Functional analysis of yersiniabactin transport genes of *Yersinia enterocolitica*

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***Yersinia enterocolitica* O:8, biogroup (BG) 1B, strain WA-C carries a high-pathogenicity island (HPI) including iron-repressible genes (*irp1–9*, *fyuA*) for biosynthesis and uptake of the siderophore yersiniabactin (Ybt). The authors report the functional analysis of *irp6,7,8*, which show 98–99% similarity to the corresponding genes *ybtP,Q,X* on the HPI of *Yersinia pestis*. It was demonstrated that *irp6,7* are involved in ferric (Fe)-Ybt utilization and mouse virulence of *Y. enterocolitica*, thus confirming corresponding results for *Y. pestis*. Additionally it was shown that inactivation of the *ampG*-like gene *irp8* did not affect either Fe-Ybt utilization or mouse virulence. To determine whether *irp6*, *irp7* and *fyuA* (encoding the outer-membrane Fe-Ybt/pesticin receptor FyuA) are sufficient to mediate Fe-Ybt transport/utilization, these genes were transferred into *Escherichia coli* *entD,F* and into non-pathogenic *Y. enterocolitica*, BG 1A, strain NF-O. Surprisingly, *E. coli* *entD,F* but not *Y. enterocolitica* NF-O gained the capability to utilize exogenous Fe-Ybt as a result of this gene transfer, although both strains expressed functional FyuA (pesticin sensitivity). These results suggest that besides *irp6*, *irp7* and *fyuA*, additional genes are required for sufficient Fe-Ybt transport/utilization. Finally, it was shown that *irp6*, *irp7* and *fyuA* but not *irp8* are involved in controlling Ybt biosynthesis and *fyuA* gene expression: *irp6* and/or *irp7* mutation leads to upregulation whereas *fyuA* mutation leads to downregulation. However, *fyuA*-dependent control of Ybt biosynthesis could be bypassed in a *fyuA* mutant by ingredients of chrome azurol S (CAS) siderophore indicator agar.**

Keywords: high pathogenicity island, siderophore, iron-repressible protein, *Escherichia coli*

INTRODUCTION

The genus *Yersinia* can be divided into non-pathogenic yersiniae (*Yersinia enterocolitica* biogroup 1A) and two pathogroups with respect to mouse virulence (Brubaker, 1991; Perry & Fetherston, 1997): the high-pathogenicity (HP) group comprising *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Y. enterocolitica* biogroup (BG) 1B, and the low-pathogenicity (LP) group of *Y. enterocolitica* BGs 2–5. In contrast to the non-pathogenic group both pathogroups harbour the virulence plasmid

pYV. However, members of the HP group additionally carry the high-pathogenicity island (HPI) (Carniel *et al.*, 1996).

The HPI consists of a conserved core region and an AT-rich variable part (Rakin *et al.*, 1999a). The core part comprises genes for the biosynthesis of the siderophore yersiniabactin (Ybt), the uptake of ferric yersiniabactin (Fe-Ybt), the AraC-like regulator YbtA and the integrase of the HPI (Bearden *et al.*, 1997; Buchrieser *et al.*, 1999; Fetherston *et al.*, 1999; Pelludat *et al.*, 1998; Rakin *et al.*, 1999a, b). Core genes are negatively regulated by the Fe²⁺–Fur complex (Carniel *et al.*, 1992; Heesemann *et al.*, 1993; Staggs *et al.*, 1994) and positively by the HPI-encoded transcriptional activator YbtA and presumably Fe-Ybt (Fetherston *et al.*, 1996).

Two evolutionary lineages of HPI have been identified: the Yen HPI of *Y. enterocolitica* BG 1B, and the Yps HPI

Abbreviations: BG, biogroup; CAS, chrome azurol S; DFOB, desferrioxamine B; EDDHA, ethylenediamine-di(o-hydroxyphenylacetic acid); Fur, ferric uptake regulator; GFP, green fluorescent protein; HDTMA, *N*-cetyl-*N,N,N*-trimethylammonium bromide; HPI, high-pathogenicity island; TTC, 2,3,5-triphenyltetrazolium chloride; Ybt, yersiniabactin.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/phenotype	Reference or source
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80lacZΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	Hanahan (1983)
S17-1 λ pir	Tp ^r Sm ^r <i>recA thi pro hsdR</i> ⁻ M ⁺ [RP4-2-Tc::Mu:Kan ^r Tn7] λ pir	Miller & Mekalanos (1988); Simon <i>et al.</i> (1988)
SM10 λ pir MOSBlue	Km ^r <i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu λ pir <i>endA1 hsdR17</i> (r _{k12} ⁻ m _{k12} ⁺) <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proA</i> ⁺ B ⁺ <i>lacI</i> ^a Z Δ M15::Tn10(Tc ^r)]	Miller & Mekalanos (1988) Amersham
H1884	<i>entD,F</i>	K. Hantke
<i>Y. enterocolitica</i>		
NF-O	Clinical isolate, serotype O:5; biogroup 1A	Heesemann (1987)
WA-C	Plasmidless derivative of strain WA-314, serotype O:8, biogroup 1B; spontaneous Nal ^r mutant	Heesemann (1987)
WA-CS	Spontaneous Sm ^r mutant derived from WA-C, Nal ^r	Pelludat <i>et al.</i> (1998)
WA-CS <i>irp1</i> ::Kan ^r	<i>irp1</i> mutant of WA-CS, Nal ^r Sm ^r Kan ^r	Pelludat <i>et al.</i> (1998)
WA-C Δ <i>irp6</i>	<i>irp6</i> in-frame deletion mutant of WA-C, Nal ^r	This study
WA-C Δ <i>irp7</i>	<i>irp7</i> in-frame deletion mutant of WA-C, Nal ^r	This study
WA-CS <i>irp7</i> ::Kan ^r	<i>irp7</i> insertion mutant of WA-CS, Nal ^r Sm ^r Kan ^r	This study
WA-C Δ <i>irp6,irp7</i>	<i>irp6, irp7</i> in-frame deletion mutant of WA-C, Nal ^r	This study
WA-CS Δ <i>irp6,irp7</i>	Spontaneous Sm ^r mutant derived from WA-C Δ <i>irp6,irp7</i> , Nal ^r	This study
WA-CS Δ <i>irp6,irp7, irp1</i> ::Kan ^r	<i>irp1, irp6, irp7</i> mutant of WA-CS, Nal ^r Sm ^r Kan ^r	This study
WA-CS <i>irp8</i> ::Kan ^r	<i>irp8</i> mutant of WA-CS, Nal ^r Sm ^r Kan ^r	This study
WA-CS Δ <i>irp6,irp7, irp8</i> ::Kan ^r	<i>irp6, irp7, irp8</i> mutant of WA-CS, Nal ^r Sm ^r Kan ^r	This study
WA <i>fyuA2</i>	<i>fyuA</i> mutant of WA-CS, Nal ^r Sm ^r Kan ^r	This study
Plasmids		
pRK290B8-5::pO:8	Cointegrate	Heesemann & Laufs (1983)
pLAFR2	Tc ^r Mob ⁺	Friedmann <i>et al.</i> (1982)
12H2	pLAFR2 carrying a DNA insert of WA-C encompassing <i>irp6-irp9</i> operon, <i>ybtA</i> and <i>irp2</i> , Tc ^r	Rakin & Heesemann (1995)
pMOSBlue	Cloning vector, Ap ^r	Amersham
pMOS1.1	pMOSBlue containing <i>ybtA6507-S360</i> PCR fragment with a 465 bp deletion between the <i>NruI</i> sites, Ap ^r	This study
pMOS2.4	pMOSBlue containing <i>S368-ybtA355</i> PCR fragment, Ap ^r	This study
pMOS2.0	pMOSBlue containing <i>ybtA355-S368</i> PCR fragment with a 402 bp deletion between the <i>EcoRV</i> sites, Ap ^r	This study
pMOS1.2	pMOSBlue containing <i>S452-8354Eco</i> PCR fragment with a 2313 bp deletion between the <i>NruI</i> and <i>EcoRV</i> sites, Ap ^r	This study
pMOS <i>irp7</i> ::Kan ^r	pMOSBlue containing <i>S368-ybtA355</i> PCR fragment with a kanamycin cassette inserted into <i>NruI</i> site, Ap ^r Kan ^r	This study
pBAD18	Cloning vector, Ap ^r	Guzman <i>et al.</i> (1995)
pBAD <i>irp8</i> ::Kan ^r	pBAD18 containing <i>irp8Xba-irp8Hind</i> PCR fragment with a kanamycin cassette inserted into <i>SphI</i> site, Ap ^r Kan ^r	This study
pKNG101	Suicide vector with <i>sacBR</i> genes, Sm ^r	Kaniga <i>et al.</i> (1991)
pKNG1.1	pKNG101 containing <i>BamHI-XbaI</i> fragment of pMOS1.1, Sm ^r	This study
pKNG2.0	pKNG101 containing <i>BamHI-XbaI</i> fragment of pMOS2.0, Sm ^r	This study
pKNG1.2	pKNG101 containing <i>BamHI-XbaI</i> fragment of pMOS1.2, Sm ^r	This study
pKAS32	Suicide vector with <i>rpsL</i> gene, Ap ^r	Skorupski & Taylor (1996)
pKAS <i>irp7</i> ::Kan ^r	pKAS32 containing <i>XbaI-SacI</i> fragment of pMOS <i>irp7</i> ::Kan ^r , Ap ^r Kan ^r	This study

Table 1 (cont.)

Strain or plasmid	Genotype/phenotype	Reference or source
pKAS <i>irp8</i> ::Kan ^r	pKAS32 containing <i>irp8</i> Xba- <i>irp8</i> Bam PCR fragment of pBAD <i>irp8</i> ::Kan ^r , Ap ^r Kan ^r	This study
pKAS-E1Kan	pKAS 32 containing <i>Eco</i> RI– <i>Sal</i> I fragment of <i>irp1</i> with a kanamycin cassette (without transcriptional terminator) inserted into <i>Eco</i> RV site, Ap ^r Kan ^r	Pelludat <i>et al.</i> (1998)
pSVfyuA	pKAS32 containing 3.3 kb <i>Eco</i> RI fragment with a kanamycin cassette inserted into <i>Sph</i> I site of <i>fyuA</i> , Ap ^r Kan ^r	This study
pSB315	Containing kanamycin cassette without transcriptional terminator, Ap ^r Kan ^r	Galan <i>et al.</i> (1992)
pT7-5	Cloning vector, Ap ^r	Tabor & Richardson (1985)
pHM6	pT7-5 with <i>fyuA</i> , Ap ^r	Rakin <i>et al.</i> (1994)
pACYC184	Cloning vector, Cm ^r Tet ^r	Chang & Cohen (1978)
pCJFY5G3	<i>fyuA5</i> – <i>gfp3</i> fragment (153 bp of the <i>fyuA</i> promoter region and 5 codons of <i>fyuA</i> fused to <i>gfp3</i>) cloned into pACYC184	Jacobi (1999)
pACYC5.1	5.1 kb <i>Sph</i> I fragment of 12H2 encompassing <i>irp6</i> and <i>irp7</i> cloned into pACYC184, Cm ^r	This study
pACYC5.2	2.6 kb <i>Xba</i> I– <i>Hind</i> III fragment of pHM6 encompassing <i>fyuA</i> cloned into pACYC5.1, Cm ^r	This study
pHM10	pACYC184 with <i>fyuA</i> , Cm ^r	Rakin <i>et al.</i> (1994)
pGEX-4T-3	Ap ^r , <i>gst</i>	Pharmacia
pGEX <i>irp6</i>	pGEX-4T-3 containing 8301Bam-6515Sma PCR product (<i>irp6</i>), Ap ^r	This study
pGEX <i>irp7</i>	pGEX-4T-3 containing 6509Bam-4727Sma PCR product (<i>irp7</i>), Ap ^r	This study

of *Y. pestis* and *Y. pseudotuberculosis*. These HPIs have high homology within the core gene sequences but unrelated AT-rich parts (Rakin *et al.*, 1995, 1999a). In Yps HPI the four core genes *ybtP*, *ybtQ*, *ybtX* and *ybtS* constitute a polycistronic operon. It has been demonstrated that YbtP and YbtQ are involved in Fe-Ybt uptake and supposedly form a heterodimeric ABC transporter (Fetherston *et al.*, 1999). YbtS participates in Ybt biosynthesis. YbtX shows homology to AmpG, which is proposed to act as a signal transducer or permease in the β -lactamase induction system (Lindquist *et al.*, 1993). The function of YbtX and its role in virulence are still unknown (Fetherston *et al.*, 1999; Gehring *et al.*, 1998). A similar operon (*irp6*, *irp7*, *irp8* and *irp9*) has been described for *Y. enterocolitica* with 98–99% homology to the corresponding sequences of *Y. pestis* (Rakin *et al.*, 1999a).

In principle, siderophore utilization by Gram-negative bacteria requires genes encoding (i) a TonB-dependent uptake system for transport through the outer membrane including a high-affinity siderophore-specific receptor, (ii) a periplasmic-binding-protein-dependent transport (PBT) system including a periplasmic binding protein and an ABC transporter for transfer from periplasm to cytoplasm, and (iii) proteins capable of releasing siderophore-bound iron (e.g. siderophore reductase/esterase) (Earhart, 1996; Boos & Lucht, 1996; Schubert *et al.*, 1999; Braun *et al.*, 1998). However, no gene encoding a periplasmic binding protein or a

protein releasing iron from Fe-Ybt has been detected so far within the HPI, suggesting that such genes might be located outside the HPI or are not required.

In spite of the high degree of similarity between Yen HPI and Yps HPI, significant HPI-associated differences between *Y. pestis* and *Y. enterocolitica* have been reported. (i) On CAS siderophore indicator agar *Y. enterocolitica* produces haloes whereas *Y. pestis* does not (Rakin *et al.*, 1999a). (ii) *Y. pestis* requires the functional Ybt system for mouse virulence when mice are challenged subcutaneously but can go without HPI when the bacteria are injected intravenously (Une & Brubaker, 1984; Bearden *et al.*, 1997; Fetherston *et al.*, 1999). In contrast, a *fyuA* mutant of *Y. enterocolitica* O:8 and an *irp2* mutant of *Y. pseudotuberculosis* (enteropathogenic *Yersinia* species) are highly attenuated for mice when the animals are challenged intravenously or orally (Carniel *et al.*, 1992; Rakin *et al.*, 1994). (iii) The promoter region of *irp6*–9 and *ybtA* of Yen HPI but not of Yps HPI carries an ERIC insertion which might have an influence on the regulation of these genes (Rakin *et al.*, 1999a). These HPI-associated differences between *Y. pestis* and *Y. enterocolitica*, the unusual Fe-Ybt transport system composed of FyuA, Irp6 and Irp7, and the unknown function of Irp8 prompted us to characterize *irp6*, *irp7*, *irp8* and *fyuA* of *Y. enterocolitica* O:8 strain WA-C with respect to mouse virulence, Fe-Ybt transport/utilization, Ybt production and Ybt-induced upregulation of *fyuA*.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the study are listed in Table 1. Strains were grown in Luria–Bertani (LB) broth or on LB agar plates (Difco) at 28 °C (*Y. enterocolitica*) or 37 °C (*Escherichia coli*). For iron-deficient growth, strains were cultivated in NBD liquid medium [nutrient broth (Difco) supplemented with 5 g NaCl l⁻¹ (NB) and 200 µM α,α' -dipyridyl (Sigma)]. Blood agar plates were used for bacterial conjugation. Antibiotics were added to the following concentrations: nalidixic acid and streptomycin, 100 µg ml⁻¹; kanamycin, 40 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹; ampicillin, 100 µg ml⁻¹ for *E. coli* and 500 µg ml⁻¹ for *Y. enterocolitica*.

Spontaneous streptomycin-resistant (Sm^r) colonies of WA-C Δ irp6,irp7 (Table 1) were selected by stepwise increments of streptomycin (10, 30, 50, 70 and 100 µg ml⁻¹) in LB medium. The resulting mutant was designated WA-CS Δ irp6,irp7.

DNA techniques. Plasmids were isolated by the alkaline lysis method and introduced into *E. coli* and *Y. enterocolitica* by electroporation (Gene Pulse Apparatus, Bio-Rad) according to the manufacturer's instructions. PCR was performed in an automated thermal cycler (Gene Amp PCR System 2400; Perkin-Elmer) as described previously (Rakin *et al.*, 1999a) with the *TaqI* polymerase and different pairs of oligonucleotides (Metabion) (Table 2). The initial denaturation step (94 °C, 7 min) was followed by 25 cycles of denaturation (94 °C), annealing and extension (72 °C), with one final extension step (72 °C, 7 min). Annealing temperatures were set according to the primers used.

Construction of mutants. Suicide vector pKNG101 (Table 1) was used for the construction of deletion mutants by reverse genetics. pKNG101 contains an R6K origin of replication that requires the *pir* gene product, *strAB* encoding streptomycin phosphotransferase and the *sacAB* genes from *Bacillus subtilis*, which render bacterial strains sensitive to sucrose. Integration of the suicide vector results in a streptomycin-resistant and sucrose-sensitive phenotype. Sucrose resistance (Suc^r) and streptomycin sensitivity (Sm^s) arise after an allelic exchange with concomitant loss of the vector.

To construct the *irp6* in-frame deletion mutant WA-C Δ irp6, the ybtA6507-S360 (Table 2) PCR product was cloned into the blunt-ended pMOSBlue vector and the 465 bp *NruI* fragment

was subsequently deleted (pMOS1.1). The *BamHI*–*XbaI* fragment from pMOS1.1 containing the in-frame deletion was ligated into the pKNG101 suicide vector to yield pKNG1.1 (Fig. 1).

To generate WA-C Δ irp7, the PCR product amplified with primers S368 and ybtA355 was ligated into pMOSBlue, resulting in pMOS2.4. The 402 bp *EcoRV* fragment was excised from pMOS2.4, generating pMOS2.0. The *BamHI*–*XbaI* fragment of pMOS2.0 was transferred into pKNG101, resulting in pKNG2.0.

To create WA-C Δ irp6,irp7, the PCR product S452–8354Eco was ligated into pMOSBlue and the 2313 bp *EcoRV*–*NruI* fragment was deleted, generating pMOS1.2. The 1.2 kb *BamHI*–*XbaI* fragment from pMOS1.2 was cloned into pKNG101, resulting in pKNG1.2 (Fig. 1).

The constructs (pKNG1.1, pKNG2.0 and pKNG1.2) were transformed into *E. coli* SM10 λ pir followed by mobilization into WA-C by conjugation.

Cells were selected on agar plates containing nalidixic acid (100 µg ml⁻¹) and streptomycin (100 µg ml⁻¹). Sm^r clones were streaked onto LB plates with 5% sucrose. Sm^s Suc^r colonies were analysed by PCR and Southern blot hybridization to identify strains containing the deletions.

pKAS32 (Table 1) contains the R6K origin of replication and the *rpsL* gene, which encodes the S12 protein of the ribosomes conferring streptomycin sensitivity (dominant allele). Insertion of the suicide vector into the chromosome results in a Sm^s dominant phenotype of a former Sm^r strain. Kan^r Sm^r derivatives arise after an allelic exchange and loss of the vector.

To generate WA-CS *irp7*::Kan^r, a *HincII*-cut kanamycin cassette without transcriptional terminator was ligated into *NruI*-digested pMOS2.4, resulting in pMOS *irp7*::Kan^r. The insert containing the kanamycin cassette was excised with *XbaI*/*SacI* and inserted into the suicide vector pKAS32 (designated pKAS *irp7*::Kan^r) (Fig. 1). The construct was transformed into *E. coli* S17-1 λ pir followed by mobilization into WA-CS.

To create WA-CS *irp8*::Kan^r, a PCR product (amplified with the primers irp8Xba and irp8Hind) containing the whole gene was cut with *XbaI* and *HindIII* and ligated into pBAD18. A

Table 2. Primers used (restriction sites underlined)

Primer	Sequence 5'–3'	Comments
ybtA6507	TCCGCAGGATTATTGTCTTTC	End of <i>irp6</i> , reverse
S360	CATTACGCGGCTTTTCATCTG	In <i>irp6</i> , forward
S368	AATCCCCCTTCTCGCCCAAAC	In <i>irp8</i> , reverse
ybtA355	CTGACCCGGAACGAAGC	In <i>irp6</i> , forward
S452	CCCCGGCGATGGTGGATAAAC	In <i>irp7</i> , reverse
8354Eco	TTTGAATTCCTGAATTGTCGCTTTTGGTG	Start of <i>irp6</i> with <i>EcoRI</i> restriction site, forward
irp8Xba	GCATCTAGACGTTATCAGGCGCTGTGGCAG	Start of <i>irp8</i> with <i>XbaI</i> restriction site, forward
irp8Hind	CGCAAGCTTTCTGTGTTATTCCCGGATCA	End of <i>irp8</i> with <i>HindIII</i> restriction site, reverse
irp8Bam	AGCGGATCCTCTGTTTATTCCCGGATCA	End of <i>irp8</i> with <i>BamHI</i> restriction site, reverse
8301Bam	GTTGGATCCATGTAATCTCAATCATC	Start of <i>irp6</i> with <i>BamHI</i> restriction site, forward
6515Sma	ATCCCCGGGATTATTGTCTTTTCATTTT	End of <i>irp6</i> with <i>SmaI</i> restriction site, reverse
4727Sma	TTTCCCCGGGTCACTCATGCACCCACTC	End of <i>irp7</i> with <i>SmaI</i> restriction site, reverse
6509Bam	TAAGGATCCATGAAAGACAATAATCCTGCG	Start of <i>irp7</i> with <i>BamHI</i> restriction site, forward
ybtA6451	CCTGACTGCCAACGCTGGAGA	In <i>irp7</i> , reverse

Fig. 1. (a) Restriction sites in *irp6*, *irp7*, *irp8*, *irp9* and *ybtA* used for genetic manipulations. Arrows indicate the directions of transcription. (b) Black arrows show primers used for amplification. (c) Suicide vectors used for construction of mutants. Dotted lines represent deletions, triangles sites of kanamycin cassette insertion. (d) Recombinant plasmids used for complementation.

HincII-cut kanamycin cassette lacking a transcriptional terminator was inserted into the *SphI* site (filled-in) of *irp8* (pBAD *irp8*::Kan^r). *irp8* containing the kanamycin cassette was amplified with *irp8Xba* and *irp8Bam*, cut with *XbaI* and *BamHI* and ligated into pKAS32, resulting in pKAS *irp8*::Kan^r. The construct was transformed into *E. coli* S17-1 λ pir followed by mobilization into WA-CS and WA-CS Δ *irp6,irp7*, generating WA-CS *irp8*::Kan^r and the triple mutant WA-CS Δ *irp6,irp7,irp8*::Kan^r.

To generate an *irp6,irp7,irp1* triple mutant suicide vector pKAS-E1Kan was mobilized into double mutant WA-CS Δ *irp6,irp7* (Table 1).

To create mutant WA *fyuA2* a 3.3 kb *EcoRI* fragment including 1.6 kb of *fyuA* was inserted into vector pKS. A *HincII*-cut kanamycin cassette lacking a transcriptional terminator was ligated into the filled-in *SphI* site of *fyuA*. The *EcoRI* fragment including the kanamycin cassette was ligated into suicide vector pKAS32. The construct was transformed into *E. coli* S17-1 λ pir followed by mobilization into WA-CS.

Mutants were selected on agar plates containing kanamycin (40 μ g ml⁻¹), streptomycin (100 μ g ml⁻¹) and nalidixic acid (100 μ g ml⁻¹). Correct insertions of the kanamycin cassettes were confirmed by Southern blot hybridization.

Construction of plasmids for complementation. The 5.1 kb *SphI* fragment of plasmid 12H2 (Table 1) carrying *irp6* and *irp7* and the promoter of the *irp6-irp9* operon was subcloned into pACYC184, resulting in pACYC5.1 (Fig. 1). pACYC5.2 additionally contains a 2.6 kb *XbaI-HindIII* fragment of pHM6 harbouring the *fyuA* receptor gene (Table 1).

irp6 was amplified by PCR using primers 6515Sma and 8301Bam (Table 2) and ligated into pGEX-4T-3 downstream of *gst* (the glutathione *S*-transferase gene) to yield pGEX*irp6* (Fig. 1). The 4727Sma-6509Bam PCR product (*irp7*) was cloned into pGEX-4T-3 downstream of *gst*, resulting in pGEX*irp7*.

Screening for production of iron-chelating compounds.

Strains to be tested were dropped (1 μ l of an overnight liquid culture) on chrome azurol S (CAS) siderophore indicator agar (Heesemann, 1987; Schwyn & Neilands, 1987) and incubated for 2 d at 28 °C. A clearly visible red-orange halo around the bacteria was indicative of siderophore production (CAS-positive phenotype).

Feeding assay with purified yersiniabactin, desferrioxamine B and culture supernatants.

Culture supernatants were obtained from yersiniae grown for 48 h at 28 °C in iron-poor NBD medium. The cells were pelleted by centrifugation and the supernatants were sterilized by filtration and used for feeding experiments. Desferrioxamine B (DFOB) (Desferal) was obtained from Ciba Geigy. Purified yersiniabactin was kindly provided by R. Reissbrodt (Wernigerode, Germany). For testing of siderophore utilization, bacteria were seeded in CDM-H agar (Flossmann *et al.*, 1985) rendered iron-deficient by addition of the chelator EDDHA. Filter papers soaked with siderophore-containing culture supernatant, Ybt or DFOB were placed on the agar layer. Bacteria were cultivated at 37 °C (*E. coli*) or 28 °C (*Y. enterocolitica*) for 16 h and colonies were visualized by using 2,3,5-triphenyltetrazolium chloride (TTC).

Pesticin assay.

The pesticin-producing strain *Y. pestis* EV76 was grown overnight at 28 °C and pesticin production was induced by adding mitomycin C (0.3 μ g ml⁻¹) and incubating for an additional 16 h. Cells were collected by centrifugation and the supernatant was used as a crude pesticin preparation after sterilization with 0.1% chloroform (Hu *et al.*, 1972).

The pesticin assay was performed as described by Heesemann *et al.* (1993). CDM-H plates without addition of EDDHA were overlaid with 0.6% agar containing 10⁷ bacteria from mid-exponential culture (double-layer technique). Ten microlitres of pesticin-containing culture supernatant was dropped on these plates followed by incubation at 37 °C for 16 h. Lysis zones were visualized by staining with TTC.

SDS-PAGE and immunoblotting. Bacteria were grown overnight in iron-limited CDM-H liquid medium, centrifuged, washed, and solubilized by boiling in Laemmli buffer (total cell lysate) (Laemmli, 1970). Equal amounts of all strains were separated on a 10% SDS-polyacrylamide gel (SDS-PAGE) and for immunoblotting, transferred electrophoretically to nitrocellulose membranes (BA85; Schleicher & Schüll) (Heesemann *et al.*, 1986). For immunostaining, a rabbit anti-FyuA antiserum (Heesemann *et al.*, 1993) (1:1000) and a peroxidase-conjugated secondary anti-rabbit antibody (1:5000) were used for chemiluminescence detection (ECL kit from Amersham).

RNA isolation and RT-PCR. Strains were grown in CDM-H liquid medium to an OD₆₀₀ of 0.5. Total cellular RNA was isolated as recommended by the manufacturer (High Pure RNA Isolation Kit; Boehringer Mannheim). DNA was digested by incubation with 2 U RNase-free DNase (Promega) for 30 min at 37 °C. DNase was heat-inactivated at 75 °C for 10 min. RT-PCR was performed with a Reverse-¹T 1st strand synthesis kit (Advanced Biotechnologies) according to the manufacturer's instructions. Products were analysed by loading 10 µl of the PCR mixture onto a 1.5% agarose gel.

GFP reporter gene studies. The plasmid pCJFY5G3 (Jacobi, 1999) containing 153 bp of the *fyuA* promoter region (Rakin *et al.*, 1994) and five codons of *fyuA* fused to *gfp3* (encoding green fluorescent protein; Jacobi *et al.*, 1998; Schubert *et al.*, 2000) was transferred into WA-C, WA-C $\Delta irp6, irp7$, WA-CS *irp8::Kan^r*, WA-CS $\Delta irp6, irp7, irp8::Kan^r, WA *fyuA2* and WA-CS *irp1::Kan^r* by electroporation (Table 1). Cells were grown in NB or NBD liquid medium or on CAS agar, and fluorescence was measured after 24 h.$

Flow-cytometric measurements were performed with a Coulter Epics Flow cytometer equipped with an argon 488 nm laser. Bacteria were diluted as required and detected by side scatter as described previously (Pelludat *et al.*, 1998). The scale was logarithmic, and fluorescence and scatter data were collected for 50000 bacteria.

Virulence testing. For testing mouse-virulence the mobilizable cointegrate pRK290B8-5::pO:8 (Heesemann & Laufs, 1983), containing the virulence plasmid (pYVO:8) of *Y. enterocolitica* O:8 was introduced into WA-C and derivatives of WA-C. The exconjugants were mouse-passaged, resulting in cointegrate dissociation and selection for pYVO:8-carrying strains. BALB/c mice were challenged intravenously with 5×10^6 c.f.u. of mouse-passaged WA-C(pYVO:8), WA-C $\Delta irp6, irp7$ (pYVO:8), WA-CS(pYVO:8) and WA-CS *irp8::Kan^r*(pYVO:8). On day 2 post-infection, mice were killed. Spleen and liver were removed and homogenized in

1 ml and 5 ml, respectively, of sterile PBS containing 0.5% Tergitol and 0.5% bovine serum albumin by the use of tissue homogenizers. Homogenates were plated on LB agar and the number of bacteria in spleen and liver was determined.

RESULTS

Role of *irp6*, *irp7* and *irp8* in Fe-Ybt utilization in *Y. enterocolitica* O:8

To investigate the function of *irp6*, *irp7* and *irp8* in *Y. enterocolitica* O:8, different mutants were constructed (Table 1, Fig. 1).

Contribution of *irp6*, *irp7* and *irp8* to Fe-Ybt uptake was shown by seeding the different mutants into iron-limited CDM-H agar and feeding from Fe-Ybt containing filter disks. Fe-Ybt supported the growth of WA-C, WA-CS and WA-CS *irp8::Kan^r* but not of the *irp6* and *irp7* single (WA-C $\Delta irp6$, WA-C $\Delta irp7$, WA-CS *irp7::Kan^r*), *irp6, irp7* double (WA-C $\Delta irp6, irp7$), *irp6, irp7, irp8* triple (WA-CS $\Delta irp6, irp7, irp8::Kan^r$) mutants or the *fyuA* (WA *fyuA2*) mutant (Fig. 2a). In contrast, filter disks with DFOB promoted growth of all strains in CDM-H agar, indicating functional TonB-dependent ferric hydroxamate transport (control test).

The *irp6* and *irp7* mutants were successfully complemented in Fe-Ybt-supported growth by plasmids pGEX*irp6* (for *irp6* mutant) and pGEX*irp7* (for *irp7* mutants) even without addition of IPTG (probably due to incomplete repression). Cross-complementation of the *irp7* mutant by the functional *irp6* gene and vice

Table 3. Numbers of c.f.u. in spleen and liver of infected mice

Strain with pYVO:8 plasmid	Spleen	Liver
WA-C	2.7×10^7	4.5×10^6
WA-C $\Delta irp6, irp7$	2.7×10^2	1.3×10^3
WA-CS	1.1×10^8	4.1×10^6
WA-CS <i>irp8::Kan^r</i>	3.3×10^7	7.5×10^7

Fig. 2. Feeding assay for strains seeded in CDM-H agar with Ybt or DFOB. (a) *Y. enterocolitica* O:8 strains WA-C and WA-C $\Delta irp6, irp7$. (b) *E. coli entD,F* strains H1884 and H1884(pACYC5.2). (c) *Y. enterocolitica* O:5, NF-O and NF-O(pACYC5.2).

Fig. 3. Detection of FyuA: (a) SDS-PAGE (10%) and (b) corresponding immunoblot using FyuA antibody. Cell lysates of *Y. enterocolitica* WA-C (lane 1), NF-O (lane 2), NF-O(pACYC5.2) (lane 3), *E. coli entD,F* strain H1884 (lane 4), H1884(pACYC5.2) (lane 5). Arrow indicates FyuA protein.

versa was inefficient. The *fyuA* mutant was complemented by introduction of plasmid pHM10 carrying *fyuA*.

These results clearly show that *fyuA*, *irp6* and *irp7* but not *irp8* are involved in Fe-Ybt uptake/utilization of *Y. enterocolitica* O:8.

Influence of *irp6*, *irp7*, and *irp8* on mouse virulence

To investigate the influence of *irp6*, *irp7* and *irp8* on mouse virulence three BALB/c mice were injected intravenously with 5×10^6 c.f.u. of WA-C(pYVO:8), WA-C $\Delta irp6, irp7$ (pYVO:8), WA-CS(pYVO:8) and WA-CS *irp8*::Kan^r(pYVO:8), respectively. Two days post-infection mice were killed and bacteria in spleen and liver were counted. The number of c.f.u. from the spleens of mice infected with WA-C(pYVO:8) was 2.7×10^7 per spleen, 10^5 -fold higher than in spleens of mice infected with the *irp6, irp7* double mutant (2.7×10^2). The difference in c.f.u. per liver between parental and mutant strain was 10^3 (Table 3). Three additional mice infected with WA-C $\Delta irp6, irp7$ (pYVO:8), were killed 10 d post-infection and no bacteria could be detected in spleen and liver. In contrast, disruption of *irp8* did not decrease virulence with respect to the parental strain WA-CS(pYVO:8), as demonstrated by similar c.f.u. counts in spleen and liver (Table 3). These results indicate that the ABC transporter genes *irp6* and *irp7* but not the *ampG*-like *irp8* are required for mouse virulence in *Y. enterocolitica* O:8.

irp6, *irp7* and *fyuA* enable *E. coli* but not non-pathogenic *Y. enterocolitica* BG 1A to utilize ferric yersiniabactin

We considered whether *irp6*, *irp7* and *fyuA* may be sufficient for Fe-Ybt uptake and utilization in an *E. coli entD,F* mutant (strain H1884 deficient in enterochelin synthesis and HPI-negative). The recombinant plasmids pACYC5.1 (carrying *irp6* and *irp7*) and pACYC5.2 (carrying *irp6*, *irp7* and *fyuA*) were able to restore Fe-Ybt-supported growth of WA-C $\Delta irp6, irp7$ and WA *fyuA2*, respectively. Plasmids carrying different combinations of transport genes were introduced into H1884 and the strains were analysed for Fe-Ybt-mediated growth support. H1884 carrying plasmids pHM10 (*fyuA*) or pACYC5.1 (*irp6* and *irp7*) were not supported in growth by Fe-Ybt-soaked filters. In contrast H1884 harbouring pACYC5.2 (carrying *irp6*, *irp7* and *fyuA*) grew significantly around the filter disk, indicating Fe-Ybt-supported growth (Fig. 2b). Obviously both ABC transporters (Irp6 and Irp7) and the outer membrane

Ybt receptor (FyuA) are sufficient for Fe-Ybt uptake and utilization in *E. coli*. All tested *E. coli* transformants showed small growth zones around DFOB-soaked filters due to low-level DFOB transport mediated by FhuE, the outer-membrane receptor protein for coprogen (Sauer *et al.*, 1990).

After introduction of pACYC5.2 (*irp6*, *irp7*, *fyuA*) into the non-pathogenic *Y. enterocolitica* O:5, BG 1A (strain NF-O), Fe-Ybt-supported growth was investigated. In contrast to WA-C and H1884(pACYC5.2), NF-O(pACYC5.2) showed no significant growth around Fe-Ybt-soaked filters when compared to the parent NF-O strain (Fig. 2c). Addition of DFOB resulted in large growth zones of NF-O and NF-O(pACYC5.2), indicating functional TonB transport (Bäumler & Hantke, 1992).

To elucidate whether these Fe-Ybt feeding differences were due to different *fyuA* expression we investigated FyuA production of *Y. enterocolitica* NF-O(pACYC5.2) and *E. coli* H1884(pACYC5.2). Equal amounts of solubilized cells were analysed by immunoblotting using anti-FyuA antiserum. WA-C was used as positive control, NF-O and H1884 as negative controls. The immunoblot revealed that NF-O(pACYC5.2) and H1884(pACYC5.2) produced FyuA in equal amounts but less than WA-C (Fig. 3). Similar results were obtained with outer membrane preparations.

As FyuA is also the TonB-dependent receptor for pesticin, functionality of FyuA was investigated with respect to pesticin sensitivity. Whereas H1884 and NF-O were pesticin-resistant, WA-C, H1884(pACYC5.2) and NF-O(pACYC5.2) showed lysis zones after pesticin addition, demonstrating functional FyuA in all three strains (results not shown).

To compare *irp6* and *irp7* transcripts, RNAs of WA-C, NF-O, NF-O(pACYC5.2), H1884 and H1884(pACYC5.2) grown in CDM-H liquid medium were isolated and RT-PCRs were performed. A primer pair (ybtA6451-ybtA355) that amplifies 350 bp (including the end of *irp6* and the start of *irp7*) was chosen for PCR. RNA of NF-O(pACYC5.2), H1884(pACYC5.2) and the positive control WA-C revealed a RT-PCR product of 350 bp, whereas RT-PCR with RNA of NF-O and H1884 turned out to be negative (Fig. 4). *irp6* and *irp7* are more weakly transcribed in NF-O(pACYC5.2) and H1884(pACYC5.2) than in WA-C, which might be due to the missing transcriptional activator YbtA in the former two strains.

In conclusion, these data demonstrate that (i) in contrast to *E. coli*, expression of *irp6*, *irp7* and *fyuA* is not

Fig. 4. Detection of *irp6*, *irp7* transcription. RT-PCR was performed with primers (ybtA6451-ybtA355) that amplify a 350 bp fragment overlapping *irp6* and *irp7*. Lanes: DNA size markers (lane 1), WA-C (lanes 2 and 3), NF-O (lanes 4 and 5), NF-O(pACYC5.2) (lanes 6 and 7), H1884 (lanes 8 and 9) and H1884(pACYC5.2) (lanes 10 and 11). The RT-PCR reaction applied to lanes 2, 4, 6, 8, and 10 contained reverse transcriptase (RT), whereas reactions in lanes 3, 5, 7, 9, and 11 lacked RT and served as negative controls.

sufficient for Fe-Ybt utilization in the non-pathogenic *Y. enterocolitica* O:5, BG 1A strain; and (ii) YbtA is not absolutely required for functional expression of *irp6*, *irp7* and *fyuA* in the plasmid construct pACYC5.2.

Influence of *irp6*, *irp7*, *irp8* and *fyuA* on regulation of Ybt production and *fyuA* expression

Recently, it has been suggested that the transcriptional activator YbtA and Fe-Ybt or a YbtA/Fe-Ybt complex might be involved in upregulation of the Ybt biosynthesis operon *irp1*–*5* and the Fe-Ybt receptor gene *fyuA* (both promoters carry repeated sequences which could serve as YbtA binding sites: Fetherston *et al.*, 1996; Rakin *et al.*, 1994). According to this model the Fe-Ybt transporter system *Irp6*, *irp7* and *FyuA* mediates the Fe-Ybt influx and thus indirectly controls the Ybt biosynthesis through YbtA and Fe-Ybt (see Fig. 5). Moreover, it is also conceivable that *Irp8*, which has

Fig. 5. Hypothetical model of ferric-yersiniabactin (Fe-Ybt) uptake and regulation of HPI core genes. OM, outer membrane; IM, inner membrane; +, transcriptional activation; –, transcriptional repression; $\diamond$, YbtA-Fe-Ybt complex.

Table 4. Yersiniabactin production of *Y. enterocolitica* WA-C and mutants

Strain	Feeding of WA-CS <i>irp1</i> ::Kan ^r by culture supernatant	<i>fyuA</i> – <i>gfp</i> upregulation in NBD medium*	CAS phenotype†
WA-C	+	+	+
WA-C Δ <i>irp6</i> , <i>irp7</i>	+	++	++
WA-C Δ <i>irp6</i>	+	++	++
WA-C Δ <i>irp7</i>	+	++	++
WA-CS <i>irp7</i> ::Kan ^r	+	++	++
WA-CS Δ <i>irp6</i> , <i>irp7</i> , <i>irp8</i> ::Kan ^r	+	++	++
WA-CS <i>irp8</i> ::Kan ^r	+	+	+
WA-CS Δ <i>irp6</i> , <i>irp7</i> , <i>irp1</i> ::Kan ^r	–	–	–
WA <i>fyuA2</i>	–	–	++

* See also Fig. 7 for GFP fluorescence intensity.

† See also Fig. 8 for halo diameter.

Fig. 6. Fe-Ybt/*fyuA*-*gfp* reporter assay: GFP fluorescence (arbitrary units) of *Y. enterocolitica* WA-CS *irp1*::Kan^r (pCJFY5G3) (reporter strain) grown in NBD medium supplemented with culture supernatants of WA-C (▲) and WA *fyuA2* (■). Culture supernatants were diluted (10⁻¹–10⁻⁶) with fresh NBD prior to cultivation of the reporter strain. GFP fluorescence was measured after 24 h growth. Graphs represent the mean of three measurements. Standard deviations are indicated by error bars.

sequence similarities to the permease AmpG, may be involved in regulation of Ybt biosynthesis.

As a first approach we investigated Ybt production of WA-C and of transport mutants by testing NBD culture supernatants of the corresponding strains for growth support of the indicator strain WA-CS *irp1*::Kan^r (disrupted biosynthesis of Ybt) seeded in CDM-H agar. As shown in Table 4, NBD culture supernatants of WA-C, WA-C Δ *irp6,irp7*, WA-C Δ *irp6*, WA-C Δ *irp7*, WA-CS *irp7*::Kan^r, WA-CS *irp8*::Kan^r and WA-CS Δ *irp6,irp7,irp8*::Kan^r grown in iron-deficient NBD medium were able to feed the indicator strain, whereas the supernatant of WA-CS Δ *irp6,irp7,irp1*::Kan^r did not support the growth as expected. Surprisingly, the NBD supernatant of WA *fyuA2* was also not able to feed significantly WA-CS *irp1*::Kan^r.

Different Fe-Ybt concentrations of WA-C and WA *fyuA2* supernatants could be determined using the Fe-Ybt-sensitive *fyuA*-*gfp* reporter strain WA-CS *irp1*::Kan^r(pCJFY5G3). GFP fluorescence intensity of the reporter strain is induced in a Fe-Ybt concentration-dependent manner. Comparison of the fluorescence intensity/supernatant dilution curves showed that WA-C supernatant contained approximately 100-fold more activity in *fyuA*-*gfp* induction than supernatant of strain WA *fyuA2* (Fig. 6). These different activities can be attributed to the different Fe-Ybt concentration in culture supernatants.

In a second approach, auto-induction of *fyuA*-*gfp* expression was tested for WA-C and the various transporter mutants harbouring pCJFY5G3 after growth in NB and NBD medium, respectively. NB-grown strains showed low background fluorescence whereas growth in NBD medium (ferrous iron

Fig. 7. GFP fluorescence (arbitrary units) of pCJFY5G3-carrying *Y. enterocolitica* strains: WA-CS; WA-CS *irp8*::Kan^r; WA-C Δ *irp6,irp7*; WA-CS Δ *irp6,irp7,irp8*::Kan^r; WA *fyuA2*; and WA-CS *irp1*::Kan^r. Solid bars represent GFP fluorescence of the strains in NB, open bars fluorescence in NBD medium. Standard deviations are indicated by error bars.

Fig. 8. CAS siderophore indicator agar plate showing halo-producing *Y. enterocolitica* WA-C, WA-CS *irp8*::Kan^r, WA *fyuA2* and WA-C Δ *irp6,irp7*.

derepression) resulted in bright fluorescing bacteria for WA-C, *irp6,irp7* and *irp8* mutants but not for *fyuA* and *irp1* mutants (Fig. 7, Table 4). The *irp6,irp7* mutant showed twofold higher induction of *fyuA*-*gfp* in comparison to the parental strain WA-C. *irp8* mutation did not contribute to *fyuA*-*gfp* expression.

Taken together the cross-feeding results (Ybt production) are in line with the *fyuA*-reporter results in so far as *irp6/irp7* mutations lead to upregulation whereas *fyuA* mutation leads to downregulation of Ybt production and *fyuA*-*gfp* expression. Thus, the outer-membrane receptor FyuA appears to be essential for upregulation of Ybt biosynthesis and *fyuA* expression under these growth conditions.

In a third set of experiments Ybt production of WA-C and mutants was checked by using CAS-siderophore indicator agar (Fig. 8, Table 4). All strains producing Ybt in NBD were also able to produce orange haloes around colonies grown on CAS agar. Interestingly, the halo diameter with respect to colony size of *irp6/irp7* mutants was larger (indicating hyperproduction of Ybt) than those of the parental strain WA-C or *irp8* mutant.

Fig. 9. GFP fluorescence (arbitrary units) of pCJFY5G3-carrying *Y. enterocolitica* strains: WA-CS; WA-CS *irp8::Kan^r*; WA-C Δ *irp6,irp7*; WA-CS Δ *irp6,irp7, irp8::Kan^r*; WA *fyuA2*; and WA-CS *irp1::Kan^r*. All strains were grown on CAS siderophore indicator agar for 24 h. Standard deviations are indicated by error bars.

Table 5. GFP fluorescence intensity of WA *fyuA2* (pCJFY5G3) grown for 24 h in NBD medium plus various supplements

Supplement*	GFP fluorescence (arbitrary units)†
None	5.5 (1.4)
HDTMA, CAS, FeCl ₃	15.1 (3.8)
CAS, FeCl ₃	9.4 (1.6)
CAS	5.5 (0.7)
Ferric citrate	9.7 (0.8)

* HDTMA, 200 μ M; CAS, 60.5 mg l⁻¹; FeCl₃, 10 μ M; ferric citrate, 10 μ M.

† Standard deviations in parentheses.

Strain WA-CS *irp1::Kan^r* was CAS negative as expected. However, the *fyuA* mutant, which was negative in cross-feeding (culture supernatant), also developed a large halo (CAS positive). This unexpected phenotype of WA *fyuA2* prompted us to check also *fyuA-gfp* upregulation of the strains grown on CAS agar. WA-C and transport mutants including *fyuA* mutant showed upregulation of the *fyuA-gfp* reporter (Fig. 9). Obviously CAS agar contains substances which are able to upregulate *fyuA-gfp* in WA *fyuA2* (pCJFY5G3). For this reason WA *fyuA2*(pCJFY5G3) was incubated for 24 h in NBD medium supplemented with components of CAS agar such as HDTMA-CAS-FeCl₃, CAS-FeCl₃ or CAS and additionally with ferric citrate. As shown in Table 5 the supplement consisting of the cationic detergent HDTMA, the ferric iron indicator dye CAS and FeCl₃ was able to induce *fyuA-gfp* upregulation in the *fyuA* mutant. A less strong inductive effect was also observed with CAS-FeCl₃ and ferric citrate. Addition of HDTMA alone (20 μ M or 200 μ M) to NBD culture turned out to

be harmful for yersiniae (bactericidal effect). In conclusion, *fyuA-gfp* in the WA *fyuA2* transport mutant can be upregulated in the presence of CAS-agar supplements, provided that Ybt biosynthesis genes are not impaired. This result indicates that FyuA is not absolutely required for upregulation of Ybt biosynthesis in the presence of complexed ferric iron and a cationic detergent.

DISCUSSION

In this study we investigated the gene cluster *irp6,7,8* of *Y. enterocolitica* WA-C with respect to Fe-Ybt utilization in yersiniae and *E. coli*, mouse virulence and Fe-Ybt-transport-dependent control of Ybt production and *fyuA-gfp* reporter gene expression in *Y. enterocolitica*.

According to the known high sequence homology between *irp6,7* of Yen HPI and *ybtP,Q* of Yps HPI we could confirm that gene products Irp6 and Irp7 are involved in Fe-Ybt transport through the cytoplasmic membrane (Fig. 2a) (Fetherston *et al.*, 1999). Consistent with the hypothesis that Fe-Ybt uptake is required for survival and replication of *Yersinia* in mice the *irp6,irp7* mutant was highly attenuated in mouse virulence in spite of Ybt production (Table 3).

The *irp8* gene downstream of *irp7* exhibits 99% homology to *ybtX* of *Y. pestis* and significant homology to *ampG*, which encodes a mucopeptide permease (AmpG) in Gram-negative bacteria (Lindquist *et al.*, 1993). Similar to AmpG we also could not identify the typical consensus sequence EAAXXXG (EAA-loop) of inner membrane proteins from periplasmic-binding-protein-dependent transport (PBT) systems within Irp8 (Braun *et al.*, 1998; Boos & Lucht, 1996). Nevertheless, it could be possible that Irp8 functions as an inner-membrane permease for Fe-Ybt in the context of Ybt-mediated signal transduction, as is known for AmpG in β -lactamase regulation (Jacobs *et al.*, 1997). However, disruption of *irp8* did not affect (i) Ybt utilization or production (similar to YbtX of *Y. pestis*; Fetherston *et al.*, 1999), (ii) Fe-Ybt-dependent *fyuA-gfp* expression in WA-CS *irp8::Kan^r*(pCJFY5G3), or (iii) mouse virulence potential of *Y. enterocolitica*. Thus, the function of Irp8 remains unclear.

We examined whether the capability to utilize Fe-Ybt can be transferred into *E. coli* H1884 and non-pathogenic *Y. enterocolitica* NF-O (both strains are HPI-negative) by introduction of the transport genes *fyuA*, *irp6* and *irp7*. Surprisingly, the *E. coli* recipient, but not the *Y. enterocolitica* NF-O recipient, showed growth support by addition of Fe-Ybt (Fig. 2b, c). Immunoblotting (Fig. 3), RT-PCR (Fig. 4) and pesticin assay revealed *irp6*, *irp7* and *fyuA* expression as well as functional FyuA in both strains, *Y. enterocolitica* NF-O and *E. coli* H1884. To explain the strains' discrepancy in Fe-Ybt uptake we may assume that *E. coli* H1884 but not *Y. enterocolitica* NF-O is able to supply a periplasmic binding protein for Fe-Ybt transport and/or a

Fe-Ybt dissociation factor, although we cannot exclude other possibilities. Nevertheless this conclusion could be of significance with respect to evolution of new pathotypes of microbes by horizontal gene transfer of the HPI. Recently, it was shown that the Yps HPI is widely distributed within the family *Enterobacteriaceae*, in particular in certain pathotypes of *E. coli* (Schubert *et al.*, 1998). Considering horizontal transfer of the HPI, it is conceivable that the only recipients that would benefit from the HPI by improved iron provision are those which already carry genes complementing the Fe-Ybt transport/utilization system.

The Fe-Ybt transport mutants of yersiniae were also studied for Fe-Ybt-dependent regulation, Ybt biosynthesis and Fe-Ybt utilization. The current model (Fig. 5) of core gene regulation includes two transcriptional regulators: (i) ferrous iron surplus in the cytosol results in gene repression by the Fe²⁺-Fur complex and ferrous iron depletion in derepression (e.g. by α,α' -dipyridyl), (ii) the transcriptional activator YbtA is an AraC-like protein which is activated by binding Fe-Ybt and thus can upregulate the *irp/fyuA* gene cluster (Fetherston *et al.*, 1996). According to this model the *fyuA-gfp* reporter gene was upregulated because of Fe-Ybt uptake in the parental strain WA-C and in the *irp8* mutant when grown in iron-depleted medium (Fur-derepressed, Fe-Ybt-induced). Under the same growth conditions the *fyuA-gfp* expression was surprisingly also upregulated in the transport mutant WA-C $\Delta irp6, irp7$ whereas it was downregulated in WA *fyuA2* (Fig. 7). Corresponding to the Fe-Ybt transport defects of the *irp6*, *irp7* and *fyuA* mutants one would expect Fur derepression and lack of Fe-Ybt/YbtA-mediated activation of *fyuA-gfp*. As Irp8 might be involved in weak transport of Fe-Ybt through the cytoplasmic membrane just sufficient for upregulation of *fyuA-gfp* but insufficient for feeding, an *irp6, irp7, irp8* triple mutant was included; it showed no differences from the *irp6, irp7* double mutant. According to these results *fyuA-gfp* upregulation requires FyuA for Fe-Ybt transport through the outer membrane or for Fe-Ybt-mediated signalling through FyuA. It is reasonable that after passing the FyuA pore Fe-Ybt is able (i) to permeate non-specifically through the cytoplasmic membrane and to activate YbtA or (ii) to use the recently described TonB-independent cytoplasmic membrane iron-transport systems *yfeABCD* of *Y. pestis* and *yfuABCD* of *Y. enterocolitica*, respectively (Bearden *et al.*, 1998; Saken *et al.*, 2000). In contrast to the cytoplasmic membrane, the outer membrane should be impermeable for Fe-Ybt and thus *fyuA* gene induction be abolished in the *fyuA* mutant.

fyuA-gfp reporter studies in NBD medium (Fig. 7) and cross-feeding results indicate high Ybt production by the *irp6, irp7* mutant and no or low Ybt production of the *fyuA* mutant. However, results from CAS agar showed high Ybt production and *fyuA-gfp* upregulation for both *fyuA* and *irp6, irp7* mutants (Fig. 9). As CAS agar contains the cationic detergent HDTMA and ferric iron bound to CAS, it is reasonable to assume that this

detergent renders the outer membrane of *Y. enterocolitica* permeable for Fe-Ybt or Fe-CAS. This hypothesis could be verified in so far as HDTMA-CAS-FeCl₃ turned out to be an effective inducer of *fyuA-gfp* expression in NBD broth. Thus, in spite of a lack of FyuA, sufficient Fe-Ybt may enter the periplasm and subsequently the cytoplasm by Irp6/Irp7 for activation of YbtA under CAS agar growth conditions. These results do not rule out the possibility that besides Fe-Ybt transport-mediated *fyuA* gene expression, FyuA signalling may play a role as has been described for ferric citrate-mediated iron transport (FecA-FecR-FecI cascade) (Braun *et al.*, 1998). Interestingly, it has been demonstrated that the outer membrane permeability to hydrophobic agents is much higher for *Y. pestis* than for *Y. enterocolitica* (Bengoechea *et al.*, 1998). If this is also correct for Fe-Ybt we would expect Ybt-dependent upregulation of the *psn*-reporter gene in *Y. pestis psn* mutant but not of the *fyuA*-reporter gene in *Y. enterocolitica fyuA* mutant (*fyuA* is orthologous to *psn* of *Y. pestis*) in detergent-free culture medium. These apparently inconsistent results have been addressed recently (Perry *et al.*, 1999).

Taken together this functional analysis of *irp6-8* and *fyuA* has revealed the following interesting new findings.

- (i) For the first time it has been demonstrated that *irp8* does not contribute to mouse virulence.
- (ii) *fyuA* or *irp6,7,8* expression is not absolutely required for Fe-Ybt-induced upregulation of *fyuA-gfp*, which indicates that these genes are probably not involved in a putative Fe-Ybt-mediated signalling pathway.
- (iii) The *irp6,7/fyuA* gene cluster is sufficient to mediate Fe-Ybt utilization in *E. coli* but not in an avirulent *Y. enterocolitica* NF-O, BG IA strain, indicating that additional genes are required for this phenotype.
- (iv) The CAS siderophore indicator supplement in CAS agar is able to induce Ybt biosynthesis and *fyuA* gene expression independent of the presence of the outer-membrane siderophore receptor. This should be considered by monitoring siderophore production of Gram-negative bacteria.

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