Conjugative plasmid DNA transfer in *Helicobacter pylori* mediated by chromosomally encoded relaxase and TraG-like proteins

Steffen Backert, Terry Kwok and Wolfgang König

Correspondence
Steffen Backert
Steffen.Backert@medizin.
uni-magdeburg.de

Department of Medical Microbiology, Otto von Guericke University, Leipziger Str. 44,
D-39120 Magdeburg, Germany

One of the striking characteristics of *Helicobacter pylori* is the extensive genetic diversity among clinical isolates. This diversity has been attributed to an elevated mutation rate, impaired DNA repair, DNA transfer and frequent recombination events. Plasmids have also been identified in *H. pylori* but it remained unknown whether conjugation can contribute to DNA transfer between clinical isolates. To examine whether *H. pylori* possesses intrinsic capability for conjugative plasmid transfer, shuttle vectors were introduced into *H. pylori* containing an *oriT* sequence of the conjugative IncP α plasmid RP4 but no mobilization (*mob*) genes. It was shown that these vectors could stably replicate and be mobilized among clinical *H. pylori* strains. It was also demonstrated that *traG* and relaxase (*rlx*) homologues carried on the *H. pylori* chromosome were important for plasmid transfer. Primer extension studies and mutagenesis further confirmed that the relaxase homologue *rlx1* in *H. pylori* encodes a functional enzyme capable of acting on the RP4 *oriT*. Furthermore, the findings of this study indicate that *traG* and *rlx1* act independently of the previously described type IV secretion systems, including that encoded by the *cag* pathogenicity island and the *comB* transformation apparatus, in mediating conjugative plasmid DNA transfer between *H. pylori* strains.

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INTRODUCTION

Helicobacter pylori infections have been linked to numerous severe gastroduodenal diseases including peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Montecucco & Rappuoli, 2001; Peek & Blaser, 2002). One of the striking characteristics of *H. pylori* is the extensive genetic diversity between strains (Akopyanz *et al.*, 1992; Alm & Trust, 1999; Salama *et al.*, 2000; Israel *et al.*, 2001; Suerbaum & Achtman, 2004). It has been proposed that both DNA transfer from other species and plasmid integration could contribute to the strain-specific diversity in *H. pylori*, particularly in the so-called plasticity zones (Alm & Trust, 1999). About 50 % of clinical *H. pylori* strains carry cryptic plasmids ranging in size from 2 to about 100 kb. The role of these plasmids is not well understood (Penfold *et al.*, 1988; Hofler *et al.*, 2004). Under laboratory conditions, *H. pylori* is naturally competent for transformation (Hofreuter *et al.*, 2001). Although transformation represents an efficient and important means of horizontal gene transfer, the mechanisms for genetic exchange among *H. pylori* strains in nature remain unclear. Kuipers *et al.* (1998) reported that chromosomally encoded

streptomycin (Str^r) and spectinomycin (Spc^r) resistance gene markers can be transferred among *H. pylori* strains via a DNase-resistant, conjugation-like mechanism. Recently, two larger *H. pylori* plasmids (11 kb and 18 kb) carrying putative genes with homology to genes involved in microcin production of *Escherichia coli* and conjugative transfer (*mob*) have been identified (Hofreuter & Haas, 2002), suggesting that *H. pylori* may be capable of transferring plasmids by a conjugative mechanism.

Additional evidence arguing for a conjugative DNA transfer mechanism in *H. pylori* is based on the identification of two putative relaxase genes, here termed *rlx1* and *rlx2*, in the *H. pylori* chromosome. These genes show significant homology particularly to the unique active sites of other known relaxases (Backert *et al.*, 1998). Relaxases are present in all conjugative DNA-transfer systems (Pansegrau & Lanka, 1996; Llosa *et al.*, 2002; Cascales & Christie, 2003; Schroder & Lanka, 2005). They play a key role in initiating conjugative plasmid DNA transfer by producing a single-stranded scission within *oriT* and binding to the 5' end of the cleavage site. Sequence alignment of relaxase proteins from different DNA transfer systems such as the mobilizable plasmids and agrobacterial Ti (tumour-inducing) plasmid show the presence of three conserved functional motifs (I–III).

Abbreviations: *cag*, cytotoxin-associated genes; PAI, pathogenicity island; RAPD-PCR, randomly amplified polymorphic DNA-PCR.

These motifs are usually located at the N-terminus of the proteins and are arranged in a specific order (Pansegrau & Lanka, 1996). Motifs I and III are involved in catalysing the nicking closing reaction at specific DNA target sequences (*oriT*) whereas motif II is thought to function as a DNA recognition domain. Motif I carries the tyrosine residue that covalently attaches the relaxase through a transesterification reaction to the 5' terminus of the cleaved single-stranded DNA (Pansegrau & Lanka, 1996). All of these motifs are well conserved in the two putative *rlx* genes identified in *H. pylori* (Backert *et al.*, 1998). The relaxase protein, its DNA substrate and a number of accessory DNA-binding proteins form a specialized protein–DNA complex at the *oriT*, termed the relaxosome. The relaxosome is specifically coupled to the transmembrane type IV secretion system (T4SS) for subsequent transfer by the so-called coupling protein. The TraG protein of RP4 and TrwB of R388 are two well-characterized examples of coupling proteins (Cabezon *et al.*, 1997; Gomis-Ruth *et al.*, 2001; Hormaeche *et al.*, 2002; Schroder *et al.*, 2002; Schroder & Lanka, 2003). The N-terminal regions of many coupling proteins have transmembrane α -helices which mediate membrane anchorage. Coupling proteins have conserved Walker motifs A and B postulated to be responsible for ATP hydrolysis to provide energy for the transfer process (Walker *et al.*, 1982; Cabezon *et al.*, 1997; Llosa *et al.*, 2002; Schroder *et al.*, 2002; Schroder & Lanka, 2003). Tato *et al.* (2005) demonstrated recently that the coupling protein TrwB indeed possesses a robust ATPase activity and a propensity to hexamerize, both of which can be stimulated in a DNA-dependent manner. Two open reading frames in *H. pylori*, namely HP0524 (a *virD4* homologue) and HP1006, have been reported to share sequence similarity with *traG*-like coupling factors (Backert *et al.*, 1998).

The set of conjugative proteins that assemble the transmembrane conjugation pore belong to the T4SS family of macromolecular transporters. *H. pylori* possesses several T4SSs which show significant homology to those of conjugation machineries (Hofreuter *et al.*, 2001; Backert *et al.*, 2002). The first T4SS identified in *H. pylori* was that encoded by the 40 kb *cag* pathogenicity island (*cagPAI*), consisting of up to 31 genes (Covacci & Rappuoli, 2000). This T4SS represents a major disease-associated determinant for the delivery of virulence factors such as the CagA protein into host target cells. The second T4SS in *H. pylori* is the *comB* system, consisting of the *comB4*, *comB7*, *comB8*, *comB9* and *comB10* genes, which has been shown to mediate the uptake of naked DNA (Hofreuter *et al.*, 2001). Components of the *comB* system display significant primary sequence and structural similarity to basic components of a T4SS showing homology to the *virB4*, *virB7*, *virB8*, *virB9* and *virB10* genes of the Ti plasmid. The third T4SS gene cluster found in certain strains of *H. pylori* is a segment named *tfs3* (type IV secretion system 3) (Kersulyte *et al.*, 2003). It encodes up to 16 ORFs, some of which are homologous to the genes *virB4*, *virB7* to *virB11* and *virD4* in *Agrobacterium tumefaciens*. No specific function has yet been ascribed to the

latter putative T4SS. It is also unclear whether the aforementioned T4SSs could play a direct role in conjugative DNA transfer in *H. pylori*.

Our aim in this study was to determine whether *H. pylori* possesses intrinsic capability for conjugative plasmid transfer and to identify the genes that might be involved in this process. For this purpose, we cloned *oriT*-containing shuttle vectors without *mob* functions and studied their mobilization among clinical *H. pylori* strains. The functional involvement of the previously identified putative *rlx* (HP0996, HP1004) and *traG* (HP1006) homologues in plasmid transfer was examined in detail using mutagenesis and primer extension studies. Our findings reveal that the shuttle plasmids can be mobilized in a conjugation-like manner in *H. pylori*. This process is dependent on specific *rlx* and *traG* homologues carried on the chromosome but independent of the *comB*- and *cagPAI*-encoded T4SSs.

METHODS

***H. pylori* strains.** The *H. pylori* strains used in this study and their origins are listed in Table 1. These strains were chosen because they do not contain any plasmids. This was verified by plasmid preparation as described by Hofreuter & Haas (2002). Genotyping was done as described previously (Kersulyte *et al.*, 2003; Gieseler *et al.*, 2005). To probe plasmid transfer and the direction of transfer, we selected *H. pylori* strains for chromosomally encoded streptomycin, spectinomycin or rifampicin-resistant (*Str^r*, *Spc^r* or *Rif^r*) clones by plating 10^{10} bacteria on GC agar plates containing streptomycin, spectinomycin or rifampicin ($10 \mu\text{g ml}^{-1}$ each), respectively (Kuipers *et al.*, 1998). Antibiotic resistance was introduced by integration of the *aphA-3* (kanamycin resistance, *Kan^r*) or *cat_{GC}* (chloramphenicol resistance, *Chl^r*) gene cassettes into the bacterial chromosomal DNA using procedures described previously (Kuipers *et al.*, 1998; Backert *et al.*, 2001). Specific knock-out mutants, including the *comB10*, *cagPAI*, *cagI*, *virD4*, *virB7*, *virB11*, *rlx1*, *rlx2* and *traG* mutants (Table 2), were generated by integration of either *aphA-3* (*Kan^r*) or *cat_{GC}* (*Chl^r*) gene cassettes into the bacterial chromosomal DNA at the genes of interest as previously described (Kuipers *et al.*, 1998; Backert *et al.*, 2001). Correct integration of each cassette was confirmed by PCR.

Construction of *oriT*-containing *E. coli*/*H. pylori* shuttle vectors and genetic complementation. The pSB13, pSB14, pSB17 and pSB18 shuttle vectors were constructed based on the plasmid pHel2, which was kindly provided by Dr Rainer Haas (LM University, Munich). pHel2 comprises the cryptic *H. pylori* plasmid pHel1 (2.93 kb; containing a series of direct repeats and a gene for the replication initiation protein RepA that binds to the vicinity of the origin of replication), an *ori_{ColEI}* for replication in *E. coli*, the *cat_{GC}* resistance gene as well as a cloned 370 bp *PstI* fragment from pMin1 representing the *oriT* sequence of the conjugative plasmid RP4 (Kahrs *et al.*, 1995; Heuermann & Haas, 1998).

This 370 bp *PstI* fragment with the *oriT* sequence is included only in the *oriT⁺* plasmids and not in the *oriT⁻* ones (Table 2). The mutant-2 *gfp* (green fluorescent protein-2) gene in a *XbaI* fragment was cloned downstream of the constitutive *H. pylori* *flaA* promoter at a unique *NdeI* site in the start codon ATG as described by Heuermann & Haas (1998). The *PflaA gfp* fusion cassette was then inserted into the *ClaI* and *KpnI* sites of pHel2, resulting in a *Chl^r*, GFP-expressing *E. coli*/*H. pylori* shuttle pSB13 (Fig. 1a). To construct a similar shuttle vector with a *Kan^r* gene cassette, the *cat_{GC}* cassette in pSB13 was replaced by the

Table 1. Origin and genotype of *H. pylori* strains used in this study

<i>H. pylori</i> strain	Origin	Genotype*						Reference
		Natural plasmid	<i>cag</i> PAI	ComB	Tfs3	VacA	IS605	
26695	UK	—	+	+	—	+	+	Tomb <i>et al.</i> (1997)
G27	Italy	—	+	+	—	+	+	Censini <i>et al.</i> (1996)
P1	Germany	—	+	+	—	+	—	Odenbreit <i>et al.</i> (1996)
P12	Germany	—	+	+	—	+	—	Odenbreit <i>et al.</i> (1996)
P300	Germany	—	+	+	—	+	—	Backert <i>et al.</i> (2004)
1061	Canada	—	—	+	—	+	—	Goodwin <i>et al.</i> (1998)

*Genotyping was done with specific PCR and absence of natural plasmids was verified as described in Methods.

aphA-3 gene, which was amplified by PCR from pILL600 with the following primers, which have *Bam*HI sites for subcloning: 52F (5'-AGGATCCGTCGGAATTCGAACCCAGCGAACCA-3'), 52R (5'-AGGATCCGGAAGATCTTTAGACATCTAAAT-3'). The resultant vector is pSB14 (Fig. 1b). For genetic complementation experiments, the *gfp* gene (0.9 kb *Nde*I–*Sal*I fragment) in pSB13 or pSB14 was replaced by *rlx1* and *rlx2* genes, respectively. The following primer pairs were used for amplification of *rlx1* and *rlx2* genes: *Rlx1*-18F (5'-CATATGAAACGCTCCCACTTAGAAAAATG-3'), *Rlx1*-18R (5'-GGA-TCCGAACAAGAATAAACCCCTTTCAAAG-3'); *Rlx2*-19F (5'-TCC-ATATGGCGTTAGAAAAAGTTATAG-3'), *Rlx2*-19R (5'-CGGGA-TCCACTTTTACTAGTATCTAAATGATC-3'). Correct insertion of the amplified fragments into pSB13/pSB14 was verified by standard RFLP and sequencing. To generate a *Rlx1Y12L* mutant, we used the

Sculptor mutagenesis kit (Amersham Biotech). The generated plasmids were named pSB17, pSB18, pSB21 and pSB22, respectively (Table 2). All *E. coli/H. pylori* shuttle plasmids were mobilized into *H. pylori* by using *E. coli* GC7 harbouring the conjugative plasmid pRK2013 as mobilizer (Heuermann & Haas, 1998). The expression of *rlx1* and *rlx2* was examined by immunoblotting of proteins using polyclonal rabbit anti-*Rlx1* or mouse anti-*Rlx2* antibodies.

Media and growth conditions. Bacterial cells were grown either on GC agar (Oxoid) plates or in brain heart infusion (BHI; Oxoid) liquid medium. GC agar plates were supplemented with 10% horse serum (Biochrom), vancomycin (10 µg ml⁻¹), nystatin (1 µg ml⁻¹) and trimethoprim (5 µg ml⁻¹) as described by Brandt *et al.* (2005). For analysis of progeny from mating experiments, bacteria were

Table 2. *H. pylori* strains and mutants used in this study

<i>H. pylori</i> strain	Mutated gene(s)*	Genotype	Shuttle vector	Reference or source
Donors				
P1wt	—	Str ^r , pSB13 Chl ^r	pSB13-GFP <i>oriT</i> ⁺	This study
P1wt	—	Str ^r , pSB13 Chl ^r	pSB13-GFP <i>oriT</i> ⁻	This study
P1wt	—	Str ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1wt	—	Str ^r , pSB22 Chl ^r	pSB21- <i>rlx1 oriT</i> ⁺	This study
P1wt	—	Str ^r , pSB21 Chl ^r	pSB22- <i>rlx2 oriT</i> ⁺	This study
P1Δ <i>comB10</i>	HP0041-42	Str ^r , <i>comB10</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>virD4</i>	HP0524	Str ^r , <i>virD4</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>virB7</i>	HP0532	Str ^r , <i>virB7</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>virB11</i>	HP0525	Str ^r , <i>virB11</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>traG</i>	HP1006	Str ^r , <i>traG</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>rlx1</i>	HP0996	Str ^r , <i>rlx1</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>rlx2</i>	HP1004	Str ^r , <i>rlx2</i> ::Chl ^r , pSB14 Kan ^r	pSB14-GFP <i>oriT</i> ⁺	This study
P1Δ <i>rlx1/rlx1</i>	HP0996	Str ^r , <i>rlx1</i> ::Chl ^r , pSB18 Kan ^r	pSB18- <i>rlx1 oriT</i> ⁺	This study
P1Δ <i>rlx1/rlx1Y12L</i>	HP0996	Str ^r , <i>rlx1</i> ::Chl ^r , pSB18 Kan ^r	pSB18- <i>rlx1Y12L oriT</i> ⁺	This study
P1Δ <i>rlx2/rlx2</i>	HP1004	Str ^r , <i>rlx2</i> ::Chl ^r , pSB17 Kan ^r	pSB17- <i>rlx2 oriT</i> ⁺	This study
Recipients				
1061 wt	—	Spc ^r , Rif ^r	—	This study
P300 wt	—	Spc ^r , Rif ^r	—	This study
26695Δ <i>comB10</i>	HP0041-42	Spc ^r , Rif ^r , <i>comB10</i> ::Chl ^r	—	Hofreuter <i>et al.</i> (2001)
G27Δ <i>cagI</i>	HP0540	Str ^r , <i>cagI</i> ::Kan ^r	—	Censini <i>et al.</i> (1996)
P12Δ <i>cagPAI</i>	HP0520-548	Spc ^r , <i>cagPAI</i> ::Kan ^r	—	This study
P1Δ <i>recA</i>	HP0153	Spc ^r , <i>recA</i> ::Chl ^r	—	This study

*Gene nomenclature according to the TIGR strain 26695 (Tomb *et al.*, 1997).

Table 3. Conjugative plasmid DNA transfer between *H. pylori* isolates

<i>H. pylori</i> donor		<i>E. coli</i> mobilizer	Medium	No. of transconjugants per donor (selected on Sp^{r} Chl^{r})		
Strain	Shuttle vector			1061 (Sp^{r} Rif^{r})	P12::Kan (Sp^{r} Rif^{r})	G27::Kan (Sp^{r} Rif^{r})
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	GC agar	8.5×10^{-8}	6.1×10^{-7}	7.3×10^{-7}
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	GC agar	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	Filter on GC agar	6.9×10^{-6}	8.4×10^{-8}	6.5×10^{-7}
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	pRK2013	Filter on GC agar	7.4×10^{-7}	5.6×10^{-6}	5.2×10^{-6}
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	BHI broth (1 ml)	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	GC agar + DNase I	2.3×10^{-8}	4.5×10^{-8}	6.9×10^{-8}
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	GC agar + Benzonase	1.9×10^{-8}	2.4×10^{-8}	4.3×10^{-8}
P1 (Str ^r)	pSB13-GFP <i>oriT</i> ⁺	–	GC agar + membrane	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1 (Str ^r) heat-inactivated	pSB13-GFP <i>oriT</i> ⁺	–	GC agar	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1 (Str ^r) cell-free extract	pSB13-GFP <i>oriT</i> ⁺	–	GC agar	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
1 μg purified plasmid DNA	pSB13-GFP <i>oriT</i> ⁺	–	GC agar	$< 1 \times 10^{-10}$	1.3×10^{-9}	$< 1 \times 10^{-10}$

[γ -³²P]ATP (0.37 MBq; Amersham Biosciences). Asymmetric PCR reactions with 333F were performed in a 30 μl reaction including 0.2 mM dNTPs, 2 mM MgCl_2 in $1 \times$ PCR buffer in the presence of 0.5 μg total DNA purified from culture of the *H. pylori* transconjugant of interest. DNA polymerase was purchased from Qiagen. Cycling was done at 94 °C for 30 s, 80 °C for 30 s and 72 °C for 15 s for 35 cycles. Extension products were resolved in denaturing polyacrylamide gels (10 % polyacrylamide, 7 M urea) in $1 \times$ TBE buffer (10 mM Tris pH 8, 1 mM EDTA and 10 mM boric acid). To determine precisely the size of primer extension products, a sequencing reaction was done in parallel using the Sequenase 2.0 kit with [³⁵S]dATP α S (1.85 MBq) and primer 333F according to the instructions of the manufacturer (Amersham Biosciences). The template DNA used was vector pSB14 containing the cloned *oriT* of RP4 (Fig. 1b). After electrophoresis, the gel was fixed by incubation in 5 % acetic acid, dried on filter paper and subsequently exposed to X-ray films (Amersham Biosciences).

Randomly amplified polymorphic DNA (RAPD) PCR fingerprinting and fluorescence microscopy. RAPD fingerprinting and fluorescence microscopy were carried out as described below to verify the successful transfer of plasmid DNA from the donors to the recipients. For RAPD fingerprinting, chromosomal DNA was prepared from parental and recombinant *H. pylori* strains using a genomic DNA preparation kit (Qiagen). RAPD-PCR was carried out in 25 μl containing 20 ng genomic *H. pylori* DNA, 3 mM MgCl_2 , 30 pmol primer, 250 μM dNTPs and 1 unit Taq polymerase in $1 \times$ reaction buffer (Qiagen). The following RAPD primers were used: 5'-CCGGATCCGTGATGCGGTGCG-3' (D9355) or 5'-GGTTGGG-TGAGAATTGCACG-3' (D14307). The cycling conditions have been described previously (Akopyanz *et al.*, 1992). The results were analysed by agarose gel electrophoresis using standard procedures. The expression of *gfp* encoded on plasmid pSB13 or pSB14 in the donor and recipient strains was verified using the following procedures. The bacteria were harvested and fixed with 4 % paraformaldehyde for 20 min at room temperature on glass coverslips. The fluorescence intensity of the specimen was examined using a DMIRE-2 fluorescence microscope. The results of RAPD fingerprinting and the fluorescence micrographs were provided to the reviewers for assessment.

RESULTS

Horizontal transfer of plasmid DNA between *H. pylori* strains

Plasmid conjugation in *H. pylori* has not been described to date. In order to investigate whether *H. pylori* has an intrinsic ability to mobilize plasmids from one strain to another, we constructed the vectors pSB13 and pSB14 based on the shuttle vector pHel2 (Heuermann & Haas, 1998) (Fig. 1). Like pHel2, both pSB13 and pSB14 contain an origin for stable replication in *H. pylori* and a cloned 370 bp *oriT* sequence of the conjugative plasmid RP4 (Pansegrau & Lanka, 1996) but carry no mobilization genes (Heuermann & Haas, 1998). The vectors are mobilizable from *E. coli* into *H. pylori* but transfer of these vectors occurs only when the Mob machinery is provided *in trans*, for example, by gene products encoded in the bacterial genome or those encoded on a mobilizer plasmid (Heuermann & Haas, 1998; Backert *et al.*, 2001). To monitor the mobilization of pSB13 between *H. pylori* strains, we electroporated the plasmid into a Str^r clone of the plasmid-free *H. pylori* P1 strain (Table 1). The resultant Str^r/Chl^r strain P1 was then used as a donor in matings with selected spectinomycin- and rifampicin-resistant (Sp^{r} /Rif^r) or Sp^{r} /Rif^r/Kan^r plasmid-free recipient strains on GC agar plates (Table 2). The frequency of plasmid transfer was assessed by quantitating the number of Sp^{r} /Chl^r double-resistant recombinants per parent. Mating routinely yielded Sp^{r} /Chl^r double-resistant recombinants at frequencies of about 10^{-7} to 10^{-8} per donor as shown for pSB13 in Table 3 (top line). All Sp^{r} /Chl^r progeny single colonies were Rif^r, GFP-expressing and exhibited a RAPD-PCR fingerprinting pattern resembling that of the parent recipients (data shown to the reviewers), indicating that the progeny were of recipient origin. Plasmid DNA isolation also demonstrated the presence of the transferred vectors in

the progeny (data shown to the reviewers), verifying that pSB13 was not integrated into the chromosome of the recipients. In addition, no Sp^r/Chl^r recombinant was obtained when each strain was incubated alone, ruling out the possibility that the Sp^r/Chl^r double-resistant recombinants arose from spontaneous mutation. Based on the above observations, we conclude that the Sp^r/Chl^r double-resistant recombinants were progeny of a horizontal transfer of pSB13 from the *H. pylori* P1 donor to the recipient strains.

Plasmid DNA transfer in *H. pylori* occurs by a conjugative mechanism

We next tested whether the plasmid transfer occurs by a conjugation-like mechanism. Given that initiation of conjugative plasmid transfer generally relies on specific nicking and processing of DNA at *oriT* by the *mob* machinery, we examined the functional importance of *oriT* and *trans*-acting *mob* genes in the observed plasmid transfer between *H. pylori* strains. Mating experiments performed with a vector which lacks the RP4 *oriT* *Pst*I fragment (designated pSB13-GFP *oriT*⁻) resulted in a drastic reduction in transfer frequency by almost three orders of magnitude (from 6.1×10^{-7} – 8.5×10^{-8} to $<10^{-10}$) as compared to transfer involving the *oriT*-containing vector (designated pSB13-GFP *oriT*⁺; Table 3, top line). The result suggests that the RP4 *oriT* sequence indeed plays a crucial role in the plasmid transfer. Conversely, when we co-incubated the *H. pylori* donor and recipient strains with the *E. coli* mobilizer strain carrying pRK2013 (which encodes a functional *mob* machinery for mobilization of RP4 *oriT*), the number of $\text{Sp}^r/\text{Chl}^r/\text{Rif}^r$ *H. pylori* progeny increased by one order of magnitude or more (Table 3). Taken together, these observations support the notion that pSB13 can be mobilized among *H. pylori* strains using a conjugation-like transfer mechanism.

The following observations further support the notion that mobilization of pSB13 between *H. pylori* strains occurred by a conjugation-like mechanism rather than transformation or transduction. First, matings carried out in 1 ml liquid BHI medium routinely produced significantly fewer recombinants as compared to matings performed on solid agar medium (Table 3). Second, no plasmid DNA transfer was detected when parents were separated by a 0.1 µm pore-size membrane that prohibited cell-to-cell contact or when the donor strain was heat-inactivated. These observations imply that exchange of pSB13 requires tight cell-to-cell contact between viable strains, a feature which is characteristic of bacterial conjugation. Third, the transfer frequency dropped only marginally in the presence of DNase I or Benzonase nuclease. Since conjugation allows DNA to be transferred in a DNase-insensitive manner whereas uptake of naked DNA by transformation is DNase-sensitive, these results argue against the possibility of plasmid transfer by transformation. Fourth, no or a very low number of recombinants were obtained when the recipients were incubated with a cell-free lysate of the donor or when the recipient strains were incubated with purified pSB13

isolated from strain P1 (Table 3), further arguing against DNA transfer by transformation or transduction. Thus, the observations presented above provide multiple lines of evidence indicating that the transfer of pSB13 between the various *H. pylori* strains occurred by a conjugation-like mechanism requiring RP4 *oriT* and tight cell-to-cell contact between two viable parent strains.

Conjugative plasmid transfer in *H. pylori* is independent of the *comB* system, *cagPAI* and *recA*

Since our pSB13 and pSB14 shuttle vectors do not carry *mob* genes, the genes involved in their transfer must be carried on the *H. pylori* chromosome. The *comB* and *cagPAI* systems are two T4SSs in *H. pylori* which have been studied intensively (Covacci & Rappuoli, 2000; Hofreuter *et al.*, 2001; Backert *et al.*, 2002). Given that both of them share significant homology with T4SSs of conjugative origin, we examined whether the *cagPAI* and the *comB* system play a role in the mobilization of the pSB vectors. As shown in Table 4, disruption of *comB10* in both donor and recipient *H. pylori* had only a minor effect on the frequency of pSB14 mobilization, although the mutation abolished uptake of free DNA by transformation (Hofreuter *et al.*, 2001 and data not shown). Thus, mobilization of pSB14 among *H. pylori* strains appeared to occur in a transformation (*comB*)-independent manner. P1 wild-type and the *virD4*, *virB7* and *virB11* isogenic mutants alike gave rise to similar mobilization frequency (Table 4, top). Given that the *cagPAI*-encoded T4SS was likely to be dysfunctional in the *virB11*, *virB7* and *virD4* mutants (Covacci & Rappuoli, 2000), this observation suggests that the *cagPAI* also did not play a significant role in the transfer of pSB14. In contrast to the requirement of RecA function in the natural transformation of *H. pylori*, the conjugative transfer of pSB14 in *H. pylori* was RecA-independent. This was shown by the fact that inactivation of *recA* in the recipient strains did not affect the number of transconjugants obtained (Table 4, top).

The relaxase and TraG homologues in *H. pylori* function in conjugative plasmid transfer

Relaxases play a key role in initiating conjugative plasmid DNA transfer by producing a single-stranded scission within *oriT* and binding to the 5' end of the cleavage site (Pansegrau & Lanka, 1996). The resultant protein–DNA complex, termed the relaxosome, is then coupled to the transmembrane T4SS by the so-called coupling protein (for example, the TraG homologues) for subsequent transfer. We have recently identified in *H. pylori* two putative relaxase genes (HP996 and HP1004; here designated *rlx1* and *rlx2*) and a putative *traG* homologue (HP1006) in a region distant from the *comB* and *cagPAI* loci (Backert *et al.*, 1998). In this study, we decided to determine by mutagenesis their functional importance in pSB14 mobilization. A knock-out mutant of HP1006 was defective in plasmid DNA transfer (Table 4, bottom), indicating that HP1006 is important for the transfer process despite the fact that its similarity to TraG of

Table 4. Conjugative plasmid DNA transfer between *H. pylori* mutants

Donor	Shuttle vector	No. of transconjugants per donor (selected on Spc ^r Kan ^r)		
		P1Δ <i>recA</i> (Spc ^r Rif ^r Chl ^r)	26695Δ <i>comB10</i> (Spc ^r Rif ^r Chl ^r)	26695 wt (Spc ^r Rif ^r)
P1wt	pSB14-GFP <i>oriT</i> ⁺	7.3×10^{-7}	4.8×10^{-7}	6.7×10^{-8}
P1Δ <i>comB10</i>	pSB14-GFP <i>oriT</i> ⁺	3.8×10^{-7}	9.5×10^{-8}	7.1×10^{-8}
P1Δ <i>virD4</i>	pSB14-GFP <i>oriT</i> ⁺	6.4×10^{-7}	3.9×10^{-7}	4.9×10^{-8}
P1Δ <i>virB7</i>	pSB14-GFP <i>oriT</i> ⁺	4.9×10^{-7}	6.7×10^{-7}	8.0×10^{-8}
P1Δ <i>virB11</i>	pSB14-GFP <i>oriT</i> ⁺	5.5×10^{-7}	7.8×10^{-7}	2.4×10^{-7}
P1wt	pSB22- <i>rlx1 oriT</i> ⁺	4.7×10^{-6}	5.4×10^{-6}	5.8×10^{-6}
P1wt	pSB21- <i>rlx2 oriT</i> ⁺	3.2×10^{-7}	4.5×10^{-7}	4.6×10^{-8}
P1Δ <i>traG</i>	pSB14-GFP <i>oriT</i> ⁺	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1Δ <i>rlx1</i>	pSB14-GFP <i>oriT</i> ⁺	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1Δ <i>rlx2</i>	pSB14-GFP <i>oriT</i> ⁺	6.5×10^{-7}	7.1×10^{-7}	5.2×10^{-8}
P1Δ <i>rlx1/rlx1</i>	pSB18- <i>rlx1 oriT</i> ⁺	7.5×10^{-7}	1.8×10^{-6}	2.3×10^{-7}
P1Δ <i>rlx1/rlx1Y12L</i>	pSB18- <i>rlx1Y12L oriT</i> ⁺	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$	$< 1 \times 10^{-10}$
P1Δ <i>rlx2/rlx2</i>	pSB17- <i>rlx2 oriT</i> ⁺	9.5×10^{-8}	5.2×10^{-7}	6.2×10^{-8}

RP4 is limited to the C-terminal region containing the conserved Walker motif B (our unpublished observation). Interestingly, mutation of *virD4* (HP0524), which is a *traG* homologue encoded by the *cagPAI*, had no effect on the transfer frequency as compared to wild-type bacteria (see above). This implies that the two different putative *traG* homologues in *H. pylori* are functionally distinct; only the HP1006 gene product appears to play an important role in plasmid mobilization.

To investigate whether the putative relaxase genes *rlx1* and/or *rlx2* play a role in plasmid mobilization, we first overexpressed them under the control of the constitutively active *flaA* promoter in wild-type *H. pylori* strain P1 and examined their effects on transfer frequency. Overexpression of *rlx1* but not that of *rlx2* led to an increase in the frequency of pSB plasmid transfer by approximately one order of magnitude (Fig. 2a, Table 4), suggesting that *rlx1* but not *rlx2* is important for the mobilization. In support of this hypothesis, inactivation of *rlx1* but not *rlx2* abolished the plasmid transfer (Fig. 2b, Table 4). Complementation of *rlx1* restored the frequency of transfer to a wild-type level (Fig. 2b, Table 4). Western blot analysis of bacterial cell lysates using anti-Rlx1 or anti-Rlx2 antisera revealed that while both Rlx1 and Rlx2 were expressed in *H. pylori* P1, only the expression level of Rlx1 correlated strictly with a transfer-positive phenotype (Fig. 2c). The Western blots also showed that (i) deletion of *rlx1* was non-polar with respect to expression of *rlx2* and vice versa, and (ii) the genetic complementation was functional.

The tyrosine residue Y22 located in the first of three highly conserved functional motifs in relaxase TraI plays a central role in catalysing the specific nicking of the *oriT* sequence in plasmid RP4 (Pansegrau & Lanka, 1996). A substitution of Y22 by leucine completely abolished the nicking activity of

TraI (Balzer *et al.*, 1994). We have previously shown that the corresponding tyrosine residue Y12 in the *rlx1* homologue of *H. pylori* is well-conserved (Backert *et al.*, 1998). Single amino acid substitution of Y12 in the *H. pylori* Rlx1 abolished transfer of pSB18 (Fig. 2b; Table 4, bottom). This observation confirms the predicted relaxase function of Rlx1 and supports the hypothesis that residue Y12 in Rlx1 plays a similar catalytic role as Y22 in TraI.

Mapping of the Rlx1 nicking site in the *oriT* sequence of RP4

Relaxases catalyse the cleavage of a specific phosphodiester bond at the *nic* site within the related *oriT* to initiate single-stranded DNA transfer during conjugation (Pansegrau & Lanka, 1996). To map the *nic* site produced by *H. pylori* Rlx1 during the transfer of pSB14, we performed primer extension assays, a sensitive technique for detecting DNA discontinuity and characterizing *nic* sites generated *in vivo* (Pansegrau *et al.*, 1990; Liu & Haggard-Ljungquist, 1994; Llosa *et al.*, 1995; Backert *et al.*, 1997). As shown in Fig. 3, wild-type bacteria but not the *rlx1* mutant produced a single band corresponding to an 86 nt primer extension product that terminates at the specific site 5'-TCC/TGCCC-3' within the *oriT* sequence in the pSB14 template. This suggests that the sequence 5'-TCC/TGCCC-3' is the *nic* site for Rlx1 in this *oriT* sequence. A functional *rlx1* gene was required for the nicking activity, as no primer extension product was obtained with a donor expressing the Rlx1-Y12L mutation (Fig. 3, lane 4). The fact that the *rlx2* gene mutant also gave rise to an 86 nt primer extension product supports the notion that *rlx2* is nonessential for conjugative transfer of pSB14 plasmids between *H. pylori* strains. Interestingly, the deduced *nic* site 5'-TCC/TGCCC-3' in our primer extension studies corresponds to a site 2 nt upstream of the *nic* site 5'-TCCTG/CCC-3' of TraI in the

Fig. 2. Effects of overexpression and mutations of *rlx1* or *rlx2* on plasmid transfer in *H. pylori*. Mating experiments were carried out between the recipient strain G27 and the donor strain P1 harbouring pSB shuttle plasmids. Transfer frequency was determined as described in Methods. (a) Abilities of the *rlx1*- or *rlx2*-overexpressing P1 strains to transfer pSB plasmids were compared with that of P1 wild-type. The *rlx1* and *rlx2* genes were overexpressed under the control of the constitutively active *flaA* promoter from the shuttle vectors pSB18 and pSB17, respectively. (b) P1Δ*rlx1* or Δ*rlx2* knock-out mutants, the corresponding complemented mutants and the P1Δ*rlx1* mutant complemented with the *rlx1*-Y12L mutant allele were analysed for their capabilities for plasmid mobilization (see Tables 2 and 4 for specification of the shuttle vectors carried by the various strains). Transfer frequency is expressed as the number of transconjugants per donor. Data are means ± SD from at least three independent experiments. The symbol ** indicates a statistically significant ($P < 0.005$) difference according to Student's *t*-test. (c) Western blots showing the expression level of Rlx1 (upper panels) and Rlx2 (lower panels) in the various strains examined. Blots were probed using polyclonal rabbit anti-Rlx1 or polyclonal mouse anti-Rlx2 antibodies. Arrowheads indicate bands corresponding to Rlx1 (upper panels) or Rlx2 (lower panels).

PRP4 system (Pansegrau & Lanka, 1996). Relaxases from different organisms have been reported to exhibit different DNA sequence specificities. Future studies are necessary to

Fig. 3. Mapping the Rlx1 nicking site in RP4 *oriT* of pSB14 by primer extension assay. The G, A, T and C lanes show the corresponding sequencing ladders obtained from sequencing of pSB14 using the *oriT*-sequence-specific primer 333F. The extension products obtained from various transconjugants using primer 333F are shown in lanes 1–4. The template DNA used was isolated from bacterial matings between *H. pylori* strain G27 as recipient and the P1 wild-type (lane 1), P1Δ*rlx1* (lane 2), P1Δ*rlx2* (lane 3) or P1Δ*rlx1*/rlx1-Y12L (lane 4) as donors. Arrowheads indicate the nicking sites determined for Rlx1 and TraI, respectively. Arrows indicate the 86 nt primer extension products obtained (lanes 1 and 3).

define in detail the sequence specificity of Rlx1 and to identify the possible cognate *oriT* sites in the *H. pylori* genome.

DISCUSSION

Although some *H. pylori* strains have been shown to carry natural plasmids of up to 100 kb (Penfold *et al.*, 1988; Kleanthous *et al.*, 1991; Heuermann & Haas, 1995; Minnis *et al.*, 1995; de Ungria *et al.*, 1999; Hofreuter & Haas, 2002; Hofler *et al.*, 2004), conjugative plasmid DNA transfer among *H. pylori* strains had hitherto not been demonstrated. In this work, we selected *H. pylori* strains having no known natural plasmid and introduced shuttle vectors into these strains. These shuttle vectors (pSB13 and pSB14) contain the *oriT* sequence of the conjugative IncPα plasmid RP4 but no mobilization genes. We showed that these vectors were mobilized in matings among clinical *H. pylori* strains at a frequency of approximately 10⁻⁷–10⁻⁸. This implies that the genes involved in the plasmid transfer must be carried on the *H. pylori* chromosome. These genes were identified in this study to include *rlx1* and the *traG*-like HP1006. The transfer of pSB13 or pSB14 occurred in a manner reminiscent of bacterial plasmid conjugation, characterized by its insensitivity to extracellular nuclease activity, requirement for viable parents and physical contact

between donor and recipient strains, and importantly, the functional dependence on both *oriT* and mobilization genes. The plasmid DNA transfer appeared to be independent of the *comB* system for natural transformation because mutation of *comB10* in both the donor and recipient strains had only little effect on their transfer frequencies. In summary, our findings provide strong evidence for the existence of a chromosomally encoded conjugative transfer system in *H. pylori*.

The T4SS encoded by the *cagPAI* or *tfs3* appeared to be unnecessary for the conjugative DNA transfer observed because, firstly, the *H. pylori* strains examined do not encode the full set of *tfs3* genes (our unpublished data), and secondly, mutations of the various *cagPAI* genes did not block plasmid transfer. Instead, we found a gene cluster in the so-called plasticity zone (Tomb *et al.*, 1997; Alm *et al.*, 1999) that encodes relaxase homologues (Rlx1 and Rlx2) and a TraG-like protein, which are typical components of conjugative T4SS systems (Pansegrau & Lanka, 1996; Cabezon *et al.*, 1997; Llosa *et al.*, 2002; Cascales & Christie, 2003; Schroder & Lanka, 2005; Tato *et al.*, 2005). Their involvement in plasmid transfer among *H. pylori* strains was supported by the following findings from our mutagenesis experiments: (i) a *traG* knock-out mutant was defective in plasmid DNA transfer; (ii) likewise, a *rlx1* knock-out mutant was completely deficient in plasmid transfer; (iii) transfer frequency was enhanced by over-expression of *rlx1*; and (iv) genetic complementation of *rlx1* in the *rlx1* mutant strain restored the plasmid transfer to wild-type levels. Taken together, our findings support a model in which plasmid DNA mobilization in *H. pylori* depends on the Rlx1 homologue acting in concert with a TraG-like protein encoded by HP1006.

Results were also obtained in this study which provide preliminary insights into the molecular mechanism by which Rlx1 might function as a relaxase in *H. pylori*. Tyrosine residue Y22 in motif I in TraI plays a key role in initiating conjugative plasmid DNA transfer of plasmid RP4 by producing a single-stranded scission within *oriT* and binding to the 5' end of the cleavage site (Pansegrau & Lanka, 1996). Analogous to the functional role of Y22 of TraI, the conserved residue Y12 in Rlx1 appeared to be essential for the relaxase function, as expression of Rlx1-Y12L in *H. pylori* abolished plasmid transfer. Consistent with this finding, the Rlx1-Y12L mutant did not produce the nicking product in our primer extension study. The Rlx1 nicking site in the *oriT* sequence of RP4 was identified and mapped to the sequence 5'-TCC/TGCCC-3', indicating that Rlx1 is able to process the *oriT* of RP4. It has been noted that relaxases that act on IncP-like transfer origins share three conserved motifs at their N-termini (Pansegrau & Lanka, 1996). Since Rlx1 contains all three of the conserved relaxase motifs (Backert *et al.*, 1998), the ability of Rlx1 to act on the RP4 *oriT* (which has an IncP origin) is not totally surprising. However, it is interesting to note that the Rlx1 nicking site determined lies 2 nt upstream of the cleavage site produced

by the TraI relaxase (5'-TCCTG/CCC-3'). The reason for this discrepancy is as yet unclear. Identification of the cognate *oriT* in *H. pylori* is one of the most important aims in future studies and may shed light into the sequence specificity of Rlx1. It will also be of interest to see if transfer of plasmids in *H. pylori* using a cognate *oriT* of Rlx1 would occur more efficiently.

A recent view of the conjugation machinery is that it consists of a DNA-processing system linked to a T4SS. The former consists of relaxases, accessory proteins and DNA, whereas the latter forms a conjugation pore through which the DNA/protein complexes of conjugative machineries are transferred across the membrane barriers (Llosa *et al.*, 2002; Schroder & Lanka, 2003, 2005). Interestingly, the mosaic nature of the conjugative machineries is often accompanied by the organization of the corresponding genes in a gene cluster. For instance, in the well-characterized RP4 conjugation system, the genes *traM*, *traL* and *traJ*, which encode components of the conjugation pore, lie in a gene cluster with the *rlx* and *traG* genes, which encode DNA-processing and coupling functions (Pansegrau & Lanka, 1996). In *H. pylori*, the genes flanking *rlx* or *traG* have no significant homology to any T4SS components, such as the *tra* or *virB* genes, although they contain smaller stretches which are homologous to genes encoding conjugative proteins (our unpublished data). This could imply that the *H. pylori* conjugative apparatus may have some atypical features unique to this organism. The fact that HP1006 shows homology with only the C-terminal portion of TraG tends to support this hypothesis. Future work is required to identify the genes involved in forming the conjugation pore and to further characterize the relaxosome and coupling process in *H. pylori* conjugation. It is also intriguing that although *H. pylori* contains two putative *rlx* genes, only *rlx1* appears to be essential for the conjugative transfer of pSB13 or pSB14. It will also be important to investigate the role of *rlx2*. We have indications that *rlx2* rather than *rlx1* is involved in mobilization of chromosomal DNA among *H. pylori* strains (our unpublished data).

In conclusion, in this study we have identified novel components of a chromosomally encoded conjugative apparatus in *H. pylori* which mediates plasmid DNA transfer among strains and is independent of the three known T4SSs in *H. pylori*, namely *comB*, *cagPAI* and *tfs3*. The genes in *H. pylori* which encode the T4SS dedicated to forming a conjugation pore are yet to be defined. An understanding of how *Helicobacter* exploits its conjugative transfer system during the course of infection will provide important insights into the evolutionary strategies of this group of clinically important pathogens. It is plausible that this phenomenon may help the bacteria to adapt to environmental stress in the acidic milieu of the stomach, where free chromosomal DNA is short-lived, and may contribute to the heterogeneity of *H. pylori* strains. In addition, transfer of genetic materials via a conjugative mechanism could be a means to overcome the interstrain

restriction barrier, assuming that the single-stranded DNA being transferred is less likely to be recognized by conventional restriction enzymes (Lin *et al.*, 2001; Nobusato *et al.*, 2000; Aras *et al.*, 2002).

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