Multiple Pathways of Plasmid DNA Transfer in *Helicobacter pylori*

Stefanie Rohrer¹, Lea Holsten¹, Evelyn Weiss¹, Mohammed Benghezai², Wolfgang Fischer¹, Rainer Haas¹*

1 Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Ludwig-Maximilians-Universität, München, Germany, 2 Ondek Pty Ltd and H. pylori Research Laboratory, Microbiology and Immunology, University of Western Australia, Nedlands, Australia

**Abstract**

Many *Helicobacter pylori* (Hp) strains carry cryptic plasmids of different size and gene content, the function of which is not well understood. A subgroup of these plasmids (e.g. pHel4, pHel12), contain a mobilisation region, but no cognate type IV secretion system (T4SS) for conjugative transfer. Instead, certain *H. pylori* strains (e.g. strain P12 carrying plasmid pHel12) can harbour up to four T4SSs in their genome (cag-T4SS, comB, tfs3, tfs4). Here, we show that such indigenous plasmids can be efficiently transferred between *H. pylori* strains, even in the presence of extracellular DNaseI eliminating natural transformation. Knockout of a plasmid-encoded *mobA* relaxase gene significantly reduced plasmid DNA transfer in the presence of DNaseI, suggesting a DNA conjugation or mobilisation process. To identify the T4SS involved in this conjugative DNA transfer, each individual T4SS was consecutively deleted from the bacterial chromosome. Using a marker-free counterselectable gene deletion procedure (ipsl counterselection method), a P12 mutant strain was finally obtained with no single T4SS (P12ΔT4SS). Mating experiments using these mutants identified the *comB* T4SS in the recipient strain as the major mediator of plasmid DNA transfer between *H. pylori* strains, both in a DNaseI-sensitive (natural transformation) as well as a DNaseI-resistant manner (conjugative transfer). However, transfer of a pHel12::cat plasmid from a P12ΔT4SS donor strain into a P12ΔT4SS recipient strain provided evidence for the existence of a third, T4SS-independent mechanism of DNA transfer. This novel type of plasmid DNA transfer, designated as alternate DNaseI-Resistant (ADR) mechanism, is observed at a rather low frequency under *in vitro* conditions. Taken together, our study describes for the first time the existence of three distinct pathways of plasmid DNA transfer between *H. pylori* underscoring the importance of horizontal gene transfer for this species.

**Citation:** Rohrer S, Holsten L, Weiss E, Benghezal M, Fischer W, et al. (2012) Multiple Pathways of Plasmid DNA Transfer in *Helicobacter pylori*. PLoS ONE 7(9): e45623. doi:10.1371/journal.pone.0045623

**Editor:** Eric Cascales, Centre National de la Recherche Scientifique, Aix-Marseille Université, France

**Received:** June 3, 2012; **Accepted:** August 20, 2012; **Published:** September 20, 2012

**Copyright:** © 2012 Rohrer et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the Deutsche Forschungsgemeinschaft (HA2697/10-1) and by 30 ERA-NET PathoGenoMics 3 HELDIVPAT (0315905C) to RH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: haas@mvp.uni-muenchen.de

**Introduction**

*Helicobacter pylori* is a highly motile, microaerophilic, Gram-negative bacterium, resident in the gastric mucus layer of about 50% of the human population. Infection with *H. pylori* is a major cause of gastroduodenal disease, including chronic active gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric carcinoma [1,2]. A remarkable feature of *H. pylori* is its panmictic population structure, reflected by an extreme genetic heterogeneity, possibly resulting from frequent recombination events after import of small pieces of foreign DNA [3–6]. Such an efficient DNA exchange has been attributed to the natural transformation competence of *H. pylori* [7], mediated by the *comB* type IV transport system [8–10], which is actually stimulated by DNA damage to trigger genetic exchange [11].

Besides natural transformation, bacterial conjugation is a further possible mechanism of lateral DNA transfer. Interestingly, about 50% of *H. pylori* isolates carry cryptic plasmids ranging between 2 and 100 kb in size [12]. Some isolates even carry multiple plasmids of different size; however, the role of these plasmids for *H. pylori* is not well understood. Chromosomal integration and excision of plasmid DNA after transfer from a donor into a recipient strain might be an alternative way to generate genome rearrangements [13].

Most *H. pylori* plasmids either replicate via the “rolling circle” mechanism [14,15], or carry direct sequence repeats, so-called “iterons”, and replicate via the theta mechanism [16] (for review see [17]). A DNaseI-resistant, conjugation-like bidirectional chromosomal DNA transfer between *H. pylori* has been reported, but the mechanism has not been explored [18]. Conjugative transfer of indigenous plasmids between *H. pylori* strains, or even more unrelated bacterial species, has not yet been demonstrated.

Plasmids are generally classified as conjugative (autotransmissible) or mobilisable (transmissible only in the presence of a helper conjugative plasmid). In contrast to conjugative plasmids, which contain all the necessary genetic information to catalyse conjugal DNA processing and DNA transport, mobilisable plasmids lack part of this machinery. Mobilisable plasmids typically have an origin of conjugative transfer (*oriT*) and code for proteins involved in conjugal processing of DNA, such as *oriT*-specific relaxases and nicking accessory proteins [19].

We have initially characterized two cryptic *H. pylori* plasmids of 10.9 and 18.2 kb in size named pHel4 and pHel5, respectively [13], and recently pHel12 [20], a 10.2 kb plasmid. They were...
assigned to the group of theta plasmids. In pHe4 and pHe12, a putative mob region was identified, showing best homologies to proteins MbeA, MbeB, MbeC and MbeD of colicogenic plasmids, such as pColE1 [13,21]. The MbeA protein of the ColE1 plasmid is an atypical relaxase, because it lacks two conserved histidine residues in the third conserved amino acid sequence motif (motif III) [22], present in most relaxases. MbeA, which reveals best sequence homology in the three conserved amino acid motifs (motif I, motif II and motif III) to the pHe4 relaxase [13], has been verified as a relaxase necessary for plasmid ColE1 mobilisation [23]. H. pylori plasmid pHe4 is 10.9 kb in size. In addition to its encoded replicase RepA, the plasmid contains a putative microcin operon, a putative mobilisation region (mobA) and a number of cryptic open reading frames (ORFs) with low homology to H. pylori chromosomal genes (Fig. 1A) [13]. Plasmid pHe12 is rather homologous to pHe4 in its gene content and primary sequence [20]. It carries a microcin region as well as a putative mobilisation region, but contains only a homologue of orfA11 (HPP12_12), but no homologues of orf4K, L, N and O. We show here that cryptic plasmids of H. pylori can be transferred by at least three different mechanisms: natural transformation, mobilisation and a DNaseI-resistant alternate pathway.

Results

Generation of a Model System to Monitor Directional H. pylori Plasmid Transfer

Several plasmids carrying putative mob regions have been described for H. pylori [17]. They are candidates for a conjugative transfer between H. pylori strains. In this study, plasmids pHe4, present in H. pylori strain P8, and pHe12, present in strain P12 (Fig. 1A), were analysed for their transfer potential. The comB T4SS (orf2) is involved in plasmid and chromosomal DNA uptake by natural transformation and is present in all H. pylori strains tested so far [9,20]. Other T4SSs, such as the cag-T4SS (orf1), tf3 or tf4, are variably present in individual H. pylori strains [20]. Plasmid transfer from a donor into a recipient strain is supposed to occur via plasmid DNA release by the donor and subsequent transformation of the recipient by the comB system [9]. Thus, any one of the known T4SSs could be involved in conjugative plasmid transfer (Fig. 1B). In strain P12, all four T4SSs are present, whereas P8 carries comB and cag-PAI only [20].

To verify conjugative transfer of plasmids pHe4 or pHe12 between H. pylori strains, a chloramphenicol acetyltransferase gene (cat) was inserted either upstream or downstream of the mobA gene [pHe4:cat] or [pHe12:cat], which leaves the mob region intact (Fig. 1A). The donor strain (P8, P12) carried a recA deletion, whereas the recipient strain (P12) was streptomycin- and kanamycin resistant (mobA::cat) (Fig. 1B). Transfer of the plasmid was monitored by screening transconjugants containing the cat gene on the plasmid and the streptomycin or kanamycin (Str/Kan) resistance in the recipients chromosome (Fig. 1B). The recA mutation in the donor avoided a possible transfer of the chromosomal resistance marker from the recipient back into the donor strain by transformation. This guaranteed a transfer of plasmid pHe4 or pHe12 into the recipient (Fig. 1B). The unidirectional plasmid transfer from donor to recipient was verified by RAPD PCR (data not shown).

Verification of Plasmid Transfer between Different H. pylori Strains

Co-cultivation of H. pylori P8 or P12 as donor strains and P12 as recipient strain resulted in transfer of plasmids pHe4 or pHe12 at a frequency between 10^{-3} and 10^{-5}, respectively (agar plate mating, see Materials and Methods) (Fig. 1C columns 1 and 5). The inactivation of the relaxase gene [pHe4::mobA::cat, pHe12::mobA::cat] reduced the plasmid transfer rate slightly but not significantly (Fig. 1C, columns 3 and 7). The transferred plasmid pHe12::cat was successfully reisolated from the recipient strain after mating. The transferred as well as the indigenous plasmids co-existed in the recipient strain, although the transferred plasmid carrying the cat resistance gene was found in higher quantities, as demonstrated by gel electrophoresis and PCR analysis (Fig. 2A-C).

To further verify and expand these data to other H. pylori strains, co-incubation experiments were performed using H. pylori strain P8 as a donor with a set of different recipient strains, such as H. pylori, P1, P8, P12, or B12. Plasmid transfer was observed with these independent recipient strains at slightly different rates (data not shown). This suggested that independent H. pylori strains can act as recipients for plasmid DNA transfer.

Plasmid Transfer Takes Place by Both, DNaseI-Sensitive Natural Transformation and a DNaseI-Resistant Transfer Mechanism

To study the mechanism of plasmid transfer, for all subsequent experiments the same strain, H. pylori P12, was used as donor (recA::erm) and as recipient (str::mobA::aphA-3) strain. This strategy ruled out potential plasmid incompatibilities or DNA restriction mechanisms [24] that might otherwise affect plasmid transfer efficiency. Furthermore, for strain P12 the complete genome sequence is available [20], so that all T4SS and relaxase genes present in the genome are known. To further corroborate the plasmid transfer as a plasmid mobilisation event, plasmid transfer was analysed with the addition of DNaseI, to remove extracellular DNA and thus to abolish natural transformation. Co-cultivation of H. pylori P8 or P12 carrying pHe4 [mobA::cat] or pHe12 [mobA::cat] (donor) and P12 [mobA::aphA-3] (recipient) with DNaseI treatment, revealed a significant reduction (p<0.05) in transfer rates between intact and mobA-defective plasmids for both P8 and P12 donor strains (Fig. 1C, compare columns 2 versus 4 and 6 versus 8). These data are best explained by natural transformation of extracellular released plasmids pHe4 or pHe12 (e.g. by lysis of bacteria), as long as no DNaseI was present (Fig. 1B, blue, dashed arrows). The addition of DNaseI destroyed extracellular plasmid DNA and abolished transformation-mediated plasmid transfer, whereas the knockout of the mobA relaxase gene is supposed to obstruct conjugative plasmid DNA transfer only. Thus, our data suggest that the relaxase MobA has a small but significant effect on DNaseI-resistant plasmid transfer of pHe4 and pHe12, which verifies MobA as a functional relaxase for H. pylori plasmids. However, since inactivation of mobA in the presence of DNaseI did not completely abolish plasmid DNA transfer, a further DNaseI-resistant transfer of pHel plasmids seems to occur, which is independent of the plasmid-encoded relaxase MobA.

Conjugative DNaseI-Resistant Plasmid Transfer is Mediated by comB, but no Other T4SS of H. pylori P12

Certain cryptic plasmids of H. pylori carry the relaxase gene mobA, but none of them contain a complete T4SS mediating its conjugative transfer. We therefore asked which T4SS is responsible for the DNaseI-resistant plasmid mobilisation between H. pylori strains. Since up to four unique T4SSs have been described in different H. pylori isolates, one (or several) of the chromosomally encoded T4SSs (orf1-orf4) might mediate plasmid mobilisation.

To study the role of these T4SSs for plasmid transfer, all T4SSs were successively removed from the genome by a marker-free
genetic deletion method, the rpsL counterselection procedure [25]. First, the complete tfs3 and tfs4 systems were deleted in the P12 donor strain and the correct deletions were verified by PCR analysis (Fig. 3) (see Materials and Methods and Tables 1, 2 and Table S1 for details of plasmid construction). Removal of TFS3 or TFS4 in the donor (Fig. 4A) or in the recipient strain (Fig. 4B) did not significantly change the plasmid transfer frequency, neither without nor with DNaseI. Also a double mutant (P12 tfs3 D tfs4) did not show a defect in plasmid transfer. In the next step the cag-PAI was removed by the same procedure (Fig. 3C), resulting in a triple mutant (P12 cag-PAI). Again, plasmid transfer rates were not reduced, as compared to the P12 wt situation (Fig. 4A, B). An additional deletion of the comB system in the donor strain (P12 cag-PAI D comB), also termed P12 ΔT4SS, or deletion of the complete comB system only, did not result in a significant change of plasmid transfer rate (Fig. 4A, columns 11 and 12). However, the same deletions in the recipient strain significantly reduced plasmid transfer rates, especially in experiments without DNaseI (transformation) but also to a lower extent with DNaseI (p<0.05) (Fig. 4B, columns 11 and 12). Knockout of the comB system only in the recipient strain gave very similar results as the quadruple mutants (Fig. 4B, columns 13 and 14). A

Figure 1. Structure of cryptic plasmids pHel4 and pHel12 and verification of their intra- and interstrain plasmid transfer. (A) Schematic map showing the gene content and basic functional regions of two cryptic plasmids pHel4 and pHel12. Both plasmids contain a microcin gene cluster with homology to E. coli mccC7 (orf4A, B, X, or orf1, 02, 03). Genes orf4C-F and orf04-orf07, respectively, show homology to the mob region of colicinogenic plasmids. Genes orf4H/H and orf08/orf09, respectively, are related in sequence to the toxin-antitoxin system RelE-RelB (TA). The plasmid replicase is encoded by orf4I (pHel4) or orf10 (pHel12). Both plasmids also carry a number of hypothetical genes (orf4-J, orf10). The insertion of the cat GC antibiotic resistance gene cassette into various orfs is shown. (B) Procedure of H. pylori co-cultivation to determine plasmid DNA transfer via natural transformation or conjugative processes. All T4SS (cag-PAI, comB, tfs3, tfs4) potentially involved in plasmid transfer between an H. pylori donor (P8, P12) and a recipient (P12) strain are indicated. Generally, natural transformation of the recipient strain by released plasmid DNA after lysis of the donor strain and conjugation processes are superimposed and are discriminated by adding of DNaseI. Plasmid transfer of pHel4(mccA:cat) or pHel12:cat from strain P8 or P12 into the P12 recipient strain was monitored by selection of the recipient strain (P12) via streptomycin (P12 str) or kanamycin (P12 mould:aphA-3). To avoid the transfer of the chromosomal marker of the recipient (str, aphA-3) into the donor strain by natural transformation, the recA gene in the donor strain was deleted (P8 recA::erm, P12 recA::erm). (C) Inter- or intratransfer of the cryptic plasmids pHel4(mccA:cat) or pHel12:cat from donor strain P8 or P12 into the recipient strain P12 in the absence (−) or presence (+) of DNaseI. Transfer rates were determined as the number of transconjugants/cfu/ml. Data shown are mean values of at least three independent experiments including standard deviations. * p<0.05, ns, not significant.
doi:10.1371/journal.pone.0045623.g001
Figure 2. Confirmation of pHel plasmid transfer into transconjugants. (A) Isolation of plasmid DNA from transconjugants after co-cultivation of H. pylori P12 donor and recipient strains. Isolation of plasmid DNA after co-cultivation of H. pylori P12 (P12[moeb::aphA-3]). Presence of the donor plasmid pHel12::cat in the recipient strain (red arrow, lane 3,4) and of the recipient plasmid pHel12 (black arrow, lanes 3 and 4) is shown. Lane 1: 1 kb ladder, lane 2: pHel12, lane 3: transconjugant 1, lane 4: transconjugant 2. (B) Scheme explaining the PCR for the specific detection of donor- and recipient plasmid in transconjugants using oligos SR57 and SR58. (C) PCR fragments obtained with primers SR57/SR58 for the donor plasmid (red arrow, 1.3 kb) and for the original pHel12 plasmid of the recipient strain (black arrow, 0.4 kb). Lane 1, 1 kb ladder, lane 2, pHel12 used as template, lane 3 and 4, plasmid DNA from transconjugants 1 and 2 used as template.

doi:10.1371/journal.pone.0045623.g002

simultaneous deletion of the comB T4SS in both, the donor as well as the recipient strain, maximally reduced plasmid transfer rates, supporting the role of the donor strain comB T4SS for DNaseI-resistant plasmid transfer (Fig. 4B, columns 15 and 16). To exclude any insufficient DNA degradation by DNaseI and therefore residual transformation, the extracellular DNA of the co-cultivation assay with and without DNaseI was extracted, sterile-filtrated and used for transformation of the H. pylori P12 wild type (wt) strain. The non-treated, but not the DNaseI-treated extract resulted in transformation of P12, indicating that DNA used was efficient during the co-incubation conditions (data not shown). Plating of the donor and recipient strains separately (without co-incubation) on double selective media did not result in any resistant bacteria, which excluded the generation of any spontaneous mutants (data not shown).

In conclusion, these data suggest that none except the comB T4SS contributes to plasmid transfer by transformation as well as a DNaseI-resistant mobilisation.

Plasmid-Encoded Relaxase MobA, but not Chromosomally Encoded Relaxases Contribute to Plasmid Transfer

The plasmid-encoded relaxase MobA contributed to DNaseI-resistant plasmid transfer of pHe4 as well as pHel12 (Fig. 1C). H. pylori P12 harbours two chromosomally-encoded VirD2-homologous relaxases, HPP12_1353 and HPP12_0451, located next to tfs3 and tfs4, respectively (Fig. 3A, B). Therefore, it could not be excluded that these putative chromosomal relaxases partially compensate for the deletion of the plasmid-encoded relaxase in strain P12. We therefore sought to analyse the effect of the plasmid- as well as chromosomally encoded relaxases in strain P12 on plasmid transfer. The original deletions of TFS3 or TFS4 described above left the VirD2-homologous relaxases in the chromosome. Therefore, the complete tfs3 and tfs4 systems, including the adjacent relaxase genes were deleted using the tfsL counter-selection procedure. Precise deletions were verified by PCR as described for the tfs3/tfs4 deletions (data not shown, see Material and Methods section and Table S1 for primers) Removal of HPP12_1353 together with tfs3 (Δtfs3ΔvirD2), as well as HPP12_0451 with tfs4 (Δtfs4ΔvirD2) did not significantly change the plasmid transfer rates (Fig. 5A). Also a double mutant (Δtfs3ΔvirD2, Δtfs4ΔvirD2) did not have a significant effect on plasmid transfer, as compared to the transfer rates between P12

strains (Fig. 5B). In conclusion, the chromosomally-encoded relaxases HPP12_1353 or HPP12_0451, located adjacent to TFS3 or TFS4, respectively, were not involved in plasmid DNA transfer, neither in a DNaseI-sensitive nor a DNaseI-resistant manner.

A T4SS-Independent Alternate DNaseI-Resistant (ADR) Mechanism of Plasmid Transfer in H. pylori

Our data provided evidence that plasmids can be transferred between H. pylori strains by natural transformation, as well as by conjugation. Both routes of DNA transfer are strictly dependent on the comB T4SS. For conjugation or mobilisation of plasmids between H. pylori, the mobA relaxase is essential. However, in our experiments DNaseI-resistant plasmid transfer was clearly seen in strains without any T4SS (P12ΔT4SS) used either as donor or as recipient (Fig. 4). To unequivocally prove the existence of such an alternate DNA transfer pathway in H. pylori operating independently of any T4SS, an experiment was designed using a P12ΔT4SS strain as a donor as well as a recipient. Such a strain cannot be transformed by plasmid DNA [9], nor can it act as a donor or recipient for conventional conjugative DNA transfer, since it is does not contain any T4SS (Fig. 6A).

Co-incubation experiments using such a pair of strains showed that plasmid pHel12::cat can be transferred between H. pylori, albeit at a lower efficiency as that seen for natural transformation or mobilization (around 1×10^5), but still at a reasonable rate (Fig. 6B). Furthermore, the additional deletion of the mobA gene of pHel12 (pHel12ΔmobA::cat) did not abrogate the transfer between the P12ΔT4SS strains. However, control plating experiments with the donor or the recipient strain alone on double-selective media did not result in any resistant bacteria, excluding spontaneous mutations of the donor or the recipient strain as a source for the double resistant bacteria. Without any T4SS extracellular plasmid DNA should not contribute to this type of plasmid transfer via transformation, which is supported by the fact that a similar low rates of DNA transfer were seen with and without addition of DNaseI (Fig 6B, C). Finally, the plasmids from the double resistant bacteria were isolated and confirmed to be pHel12::cat.

In conclusion, these experiments clearly show that an alternative pathway for plasmid exchange is existent in H. pylori, which is
distinct from natural transformation and conventional conjugation. We suggest naming this pathway as ADR pathway.

Discussion

The existence of plasmids in *H. pylori* has been known for more than twenty years [12]. More recently, a subgroup of *H. pylori* plasmids has been described that carries putative mobilisation genes [13,17,20,26], which might be involved in conjugative transfer between different *H. pylori* strains. This is of particular interest, since such plasmids might be involved in the generation of genetic variation in *H. pylori* [13]. Here we studied the transfer of such plasmids between identical and different, unrelated *H. pylori* strains. Since *H. pylori* is naturally competent for DNA transformation, the analysis of conjugative transfer of plasmid DNA between *H. pylori* is more complicated than in bacteria without natural transformation competence, since both processes are superimposed (Fig. 1B). A simple transfer of the chromosomal resistance gene marker (*str, aphA-3*) from the recipient bacteria into the donor strain by natural transformation, which would result in double resistant pseudo-transconjugants should be avoided. We

Figure 3. Deletion of individual T4SS from the genome of *H. pylori* strain P12 by the marker-free counterselection procedure. (A) Schematic representation of Tfs3 and verification of its deletion. For deletion of the complete Tfs3 (16 kb region), the flanking sequences were amplified using oligos SR17/SR18 and SR30/SR35 (see Table S1 for primers) and cloned into the pBlueScript vector either with or without the rpsL-erm cassette in-between. Verification of the correct deletion was obtained by DNA amplification of a genomic fragment using oligos SR24/SR25 resulting in a PCR fragment of 0.77 kb, as expected (gel I). The PCR fragment was further verified by DNA sequencing. Furthermore, amplification of an internal region of Tfs3 (primers W5642/355) resulted in the expected PCR product from wt, but not P12AT4SS chromosomal DNA (gel II) (B) For deletion of the complete Tfs4 (14 kb region) the flanking sequences were amplified using oligos SR13/SR14 and SR32/SR34 (see Table S1 for primers) and cloned into the pBlueScript vector either with or without the rpsL-erm cassette enclosed. Verification of the correct deletion was obtained by DNA amplification of a genomic fragment using oligos SR22/SR34, resulting in a 2.6 kb fragment, as expected (gel I). The PCR fragment was further verified by DNA sequencing. Amplification of an internal region of Tfs4 (primers SR48/49) resulted in the expected PCR product from wt, but not P12AT4SS chromosomal DNA (gel II) (C) The complete cag-PAI was deleted by the rpsL-counterselection procedure. Flanking fragments were cloned using oligos JP22/23 and JP24/25 for PCR (see Table S1 for primers). The correct deletion was verified by the generation of a PCR fragment of 300 bp spanning the deletion, as generated by primers hp519 and hp549. The PCR fragment was further verified by DNA sequencing. Amplification of an internal region of the cag-PAI (primers W5418/HPS421) resulted in the expected PCR product from wt, but not P12AT4SS chromosomal DNA (gel II). (D) Deletion of the comB6–comB10 genes of the comB T4SS by the rpsL-counterselection procedure (4.3 kb). Flanking regions were cloned using oligos AK59/AK65 and DHO10/DHO11 for PCR (see Table S1 for primers). The deletion was verified by the generation of a 400 bp fragment, whereas the intact comB locus resulted in a 4300 bp fragment. Amplification of an internal region of comB (primers DHO14/DHO15) resulted in the expected PCR product from wt, but not P12AT4SS chromosomal DNA (gel II). Components with sequence homology to the VirB/D4-System of *A. tumefaciens* are shown in red; other elements important for the potential function of the T4SS are depicted in blue or orange. Lane M, 1 kb ladder; lane 1 donor strain P12AT4SS; lane 2, recipient strain P12AT4SS; lane 3, P12 wt strain; lane 4: water control.

doi:10.1371/journal.pone.0045623.g003
Therefore generated a recA mutation in the donor strains making them unable to integrate chromosomal markers (Fig. 1B).

Generally, plasmid mobilisation is defined by the DNaseI-resistant transfer of DNA and its dependence on a functional T4SS, mediating the conjugative transfer. Classical conjugative transfer is also dependent on a functional relaxase, which nicks one strand of the plasmid DNA at a specific sequence in the mob site. Rolling circle replication elongates the DNA strand and the relaxase bound to the 5'-end of the single-stranded DNA then mediates the specific transfer of the nucleoprotein complex into the T4SS and the recipient cell [27].

We show here for the first time that the comB T4SS, which up to now has been associated with natural transformation competence only, can accomplish DNaseI-resistant plasmid DNA transfer as well. We identified two distinct mechanisms of DNaseI-resistant plasmid transfer, one mechanism being dependent on a functional plasmid-encoded relaxase [plasmid mobilisation] and one transfer mechanism completely independent of any relaxase, and even any T4SS, but nevertheless resistant to DNaseI [ADR]. Conjugative transfer of H. pylori shuttle vectors carrying an RP4 oriT sequence has been reported to occur between different H. pylori isolates [28].

These plasmids did not carry a relaxase gene on the plasmid, but contained an origin of transfer (oriT) from the broad host range plasmid RP4. Conjugative transfer of these plasmids was reported to be dependent on a chromosomal relaxase gene (rlx1, hp0996) and the coupling protein HP1006 [28]. Plasmid transfer with the endogenous plasmid pHel12, which carries a H. pylori-specific relaxase gene and no RP4 oriT, has been shown here to be independent of chromosomal relaxases.

The comB T4SS is well established as being absolutely necessary for transformation-mediated DNA uptake into H. pylori [9]. Our data show that in addition to transformation, comB is also important for DNaseI-resistant plasmid DNA transfer between H. pylori strains, generally designated as conjugative transfer or mobilisation. First, a comB deletion in the recipient strain significantly reduced plasmid DNA transfer rates, both for transformation and DNaseI-resistant transfer (Fig. 4B, columns 11–16). Second, a moeA deletion in pHel12 resulted in a significant reduction of DNA transfer (Fig. 1C), whereas other T4SS did not have any effect. Thus, we showed here for the first time that a comB-dependent mobilisation of pHel12 plasmid is an obvious pathway of plasmid transfer between H. pylori.

Table 1. Bacterial strains used in this study.

| Strain | Plasmid | Genotype and reference |
|--------|---------|------------------------|
| DH5α  |         | Escherichia coli K12    |
| ATCC43526 |       | H. pylori reference strain, ATCC |
| Hp P1  |         | H. pylori clinical isolate (69A) University Hospital Amsterdam, The Netherlands |
| Hp P1  |         | moeB::aphA3             |
| Hp P8  |         | H. pylori clinical isolate (196A) University Hospital Amsterdam, The Netherlands |
| Hp P8  | pHel12 [mccC::cat] | recA::erm |
| Hp P8  | pHel12 [mobA::cat] | recA::erm |
| Hp P12 | pHel12 | recA::erm; Δfs3 |
| Hp P12 | pHel12 [cat] | recA::erm; Δfs4 |
| Hp P12 | pHel12 | recA::erm; Δfs5 |
| Hp P12 | pHel12 | Δfs6; ΔvirD2 (at3) |
| Hp P12 | pHel12 | Δfs5; ΔvirD2 (at3) |
| Hp P12 | pHel12 | Δfs6; ΔvirD2 (at3) |
| Hp P12 | pHel12 | Δfs5; ΔvirD2 (at3) |
| Hp P12 | pHel12 | Δfs5; ΔvirD2 (at3) |
| Hp P12 | pHel12 [mobA::cat] | Δfs6; ΔvirD2 (at3) |
| Hp P12 | pHel12 | moeB::aphA3 |
| Hp P12 | pHel12 | moeB::aphA3 |
| Hp P12 | pHel12 | moeB::aphA3 |
| Hp P12 | pHel12 | moeB::aphA3 |

doi:10.1371/journal.pone.0045623.t001

Plasmid Transfer in Helicobacter pylori
Surprisingly, a P12 recipient strain without any T4SS (P12ΔT4SS), for which DNA transformation was completely abolished, did not exhibit a significantly different efficiency of DNaseI-sensitive or resistant plasmid transfer rate under contact-dependent co-incubation conditions (Fig. 4B, columns 11/12) than a P12ΔcomB strain (Fig. 4B, columns 13/14). Definite proof for pHel12 plasmid transfer in the absence of any T4SS allowing natural transformation or conjugation was coming from a co-incubation experiment with donor and recipient strains devoid of any T4SS (P12ΔT4SS). Since plasmid transfer could be demonstrated under these conditions, our data prove that plasmid transfer between H. pylori can occur by a novel, hitherto not characterized T4SS-independent, but DNaseI-resistant pathway. We propose to designate this pathway as alternate DNaseI-Resistant pathway (ADR pathway). The mechanism for plasmid DNA transfer via the ADR pathway is unclear and currently investigated in detail in our lab. From the current literature we would envisage two possible mechanisms for plasmid DNA transfer in H. pylori via the ADR pathway, (i) either outer membrane vesicles (OMVs) [29] or (ii) nanotubes [30]. Nanotubes have been recently described as variously sized tubular extensions connecting Gram-positive or Gram-negative bacteria, allowing non-conjugative DNaseI-resistant plasmid transfer between the same or even different bacterial species. The production of OMVs has been described for H. pylori, especially for a potential delivery of proteins (e.g. VacA) or peptidoglycan into host cells [31,32], but not for transfer of plasmid DNA.

Taken together, these data demonstrate that three different mechanisms of plasmid DNA exchange are operating in H. pylori. Whether these different mechanisms are active at the same time, or whether there is a spatial or temporal control for one or the other mechanism, is currently not known. However, it is possible that by knockout of the comB system, e.g. in the donor strain, the ADR pathway may compensate for the defect in plasmid transfer, which might explain the small effect of a P12ΔcomB donor strain on plasmid DNA transfer efficiency (Fig. 4A). Furthermore, our data were exclusively generated under in vitro conditions. The in vitro plasmid transfer rates cannot easily be transferred to the situation of the bacteria under in vivo conditions in the stomach mucosa. It is well possible that one or the other pathway might be turned on or off under in vivo conditions. In a recombination-based in vivo expression technology (RIVET) screen, a promoter in the mG27 H. pylori strain was identified, which turned on the expression of the plasmid-encoded mobA gene under in vivo conditions in the mouse stomach [33], indicating that plasmid transfer might be enhanced when the bacteria are in their natural environment, the stomach mucosa. In the in vivo situation, natural transformation seems to represent the most efficient way of plasmid transfer, followed by conjugative transfer, whereas the ADR pathway contributes only minimally. The relative contribu-

### Table 2. Plasmids used in this study.

| Plasmid     | Characteristics                                                                 | Reference        |
|------------|----------------------------------------------------------------------------------|------------------|
| pAK23      | pMin1, upstream region of comB, downstream region of comB10, separated by aphA-3 cassette, TetR, ErmR | [10]             |
| pBluescript SK+ | orf12GH, orf103, lacZ, M13 forward-reverse primer binding sites, AmpR, KanR | Invitrogen       |
| pCR2.1-TOPO | orf12GH, orf103, lacZ, M13 forward-reverse and T7 promoter/primer binding sites, AmpR, KanR | Invitrogen       |
| pDH29      | pBluescript II K5' recA-erm, AmpR, ErmR | [40]             |
| pJP44 (rpsL-erm) | ΔcagPAI::rpmL-erm | This study       |
| pSR11      | pBluescript II SK rpmL-erm-cassette in BamHI | This study       |
| pSR12      | Deletion of T4SS tsf3::rpmL-erm | This study       |
| pSR13      | Deletion of T4SS tsf3 rpmL-erm deleted | This study       |
| pSR14      | Deletion of T4SS tsf4::rpmL-erm | This study       |
| pSR15      | Deletion of T4SS tsf4::rpmL-erm deleted | This study       |
| pSR18      | Deletion of T4SS of cagPAI; rpmL-erm (pJP44::rpmL-erm) | This study       |
| pSR19      | Deletion of T4SS comB; rpmL-erm (pAK23::rpmL-erm) | This study       |
| pSR20      | pSP76 carrying aphA-3 cassette; in moeB locus | This study       |
| pSR21      | Deletion of virB4::topA; Insertion of rpmL-erm cassette | This study       |
| pSR23      | Insertion of virB4::topA [ATCC43526] | This study       |
| pSR24      | Deletion of orf12GH in pHel12; Insertion of aphA-3 cassette | This study       |
| pSR25      | Deletion of orf12GH in pHel12; Insertion of catGC-cassette | This study       |
| pSR26      | Deletion of orf12C in pHel12; Insertion of catGC-cassette | This study       |
| pSR27      | Deletion of orf12C in pHel12; Insertion of aphA-3-cassette | This study       |
| pSR28      | Insertion of catGC cassette between orf4M and orf4A in pHel12 | This study       |
| pSR29      | Deletion of tsf3 including chromosomal relaxase using rpmL-erm | This study       |
| pSR33      | Deletion of comE3; Insertion of aphA-3-cassette | This study       |
| pWS48      | pBluescript II K5' carrying recA (partial::cat, AmpR, CamR | [40]             |
| pWS102     | Deletion of vacA; Insertion of aphA-3-cassette | This study       |
tion of one or the other pathway under in vivo conditions has to be determined in future.

Materials and Methods

Bacterial Strains and Growth Conditions

In this study, H. pylori strains P8 (originally isolated as 196A) and P12 (originally isolated as 888-0) [34] were used. For routine culture, H. pylori strains were grown on GC agar plates (Oxoid) supplemented with horse serum (8%), vancomycin (10 μg/ml), trimethoprim (5 μg/ml), and nystatin (1 μg/ml) (serum plates). Erythromycin (10 μg/ml), chloramphenicol (6 μg/ml), kanamycin (8 μg/ml), and streptomycin (250 μg/ml) were added to select for transformants or screen colonies for resistance to either drug. Inoculated plates were incubated for 24 to 48 h under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) at 37°C. Escherichia coli DH5α was grown on Luria-Bertani (LB) agar plates or in LB liquid medium [35] supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), crythromycin (250 μg/ml), or kanamycin (40 μg/ml), as appropriate.

Electroporation, Natural Transformation and Conjugation of H. pylori

Transformation of H. pylori strains was performed with plasmid or chromosomal DNA as described earlier [34]. Bacteria were harvested from serum plates and suspended to an optical density at 550 nm (OD₅₅₀) of 0.2 in Brucella broth (BB) containing 10% fetal calf serum. DNA was added (1 μg), and incubation was extended for 4 h under microaerophilic conditions before the suspension was plated on selective serum plates. For electroporation of H. pylori, bacterial cells were harvested from serum plates and suspended in 1 ml of phosphate-buffered saline (PBS) solution. For each electroporation, bacteria were diluted to an OD₅₅₀ of 1.
and 1 ml bacterial suspension was washed twice with PBS and suspended first in 500 μl and then in 40 μl electroporation buffer [36]. Forty microliters of *H. pylori* competent cells was mixed with 1 to 2 μl DNA in pre-chilled 0.2 cm electroporation cuvettes. Electroporation was performed at 2.5 kV, 200 Ω, and 25 μF by a Gene Pulser (Bio-Rad, Munich, Germany). After electroporation, 1 ml of BB containing 10% fetal calf serum was added immediately to each sample. The aliquots were incubated for 1 h in a CO₂ incubator before being plated on selective agar plates.

**DNA Manipulations and Plasmid and Strain Constructions**

Cloning and DNA analysis procedures were performed according to Sambrook et al. [35]. Chromosomal DNA from *H. pylori* was isolated with the QIAamp tissue kit (QIAGEN, Hilden, Germany). Plasmid DNA was purified from *E. coli* using the QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany).

Deletion and replacement of genes or genomic regions were achieved using the streptomycin susceptibility counterselection strategy [25]. A *tpsl-erm* cassette cloned into the *BamHI* restriction site of pBluescript IISK was used for all cloning procedures. For the deletion of the *TFS3* system, the corresponding upstream and downstream regions were amplified by PCR using primers SR17/SR18, and SR30/SR35, respectively (Table S1). Likewise, *tsf4* upstream and downstream regions were amplified using primers SR13/SR14 and SR32/SR34, respectively. Upstream and downstream regions were cleaved with *XhoI/ClaI* and *NdeI/SceI*, respectively, and cloned with or without the *tpsl-erm* cassette into the corresponding sites of pBluescript IISK+.

To inactivate the ComB system, *comB6-comB16* were deleted using the *tpsl-erm* system and plasmid pAK23 [10]. For deletion of the *cag-PAI*, a plasmid containing the left and right flanking gene regions of the *cag-PAI* [37], was modified by insertion the *tpsl-erm* cassette. To monitor plasmid transfer between *H. pylori* strains, a chloramphenicol resistance cassette (*erm*) was inserted between *orf4M* and *orf4K* in *Hel12* using an inverse PCR reaction with primers SR33 and SR34. The *catGC* cassette (pWS48) was cloned via a *BamHI* restriction site. Homologous recombinination in plasmid *Hel12* resulted in *Hel12-catGC*.

For deletion of the plasmid-encoded relaxase of *Hel12*, a plasmid from a shotgun library was used as template and after inverse PCR using primers SR41/SR52, the *catGC* cassette was inserted via a *BamHI* restriction site. In *Hel12* the *catGC* cassette was inserted within *orf45* to disrupt the corresponding gene. To delete the chromosomal relaxases within *tsf3* and *tsf4*, plasmids pSR29 and pSR31 were generated using primer pairs SR17/ SR18 and SR69/SR70 (tsf3/Rel deletion), or SR71/SR72 and SR28/SR34 (tsf4/Rel deletion). As already described above, the *tpsl-erm* counterselection strategy was applied to generate marker-free *H. pylori* mutant strains.

**Mating Experiments**

To monitor transfer of the donor plasmid into the recipient strain, donor strains were provided with a *catGC* cassette in the plasmid. For selection of the recipient strain, either an *aphB-3* cassette was inserted into the recipients chromosomal *moaB* locus, using plasmid pSP76-aphB-3, or a streptomycin resistant *P12* mutant (pEG21) was used. To prevent transformation of donor strain with chromosomal DNA from recipient strain, the *recA* gene was deleted in the *H. pylori* donor strains by transformation with...
the plasmid pH29 (recA::erm). Donor and recipient strains were harvested after 24 h of growth on selective GC agar plates and suspended in 1 ml of BB. For mating experiments between H. pylori 1.5 × 10⁷ cells were used. 25 μl donor and 25 μl recipient cell suspension were either mixed with 25 μl DNaseI (Roche; 1 mg/ml in 20 mM Tris-Cl (pH 7.5), 1 mM MgCl₂) each in separate tubes and pre-incubated for 30 min at 37°C and 10% CO₂, or left untreated. Subsequently donor and recipient cells were mixed, collected at 1000 g for 5 min and resuspended either in 50 μl DNaseI or 50 μl BB. The cells were spotted onto non-selective GC agar plates directly and incubated over night at 37°C under microaerobic conditions. After incubation the cells were harvested and resuspended in 1 ml BB. The cell suspension was serially diluted and 100 μl were plated on GC agar plates containing chloramphenicol to determine the CFU/ml of the donor strain and 100 μl and harvested ~800 μl cell suspension were plated on appropriate double selective GC agar plates (chloramphenicol and kanamycin or streptomycin) to determine the number of transconjugants. The plasmid transfer rates were determined by colony counting and are presented as numbers of transconjugants per donor cfu. Plasmid sequence accession numbers: pHel14: NC_004950; pHel12: CP001218.

PCR-based RAPD Fingerprinting

To differentiate between donor (P8) and recipient (P12) H. pylori strain in mating experiments a RAPD DNA fingerprinting method was applied [38]. PCR was carried out in 25 μl containing 20 ng of H. pylori genomic DNA, 3 mM MgCl₂, 20 pmoles of primer, 1 U of Takara® Taq DNA polymerase, 250 μM each of dCTP, dGTP, dATP and dTTP. The cycling program was four cycles of [94°C, 5 min; 40°C, 5 min; and 72°C, 5 min; low stringency amplification], 30 cycles of [94°C, 1 min; 55°C, 1 min; and 72°C, 2 min; high stringency amplification], and a final incubation at 72°C for 10 min.

Statistical Analysis

The values shown are means ± SD from at least three independent experiments. Students t-test was used to analyze the data. P values are indicated in the figures and were considered significant if they were <0.05.
Supporting Information

Table S1 Oligonucleotide Primers used in this study. Different types of oligonucleotide primers are listed which were used for amplification of genes for cloning purposes, for verification of chromosomal deletions (e.g. complete T45S systems) or for sequencing. The cloning procedures are described in the methods section.

References

1. Suerbaum S, Michetti P (2002) Helicobacter pylori infection. N Engl J Med 347: 1175–1186.
2. Peek RM, Blaser MJ (2002) Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2: 28–37.
3. Suerbaum S, Smith JM, Bapumia K, Morelli G, Maynard Smith J, et al. (1998) Free recombination within Helicobacter pylori. Proc Natl Acad Sci USA 95: 12619–12624.
4. Falsch D, Kraft C, Taylor NS, Correa P, Fox JG, et al. (2001) Recombination and mutation during long-term gastric colonization by Helicobacter pylori. Estimates of clock rates, recombination size, and minimal age. Proc Natl Acad Sci USA 98: 15056–15061.
5. Suerbaum S, Jostenhaus C (2007) Helicobacter pylori evolution and phenotypic diversification in a changing host. Nat Rev Microbiol 5: 441–452.
6. Lin EA, Zhang XS, Levine SM, Gill SR, Falush D, et al. (2009) Natural transformation of Helicobacter pylori involves the integration of short DNA fragments interrupted by gaps of variable size. PLoS Pathog 5: e1000337.
7. Nedenkov-Sorensson P, Buhkholm G, Boye K (1990) Natural competence for genetic transformation in Campylobacter pylori. J Infect Dis 161: 356–366.
8. Hofreuter D, Odenbreit S, Henke G, Haas R (1998) Natural competence for DNA transformation in Helicobacter pylori: identification and genetic characterization of the comB locus. Mol Microbiol 28: 1027–1038.
9. Hofreuter D, Odenbreit S, Haas R (2001) Natural transformation competence in Helicobacter pylori is mediated by the basic components of a type IV secretion system. Mol Microbiol 41: 379–391.
10. Karmohl A, Heidler C, Odenbreit S, Fischer W, Hofreuter D, et al. (2006) Functional and topological characterization of novel components of the comB DNA transformation competence system in Helicobacter pylori. J Bacteriol 188: 892–893.
11. Dorer MS, Fero J, Salama NR (2010) DNA damage triggers genetic exchange in Helicobacter pylori. PLoS Pathog 6: e1000266.
12. Penfold SS, Lastovica AJ, Elsila BG (1988) Demonstration of plasmids in Campylobacter pylori. J Infect Dis 157: 850–851.
13. Hofreuter D, Haas R (2002) Characterization of two cryptic Helicobacter pylori plasmids: a putative source for horizontal gene transfer and gene shuffling. J Bacteriol 184: 2753–2766.
14. Kleanthous H, Clayton GL, Tabaschali S (1991) Characterization of a plasmid from Helicobacter pylori encoding a replication protein common to plasmids in Gram-positive bacteria. Mol Microbiol 5: 2357–2369.
15. Song JT, Park SG, Kang HL, Lee WK, Cho MJ, et al. (2003) pH499, a Helicobacter pylori small cryptic plasmid, harbors a novel gene coding for a replication initiation protein. Plasmid 50: 236–241.
16. de Ungria MC, Kolenikov T, Cox PT, Lee A (1999) Molecular characterization and interstrain variability of pHS1, a plasmid isolated from the Sydney strain (SS1) of Helicobacter pylori. Plasmid 41: 97–109.
17. Höller C, Fischer W, Hofreuter D, Haas R (2004) Cryptic plasmids in Helicobacter pylori: putative functions in conjugative transfer and microcin production. Int J Microbiol 2004: 141–148.
18. Kuipers EJ, Israel DA, Kusters JG, Blaser MJ (1998) Evidence for a conjugation-like mechanism of DNA transfer in Helicobacter pylori. J Bacteriol 180: 2901–2905.
19. de la Cruz F, Frost LS, Meyer RJ, Zeicher EL (2010) Conjugative DNA metabolism in Gram-negative bacteria. FEMS Microbiol Rev 34: 18–40.
20. Fischer W, Wüthrich L, Rohrer S, Zeilier M, Karmohl A, et al. (2010) Strain-specific genes of Helicobacter pylori: genome evolution driven by a novel type IV secretion system and genomic island transfer. Nucleic Acids Res 38: 6089–6101.
21. Boyd AC, Archer JA, Sherratt DJ (1989) Characterization of the CroE1 mobilization region and its protein products. Mol Gen Genet 217: 480–489.

Acknowledgments

The authors are grateful to Luisa Jimenez-Soto for critical reading of the manuscript and helpful discussions, and to Douglas Berg for the gift of the rpsL-erm counterselection system.

Author Contributions

Conceived and designed the experiments: RH SR WF. Performed the experiments: SR LH EW. Analyzed the data: SR LH WF RH. Contributed reagents/materials/analysis tools: MB. Wrote the paper: SR RH.