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Genetic Organization and Conjugal Plasmid DNA Transfer of pHP69, a Plasmid from a Korean Isolate of *Helicobacter pylori*

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We isolated pHP69, a 9,153 bp plasmid from *Helicobacter pylori* with a 33.98% (G+C) content. We identified 11 open reading frames (ORFs), including replication initiation protein A (*repA*), *fic* (cAMP-induced filamentation protein), *mccC*, *mccB*, *mobA*, *mobD*, *mobB*, and *mobC*, as well as four 22 bp tandem repeat sequences. The nucleic acid and predicted amino acid sequences of these ORFs exhibited significant homology to those of other *H. pylori* plasmids. pHP69 *repA* encodes a replication initiation protein and its amino acid sequence is similar to those of replicase proteins from theta-type plasmids. pHP69 contains two types of repeat sequences (R1 and R2), a MOB_{HEN} family mobilization region comprising *mobC*, *mobA*, *mobB*, and *mobD*, and genes encoding microcin B and C. Among the 36 *H. pylori* strains containing plasmids, *mobA* or *mccBC* are present in 12 or 6, respectively and 3 contain both genes. To examine intrinsic capability of *H. pylori* for conjugative plasmid transfer, a shuttle vector pBHP69KH containing pHP69 and replication origin of pBR322 was constructed. It was shown that this vector could stably replicate and be mobilized among clinical *H. pylori* strains and demonstrated to gene transfer by natural plasmid.

Keywords: *Helicobacter pylori*, pHP69, relaxase, conjugation

Introduction

Helicobacter pylori is a Gram-negative, spiral shaped, microaerophilic bacterium that is a causative agent of human chronic gastritis, gastro-duodenal ulcers and gastric cancer (Marshall and Warren, 1983; Parsonnet *et al*., 1991). RFLP of Korean *H. pylori* clinical isolates indicated extensive genetic diversity (Lee *et al*., 1995) and the differences between strains 26695 and J99 included several large genomic inversions. This diversity is thought to arise through frequent recombination events, mutation and impaired DNA repair (Alm and Trust, 1999; Occhalini *et al*., 2000; Kersulyte *et al*., 2003). Although the extent of horizontal DNA transfer in *H. pylori* via transmissible plasmids remains unclear, recent reports have indicated that some conjugation may occur (Kuipers *et al*., 1998; Christie and Vogel, 2000; Backert *et al*., 2005).

Many plasmids have been isolated from various *H. pylori* strains (Heuermann and Haas, 1995; Lee *et al*., 1995; Minnis *et al*., 1995; de Ungria *et al*., 1998; de Ungria *et al*., 1999; Quiñones *et al*., 2001; Hofreuter and Haas, 2002; Hosaka *et al*., 2002); some are small and cryptic, containing a single open reading frame (ORF), while others are large and have many genes that encode proteins such as those required for mobilization or antibiotic synthesis (de Ungria *et al*., 1999; Quiñones *et al*., 2001). The idea that plasmids may mediate recombination between different *H. pylori* strains is supported by recent reports identifying genes in *H. pylori* plasmids that are required for conjugal DNA transfer (Kuipers *et al*., 1998; Backert *et al*., 2005). In addition to other ORFs, all of these plasmids contain a gene encoding a replication protein and a Rep binding site comprising tandem direct repeats or "iterons" (Chattoraj, 2000). Here, we show the genetic organization of pHP69, a 9,153 bp cryptic plasmid isolated from the Korean strain of *H. pylori*. This plasmid contains genes encoding proteins for mobilization (*mob*) and antibiotic synthesis (*mcc*), and exhibits conserved features common to *H. pylori* plasmids isolated from western regions as well as Korean clinical isolates.

Materials and Methods

Bacterial strains and culture conditions

H. pylori strain 69 that was from a Korean patient with chronic gastritis and other clinical isolates used in this study were obtained from the *H. pylori* Korean Type Culture Collection (http://hpktcc.knrrc.or.kr). Cells were cultured as described previously (Rhee *et al*., 1988). Frozen cells were thawed and streaked onto *Brucella* agar (BA) containing vancomycin (10 μg/ml), nalidixic acid (25 μg/ml), amphotericin B (5 μg/ml), and 10% bovine serum, then incubated at 37°C, under 5% O_2, 10% CO_2, and 100% relative humidity. One loop of overnight culture was used to inoculate *Brucella* agar plates enriched with 10% bovine serum, which were then grown overnight under the conditions described above.

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Plasmid DNA isolation, recombination techniques, and DNA sequence analysis

Natural *H. pylori* plasmids were isolated by alkaline lysis and purified using the Qiagen plasmid purification kit (Qiagen, USA). Purified pHP69 was linearized with *Hin*dIII, then cloned into pBluescript II SK(+). All clones and PCR fragments were sequenced (Bionex Co. Ltd, Korea).

Identification of *mob*A and *mcc* in plasmids isolated from different *H. pylori* strains using Southern hybridization

Plasmids (40 ng) were prepared from 36 clinical isolates of *H. pylori*, then spotted onto a Hybond-N® membrane (Amersham Pharmacia Biotech, USA). The plasmids pHP69 and pHP51 were included as positive and negative control, respectively. We PCR-amplified conserved sequences from *mobA* and *mccBC* (152 and 439 bp, respectively) using two sets of primers (*mobA* Forward, 5′-GGGGCATACTTYCK CATCAT-3′ and Reverse, 5′-TYYCCTTATTTCTTGGTCG G-3′; and *mccBC* Forward, 5′-GGGTAGATAAGGATACC GTTGATCCC-3′ and Reverse, 5′-GATGGTG CCTGCAT GCGGTCATTTAT-3′). Probes were labeled using the ECL Direct Nucleic Acid Labeling and Detection kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

Construction of shuttle vectors for conjugation

Kanamycin resistance marker (Km) was PCR-amplified from pBHP489K (Song *et al.*, 2003b) using the following primer set (km1; AACCCAGCGAACCATTTGAGGTGA, km2; G GGATATCAAGCTAGCTTTTTAGACA) and inserted into the Klenow-treated *Nde*I site of pBR322. The 2.3 kb fragment (ori-Km fragment) harboring the replication origin of ColEI and the Km marker was amplified (orf2; GGGAC TGAGCGTCAGACCCCGTAGAA, km2; GGGATATCAA GCTAGCTTTTTAGACA). Two parts of the pHP69 *mcc* were amplified using the following two primer sets (mcc1stF; GGGTAGATAAGGATACCGTTGATCCC, mcc1stR; GAT GGTGCCTGCATGCGGTCATTTAT, mcc2dnF, GGCAA GCCTACTTTCTAGAC, mcc2ndR; CCCGGGTGGTAGG AATAATCGTTT) to generate 442 bp and 533 bp segments, respectively. Two fragments were ligated and amplified using the primers mcc1stR and mcc2dnF to yield the mcc fragment). The ori-Km fragment and the *mcc* fragment were ligated in order to generate pBKmcc. This vector was then digested with *Sma*I, which was located in the middle of the *mcc* fragment, and introduced into *H. pylori* 69. After screening the kanamycin-resistant colonies, the plasmid was isolated and transformed into *E. coli* DH10B/r. The purified plasmid was fingerprinted with the restriction enzyme, *Xba*I. We designated this recombinant plasmid pBHP69KH. pBHP69KH was digested with *Swa*I, 7,494 bp fragments was eluted and relegated to generate pBHP69KH*mob*$^-$ (Table 1).

Construction of chloramphenicol-resistant mutant strain of *H. pylori*

A pBluescript II SK containing the *H. pylori* gamma-glutamyl transpeptidase (*ggt*) ORF was used to construct a chloramphenicol-resistant mutant (Kim *et al.*, 2007). pBluescript/GGT3 which contains whole ORF of *H. pylori ggt* was linearized via treatment with *Hin*dIII, which was located in the middle of *ggt* ORF, and flushed both of its termini via Klenow enzyme treatment and ligated with a blunt-ended chloramphenicol-resistant marker (Cm). This vector was transformed into *H. pylori* strain 219. Some colonies (*H. pylori* 219Cm) grown on the chloramphenicol-containing Brucella agar plates were cultured and assessed via PCR to determine the homologous recombination of the plasmid into the genome by the primers (GGT4F; AACAGATGAGACG GAGTTTTTTGAA, GGT4R; CTCGAGAAATTCTTTCCT TGGATCCGTTGA) (Table 1).

Conjugation experiment with *H. pylori*

A donor strain, *H. pylori* 219, which harbors pBHP69KH or pBHP69KH*mob*$^-$ and a recipient *H. pylori* 219Cm, was cultured on Brucella agar for 12 h. Conjugation was performed as described previously (Balzer *et al.*, 1994). Cells were harvested and suspended in 1 ml Brucella broth. Aliquots (contained 10^9 *H. pylori* of each strain) of cultures of the parent strains and mixed cultures were filtered by a 0.45 μm pore-size membrane (Millipore, USA). The membranes were incubated for 12 h at 37°C, under 5% O_2, 10% CO_2 as follows; 1, donor *H. pylori* 219 containing pBHP69KH alone; plate 2, recipient *H. pylori* 219Cm alone; plates 3, donor *H. pylori* 219 containing pBHP69KH and recipient *H. pylori* 219Cm; plate 4, donor *H. pylori* 219 containing pBHP69KH*mob*$^-$ and recipient *H. pylori* 219Cm; plate 5, the heat-inactivated

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmid	Genotype or phenotype	Reference or source
E. coli		
DH10B	F$^-$ *end*A1 *rec*A1 *gal*E15 *gal*K16 *nup*G *rps*L Δ*lac*X74 Φ80*lac*ZΔM15 *ara*D139 Δ(*ara,leu*)7697 *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) λ$^-$	Durfee *et al.* (2008)
H. pylori		
69	Wild type	This study
219	Wild type	This study
219Cm	*ggt*::Cm	This study
Plasmid		
pBluescript SK II	Amp	Alting-Mees and Short (1989)
pHP69	Wild type, *mob*	This study
pBHP69KH	ColEI$_{ori}$ *mob* Km	This study
pBHP69KH*mob*$^-$	ColEI$_{ori}$ *mob*-defective Km	This study
pBK*mcc*	ColEI$_{ori}$ Km contain 985 bp of *mcc*	This study

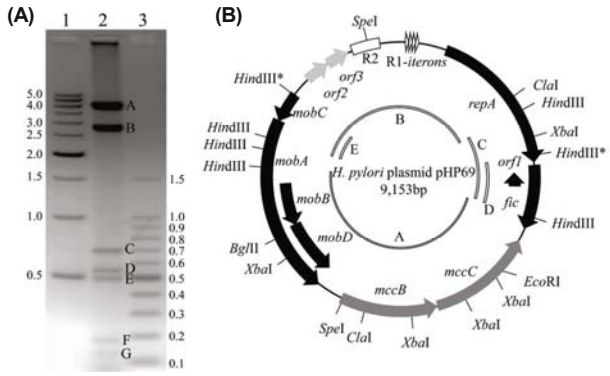

Fig. 1. (A) Restriction analysis of pHP69 fragments performed using 1.0% agarose gel electrophoresis. 1, 500 bp ladder; 2, pHP69 digested with *Hind*III; 3, 100 bp ladder. (B) Physical and genetic map of pHP69 indicating the location and orientation of 11 ORFs and 2 conserved sequences. H, *Hind*III.

donor *H. pylori* 219 containing pBHP69KH and recipient *H. pylori* 219Cm, plate 6, cell free extract of donor *H. pylori* 219 containing pBHP69KH and recipient *H. pylori* 219Cm; plates 7, 1 μg of purified pBHP69KH and recipient *H. pylori* 219Cm. Cells on each membrane were harvested in separate 1 ml aliquots of Brucella broth, plated on the Brucella agar plate containing kanamycin and chloramphenicol. All plates were incubated for 72 h, after which the number of single colonies was counted.

Results

Cloning and sequence analysis of pHP69

The digestion of pHP69 with *Hind*III generated seven fragments: A (4.2 kb); B (2.9 kb); C (0.7 kb); D (0.6 kb); E (0.5 kb); F (0.18 kb); and G (0.13 kb; Fig. 1A). All fragments were cloned into pBluescript II SK(+) at the *Hind*III site, and then sequenced. Fragment A harbored *mobA*, which encodes for the relaxase protein and the 3′-end of *fic*, which encodes for a cAMP-induced filamentation protein. Fragment B contained the middle region of *repA* and the promoter of *mobA*. Fragments C, D, and E harbored *fic*, *repA*, and *mobC*, respectively. Both fragments F and G harbored a part of *mobA*. In an effort to connect seven sequences, we designed the sequencing primers from both ends of the cloned pHP69 fragments (Table 2).

The 9,153 bp sequence of pHP69 contained directed repeat sequences and eleven ORFs, including *repA* (1,617 bp), *fic* (720 bp), the microcin genes *mccB* (1,053 bp) and *mccC* (1,164 bp), the relaxase *mobA* (1,983 bp), *mobB* (531 bp), *mobC* (345 bp), *mobD* (702 bp), *orf1* (153 bp), *orf2* (279 bp), and *orf3* (243 bp) (Fig. 1B and Table 3). The (G+C) content of pHP69 was 33.98%, lower than that of the *H. pylori* genome (strains 26695 and J99 are 38.87 and 39.19%, respectively).

Table 2. List of the oligomers for confirming the sequence of the pHP69

Name	Sequence
conA2	GGTTTTAGGTTCGTTGGGTAGC
conB1	GACAGGCGCAAAGTAACGCATA
conC1	GTGATAGCGCCACAACAAGA
conC2	GGCATGCATGTCCTTTCAAG
conD1	CATCGCCTCCCCCTTGATTAAT
conD2	GTGATGGGAGCGTTAATTACTTGC
conE1	CGGATCGTGAAACTGAACGA
conE2	ATGTCTTGTTGGACACACGC
conF2	GCACAACATACAACAACACC
conG1	CTTGTCAGTGTGTTCTACCC

Nucleotide sequence accession number

The whole nucleotide sequence of pHP96 has been deposited in the GenBank database under accession number DQ915941.1.

Replication initiation protein

The plasmid pHP69 contained *repA*, which encodes a replication initiation protein. The 1,617 bp *repA* sequence exhibited a high level of homology (>88%) with theta-type plasmids of *H. pylori* such as pHPS1 (de Ungria *et al.*, 1998), pHel5 (Hofreuter and Haas, 2002), pHPM180 (Minnis *et al.*, 1995), pHPM8 (Quiñones *et al.*, 2001), pHP666 (DQ198799) and pAL226 (DQ239897), suggesting that it replicates in a similar manner. However, the peptide sequence of pHP69 RepA showed no homology to those of rolling circle replicating plasmids such as pHP489 (Song *et al.*, 2003b) or pHPK225 (Kleanthous *et al.*, 1991) and its homology to those of other bacterial genera was <57% (*Campylobacter* pUPTC237 RepA [BAE93259] and staphylococcal pIP1629 RepA [AAD02381]), indicating that *H. pylori* and its plasmids evolved in a physically-isolated habitat.

Conserved repeat sequences

R1 iterons were identified 420 bp upstream of the *repA* start codon in an 89 bp region comprising four 22 nucleotide direct repeats (TTCTTNCNANNNNTANNNGNAN). The R1-*repA* replicon demonstrated significant sequence identity with corresponding regions in other *H. pylori* plasmids, including pHel4, pHPM186, pHPAG1, pHP666, pHPM180, pAL202, and pHPM8 (72, 90, 77, 75, 77, 63, and 81%, respectively). These *iterons* are proposed to function as binding sites for initiation and regulation of plasmid replication (Chattoraj, 2000).

H. pylori plasmids such as pHPM180 and pHPS1 contain two long R2 repeat sequences comprising 357 and 232 bp, respectively. However, pHP69 contains only a single 279 bp R2 sequence, which exhibits a high sequence similarity to the corresponding regions in pHPM8, pAL226, pHPO100, pHPS1, and pHP51 (89, 88, 89, 89, and 90%, respectively). In addition, common region 1 (C1, 188 bp) is a conserved sequence that was identified within R2, and the nucleotide sequences between R1 and R2 exhibited 87 and 89% identity with the corresponding regions in pHPM8 and pAL226, respectively. Although not present in other bacterial plasmids, the R2, C1 and other conserved sequences are found in most *H. pylori* plasmids. Their functions remain unclear.

Region encoding proteins for conjugation

Five *H. pylori* plasmids (pHel4, pAL202, pAL226, pHPAG1,

Table 3. List of ORFs identified in pHP69

orf	Codon position (start-stop)	Molecular mass (kDa)	Proposed function	Identities and percent identity	
repA	0521-2137	63.736	Plasmid replication initiation	RepA of pHPM8 RepA of pHP666	87 90
fic	2172-2891	28.015	Replication associated protein	ORF2 of pHP51 ORF2 of pHPM8	96 95
orf1	2253-2405	5.605	Hypothetical protein	Orf4N of pHel4 ORF7 of pAL202	78 78
mccC	4182-3019	43.867	Peptide exporter	ORF3 of pHPM8 MccC-like protein of pHel4	98 95
mccB	5246-4194	40.013	Peptide modification	ORF4 of pHPM8 MccB-like protein of pHel4	95 95
mobA	7508-5526	78.491	Single strand plasmid DNA transfer	ORF10 of pAL202 MobA-like protein of pHel4	85 83
mobD	6265-5564	27.701	*oriT* recognition-like protein	ORF11 of pAL202 MobD-like protein of pHel4	86 82
mobB	6805-6275	20.666	Formation of *nic*	ORF12 of pAL202 MobB-like protein of pHel4	83 75
mobC	7842-7498	13.278	Formation of *nic*	MobC-like protein of pHel4 ORF13 of pAL202	96 94
orf2	8038-8316	10.720	Hypothetical protein	ORF4G of pHel4 R4 of pHP666	92 89
orf3	8327-8569	9.638	Hypothetical protein	ORF4H of pHel4 R3 of pHP666	92 92

and pHP666) were reported to contain mobilization regions. The deduced amino acid sequences of each of the pHP69 *mob* genes exhibited a high level of identity with those from other *H. pylori* mobilizable plasmids (73–85%), which belongs in the ColEI superfamily. Although the C-terminal region of pHP69 MobA showed no homology with Col factor MobA, the N-terminal half showed 45–47% amino acid identity to MobA from Col factors such as ColA, ColD ColEI, and ColK. Alignment of the N-terminal MobA sequences from pHP69 and other MOB_{HEN} relaxases (Fig. 2) indicated the presence of three conserved motifs, I (catalytic Tyr residue), II (Ser-Phe-X-Glu), and III (H-X-D-Xn-E-X-N).

Region encoding the cAMP-induced filamentation protein

The deduced amino acid sequence encoded by *fic* showed >90% identity with the corresponding sequences from other *H. pylori* plasmids (Table 3). Fic is involved in regulation of cell division and synthesis of PAB (p-aminobenzoate) or folate, and *fic* may be part of the *pab* operon (Komano *et al.*, 1991). This suggests that Fic and cAMP are involved in regulation of cell division via folate metabolism. The Fic protein family contains a central conserved motif (HPFXXGNGR) and retains significant amino acid sequence similarity with bacterial homologs that are encoded genomically.

Other coding regions

pHP69 contains genes encoding microcin synthesis proteins (*mccB* and *mccC*) as well as unknown proteins (*orf1*, *orf2*, and *orf3*). The amino acid sequences of pHP69 *mccB* and *mccC* shared 95% similarity with their homologs in pHel4 and pAL202, respectively. Microcins are a group of low molecular weight peptide antibiotics (<10 kDa) produced by certain members of the Enterobacteriaceae, primarily *E. coli* strains of fecal origin, which inhibit growth of phylogenetically-related genera and species. The cytoplasmic targets of Microcin B and C are DNA gyrase (Vizán *et al.*, 1991) and ribosomes (González-Pastor *et al.*, 1995), respectively. The gene encoding ORF1 is overlapped by *fic*, and its amino acid sequence shared 78% identity with *orf4N* of pHel4 and *orf7* of pAL202. The amino acid sequence encoded by *orf2* exhibits 86–92% identity to those of *H. pylori* mobilizable plasmid genes such as *r4* of pHP666, *orf*4G of pHel4 and *p006* of pHPAG1. It shows 31% identity and 63% similarity to the spermidine/putrescine ABC transporter, permease and the substrate-binding component of *Mycoplasma mycoides* subsp. *mycoides* (CAE76869). The amino acid sequence encoded by *orf3* showed 93–99% identity with the corresponding genes of pHel4, pHPAG1 and other *H. pylori* plasmids. It contains a domain of unknown function (DUF332) that shares 43–45% identity and 67–69% similarity to a number of uncharacterized proteins of about 90 amino acid residues, the RelE/StbE family addiction module toxins of *Desulfitobacterium hafniense* DCB-2 (ZP_01369432) and the plasmid stabilization system of *Sphingomonas* sp. SKA58 (EAT08487)

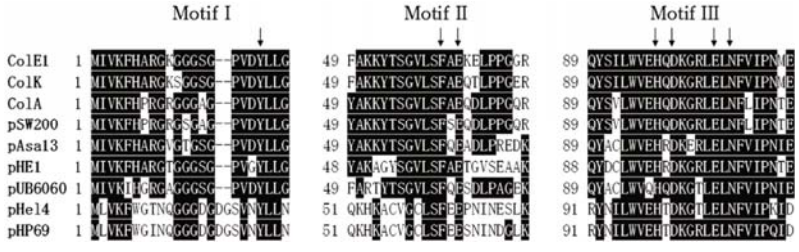

Fig. 2. Amino acid sequence alignment of pHP69 MobA and related proteins. The figure shows a CLUSTALW alignment of the N-terminal sequences from pHP69 MobA and MOB_{HEN} family relaxases. The arrows indicate locations of conserved residues in the three conserved motifs of MOB_{HEN} family relaxases. Accession numbers of the MobA proteins are as follows: ColE1 (J01566); ColK (AY929248); ColA (M37402); pSW200 (L42525); pAsa13 (NC_004340); pHE1 (AJ243735); pUB6060 (AJ249644); and pHel4 (AF 469112).

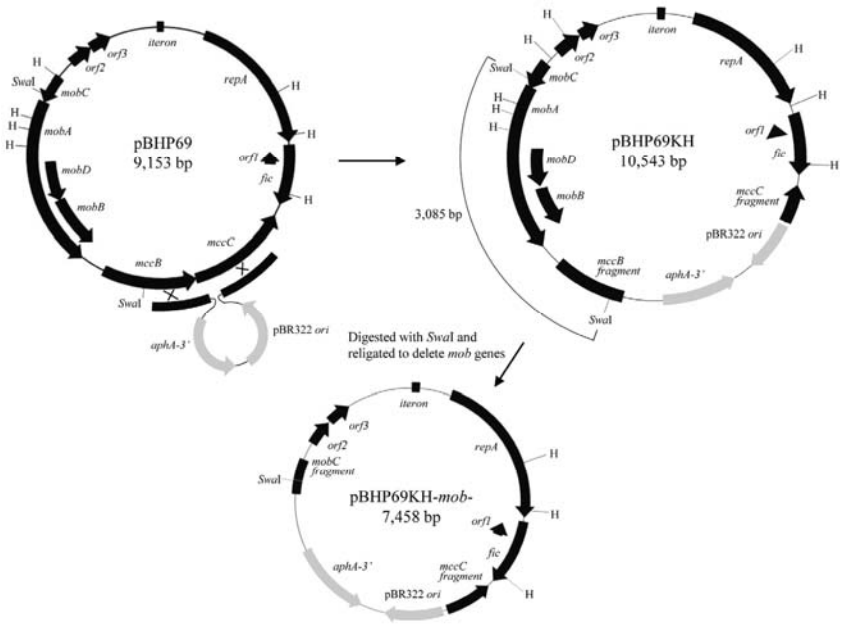

Fig. 3. Construction of conjugative plasmid. H, *Hin*dIII.

Identification of *mob*A and *mcc* in plasmids isolated from different *H. pylori* strains

Southern hybridization was used to identify the presence of *mobA* and *mccBC* in *H. pylori* plasmids. The *mobA* and *mccBC* probes were hybridized to *H. pylori* plasmids from 36 clinical isolates, which contained one or more plasmids. One third (15 strains) of the *H. pylori* isolates contained plasmids with *mobA*, 3 of these also contained *mccBC*, and 9 isolates had *mccBC*-containing plasmids (data not shown).

Mobilization of recombinant plasmid DNA of *H. pylori* by conjugation

In order to assess the intrinsic capabilities of *H. pylori* for conjugative plasmid transfer, the shuttle vector pBHP69KH, which harbors pHP69, was constructed (Fig. 3). To facilitate *H. pylori* transformation and propagation of the plasmid, the shuttle vector pBHP69KH isolated from *H. pylori* 69 was introduced into the highly competent *H. pylori* strain 219. The transformant 219(pBHP69KH) was used as a donor in mating experiments with 219Cm, in which the gamma-glutamyl transpeptidase gene was disrupted by a chloramphenicol-resistant gene (*ggt*::Cm) (Table 1). The plasmid transfer frequency was calculated via the quantitation of the number of Km^r/Cm^r double-resistant recombinants per parent (Table 4). The Km^r/Cm^r progeny colonies were then analyzed by PCR for *ggt* and plasmid DNA isolation. The isolated plasmid was transferred to *E. coli* DH10B/r and demonstrated by restriction enzyme fingerprinting, due to the difficulty of cutting of plasmids isolated from *H. pylori*. In addition, no KM^r/CM^r recombinants were obtained when each strain was incubated alone, thereby ruling out the possibility that the KM^r/CM^r recombinants arose from spontaneous mutation. Therefore, we conclude that the KM^r/CM^r recombinants were the progeny of a horizontal transfer of pBHP69KH from the donor to the recipient (Table 4). Also, the ratio of natural transformation by cell-free plasmids from donors was assessed in the following three types: 1) purified 1 μg plasmid from donor, 2) heat-inactivated cells and 3) cell-free extract (three freezing and thawing cycles). We obtained natural transformants from 1) and 3). Almost no natural transformants were not obtained from 2) which indicated conjugal plasmid transfer needed plasmid-encoded gene(s) (*mob*) and chromosomally-encoded machinery.

It was shown that transfer efficiency, using a pBHP69KH-*mob*$^-$ was just 6.7% as compared with pBHP69KH.

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

H. pylori donor	No. of 219Cm transconjugants per donor
219(pBHP69KH)	1.2×10^{-4}
219(pBHP69KH *mob*$^-$)	1.8×10^{-5}
1 μg of purified pBHP69KH from 219	4.5×10^{-5}
Heat inactivated 219(pBHP69KH)	$<1.0\times10^{-9}$
Cell free extract of 219(pBHP69KH)	8.3×10^{-5}

Discussion

Previously, we demonstrated that ca. 77% of Korean *H. pylori* clinical isolates contain one or more plasmids, ranging between 1 and >60 kb in size (Lee *et al*., 1997). Two small plasmids (pHP489 and pHP51) isolated from Korean *H. pylori* strains were found to contain ORFs (one and two), respectively involved in replication initiation (Song *et al*., 2003a, 2003b). In this study, we isolated plasmid pHP69 of about 10 kb, included functional genes, from *H. pylori* 69 and assayed the sequence. In result, we identified containing eleven ORFs and one iteron. These genes show a high degree of homology at both the nucleotide and amino acid sequence levels (>89 and 78%, respectively) with genes on other *H. pylori* plasmids including pHel4 (AF469112), pHPAG1 (CP000242), pAL202 (AY584531), pAL226 (DQ239897), and

pHP666 (DQ198799). However, the amino acid sequence homology between pHP69 proteins and those of other bacterial genera was <60%, suggesting that *H. pylori* or its plasmids have been isolated evolutionarily.

The *H. pylori* plasmids pHel4, pHP666, pAL202, pHPAG1, and pAL226 have been reported to contain mobilization regions. Their organization indicates that they belong to the MOB_{HEN} family, which includes ColA, ColD, ColE1, and ColK. The most interesting feature of pHP69 is its orientation and gene order (Fig. 4). Although these *H. pylori* strains were isolated from geographically-separated areas (Korea, Germany, Italy, and USA), the orientation and order of the R1-repA replicon, *fic* and *mobCABD* remained substantially identical, with the exception of additional genes between *fic* and *mobA*. Instead of *mccBC* in pHP69, the simplest plasmid (pHP666) contains a small hypothetical ORF comprising 44 amino acid residues and an additional putative *repA* gene upstream of *mobC*. The putative *repA* in pHP666 shares no homology with any other *H. pylori* plasmid *repA* genes. However, its amino acid sequence is 50–70% identical to a putative RepA from other bacteria such as *Campylobacter coli* RM2228 (EAL55758) and *Staphylococcus sciuri* (CAE18149). In pHPAG1, an additional R2-R1-repA replicon was located between the R1-repA replicon and *mobA*. When compared to the primary *repA* gene, it showed 95 and 89% homology at the nucleotide and amino acid sequence levels, respectively. In pAL266, the insertion sequence ISHP606 is located between *fic* and *mobA*, and contains four ORFs and a C-terminal region of *H. pylori repA* in which the amino acid sequence is almost identical to that of pHP666 RepA. Apart from an additional four genes between R1-repA and *fic*, the organization of pHel4 and pAL202 was similar to that of pHP69. Thus, the reported mobilizable *H. pylori* plasmids and pHP69 show conservation of genetic organization (ORFs and repeat sequences), suggesting that *H. pylori* and its plasmids evolved separately from other bacterial genera. We expect that these mobilizable plasmids could transfer gene(s) such as ISHP606 or those encoding microcin synthesis to other *H. pylori* strains and possibly other bacterial genera residing in the human stomach. The pHP69 *mobCABD* genes are necessary for relaxosome formation and processing. These mobilization genes are conserved within the MOB_{HEN} family of the ColEI superfamily, which includes ColA, ColD, and ColK (Francia *et al.*, 2004). Although *mobB* and *mobD* are conserved in most of the MOB_{HEN} family, they were overlapped entirely by *mobA* in pHP69. We did not identify a homolog to the fifth *mob* gene of ColEI (*mobE* or *mbeE*) in pHP69. Other plasmids contained a number of genes, including one encoding a mobilizing protein (Hofreuter and Haas, 2002). Although horizontal gene transfer among *H. pylori* strains has been investigated using a chromosomally-encoded relaxase homolog (Rlx) and TraG-like protein and artificial plasmid included oriT of RP4, molecular evidence for conjugation mediated by a plasmid-encoded Rlx were not clear (Backert *et al.*, 2005). We tested conjugal plasmid DNA transfer by using pBHP69KH and pBHP69KH-*mob*$^-$. We confirmed that donor *H. pylori* 219 contained *traG*-like gene but not chromosomally-encoded Rlx by Southern blotting. Efficiency of Rlx-free conjugal DNA transfer by pBHP69KH was higher than that of the *mob*-deletion mutant plasmid, indicating *mob* of pHP69 played critical role for conjugal plasmid transfer between *H. pylori*.

Two sequence repeats R1 and R2 were identified on pHP69 by comparison of reported *H. pylori* plasmids. R1 correspond to "iteron" sequence, located upstream of *repA*. R2 repeat has been suggested to be a target sequence for site-specific recombination (Minnis *et al.*, 1995; de Ungria *et*

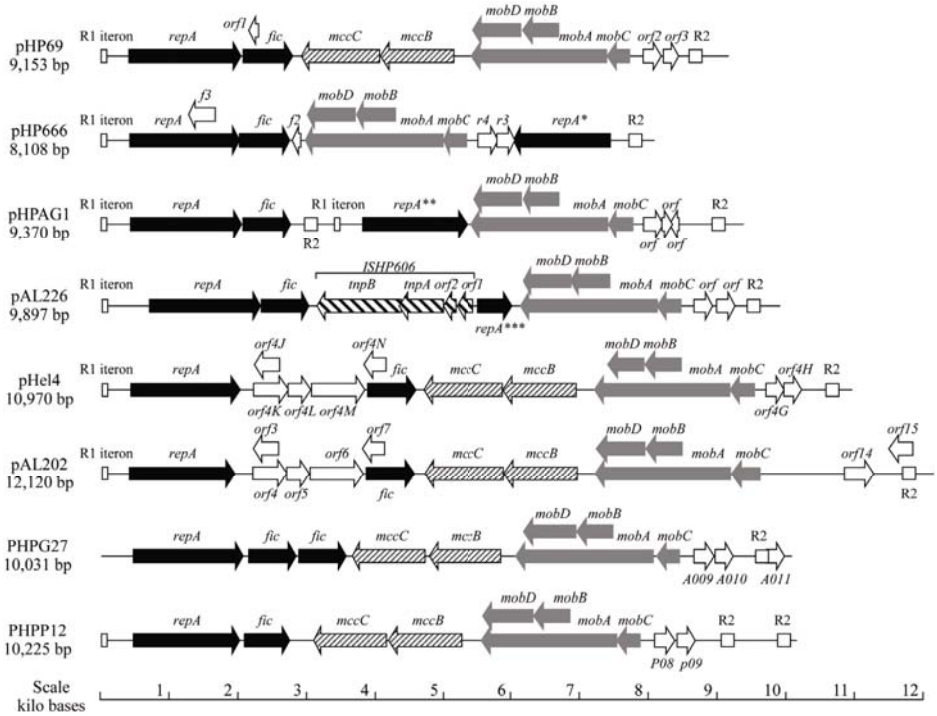

Fig. 4. Genetic organization of *H. pylori* mobilizable plasmids. The location and orientation of ORFs are shown. White arrows indicate putative ORFs. *repA** is a putative replicase. *repA*** exhibits 95 and 89% identity to the primary *repA* gene at the nucleotide and amino acid sequence levels, respectively. The amino acid sequence of *repA**** has 95% identity to the C-terminal region the primary RepA.

al., 1999). Mostly conserved location and direction of repeat sequences and genes in the plasmid were suggested as a "modular structure" that may involved in intra-plasmid recombination or integration event into *H. pylori* chromosome (Hofreuter and Haas, 1995). This characteristic of pHP69 might get some chromosomal genes by recombination or transfer foreign genes by conjugation or natural transformation. The rapid distribution of DNA sequences by conjugative plasmid might be one of good explanation of macrodiversity found among *H. pylori* strains.

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