Complementation System for Helicobacter pylori

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Previously Langford et al. (2006) developed the pIR203C04 complementation system for Helicobacter pylori, which can be used to complement and restore phenotypic effects in H. pylori mutant, and furthermore they used the complementation system in vivo experiments to animals without altering the ability of strain SS1 to colonize mice. In their previous study, the pIR203C04 was able to transform 26695, SS1, J99, and 43504 H. pylori strains by an electroporation method. However, in the present study using a natural transformation the pIR203C04 transformed only 26695 H. pylori but not SS1, J99, 7.13, and G27 H. pylori strains. Since the useful complementation system has a limitation of narrow selection among H. pylori strains, we redesigned the complementation system for the improvement. The same intergenic chromosomal site between hp0203 and hp0204 was utilized for the new complementation system because the insertion at the intergenic site didn't show any polar effects and disruption of other H. pylori genes. The genome sequence analysis showed that the intergenic regions among H. pylori strains may have too low homology to each others to do a homologous recombination. Thus, in addition to the short intergenic region, the fragments of the new complementation system included 3' conserved parts of hp0203 and hp0204 coding regions. Between the fragments there are a chloramphenicol acetyltransferase cassette and multicloning sites, resulting in pKJMSH. DNA fragment of the interest can be cloned into the multicloning sites of pKJMSH and the fragment can be integrated at the intergenic region of H. pylori chromosome by the homologous recombination. Indeed, by the natural transformation, pKJMSH was able to transform all five H. pylori strains of 26695, SS1, J99, 7.13, and G27, which are common for the investigation of molecular pathogenesis. Thus, the new pKJMSH complementation system is applicable to most *H. pylori* wild-type stains.

Keywords: H. pylori, intergenic region, complementation system

Helicobacter pylori is a Gram-negative microaerophilic, spiralshaped bacterium (Marshall and Warren, 1984), which infects over 50% of the world's population, and causes one of the most common infections of mankind (EUROGAST study group, 1993; Matysiak-Budnik and Megraud, 1997). H. pylori is an etiological agent of gastritis, peptic ulcer (duodenal and gastric ulcers), and two types of gastric cancers (adenocarcinoma and mucosa-associated lymphoid tissue lymphoma) (Parsonnet et al., 1991; Dunn et al., 1997; Blaser 1998; Covacci et al., 1999; Ernst and Gold, 2000). H. pylori is classified as class I carcinogen by World Health Organization because of the association of H. pylori and gastric cancer (Neugut et al., 1996; Ernst and Gold, 2000). High prevalence and association of gastric cancer give the second most common cause of cancer-associated death (Neugut et al., 1996). It has been proposed that the virulence factors of the infecting strains are associated with the severity of the clinical outcome. Also, an extensive genetic diversity of H. pylori contributes important in pathogenesis.

Molecular biological research on *H. pylori* pathogenesis is important to find out the mechanism of gastric disease devel-

opment including gastric cancer. Scientists have tried intensively to develop animal models of H. pylori infection, and recently the animal models of mouse and gerbil are available for H. pylori in vivo research (Hirayama et al., 1996; Arnold et al., 2011). However, only certain H. pylori strains, 7.13 for gerbil and PMSS1 for mouse, can be used for in vivo research. Also, the extensive genetic diversity of H. pylori makes the selection of H. pylori strain limited and difficult even for the in vitro research. Other limitation of research on H. pylori pathogenesis would be a lack of useful tools for molecular biology such as the complementation system. Previously Langford et al. (2006) developed the pIR203C04 complementation system for H. pylori. They complemented, and restored phenotypic effects in arginase mutant H. pylori, and furthermore used the pIR203C04 complementation system in vivo experiments to animals without altering the ability of strain SS1 to colonize mice. When they used an electroporation method to introduce the pIR203C04 complementation system, of H. pylori strains 26695, SS1, J99, and 43504 could be transformed. Another transformation method is a natural transformation that is the most common for H. pylori research because a special equipment is not required unlike the electroporation. Unfortunately, the natural transformation of pIR203C04 failed with G27 and 7.13 H. pylori strains and it gave us a rationale to redesign the complementation system to be appli-

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Primer	Sequence				
IR1	GGgageteAAGTCGCAAAGCAGTTATTGCAGCG				
	a				
IR2	AAAATATTAActcgagCCTggatccTTTATCTATACTTTACTTGG				
	b c				
IR3	TATAGATAAAggatccAGGctcgagTTAATATTTTTTTAGTCTGT				
	c d				
IR4	GGgtcgac TTGTGTGGGGGGCAAAGGATTTGG				
	d				
HP 0203-F	GATTGACTTGGGGTTCAGCGTTGGTG				
HP 0204-R	GTTTGAGCTTGCTAATGATAAGCGG				
estriction enzyme sites (a, SacI; b, X	noI; c, BamHI; and d, SalI) were inserted in the primer sequences.				

Table 2. Transformation of pIR203C04

DNA (method)	26695	SS1	J99	7.13	G27	reference
pIR203C04 (Natural transformation)	7.0×10^{3a}	0	0	0	0	This study
pIR203C04 (Electroporation)	2.2×10 ^{-4b}	< 10 ⁻¹⁰	< 10 ⁻¹⁰	NA	NA	Langford et al. (2006)

^a Transformation efficiency

^b Transformation frequency; NA, not applicable

cable to more H. pylori strains containing G27 and 7.13.

Materials and Methods

H. pylori strains and culture conditions

All *H. pylori* strains (26695, G27, 7.13, SS1, and J99) were preserved at -80°C as stocks, grown, and cultured on antibiotic-supplemented horse blood agar plates under microaerophilic conditions created by an Anoxomat evacuation/replacement system (Sprial Biotech, USA) as previously described (Carpenter *et al.*, 2007; Jones *et al.*, 2009, 2011; Jang *et al.*, 2010). For liquid cultures, brucella broth (Acumedia, USA) containing 10% fetal bovine serum (Invitrogen, USA) and 10 μ g/ml vancomycin (AMRESCO, USA) was used and grown under the microaerophilic conditions.

Preparation of plasmid constructs

Chromosomal bacterial DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, USA). PCR amplifications were carried out in a DNA thermal cycler (Biometra, Germany) using the Expand High Fidelity PCR System (Roche Applied Science, Germany) as follows: 1 cycle at 95°C for 2 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; a final elongation step at 72°C for 10 min. Primer sequences are listed in Table 1. An QiaQuick Gel Extraction kit (QIAGEN, Germany) was used for the extraction of PCR fragments from 1% agarose gel. Restriction enzymes of *SacI*, *Bam*HI, *XhoI*, and *SalI* were purchased from Promega (USA). A ligation was performed using Rapid DNA Ligation kit (TaKaRa, Japan). For the transformation of pKJM and pKJMSH to *Escherichia coli*, DH5 α strain was used. Plasmid DNA was isolated by Plasmid DNA Purification kit (iNtRON, Korea).

Natural Transformation for H. pylori

H. pylori was transformed with 1 μ g plasmid by natural transformation as described earlier (Clayton and Mobley, 1997) with the following

modification (Kim *et al.*, 2000, 2009). The *H. pylori* strain was struck and incubated for 3 days. Sweeps of the bacteria colonies from the initial plate were then restruck on a fresh Columbia blood agar plate without chloramphenicol antibiotics and incubated. After 24 h, these cells were inoculated as a circle on a fresh Columbia blood agar plate with four 1-inch scrapes. After 6 h, 1 µg DNA of pIC203C04 and pKJMSH was added to the *H. pylori* circle. After 24 h, the whole circle was restreaked onto Columbia blood agar containing 25 µg/ml chloramphenicol antibiotics and incubated for 3-5 days until transformants appeared. Transformation efficiency was determined by calculating the number of transformant colonies per microgram of DNA (CFU/µg of DNA).

DNA sequence analysis

The DNA sequences were analyzed using Vector NTI version 9.1 (Invitrogen, USA) and Sequencher 4.5 (Gene Codes Corp., USA). The accession numbers of GenBank for 26695, SS1, J99, and G27 were AE000511, DQ538311, AE001439, and CP001173, respectively. The DNA sequence of 7.13 strain was kindly provided by Richard M. Peek Jr. (Vanderbilt University, USA)

Results

Delivery of pIR203C04 complementation system to various *H. pylori* via natural transformation

To determine whether the pIR203C04 complementation system can transform 26695, SS1, J99, 7.13, and G27 *H. pylori* strains, the method of standard natural transformation was used. The

Table 3. Natural Transformation of pKJMSH

Strain DNA	26695	SS1	J99	7.13	G27
pKJMSH	8.7×10^{3}	1.9×10^{3}	0.3×10^{3}	4.1×10^{3}	1.5×10^{4}

						ID 1					
7.13 26695 J99 G27 SS1	1 ATGAAAAAGA ATGAAAAAGG ATGAAAAAGG ATGAAAAAGG	TAATTTTTTT TTGTTTTTTT TTGTTTTTTT TGATTTTTTT	ATTITTAATG ATTIGTTAGTT ATTATTATTC ATTITTAGTG	GTGTTGGGGG ATACTAGGGG ATGCTAGGGG GTGTTGGGGG	GGTTAAAGTC GTTTAGAAGC GTTTAGAAGC G-TTAAAGTC	GCAAAGCAGT GCAAAGTACT GCAAAGTGAT GCAAAGCAGT	TATTGCAGCG TATTGCAGTG TATTGTAGTG TATTGCAGCG	ATTTTTGCGA ATCATTGCGA ATCATTGTGA ATTTTTGCGA	AGGCACTCCA AGGCACGCCA AGGCACGCCA AGGCACGCCA	100 GATAGCCGTA GATAGCCGTA GATAGCCGTA GATAGCCGTA	
7.13 26695 J99 G27 SS1	101 TCCCTCCTAT TCCCTCCTAT TCCCTCCTAT TCCCTCCTAT	GGGGTTTCAT GGGGTTTCAT GGGGTTTCAT GGGGTTTCAT	ТТСАСТТТТС ТТСАСТТТТС ТТСАСТТТТС ТТСАСТТТТС ТТСАСТТТТС	TGCATTCAGT TGCATTCAGT TGCATTCAGT TGCATTCAGT	GAAATATTAT GAAATATTAC GAAATATTAC GAAATATTAC AATATTAT	TTGCAAGATC TTGCAAGATC TTGCAAGACC TTGCAAGATC TTGCAAGACC	CGCAAGAGCG CGCAAGAGCG CACAAAAAGCG CACAAGAACG CGCAAGAGCG	CGATCACAAG CGATCACAAG TGATCACAAG CGATCACAAG CGATCACAAG	СТТАААААА СТТДАААААА СТТДАААААА СТТАААААА СТТААААААА СТТААААААА	200 GCCATCAAGC GCCATCAAGC GCCACAAAGC GCCATGAAGC GCCATCAAGC	<i>hp0203</i> (258 bp)
7.13 26695 J99 G27 SS1	201 CTTTGATTCG CTTTGATTCG CTTTGACTCG CTTTGATTCG	ACGCTTAAGG ACTCTTAAGG ACGCTTAAGG ACCCTTAAGG ACGCTTAAGG	ТТААТТТТАТ ТТААТТТТАТ ТТААТТТТАТ ТТААТТТТАТ ТТААТТТТАТ	TACGAAGTCT TACGAA-TCT CACGAAGTCT TACGAAGTCT CACGAAGTCT	ТТТАААААGG ТТТААААААGG ТТТААААААGG ТТТААААААGG ТТТААААААGG	ATTGCAAGCA ATTGCAAGCA ATTGCAAGCA ATTGCAAGCA ATTGCAAGCA	TGCACAAATG TGCGCAAATG TGCGCAAATG TGCGCAAATG TGCGCAAATG	GCTTTAGAGC GCTTTAGAGC GCTTTAGAGC GCTTTAGAGC GCTTTAGAGC	ААGCTСАЛАА ААGCCCАЛАА ААGCTCАЛАА ААGCTCАЛАА ААGCTCАЛАА	300 AGGAACTCCA AGGAACTCCA AGGAACTCCA AGGAACTCCA	
7.13 26695 J99 G27 SS1	301 TAA-AAGGGT TAAAAGGGGT TGA-AGGGGT TAA-AAGGGT TAAAAGGGGT	TTCTTTÅGGG TTCTTTÅGGG TTCTTTÅGGÅ TTCTTTÅGGG TTCTTTÅGGG	ATTTTATTTC ATTTTATTTC ATATTATTTC ATTTTATTTC ATTTTATTTC IR2	TTATAGCAGA TTATAGCAGA CTATAGCAGA TTATAGCAGA TTATAGCAGA	ААТТАТТТТТ ААТТАТТТТТ ААТТАТТТТТ ААТТАТТ	AAAGCAAAAG AAAGTAAAAG AAAGTAAAAG AAAGCAAAAG AAAGCAAAAG	АСАААТТАТС АСАААТСАТС АССААТААТС АССАААТААТС ССАААТСАТС	ТТТСТАТТАА ТТТ ТТТ ТТТСТАТТАА ТТТСТАТТАА	TTAGAGATAT AGAGATAT AAAGGCAT TTAGAGATAT TTAGAGATAT	400 AGTAATTATT AGTAGATATT TCTAGTTTTT AGTAGTTATT ATTGGTTATT	IR203 in pKJMSH (132 bp)
7.13 26695 J99 G27 SS1	401 ТТАСТАССТА ТТААТАТСТО ТТААТАССТА ТТАСТАССТА ТТАСТАССТА	ТGAGAACCA- ТТGTТАТТАТ ТТGTTCTTAT ТТATGGCTAT ТТATGGCTAT	-AGTAAAGTA TGGATTAGTA TGGGTTAGTA TGGATTTGTA TGGATTTGTA	ТАGАТААА ТТААСАССТА ТТААТАССТА ТТАССССС ТТАССССС	TTAGAGCTAC TTAGAGCTAC	TATAATGGCG TATAATGGCA	TTTAAAGGCG	ATACTGATGA ATACTGATG-	TGATGAAGTT	500 GAGAGTGATG	
7.13 26695 J99 G27 331	501 GGTTTTTTAG -GTTTTTTAG	TAGAATATGG TAGAATATGG	GATAAATTCG GATAA-TTCG	TTGAATATTT TTGAATATTT	CGGCTATACT CGGCTATACT	CTAGTTACTA CTAGTTACTA	TA-TAATGTT TAATAATGTT	TTTTCCTTAT TTTTCCTTAT	ATAATTGGAC ATAATTGGAC	600 CAGTTATCG- CAGTTATTGT	
7.13 26695 J99 G27 SS1	601 CATAATATTT	ATACTTATCT	GAGTTAGGTT	ATATAAGCTT	CTTTAA CTTTAACTTT	ATAAGATGAA	ТААСТБАСТА ТТБТСАБ	TTTTTA TTATTTTTTA AAGTGGTGTA	TATTTTTTG TATTTTTTCTA CAAACATTTA TTGTCAG	700 AAGCCAATTC AACCCAACTC AAGGTAGAGA AAGGTAGAGA	
7.13 26695 J99 G27 S31	701 TTCCAATGAC TTCCAATGAT TGGTGATGAC TGGTGATGAC	TAGTAGCGTC TAGTAGCGTC GATAATGATG GATAATGATG	ТСААССАААА ТСААСТАААА ТТААТБАТББ ТТААТБАТББ	AC ACGCAGAAAC TTTT TTTT	TCAACCAACA TCAACCAACA TTAGTAGATT TTAGTAGATT	ACCAATTGAG ACCAATTGAG ATGGGATAAG ATGGGATAAG	ААСССААТАТ ААСССААТАТ ТБССТАБААТ ТБССТАБААТ	AGAAATTAAT AGAAATTAAT GGTTAGGAAT GGTTAGGAAT	ATTCTTTAGT ATTTTTTAGT AATTT AATTT	800 CTGTTTTTTA CTGTTTTTTTT CGGTATTTAT CGGTATTTAT	
7.13 26695 J99 G27 SS1	801 AAAAAGGGGG AAAAATAGGG AGGCATATTT AGGCATATTT	CTTTGGTTGC CTTTGGTTGC TTCCCTTATA TTCCCTTATA	TTTATTTTAT TTGGTTTGAT TAGCCGGTAT TAGCCGGTAT	GGCAAA.CTT AGCAAAACTC AGTCTTACTC AGTTTTACTC	TTAAAGGGAT TTAAGGGGAT TTAATTGTTT TTAATTGTTT	AGGGGGGATAT AGGGGGGATAT TATTC TATTC	TTTGCGCTTT TTTGCGCTTT AGTAATGATT AGTAATGATT	AATATCCCTT AATACCCCTT AGTAGCTTCT AGTAGCTTCT	TAACCCCC-A TAACCCCCCA CAACTAAA-A TAACTAAA-A	900 ACTAAATCCC ACTAAATCCC ACTCAAC- ACTCAAC-	
7.13 26695 J99 G27 SS1	90 <u>1</u> TTAATATT CCCTAACCCC CCCTAACCCC CAACAACCCA CAACAACCCA	IR3 TTTTTAGTCT ATAATACT ATAATACC TCGAGA TTGAGA	GTTTTTTAAA GCATTACAAG GCATTACAAG ACCAAG ATCAAG	AAGTCATCGC AAGCTATCGT AAGTTACCGC TA TA	ТТССТАВАСА ТТССТАВАСА ТТССТАВАСА -ТАААСА -ТАААСА	AGCTTTTTGA AACTTTTTGA AACTTTTTGA AGCTTTTTGA AGCTTTTTGA	ТАТТАААААА ТАТТАААААА ТАТТАААААА ТАТТАААААА	GCTTTTAGCA GTTTTTAGCG GTTTTTAGCA GCTTTTAGCA GCTTTTAGCA	TTTTTGAATT TTTTTAAACT TTTTTAAATT TTTTTGAATT TTTTTGAATT	1000 TTATGACCAA TCATTACCAA TTATATCCAA TTATGACCAA TTATGACCAA	IR204 in KJMSH (130 bp)
7.13 26695 J99 G27 SS1	1001 CACCCCATTA CACCCCATTA CACCCACATTA CACCCCCATTA CACCCCATTA	GAACAGAAAT GAACAAAAAC GAACAAAGAC GAACAGAAAT AAACAGAGAT	ТАДАААСТСТ САААТТ САААТТТ ТАДАААСТСТ ТАААААСТСТ	TT-TTATTGA TTATTTG CTATTTG TT-TTATTGA TT-TTATTGA	GGAAACTTGT GAAAATTTAT GAAAATTTAT GAAAACTTGT GAAAACTTGT	GGTAATACTC GGTAATACTC GGTAATATTC GGTAATACTC GGTAATACTC	GCCCTTGTCC GCCCTTGTCT GCCCTTGTCC GCCCCTGTCC GCCCCTGTCC	GTGATGATTT GTGATGATTT GTGATGATTT GTGATGATTT GTGATGATTT	ТААСТТСТАА ТААСТТСТАА ТААСТТСТАА ТААСТТСТАА ТААСТТСТАА	1100 TAATTGGTTA CAATTGGTTA CAATTGGTTA CAATTGGTTA CAATTGGTTA	
7.13 26695 J99 G27 SS1	1101 GGTTTGCAAT GGCTTGCAAT GGCTTGCAAT GGCTTGCAAT	TCAGGCGGTA CCAAGCGGTA CCAGGCGGTA TCAGGCGGTA TCAGGCGGTA	АААТАТСТСТ АААТАТСТСТ АААТАТСТСТ АААТАТСТСТ АААТАТСТСТ АААТАТСТСТ	TGCGAGTATT TGCGAATACT TGCGAGTATT TGCGAGTATT TGCGAGTATT	ТТААСТСАТС ТТААСТСАТС ТТААТТСАТС ТТААСТСАТС ТТААСТСАТС	ATCAAAGAGC ATCAAAGAGT ATCAAAGAGT ATCAAAGAGT ATCAAAGAGT	TTTTCTTTTT TTTTCTTTTT TTTTCTTTTT TTTTCTTTTT	ТАААСТТАТС ТАААСТТАТС ТАААСТТАТС ТАААСТТАТС ТАААСТТАТС	GCCCAATTTG GCCCAATTTG GCCCAATTTG GCCCAATTTG GCCCAATTTG	1200 GGTTCTACTT GGTTCCACTT GGTTCCACTT GGTTCCACTT GGTTCTACTT	b=0204
7.13 26695 J99 G27 SS1	1201 TTTTAGTCGC TTTTAGTCGC TTTTAGTCGC TTTTAGTCGC TTTTAGTCGC	TTCGCATGAA TTCGCATGAA TTCGCATGAA TTCGCATGAA TTCGC	TTTCCCCTAT TTTCCCCTAT TTTCCCCTAT TTTCCCCTAT	ТСАСААТААС ТСАСААТААС ТСАСААТААС ТСАСААТААС	ATTTTTAATC ATTTTTGATC GTTTTTAATC ATTTTTAATC	ACCACCGGTT ACCACCGGCT ACCACCGGTT ACCACCGGCT	CATCGTTCAT CATCGTTCAT CATCGTTCAT CATCGTTCAT	AGAAGTGATG GGAAGTGATG AGAAGTGATA GGAAGTGATG	СТТААААGAC СТТААААGAC СТТААААGAC СТТААААGAC	1300 TAGAGTCCGT TAGAATCCGT TAGAGTCCGT TAGAATCCGT	(340 bp)
7.13 26695 J99 G27 SS1	1301 CATTTTCCCC CATCTTCCCC CATTTTCCCC CATTTTCCCC	ATGAGTTTCC ATGAGTTTCC ATGAATTTCC ATGAGTTTCC	CCCCAGTCGC CCCCAGTCGC CCCCAGTCGC CCCCAGTCGC	GCGATAATCC ACGATAATCC GCGATAATCC ACGGTAATCC	АGCTTGAAAT АGCTTGAAAT АGCTTGAAAT АGCTTGAAAT	CCAAATCCTT CCAAATCCTT CCAAATCCTT CCAAATCCTT	IR4 TGCCCCCACA TGCCCCCACA TGCCCCCACA TGCCCCCGCA	CAAACACCGC CAAACACCGC CAAACACCGC TAAATACCGC	TTATCATTAG TTATCATTAG TTATCATTAG TTATCATTAG	1400 CAAGCTCAAA CAAGCTCAAA CAGGCTCAAA CAAGCTCAAA	
7.13 26695 J99 G27 SS1	1401 CACATCTTTT CACATTTTTT CACATTTTTT CACATCTTTT	1417 TARACAT TARACAT TARACAT TARACAT									

Fig. 1. Comparison of DNA sequences in hp0203 and hp0204 including intergenic region of *H. pylori* wild type strains of 7.13, 26695, J99, G27, and SS1. The structural genes of hp0203 and hp0204 are indicated with gray boxes. IR203 and IR204 were indicated on the right side. The arrows indicate primers used for construction of fragments in the pKJMSH. The sequence of SS1 started at 143 nucleotides and ended at 1215 nucleotides because there is no available sequence at the flanking regions. A dash means a missing nucleotide to match.

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transformation efficiency was shown in Table 2. The natural transformation showed the different result from the previous result of the electroporation method (Langford *et al.*, 2006). Only 26695 *H. pylori* was successfully transformed by pIR203C04 via the natural transformation but the other SS1, J99, 7.13, and G27 *H. pylori* strains failed. It gave us a rationale to redesign the complementation system to be applicable to more *H. pylori* strains.

Variation of intergenic region in different H. pylori To identify a problem why SS1, J99, 7.13, and G27 H. pylori strains failed to take up pIR203C04 by the natural transformation, the DNA sequence alignment of the intergenic region was performed (Fig. 1) because the system requires the homologous recombination. The intergenic region of pIR203C04 shares the perfect match with 26695 H. pylori because 26695 H. pylori genome was template to construct pIR203C04. However, except approximately 130-bp intergenic region near to hp0204, SS1, J99, 7.13, and G27 H. pylori strains showed low DNA sequence homology to that of 26695 H. pylori. Especially the intergenic regions between hp0203 and hp0204 of 7.13 and G27 are short because of the deletion. This made us postulate that the natural transformation was problematic due to the low homology between the intergenic regions of H. pylori genome and pIR203C04.

Because the intergenic regions of 7.13 and G27 may not be enough for the homologous recombination, the conserved 3' parts of coding regions of *hp0203* and *hp0204* were included in the fragments of newly designed construct. To construct the new complementation system, the PCR amplicons with a template of 7.13 chromosomal DNA using primer sets of IR1 and IR2, and of IR3 and IR4 (Fig. 2A) were gel-extracted and used as a template for PCR of Splicing by Overlapping Extension (SOE) ing with primers of IR1 and IR4. The PCR SOEing amplicon was cloned into pGEM T-easy vector and the resulting plasmid was digested with *SalI* and self-ligated to make *PstI* site unique in the construct, resulting in pKJM (Fig. 2B). The pIR203C04 was digested with *Bam*HI and *XhoI* restriction enzymes to get a DNA fragment of a *cat* containing multicloning sites. The *Bam*HI and *XhoI* sites of the pKJM were used to clone the DNA fragment containing a *cat* with multicloning sites, resulting in pKJMSH.

Delivery of the pKJMSH complementation system to various *H. pylori* strains via natural transformation To determine whether the new pKJMSH complementation system can transform 26695, SS1, J99, 7.13, and G27 *H. pylori* strains, the method of standard natural transformation was used. The transformation efficiencies to 26695, SS1, J99, 7.13, and G27 *H. pylori* strains were 8.7, 1.9, 0.3, 4.1, 14.9×10^3 CFU/µg of DNA, respectively. The correct integration location at the intergenic site was confirmed by PCR with two sets of primers; HP0203-F and IR2, and IR3 and HP0204-R. The primers of HP 0203-F and HP0204-R are aligned at



Fig. 2. Construction of pKJMSH complementation system. (A) Wild-type 7.13 *H. pylori* chromosome region of *hp0203* and *hp0204* genes including the intergenic regions IR203 and IR204. The intergenic region was divided into two fragments for cloning purposes indicated by primers of IR2 and IR3. (B) A 400-bp amplicon using primers of IR1 and IR2 and a 480-bp amplicon using primers of IR3 and IR4 were cloned by SOEing PCR into pGEM T-easy vector. (C) The DNA fragment containing a *cat* with multicloning sites (MCS) was from the pIR203C04 by digestion of *Bam*HI and *Xho*I restriction enzymes. The cat with MCS was cloned at *Bam*HI and *Xho*I sites of the pKJM, resulting in pKJMSH. Arrow indicates a direction of transcription for the coding regions.

hp0203 and *hp0204* coding regions, respectively, but outside from the construct fragments (Fig. 2). All five wild-type *H. pylori* used in this study were able to incorporate the pKJMSH complementation system at the intergenic site of *H. pylori* gene. When the transformation efficiencies of 26695 with pIR203C04 and pKJMSH were compared, pIR203C04 has approximately 2.5-fold better transformation efficiency. The results suggested that by natural transformation, pKJMSH can transform *H. pylori* strains of 26695, SS1, J99, 7.13, and G27 which are common for the investigation of molecular pathogenesis. Thus, the pKJMSH complementation system is applicable to more *H. pylori* wild-type stains than the pIR203C04.

Discussion

Langford et al. (2006) developed the pIR203C04 complementation system that is useful for identifying functions of specific genes in vitro and even in vivo studies. Their data using this system convinced us to apply this system to G27 for in vitro experiment and to 7.13 for in vivo gerbil experiment. G27 H. pylori strain is one of the common strains for H. pylori in vitro research, and 7.13 H. pylori strain is a gerbil-adapted strain for the gerbil infection. However, as this study indicated, when the natural transformation is used, their complementation system is limited to use for narrow range of H. pylori strains; only 26695 H. pylori among five common strains can be used. Because Langford et al. (2006) used 26695 H. pylori chromosomal DNA to construct the intergenic region of pIR203C04, the perfect match of intergenic region between pIR203C04 and H. pylori chromosomal DNA may increase the possibility of homologous recombination event and thus only 26695 H. pylori can be transformed by the natural transformation. Even though we redesigned the complementation system of pIR203-C04 for application to wider range of H. pylori strains containing 7.13 and G27, the same intergenic chromosomal site in H. pylori was utilized because it has been proved that the insertion to the intergenic site can be done without the polar effects and disruption of other H. pylori genes. Thus, to improve the efficiency of homologous recombination to various H. pylori, the conserved regions among these H. pylori strains were selected and employed to construct the new complementation system. The fragments of the new pKJMSH complementation system contain some intergenic regions and the conserved 3'coding sequences of hp0203 and hp0204 because the intergenic regions of 7.13 and G27 chromosomal DNA share too low homology with the intergenic fragments of other H. pylori strains to do the homologous recombination especially because the intergenic region near to hp0204 in 7.13 and G27 H. pylori genomes was deleted mostly and showed poor homology. Since their coding regions have the similar homology to the fragments of pKJMSH, the different transformation efficiency may reflect the competency of each H. pylori strain for the natural transformation. It has been known that G27 has high transformation efficiency and it was the case in this study. The transformation efficiencies of pIR203C04 to 26695 H. pylori was an approximately 2.5-fold higher than that of pKJMSH probably because the fragment of pIR203C04 has higher homology to the longer region of 26695 H. pylori genome.

The transformants were selected by the resistance to chloramphenicol antibiotics, and therefore the transformants were able to express the *cat* gene that is located between the fragments of pKJMSH. It suggests that the gene integrated at the intergenic region in all five *H. pylori* strains has no problem to express. Therefore, the new pKJMSH complementation system may keep all strong points of pIR203C04 with better selection for *H. pylori* strains.

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