestion with Xhol, the plasmid was religated and used to transform *E. coli* S17-1:pir to chloramphenicol resistance, resulting in plasmid pEP-Δflp. The plasmid was transferred to *Y. enterocolitica* by mating. Following analysis of proper chromosomal integration, cycloserine enrichment was used to identify Cam$^S$ exintegrants with a deletion in the flp gene. Western blot and PCR confirmed the correct genotype.

The construction of 3' nested deletions of the flp and pypB promoter regions fused to lacZ was as follows. DNA fragments comprising the flp upstream region were amplified by PCR using GH-flp150f, GH-flp250f and GH-flp500f as forward primer and SF-flp1-10 as the reverse primer, respectively, and ligated into XbaI/EcoRI-digested pKW1 to result in pKW-flp150, pKW-flp250 and pKW-flp500. The constructs contain 150, 250, or 500 bp, respectively, upstream of the ATG start codon and the first 50 bp of the flp coding region fused to the promoterless lacZYA genes. Similarly, DNA fragments comprising the pypB upstream region were amplified by PCR using JS-pypB1 as forward and JS-pypB6 as the reverse primer, resulting in pKW-pypB500 (containing 500 bp upstream of the pypB ATG start codon and the first 27 bp of the coding region) or JS-pypB244 and JS-pypB126 as forward primer and JS-pypB-200 as the reverse primer, respectively, and ligated into XbaI/EcoRI-digested pKW1 resulting in pKW-pypB126 and pKW-pypB244 (containing 244 or 126 bp upstream of the pypB ATG start codon, respectively, and the first 200 bp of the coding region).

To complement the flp deletion strain (GHY545), the primers JS-pypB.for and JS-flp1.rev were used for PCR amplification of a 1.5-kb DNA fragment containing the pypB promoter region, pypB and flp. The PCR product was digested with XbaI and
EcoRI and ligated into XbaI/EcoRI-digested pWSK129, resulting in plasmid pWSK-

kflp. To construct a C-terminally HA (hemagglutinin epitope)-tagged version of the Flp protein, the primers JS-pypB1 and JS-flp2 were used in a PCR reaction to amplify the pypB and flp genes including $P_{pypB}$. The DNA sequence encoding the HA-epitope was inserted via the reverse primer. The 1.5-kb PCR product was digested with XbaI and EcoRI and ligated into pWSK129, resulting in pWSK-pypB/flpHA.

RNA isolation and RT-PCR
An overnight culture of Y. enterocolitica carrying pBAD-pypB was diluted 1:10 in fresh medium, and PypB expression was induced for 3 hours with 0.2 % (w/v) arabinose at 26°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Residual DNA contaminations were removed by digestion with DNase (Ambion, Turbo DNA-free). Subsequently, RNA was precipitated with 3 M sodium acetate and quantified using a UV spectrophotometer at 260 nm. Synthesis of cDNA was carried out using AMV Reverse Transcriptase (Promega) by random priming. The derived cDNA was used as a PCR template using the following primer pairs for the indicated gene junction: JS-flp1/SF-flp1-RT2 (pypB-flp), SF-flp1-

RT1/JS-tadV1 (flp-tadV), JS-tadV2/JS-rcpC1 (tadV-rcpC), JS-rcpC2/JS-rcpA1 (rcpC-

tadB1 (tadA-tadB), JS-tadB2/JS-tadC1 (tadB-tadC), JS-tadC2/JS-tadD1 (tadC-tadD),

JS-tadD2/JS-tadE1 (tadD-tadE), JS-tadE2/JS-tadF1 (tadE-tadF), JS-tadF2/JS-tadG1

(tadF-tadG). The cDNA was also used as a PCR template to detect the indicated longer transcripts using primer pairs JS-flp1/JS-rcpC1 (pypB-flp-tadV-rcpC), JS-
tadV2/JS-rcpA1 (tadV-rcpC-rcpA), JS-rcpA2/JS-tadA1 (rcpA-tadZ-tadA), JS-
tadA2/JS-tadC1 (tadA-tadB-tadC), JS-tadC2/JS-tadE1 (tadC-tadD-tadE), and JS-
tadE2/JS-tadG1 (tadE-tadF-tadG) to detect longer transcripts of the tad operon. As a negative control, the primer pair JS-alkB2for/SF-flp1-RT2 was used spanning a region upstream of pypB (in YE3631) to flp. As a further control, 1 µg of total RNA was used as template. RT-PCR products were separated on 1 % agarose gels.

Northern blot analysis
For Northern blot analysis, total RNA (15 µg) was separated on a 2 % formaldehyde-containing agarose gel. The RNA was transferred from the gel to positively charged
nylon membrane and UV-cross-linked to the membrane. For hybridization and
detection, we used the DIG labeling and detection system (Roche). After pre-
hybridization, the blot was hybridized using a 170-bp flp DNA fragment that was
amplified by PCR using primers SF-flp1-RT1 and JS-flp.dig (DIG-labeled at its 3’
end) (Table 2) according to the protocol (DIG Easy Hyb Granules, Roche). 500 ng of
the PCR product was used for hybridization at 50°C overnight. Subsequently, the
membrane was washed and developed using anti-DIG-AP-solution (1:15000;
Roche). Chemiluminescence was detected using a Lumi Imager F1 (Roche).

β-Galactosidase assay
β-Galactosidase assays of the lacZYA fusion strains were performed as previously
described (38). Briefly, overnight cultures were diluted 1:20 in fresh medium and
grown for 3 hours with aeration at 26°C. The cultures were then collected by
centrifugation at 4 °C and washed in cold 0.85 % (w/v) NaCl before enzyme activity
assays. β-Galactosidase enzyme activities are expressed in arbitrary units, which
were determined according to the formula of Miller (24). The reported values are the
mean and standard deviation from experiments that have been repeated at least
three times, each in triplicate.

Production of antibodies directed against Flp
For the production of a Flp-specific antiserum, a peptide was synthesized containing
the amino acids KTPLKEIVDTSMTNIKDMVK (corresponding to aa 20-39 of the
mature Flp protein, Fig. 1) and conjugated to keyhole limpet hemocyanin. The
peptide was used to immunize rabbits according to standard procedures (Seqlab,
Göttingen, Germany) to obtain polyclonal antiserum against Flp.

Detection of Flp expression by immunoblotting
To analyze Flp expression in Y. enterocolitica, the wild type, the Δflp (GHY545) and
the complemented Δflp (GHY545, pWSK-kflp) strain, each carrying pBAD33-pypB to
overproduce PypB, were grown overnight at 26°C. The next day, the strains were
diluted 1:10 in fresh LB medium containing 0.1 % arabinose to induce expression of
the PypB protein and grown for 5 hours at 26°C. For the preparation of bacterial
supernatants, cultures normalized to an OD$_{600nm}$ of 0.5 were centrifuged three times
at 4°C 3500 x g. Between each centrifugation the supernatant was carefully removed
and transferred to a fresh microcentrifuge tube, leaving a residual volume behind, to avoid contamination of the supernatant fraction with bacteria. After addition of 4x protein loading buffer, 20 µl of supernatant were boiled and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To analyze whole cells, bacteria normalized to an OD$_{600nm}$ of 0.25 (corresponding to approximately 1.2x10$^6$ bacteria) were resuspended in 4x protein loading buffer, boiled and separated by SDS-PAGE. Proteins were transferred to nitrocellulose by Western blotting, blocked with 5 % (w/v) milk powder and incubated with Flp antiserum (1:5000) and goat anti-rabbit PO-coupled secondary antibody (Dianova, 1:5000). As a control for the contamination of the bacterial supernatants with cytoplasmatic proteins, streptavidin-PO (1:5000, Roche) was used to detect the cytoplasmatic acetyl-CoA carboxylase biotin carboxyl carrier protein (YE3811, AccB).

To analyze Flp-HA expression in *Y. enterocolitica* and in *E. coli* DH5α, strains containing the plasmids pBAD33-pypB and pWSK-pypB/flpHA were grown overnight at 26°C and 37°C, respectively. The next day, the strains were diluted 1:10 in fresh LB medium containing 0.1 % arabinose to induce expression of the PypB protein from $P_{BAD}$ and grown for 5 hours at 26°C or 37°C, respectively. Bacteria normalized to an OD$_{600nm}$ of 0.25 (corresponding to approximately 1.2x10$^6$ bacteria) were resuspended in 4x protein loading buffer, boiled and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane by Western blotting. The membrane was blocked with 5 % (w/v) milk powder in phosphate-buffered saline (PBS), washed with PBS and incubated with mouse monoclonal anti-HA-tag antibody (1:1000, Cell Signaling) and goat anti-mouse PO-coupled secondary antibody (1:5000, Dianova). Bound antibodies were detected with enhanced chemiluminescence substrate (Pierce) using a Lumi Imager F1 (Roche).

**Electrophoretic mobility shift assay**

For the electrophoretic mobility shift assay (EMSA), we employed purified C-terminally His-tagged PypBΔc in elution buffer (50 mM Tris-HCl, pH 8.0; 250 mM NaCl; 250 mM imidazole; 10 % (v/v) glycerol; 0.1 % (v/v) Triton X-100) that was affinity-purified as described elsewhere (38). As probes, we employed a PCR fragment encompassing 250 bp upstream of the *flp* ATG start codon and the first 50 bp of the *flp* coding region (primer pair: GH-flp250f/SF-flp1-10) or a PCR fragment encompassing 500 bp upstream of the *pypB* ATG start codon and 27 bp of the *pypB*
coding region (primer pair: JS-pypB500/SS-pypB6). For the binding reaction, 3 ng of
the respective DIG-labeled PCR product (DIG Gel Shift Kit, 2\textsuperscript{nd} Generation, Roche)
was incubated at room temperature for 45 min with different amounts of PypB\textDelta c. To
test specificity of the protein-DNA interaction, we used specific (unlabeled PCR
fragment used as a probe) and unspecific (200-bp PCR fragment generated using
primers KR-cpxA1 and GH-cpx9 encompassing an internal fragment of the Y.
\textit{enterocolitica} cpxA gene) unlabeled competitor DNA. For the \textit{pypB} EMSA,
competitor DNA was used in 300-fold excess. For the \textit{flp} EMSA, competitor DNA was
used in a 500-fold excess. After the binding reaction, the samples were separated on
a 6 \% native polyacrylamide gel and transferred to a positively charged nylon
membrane by electroblotting. For detection by chemiluminescence we employed an
anti-DIG-AP-coupled antibody (1:15.000, Roche) and a Lumi Imager F1 (Roche).

Biofilm assays
For biofilm assays, overnight cultures of \textit{Y. enterocolitica} wild type and \textit{\Delta flp} strains,
both containing pBAD-pypB but lacking the virulence plasmid pYV, were diluted 1:20
in fresh LB medium containing 0.1 \% arabinose or glucose for induction or repression
of PypB expression and grown in polyvinyl chloride (PVC) 96-well microtiter plates for
different time periods (3 h - 50 h) at 26\degree C or 37\degree C. Subsequently the supernatants
were discarded, and the 96-well plates were washed three times with ddH\textsubscript{2}O. After
incubation with 1 \% crystal violet for 15 min, plates were again washed three times
with ddH\textsubscript{2}O. To solubilize the crystal violet, DMSO was added for 60 min, and the
degree of staining was quantified at a wavelength of 590 nm. Uninoculated wells
were used as a blank and subtracted from each value. Alternatively, bacteria were
grown overnight in LB medium lacking NaCl and subcultured in PVC 96-well
microtiter plates in M63 minimal medium as described by Kim et al (18).

Microcolony formation
Overnight cultures of \textit{Y. enterocolitica} wild type and \textit{Y. enterocolitica} \textit{\Delta flp} strains
carrying pBAD-pypB grown at 26\degree C in LB medium were diluted 1:20 in fresh LB
medium containing 0.1 \% or 0.2 \% arabinose to induce PypB expression from the
\textit{P\textsubscript{BAD}} promoter in microtiter plates for 24 h - 48 h at 26\degree C. Microcolony formation was
detected by light microscopy.
**Immunofluorescence analysis**

For immunofluorescence analysis, *Y. enterocolitica* wild type, ∆flp (GHY545), and the complemented ∆flp strain (GHY545 carrying pWSK-kflp) all carrying pBAD33-pypB were grown overnight at 26°C. The next day, strains were diluted 1:10 in LB medium containing 0.1 % arabinose and grown for 5 h to induce PypB expression from the \( P_{BAD} \) promoter. Subsequently bacteria were washed with D-PBS, fixed with 4 % (w/v) paraformaldehyde (PFA) and incubated with 50 mM NH₄Cl. After blocking unspecific binding sites by incubation with 1 % (w/v) bovine serum albumin (BSA), Flp-specific antiserum (1:200) was added, and incubation continued overnight at 4°C. After washing the cells several times with PBS, secondary antibody (goat anti-rabbit-Cy3, 1:200, Dianova) was added for 1 h at room temperature. DNA was stained with DRAQ5 (Biostatus Limited). Bacteria were then transferred to a microscope slide, mounted with fluorescence mounting medium (Dako Cytomation) and analyzed by confocal laser scanning microscopy (CLSM Axiovert 200M).

**Determination of cell-associated bacteria**

To determine the number of bacteria associated with eukaryotic cells, *Y. enterocolitica* wild type, ∆flp (GHY545), and the complemented ∆flp strain (GHY545 carrying pWSK-kflp) all carrying pBAD33-pypB but lacking the pYV virulence plasmid were grown as described above. HeLa, CHO-K1 and Caco-2 cells were infected with 10 µl of bacterial suspension for 1-3 h, washed three times with PBS and lysed with PBS containing 1 % (v/v) Triton X-100. The % cell-associated bacteria was determined by plating appropriate dilutions on LB agar and compared to the initial inoculum used for infection.

**Electron Microscopy**

For transmission electron microscopy (TEM), overnight cultures of *Y. enterocolitica* wild type and ∆flp (GHY545) strains carrying pBAD-pypB were diluted 1:20 in LB medium containing 0.1 % (w/v) arabinose to induce PypB expression from the \( P_{BAD} \) promoter for three hours at 26°C. Bacteria were negatively contrasted with 1 % (w/v) phosphotungstic acid and applied to formvar-coated copper grids. The samples were analyzed using a Tecnai G2 Spirit Twin electron microscope (Fei, Tecnai Spirit Twin). For ultracryo-electron microscopy, overnight cultures of *Y. enterocolitica* wild type, ∆flp (GHY545), and the complemented ∆flp strain (GHY545 carrying pWSK-kflp) all
carrying pBAD33-pypB were diluted 1:20 and expression of PypB was induced with 0.1 % (w/v) arabinose for three hours at 26°C. Cells were fixed with 2 % (w/v) PFA, 2 % (w/v) glutaraldehyde in D-PBS, pH 7.4. Ultrathin frozen sections were prepared by the Tokayasu method (70 nm samples), immunostained using gold-coupled Flp antiserum and observed by electron microscopy (Fei, Tecnai Spirit Twin).

**RESULTS**

**The tad locus of Y. enterocolitica**

In a previous analysis we could show that PypB is a transmembrane transcriptional regulator similar to ToxR of *Vibrio cholerae* and CadC of *E. coli* (38). Characteristic for this class of transcriptional regulators is a cytoplasmatic OmpR-like winged helix-turn-helix DNA binding motif that is separated from a carboxyterminal periplasmatic domain by a transmembrane domain. In PypB, this periplasmatic domain is unusually short (~9 aa compared to 95 aa in ToxR of *V. cholerae*). Analysis of the genomic localization of *pypB* revealed that it is located on the YGI-1 genomic island of *Y. enterocolitica* (Fig. 1A) (35). This island harbors genes with similarity to *tad* loci, encoding genes for the biogenesis of the Flp subfamily of T4b pili. Further analysis of completed and unfinished genomes revealed that this locus is conserved throughout the *Yersiniae*, but as already reported by Thompson *et al.* (35) is inactivated in *Y. pestis*. Interestingly, analysis of available genome sequences revealed that all YGI-1 homologues in *Yersinia* show some specific characteristics. First, they are all inserted at the same genomic locus 5’ of the *exbD* gene. Second, in contrast to most *tad* loci described so far, it contains only one instead of two *flp* genes; third, all *tad* loci are associated with a gene at their 5’ end coding for a transcriptional regulator homologous to PypB.

Although the *tad* locus is widespread among bacteria and archaea, an association with a transmembrane transcriptional regulator has so far not been described. Analysis of available completed as well as unfinished prokaryotic genomes using the BLAST algorithm did not reveal a similar association of the *tad* locus with a transcriptional regulator besides the genus *Yersinia* with one exception. We identified a transcriptional regulator similar to PypB in the genome of *Shigella flexneri* 2A strain 301. In this strain, SF3009 is 41% identical on protein level to PypB of *Y.
enterocolitica. Similar to the genomic organization in Y. enterocolitica, a flp (SF3008) and a tadV (SF3007) gene are immediately downstream of SF3009. However, additional genes of the tad locus are not present. Instead, the genes SF3007-3009 are flanked by genes encoding IS elements and transposases, indicating a mobile genetic element. As shown in Fig. 1, homologues of Y. enterocolitica PypB are conserved between pathogenic as well as non-pathogenic Yersinia strains. Similarity is high in the DNA binding as well as the transmembrane domains of PypB, while the region of the PypB sequence linking both domains is relatively variable (Fig. 1B). A conserved DNA binding domain suggests that similar promoter regions are recognized in all species, while conservation of the transmembrane domain might be an indication for a role in signal perception.

A comparison of tad loci in different genomes revealed that probably not all genes are essential for functionality in all prokaryotes. RcpA, RcpB and TadD are predicted outer membrane proteins not found in Gram-positive bacteria due to a lack of an outer membrane (37). Most genes of the tad locus from A. actinomycetemcomitans can be identified in the same genetic organization in the genome of Y. enterocolitica with the exception of rcpB (35). The rcpB gene has so far only been identified in tad loci of Pasteurellaceae and encodes an outer membrane protein (30). A specific feature of the tad locus of Yersinia is related to the Flp structural protein. As already mentioned, the tad locus of A. actinomycetemcomitans encodes a pilus with specific characteristics. These include a long leader peptide and a relatively short mature pilin. Processing occurs by the TadV prepilin peptidase after a Gly residue in the conserved G/(X4)EY processing site (the slash indicates the processing site) (17, 36). In addition, a shared “Flp motif” has been identified at the amino terminus of the mature pilin that is characterized by adjacent Glu and Tyr residues within a stretch of approximately 20 hydrophobic, non-polar, aliphatic amino acids. In addition, most predicted Flp proteins contain a conserved phenylalanine residue towards their C-terminus (16, 17). All predicted Flp proteins of the Yersiniae show differences in these features that are conserved in the genus Yersinia. Besides the missing conserved Phe in the C-terminus, the N-terminus of the mature Y. enterocolitica Flp protein shows a different sequence at the putative prepilin peptidase cleavage site, which is G/(X3)EY. Therefore, the Glu of the mature pilin is located at position 4 in the Y. enterocolitica processed Flp instead of position 5 in other T4P (Fig. 1C). An alignment with Flp-1 of A. actinomycetemcomitans suggests that the conserved N-
terminal residue of the mature pilin, which is usually Val, Met, or Leu in typical T4b pili has been omitted to result in Thr as the N-terminal aa in the mature Yersinia Flp (Fig. 1C) (6). However, the putative pseudopilins TadE and TadF of Yersinia that are presumably also processed by the TadV prepilin peptidase as in A. actinomycetemcomitans (36) have a consensus cleavage site (G/(X4)EY) of T4b pili (data not shown).

PypB overproduction results in flp transcription

In previous analyses we have shown that pypB is autoregulated and regulated by PypC(38). As pypB is associated with the tad locus in Yersinia, we hypothesized that PypB might be involved in transcriptional regulation of the tad locus. To analyze if a promoter exists upstream of flp and if PypB activates this promoter, we constructed a transcriptional fusion of the flp 5’ region with the lacZ gene as a reporter. To avoid potential read-through from the promoter upstream of pypB, we did not construct a strain with a reporter homologously integrated into the chromosome of Y. enterocolitica, but instead used the episomal β-galactosidase reporter plasmid pKW-flp500. The plasmid contains approximately 500 nt 5’ of the ATG start codon of the flp gene. In this reporter construct, transcription of lacZ is independent of PpypB and depends only on a putative promoter upstream of flp. Overproduction of PypB from a PBAD promoter in this strain resulted in a strong increase in β-galactosidase activity (Fig. 2). Similar results can be obtained when the pKW-pypB500 reporter plasmid is used, containing 500 bp 5’ of the pypB ATG start codon fused to lacZ, to monitor pypB transcription, confirming previous results with a chromosomal pypB-lacZ fusion(38). To identify regions at the pypB and flp promoter that might be necessary for activation by PypB, we constructed transcriptional fusions with lacZ containing fragments of the pypB or flp promoter region deleted from the 5’ end. As shown in Fig. 2, a region between 250 and 500 nt 5’ of the ATG start codon of pypB and 150 nt upstream of the start codon of flp, respectively, are required for transcriptional activation by PypB. From these data we conclude that PypB is a positive transcriptional regulator of the pypB as well as the flp gene of Y. enterocolitica, and that a PypB-responsive promoter exists upstream of the flp gene.

The tad locus of Y. enterocolitica is organized as an operon including pypB
The analysis of tad loci in other bacteria revealed that it is organized as an operon (12, 27). Our data indicate that promoters exist upstream of pypB and of flp in Y. enterocolitica. Transcription from these promoters might result in mono- and/or polycistronic transcripts. To study this in more detail, total RNA was reverse transcribed into cDNA and used as a template in a PCR (RT-PCR). The primers were designed to bind in the 3' region of a gene and the 5' region of the adjacent gene, so that a PCR product would only result when the genes are co-transcribed. As shown in Fig. 3A, we obtained PCR products in all cases, indicating that all genes of the tad locus, including pypB, are co-transcribed into a polycistronic mRNA. PCR products could also be obtained for larger transcripts, including the pypB-flp-tadV-rcpC, tadV-rcpC-rcpA, rcpA-tadZ-tadA, tadA-tadB-tadC, tadC-tadD-tadE, and tadE-tadF-tadG regions (Fig. 3). In contrast, PCR products could not be obtained when total RNA was used as a template or when a primer pair spanning a region upstream of pypB (in YE3631) to flp was used (Fig. 3).

In A. actinomycetemcomitans a monocistronic transcript has been identified that corresponds to the flp gene (20). To analyze if a transcriptional terminator might exist downstream of flp, we performed Northern blot analysis using a flp specific probe. As shown in Fig. 3B, we were able to detect a transcript with a size of approximately 400 nt. The size of this fragment corresponds well with a monocistronic flp transcript starting from Pflp, indicating a transcriptional terminator downstream of the flp gene as described for A. actinomycetemcomitans (20). Indeed, we identified a putative stem-loop structure in the intergenic flp-tadV region that resembles classical terminators and has a calculated $\Delta G$ of -17.9 kcal (Fig. 3C). Similar to A. actinomycetemcomitans, larger transcripts could not be identified by Northern blot, indicating that they are only transcribed in small amounts or are unstable.

**PypB directly interacts with the pypB and the flp promoter region**

In a previous study we could show that PypB is able to activate and directly interact with the hreP promoter(38). To show that the effect of PypB$^{OP}$ on pypB and flp activation is also direct, we performed electrophoretic mobility shift assays (EMSA). For this purpose we employed a recombinant version of PypB that is lacking its C-terminal membrane domain (PypB$^{\Delta c}$), but has an intact winged helix-turn-helix motif necessary for DNA interaction (38). To analyze if this protein is able to activate pypB and flp, we introduced a plasmid to express PypB$^{\Delta c}$ including a C-terminal 6-His tag.
from the $P_{BAD}$ promoter in *E. coli* carrying pKW-flp250 or pKW-pypB500 reporter plasmids, respectively, and determined the $\beta$-galactosidase activity. Overproduction of PypBΔc resulted in a comparable activation of gene expression as observed for wild type PypB (for *pypB-lacZ*, control: 38±8 Miller units, PypBΔc<sup>OP</sup>: 891±341 Miller units; for *flp-lacZ*, control: 196±165 Miller units, PypBΔc<sup>OP</sup>: 9820±5252 Miller units), indicating that membrane localization of PypB is not required for activation of *flp* and *pypB*. For EMSA assays, recombinant PypBΔc was incubated with DIG-labeled DNA fragments encompassing 500 nt upstream of the *pypB* start codon or 250 bp upstream of the *flp* start codon, respectively. Both fragments have been shown to contain regions responsive to PypB (Fig. 2). Increasing amounts of PypBΔc result in a mobility shift of the *pypB* as well as of the *flp* probe (Fig. 4). The shift can be blocked by adding an excess of specific competitor DNA (unlabeled promoter fragment of *pypB* or *flp*, respectively). In contrast, addition of an excess of unspecific competitor DNA (an internal fragment of the *Y. enterocolitica cpxA* gene) had no effect on the DNA mobility shift. From these data we conclude that PypB is able to directly and specifically bind to the *pypB* and the *flp* promoter, resulting in transcriptional activation. Sequence similarities indicative of a PypB binding site could not be identified in the *pypB*, *flp* and *hreP* promoter regions.

**Flp is expressed and processed after PypB overproduction**

Our data indicate that PypB activates transcription of promoters upstream of *pypB* and *flp*, thereby activating the whole *tad* locus. To analyze if the *tad* locus is expressed after PypB<sup>OP</sup>, we used Western blotting to detect Flp in *Y. enterocolitica*. Whole cell fractions as well as supernatants of *Y. enterocolitica* strains carrying $P_{BAD}$-*pypB* plasmids grown in the presence of arabinose were separated by SDS-PAGE and subsequently processed for Western blotting using Flp-specific peptide antiserum. As shown in Fig. 5A, Flp is expressed in the *Y. enterocolitica* wild type, but not in a strain deleted for the *flp* gene. Two bands with apparent molecular masses of 4 and 6 kDa specifically reacting with Flp antiserum can be detected in whole cell lysates (calculated molecular mass of 7 kDa for full-length Flp and 4.2 kDa for processed Flp, respectively). Only the 4-kDa band appears in the supernatant, indicating that Flp is processed and that the 4-kDa protein represents mature Flp that is secreted most likely by the Tad-encoded secretion system. When complementing...
the \( \Delta \text{flp} \) deletion from a plasmid, both, the 4-kDa as well as the 6-kDa proteins missing in the mutant strain appeared in whole cell lysates, indicating that they correspond to Flp. The complemented \( \Delta \text{flp} \) strain also secreted Flp into the supernatant, although in lower amounts compared to the wild type. As a control for cytoplasmatic contamination of the supernatants, streptavidin-PO was used to detect the cytoplasmatic acetyl-CoA carboxylase biotin carboxyl carrier protein. The protein could only be detected in whole cell lysates, indicating that cytoplasmatic proteins do not contaminate the supernatants. As a further control we expressed a HA-tagged version of Flp from a plasmid in \textit{E. coli}. While Flp-HA is expressed in \textit{E. coli} and in \textit{Y. enterocolitica}, it is only processed in \textit{Y. enterocolitica}, indicating that processing of Flp occurs by a \textit{Y. enterocolitica}-specific peptidase, presumably TadV (Fig. 5B).

As processing of preplins by their cognate preplin peptidase occurs at the cytoplasmatic membrane (21, 34), we analyzed by cryo-electron microscopy if we can detect Flp associated with the inner membrane of \textit{Y. enterocolitica}. Indeed, using a Flp-specific gold-labeled antibody we found Flp at the inner membrane of \textit{PypB}\textsuperscript{OP} wild-type, but not \( \Delta \text{flp} \) bacteria (Fig. 5C and data not shown). We conclude that Flp is expressed in \textit{PypB}\textsuperscript{OP} \textit{Y. enterocolitica}, processed by a preplin peptidase missing in \textit{E. coli} DH5\textalpha, and is subsequently transported through the bacterial outer membrane.

**Phenotypic characterization of the \textit{Y. enterocolitica} tad locus**

The \textit{tad} locus has been shown to encode a type IVb pilus that is implicated in the formation of biofilms and microcolonies, and the adherence to eukaryotic cells in \textit{A. actinomycetemcomitans}, \textit{Haemophilus ducreyi} as well as \textit{Pseudomonas aeruginosa} (7, 15-17, 27). As we could show that the \textit{tad} locus is expressed in \textit{Y. enterocolitica} after \textit{PypB}\textsuperscript{OP}, we were interested in analyzing if it mediates similar phenotypes. As an initial attempt to analyze whether Flp is not only expressed but also assembled into pili, we tried to detect Flp pili on the surface of \textit{Y. enterocolitica} by immunofluorescence microscopy. The wild type and the \( \Delta \text{flp} \) mutant strain as well as a complemented \( \Delta \text{flp} \) strain were grown for 5 h at 26 °C in the presence of 0.1 % arabinose to induce \textit{PypB} expression from the \( P_{\text{BAD}} \) promoter. Cells were incubated with Flp-specific peptide antibody and Cy3-labeled secondary antibody and analyzed by confocal laser scanning microscopy. As shown in Fig. 6A, only a fraction of the wild type bacteria are expressing Flp at their surface, while no signal could be detected in the \( \Delta \text{flp} \) mutant, indicating specificity of the antiserum for Flp. The signals
obtained for Flp are distributed in a patchy pattern on the bacterial surface. Similar
Flp signals were observed in the complemented ∆flp strain, however, only a fraction
of all cells show Flp expression. This is in agreement with data obtained by Western
blotting, which revealed only weak secretion of Flp in the complemented ∆flp mutant
strain (Fig. 5A). To follow on these results, we analyzed the bacteria by negative-
controlling transmission electron microscopy. We were able to detected thin pilus-
like structures on the bacterial surface of wild type, but not ∆flp bacteria, indicating
that they might represent Flp pili (Fig. 6B). However, only a very small fraction of all
analyzed bacteria were found to carry these pili. As subsequent transmission
electron microscopy coupled with immunogold labeling did not result in the detection
of gold-labeled pili or any other specific signal associated with the bacterial surface,
even after several attempts (data not shown), we cannot unequivocally state that the
observed structures are indeed Flp pili. Our results might either indicate that the
amount of pili expressed is too low to be detected, or that the Flp protein is not
assembled into a functional pilus. We also cannot exclude that the Flp pili are
instable and disassemble during processing for electron microscopy.

Next we determined if the tad locus of Y. enterocolitica is associated with
microcolony formation. Wild type and ∆flp bacteria were grown in 96-well plates at 26
°C in the presence of 0.1 % arabinose to induce PypB<sup>op</sup> from P<sub>BAD</sub>. After 24 h, small
bacterial aggregates were visible in the wild type culture, but not in the ∆flp mutant
strain. After prolonged growth (up to 50 h), we detected larger microcolonies that are
dependent on the presence of the flp gene, similar to what has been described for
other bacteria (Fig. 7) (16).

To determine whether the tad locus is also involved in the attachment of Y.
enterocolitica to plastic surfaces and biofilm formation, we grew pYV-cured wild type
and ∆flp bacteria overproducing PypB from P<sub>BAD</sub> at 26°C and 37°C in 96-well plates
for various time points. After removal of the growth medium and washing away
unbound bacteria, attached cells were stained with crystal violet and quantified.
However, we were not able to detect any significant difference in the attachment of
bacteria to plastic surfaces (data not shown). In addition, we analyzed whether the
expression of Flp contributes to binding of Y. enterocolitica to cultured eukaryotic cell
lines. To this end, we infected HeLa, CHO-K1, and Caco-2 cells for various time
points (1 h to 3 h) with wild type bacteria, the ∆flp, or the complemented ∆flp strain,
each overproducing PypB, and determined the amount of cell-associated bacteria.
As we expected that the well-described invasion factors Inv and the pYV-encoded YadA might mask our results, we also performed the experiments with inv-negative and pYV-cured strains. However, although in some experiments the presence of Flp seemed to positively affect the number of cell-associated bacteria, the values we obtained were highly variable. Even after several attempts, also by directly counting cell-associated bacteria by microscopic examination, we were not able to detect a significant difference in cell adhesion dependent on the flp gene (data not shown).

**DISCUSSION**

T4P mediate phenotypes associated with bacterial surface interactions and have been described as virulence factors in various bacteria (6). The expression of pili by pathogens has to be tightly regulated especially during an infection, as pili are not only important for colonization, but also are antigens that can be recognized by the immune system of the host. Pili have been identified before in the genus Yersiniae, but T4P have not been characterized in Y. enterocolitica (3, 4, 13). Here we describe transcriptional activation of the tad operon encoding Flp T4b pili of the human pathogen Y. enterocolitica by the transcriptional regulator PypB. The pypB gene is part of the tad operon, a genetic organization that is specific for Yersiniae. PypB activates transcription of the tad locus by binding to promoter regions upstream of pypB as well as flp, resulting in expression, processing and secretion of the Flp pilin protein. Although we describe Flp-dependent microcolony formation, other typically tad-associated phenotypes as biofilm formation and host cell attachment could not be detected for the Flp pili of Y. enterocolitica.

In previous studies we could show that PypB acts as a transcriptional regulator of hreP, pypC and pypB (38). A closer examination of the pypB locus revealed that pypB is associated with the tad locus that has been identified in diverse bacteria, but is best characterized so far in A. actinomycetemcomitans (16, 37). Although many bacterial species carry the tad locus, it has never been shown to be associated with a transmembrane transcriptional regulator similar to PypB. However, analysis of so far available genome sequences of the genus Yersinia reveal that they all possess the tad locus associated with the pypB gene at their 5' end, indicating that Yersiniae have evolved to employ PypB as a regulator activating the tad locus in response to specific environmental conditions. Similar to what has been described in Y.
enterocolitica (38), PypB might be involved in coordinating the expression of other
genes in other Yersinia species. Interestingly, pypC and its homolog, pclR, encode
transcriptional regulators that are associated with type-II secretion system gene
clusters in Yersiniae, indicating a common theme of activating transcription of
molecular export machines by associated regulators (32).

P. aeruginosa has also been shown to possess a fip-tad-rcp gene cluster. Its
transcriptional activation is controlled by a genetically linked two-component
regulatory system (1). However, the genetic organization of the locus is different from
that in Y. enterocolitica or A. actinomycetemcomitans, as the cluster is composed of
five independent transcriptional units (1).

PypB activates transcription of the tad locus by directly binding at regulatory regions
upstream of pypB as well as flp. The genes of the tad locus are organized in one
large transcriptional unit, and our Northern blot experiments furthermore reveal a
shorter flp-containing transcript, indicating a transcriptional terminator 3’ of the flp
gene. This is similar to what has been described in A. actinomycetemcomitans and,
to a certain extend, in H. ducreyi (20, 27), with the exception that in Y. enterocolitica
the pypB gene is part of the operon but is missing in other species outside the genus
Yersinia.

Besides the association of the tad locus with a transcriptional regulator, there are
further peculiarities associated with the Flp pilin protein. Kachlany et al. described
that the tad locus of A. actinomycetemcomitans encodes a subtype of T4b pili that
has specific characteristics (17). The tad locus of Y. enterocolitica, however, is again
different. First, there is only one flp gene instead of two in most other tad loci;
second, the mature Flp of Y. enterocolitica is unusually short (only 39 aa); third, Flp is
missing a conserved Phe residue in its hydrophilic C-terminal part; and fourth, the
conserved amino acids Glu-Tyr usually found in the hydrophobic N-terminus at
position 5 and 6 of the mature pilin are shifted to position 4 and 5, respectively (Fig.
1C). These characteristics are conserved throughout the Flp proteins of Yersiniae.

Interestingly, the pseudopilins TadE and TadF are presumably also processed by the
TadV prepilin peptidase, and the amino acids at position 5 and 6 (Glu-Phe) in the
mature pseudopilins are conserved also in Y. enterocolitica (36). Obviously the
unalusual features of the Flp protein do not interfere with processing. All T4b pili are
processed by prepilin peptidase after a Gly residue that is also conserved in the Flp
pili and the TadE/TadF pseudopilins of Yersiniae. If processing indeed occurs by the
TadV prepilin peptidase, or by another so far not identified enzyme, has to be elucidated in the future. At least, we did not detect Flp processing in *E. coli* DH5α, where the TadV peptidase is absent. We clearly demonstrate that transcriptional activation of the *tad* locus after PypB<sup>OP</sup> results in the expression and secretion of Flp; however, the associated phenotypes are different from what we expected. Although we detect microcolony formation dependent on Flp, we did not observe a characteristic biofilm or adhesion to eukaryotic cells. Also we were not able to detect Flp pili by TEM after immunogold labeling, so that we cannot say with certainty that the pilus-like structures detected on the *Y. enterocolitica* surface after negative contrasting TEM are indeed Flp pili. Similar observations have been made in *H. ducreyi*; although the *tad* genes are expressed and mediate microcolony formation, no Flp pili could be detected on the bacterial surface (27). In contrast, however, we localize Flp after cryo-immuno-EM at the bacterial inner membrane and also detect Flp-specific signals at the bacterial surface by immunofluorescence microscopy. Our findings might be explained by the specific characteristics of the *Y. enterocolitica* Flp protein. As it has been suggested that especially the N-terminal amino acids of the mature pilin (the Glu-Tyr motif, see above) are important for the proper assembly of pilin subunits into a pilus (23, 28, 33), this might be a reason why we were not able to clearly demonstrate pilus assembly by (immunogold labeling) TEM. However, as we detected Flp-specific signals at the bacterial surface by immunofluorescence microscopy, additional biochemical studies have to be performed before we can make a final statement on this aspect. In preliminary experiments, we detected Flp multimers in the supernatant of PypB<sup>OP</sup> bacteria after gel filtration chromatography, further indicating that Flp is able to polymerize (data not shown). These multimers might be instable due to decreased hydrophobic interactions via the N-termini of the Flp protein that are necessary for pilus assembly (6). Flp pili in other bacteria are stable, and the question remains what the function of *Y. enterocolitica* Flp might be, if not assembly into a pilus associated with biofilm formation or adhesion to surfaces. Flp is able to mediate *Y. enterocolitica* microcolony formation, and this might result in a growth advantage under specific conditions compared to planktonic bacteria. It might be possible that Flp acts as “glue” mediating cell-cell contacts due to an increased availability at the bacterial surfaces.
In summary, although we show that Flp is expressed in *Y. enterocolitica* after PypB^OP^, we could not unequivocally show that it is assembled into a pilus. Therefore, future studies will be aimed at identifying factors that might play a role in pilus biogenesis. To this end, we will identify in vitro conditions that result in *tad* activation, as this might also result in pilus expression and assembly, but also study the Flp protein and other *tad*-encoded proteins necessary for pilus biogenesis using molecular and biochemical approaches. Alternatively, we cannot rule out that pili are not formed and that the *tad* locus encodes a secretion system determined to secrete Flp subunits into the supernatant. In this case, Flp might have a different role independent of pilus-mediated functions that needs to be determined. As the *tad* locus is found in pathogenic as well as non-pathogenic species of the genus *Yersinia*, the function of the *tad* locus might not be associated with virulence, but with a *Yersinia*-specific environment outside the human host.

ACKNOWLEDGEMENTS
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REFERENCES


Table 1: Bacterial strains and plasmids

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**FIGURE LEGENDS**

Fig. 1: The pypB gene is part of the tad locus in Y. enterocolitica and encodes the PypB transmembrane transcriptional regulator that is conserved in Yersinia. (A) Genomic organization of the tad locus of Y. enterocolitica. Arrows indicate open reading frames and their direction of transcription. (B) Alignment of PypB of Y. enterocolitica with orthologs from other Yersinia species. The locus tags of the respective genes have been indicated (yberc, Y. bercovieri; ykris, Y. kristensenii; yrohd, Y. rohdei; yfred, Y. frederiksenii; yinte, Y. intermedia; ymoll, Y. mollaretii; yruck, Y. ruckeri; yaldo, Y. aldovae; ypsIP31758, Y. pseudotuberculosis IPS31758). Grey bars above the sequence indicated the DNA binding domain and the transmembrane (TM) domain of PypB. (C) Alignment of the Flp protein of Y. enterocolitica (Ye) and Flp-1 of A. actinomycetemcomitans (Aa). The arrow indicates the predicted processing site. The conserved G/(X)4EY and G/(X)3EY motifs, respectively, are underlined. An asterisk (*) indicates identical residues in all sequences; a colon (:) indicates conserved residues; a full stop (.) indicates semi-conserved residues. Alignments have been generated using the ClustalW algorithm.

Fig. 2: PypB activates transcription of flp and pypB. Y. enterocolitica containing the indicated flp-lacZ or pypB-lacZ reporter plasmids and either pBAD18Kan (-) as a control or pBAD-pypB (+) to induce PypB\textsuperscript{OP} from the P\textsubscript{BAD} promoter were grown in the presence of 0.2 % arabinose for 3 h at 26°C before determination of β-galactosidase activity (in arbitrary Miller units). Data are means and standard deviations from at least three experiments, each performed in triplicate. 150 nt upstream of the flp ATG start codon are sufficient for flp activation by PypB, while nt 245-500 upstream of the ATG start codon of pypB are necessary for pypB activation by PypB.

Fig. 3: Transcriptional analysis of the tad locus. (A) RT-PCR analysis of the tad locus using primers binding in the 3' region of a gene and the 5' region of the adjacent gene or a gene further downstream so that the transition region between genes as indicated in the figure is amplified. (-), RNA as template; (+), cDNA as template; (chr); chromosomal DNA of Y. enterocolitica as template. (B) Northern blot analysis of total...
RNA isolated from PypB<sup>OP</sup> *Y. enterocolitica* that was probed with a *flp* fragment reveals a *flp* transcript with an approximate size of 400 nt. (C) Stem-loop structure in the *flp-tadV* intergenic region as predicted by Mfold (40).

**Fig. 4:** Electrophoretic mobility shift assays demonstrate a direct interaction of PypB with the *pypB* and *flp* promoter region. Increasing amounts of recombinant PypBΔc were incubated with a DIG-labeled DNA fragment encompassing 500 nt upstream of the *pypB* start codon (A) and 250 bp upstream of the *flp* start codon (B), resulting in a mobility shift of the DNA fragment. Shifting of the probe can be inhibited by specific, but not by unspecific unlabeled competitor DNA.

**Fig. 5:** Flp is secreted and processed by *Y. enterocolitica*. (A) Whole cell extracts (CE) and supernatants (SN) of *Y. enterocolitica* wild type, Δ*flp*, and the complemented Δ*flp* strain and overproducing PypB from pBAD33-pypB were analyzed by Western blotting using Flp-specific antiserum. The upper part of the blot was probed with streptavidin-PO to detect the cytoplasmatic acetyl-CoA carboxylase biotin carboxyl carrier protein (AccB) to exclude contamination of supernatants with cytoplasmatic proteins. (B) Whole cell extracts of *Y. enterocolitica* wild type and *E. coli* DH5α carrying pWSK-pypB/flpHA and pBAD33-pypB to overproduce PypB were analyzed by Western blotting with HA-specific antibody to detect Flp-HA. Flp-HA is expressed in both strains, but only processed in *Y. enterocolitica*. (C) Ultracyro-EM of *Y. enterocolitica* overproducing PypB indicates that Flp is expressed and transported to the bacterial inner membrane. Flp was detected using gold-labeled Flp antiserum (bar: 1 µm).

**Fig. 6:** Flp is associated with the bacterial surface. (A) Confocal fluorescence microscopy indicates that Flp is expressed by and transported to the surface of the PypB<sup>OP</sup> *Y. enterocolitica* wild type and the complemented Δ*flp* mutant strain, but not by the Δ*flp* mutant. Flp was detected using Flp-specific antiserum and a Cy3-conjugated secondary antibody (left panel), and DNA was stained with DRAQ5 (right panel). (B) TEM of a negative-contrasted sample indicates the presence of short pili (arrows) on the surface of the wild type strain overproducing PypB (bar: 0.5 µM).
Fig. 7: Microcolony formation of PypB<sup>OP</sup> *Y. enterocolitica* depends on Flp. Wild type bacteria cultured in microtiter plates for 48 h at 26°C and expressing PypB from the <sup>P<sub>BAD</sub></sup> promoter form microcolonies, while the <sup>Δflp</sup> mutant strain does not.
Fig. 1

DNA binding domain

TM domain

PypB Y.e. 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yberc0001_35170 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
ykr is0001_26480 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yrohd0001_16440 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yre0001_20940 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yred0001_29840 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yinte0001_2360 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
ymol l0001_34120 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yruck0001_3230 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
yaldo0001_3370 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG
YpsIP31758_0605 1 MEKNEIMFKLEGAVLFSPAFQRCLNPGDSVVMNRENRLFRQLLNLGTVKEIKVQWEKQAQGESSYSG

PypB Y.e. 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yberc0001_35170 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
ykr is0001_26480 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yrohd0001_16440 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yre0001_20940 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yred0001_29840 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yinte0001_2360 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
ymol l0001_34120 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yruck0001_3230 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
yaldo0001_3370 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL
YpsIP31758_0605 74 QLYMLRKAFLQVGLKESLIHTIPRKGVRYTGSVESNAWQTDCDEATEQQSHETMLSATAPF------SLQLYL

PypB Y.e. 140 PFSNAMPSQGFPAQ--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
yberc0001_35170 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
yre0001_20940 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
ymol l0001_34120 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
yruck0001_3230 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
yaldo0001_3370 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP
YpsIP31758_0605 138 SPFNASQVFQALPAF--NKQSFQLSYRSWKLILS-LAFFSCWLSFLSGLILFQEPGNFNP

Ye MNM-16TGYTYAQVAQVAVFQPDGEKFGG----64
Aa MNLGIYANTKAEASIAFQVEKAYQELAAYAAYVVFYNSNGPANLQQKFNLSLASTVASANVTP 75
β-galactosidase activity (Miller units)

Fig. 2