Doctoral Thesis

Function of base-excision restriction enzymes

（塩基切り出し型制限酵素の機能）

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Summary

Restriction enzymes cleave DNA judged as non-self based on its epigenetic modification. All the restriction enzymes examined so far were phosphodiesterases that cleave a phosphodiester bond linking the monomer units and generate 3' hydroxyl (3’ OH) and 5' phosphate (5’ P) ends. Our group identified a restriction enzyme that has a DNA glycosylase activity that excises a base (A) from its recognition sequence (5’GTAC) if it is not methylated. This enzyme, R.PabI, is from a hyperthermophilic archaeon, *Pyrococcus abyssi*. In the initial report, the enzyme was concluded not to be an endonuclease: the resulting AP site was assumed to be cleaved by heat but not by the enzyme. The following works, however, detected its weak and uncoupled AP lyase activity at a high temperature (> 60ºC), which generates a 3’-phospho-α, β-unsaturated aldehyde (3’-PUA) and 5’P end pair and then a 3’P and 5’P end pair. In this work, we examined role of the AP lyase in restriction *in vivo* using *E. coli*. We purified two R.PabI homologs from mesophilic eubacteria, *Campylobacter coli* (R.CcoLI) and *Helicobacter pylori* (R.HpyAXII). We demonstrated their DNA cleavage, glycosylase and AP lyase activities *in vitro* at 37ºC. The AP lyase and glycosylase were more coupled in R.CcoLI than in R.PabI. R.CcoLI as well as R.PabI promoted restriction of incoming bacteriophage/plasmid DNA and chromosomal DNA in *E. coli* at 37ºC, although the R.PabI-mediated restriction was promoted by AP endonuclease action *in vivo* or *in vitro*. These results reveal involvement of DNA breakage in the restriction and diversity by their intrinsic AP lyase activity or an AP endonuclease. Plasmid transformation assay revealed that the ends generated by R.CcoLI and R.PabI *in vitro* are difficult to repair by rejoing *in vivo*, which suggests biological significance of this family. The resulting high toxicity may explain strong avoidance of its recognition sequences in Helicobacter genomes. The difficulty in repairing ends may be useful in targeted DNA inactivation.

Introduction

Restriction enzymes cleave DNA that lacks proper epigenetic modification (base methylation in most of the cases studied) to abolish its biological activity (1). All known restriction enzymes hydrolyze a phosphodiester bond linking the monomer nucleotide units and generate 3'-OH and 5'-P ends (Figure 1, ii). They fall into several superfamilies, each characterized by a unique fold and motifs. We earlier looked for restriction enzymes of novel structure and function by taking advantage of the nature of restriction-modification (RM) systems as mobile genetic elements (2). In comparing genomes, we looked for genes that lack any known motif of restriction enzymes but move together with another gene with the motifs of DNA methyltransferases (3,4). Those restriction enzyme candidate genes were expressed *in vitro* and tested for DNA cleavage activity (4). The product of one such gene, from the hyperthermophilic archaeon *Pyrococcus abyssi*, was an endonuclease recognizing 5'GTAC (4) and designated as R.PabI. It forms an RM system together with a linked DNA methyltransferase generating 5'GTm6AC (5). This restriction enzyme turned out to have a novel fold, designated as 'half pipe' (6). In addition to its preference for high temperature (85ºC), this enzyme displayed several exceptional properties. One is independence from divalent metal ions (6). Structural analysis of the co-crystal with cognate DNA and biochemical analysis demonstrated that this enzyme is a DNA glycosylase that excises adenine bases from its target sequence (Figure 1 i, iii) (7). Its superfamily members are called *restriction glycosylases* (11).

This finding started a new phase in the study of restriction enzymes and led us to propose generalization of the concept of RM systems to general RM systems or self-recognizing epigenetic systems (12). Any agent that damages DNA with a particular epigenetic status can be called a general restriction enzyme. This generalization relates RM systems to toxin-antitoxin systems (8). Indeed, there are systems (BREX and others) that limit DNA bacteriophage propagation by means other than DNA breakage (9) (10). Such generalized systems may also include uracil N-glycosylases in prokaryotes and eukaryotes and demethylation systems in eukaryotes.

What could be the relationship between base excision and DNA strand cleavage in the restriction process of these restriction glycosylases? In one report (7), the enzyme R.PabI was concluded not to be an endonuclease: strand cleavage both *in vitro* and *in vivo* was ascribed to heat-promoted processing of the AP sites (= abasic sites). Another study, however, demonstrated that R.PabI itself has a type of endonuclease activity called AP lyase (Figure 1 iv) that generates unique ends (11). This activity is, however, weak and uncoupled with the glycosylase activity, and its role in restriction remains unclear.

Indeed, DNA treated with R.PabI at a low temperature (37°C) remained free of any strand breaks but lost transformation activity with *Escherichia coli* cells at 37°C (11).

In the present work, we aimed to clarify the role, if any, of DNA strand cleavage in the restriction action of the restriction glycosylases. We analyzed PabI homologs from mesophilic bacteria (4) (7) (12) (20) and used an *E. coli* DNA transfer system at 37°C. Our results suggest the critical role of endonucleolytic breakage in the restriction process but unexpected diversity in its origin.

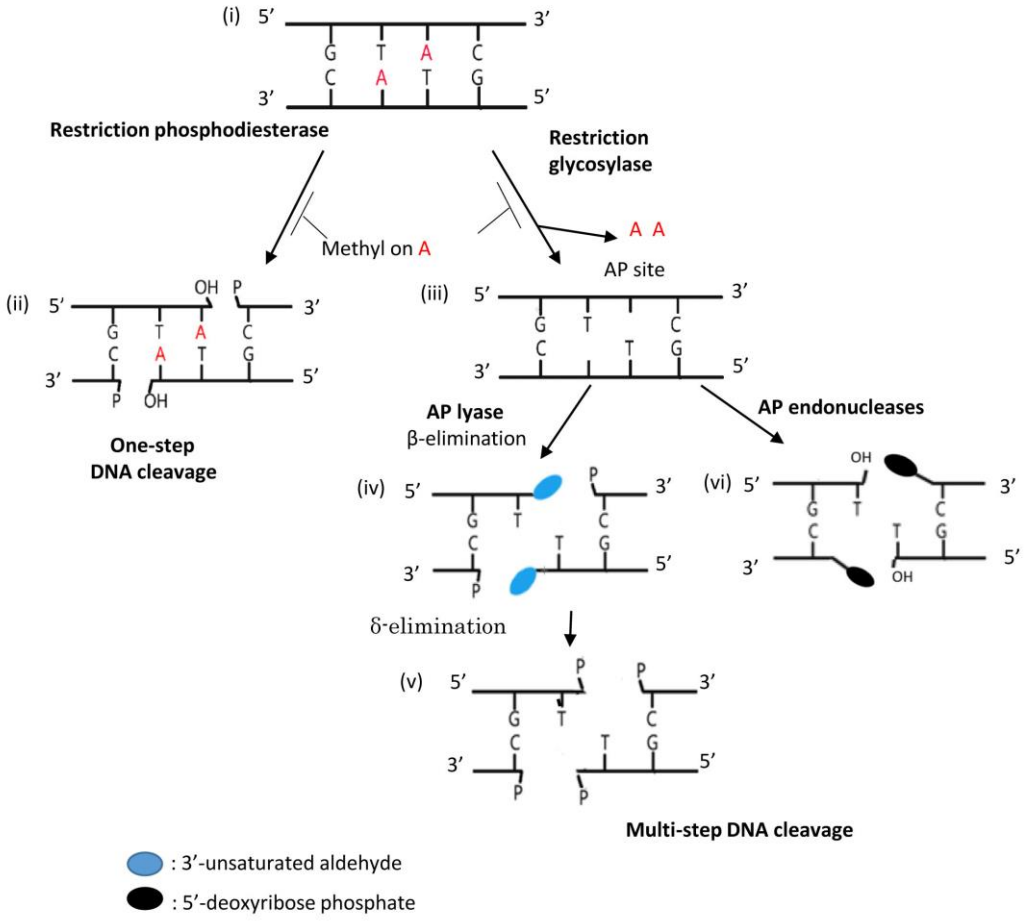

Figure 1. DNA cleavage pathways for restriction enzymes.

(i) A double-strand DNA with the recognition sequence for PabI. A in red, adenine base to be excised unless methylated by a paired modification enzyme. (ii) Hydrolysis of phosphodiester bonds to generate two 3'-OH and 5'-P end pairs. (iii) Generation of AP sites by base excision. (iv) Cleavage with AP lyase generates two breaks with 5'-P and 3'-modified sugar (blue oval) ends by β-elimination. (v) Further δ-elimination. (vi) AP site cleavage by AP endonucleases, generating 3'-OH and 5'- deoxyribose phosphate ends but not a sticky end.

Materials and methods

Bacterial strains, bacteriophages, plasmids, and oligonucleotides

All *E. coli* strains and plasmids used here are listed in Table S1. The synthetic oligonucleotides used are listed in Tables S2 and S3.

Bacteriophage lambda *vir* and bacteriophage P1 *vir* from our laboratory collection were used for the restriction assay and construction of *E. coli* mutant strains, respectively. We used *E. coli* HST08 (TaKaRa) for standard DNA cloning. *E. coli* strains were grown in LB broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per 1 L) at 37°C. Antibiotics were used at the following concentrations: kanamycin (Km), 30 ug/mL; ampicillin (Ap), 50 ug/mL; chloramphenicol (Cm), 25 ug/mL. Agar plates were made by adding 1.5 % agar to LB broth.

P1 phage-mediated transduction was carried out as previously described (13). The donor strains *E. coli* JW2146 (*Δnfo*::*kan*), JW1738 (*Δxth*::*kan*), JW0221 (*ΔdinB*::*kan*), JW0059 (*ΔpolB*::*kan*), JW2669 (*ΔrecA*::*kan*), and JW2788 (*ΔrecB*::*kan*) are from the KEIO collection (13), and the recipient strain was *E. coli* T7 Express *lysY/I*^{q} (New England Biolabs, Ipswich, MA, USA). After transduction, pFLP3 plasmid was introduced into the transductant strain to remove the kanamycin resistance gene (14). The plasmids used for expression of restriction enzymes and the DNA methyltransferase were introduced into these strains.

A substrate plasmid that carries a single GTAC site was constructed by two cycles of site-directed mutagenesis. First, plasmid pHSG398 was PCR-amplified using primer pairs m-phsg398-F / m-phsg398-R and KOD FX Neo polymerase (Toyobo), and the PCR product was purified using a Gen-Elute PCR Clean-Up Kit (Sigma). 10 uL (≈ 200 ng) PCR product was digested with 10 U DpnI (Fermentas) in 1×FastDigest reaction buffer, and the mixture was used for heat-shock transformation into *E. coli* HST08 competent cells, which were prepared with 0.1 M $CaCl_2$ and spread on an LB agar plate (Cm, 25 ug/mL) for overnight incubation. Plasmid from the resulting single colony was used as a template for PCR with another primer pair (m-phsg398-F-2 / m-phsg398-R-2), as above. These two steps introduced two mutations in the *cat* gene of pHSG398 as confirmed with RsaI (New England Biolabs) and KpnI (Fermentas). The resulting construct was designated as pHSG398m.

The synthetic genes for R.PabI homologs, R.HpyAXII and R.CcoLI, were designed, considering the protein sequences in NCBI resources (WP_000052868.1, WP_002830209.1) and codon optimization for expression in *E. coli*, by Funakoshi. The plasmids carrying the synthetic genes were designated pTAKN-2-*ccoLI* and pTAKN-2-*hpyAXII*. The coding regions of the two homologs were PCR-amplified with primer pairs CcoLI-F/ CcoLI-R for CcoLI and HpyAXII-F/ HpyAXII-R for HpyAXII (Table S2) and then cloned into plasmid pET28a using an In-Fusion® HD Cloning Kit (TaKaRa). The resulting plasmids were designated as pET28a_*ccoLIR* and pET28a_*hpyAXIIR*. The mutation D214A of R.PabI

coding region was amplified with the primer pairs P-D214A-R/ P-D214A-F (carrying mutation site) and pET28a_*pabIR* plasmid as template, and then the coding region was cloned into pET28a. The resulting construct was designated as pET28a_*pabIRD214A*.

Restriction enzyme expression *in vitro*

Two protein-coding regions of the *pabIR* homologs from plasmids pTAKN-2-*ccoLI* and pTAKN-2-*hpyAXII* were inserted into pEU3-NIIb, a plasmid for cell-free protein synthesis (4). The R.PabI homolog coding region was amplified by PCR with KOD-plus (Toyobo) from plasmids with a BamHI site attached at their 3' ends by using primers CF-CcoLI-F/ CF-CcoLI-R and CF-HpyAXII-F/ CF-HpyAXII-R (Table S2). The resulting plasmids were designated as pEU3-NIIb_*ccoLIR* and pEU3-NIIb_*hpyAXIIR*. This connected the coding region to an SP6 promoter (4) and a translation promotion signal, omega, at its 5' end. The amplified DNA fragment was inserted between the EcoRV and BamHI sites of pEU3-NIIb.

The putative restriction enzymes were expressed in a wheat-germ-based cell-free protein synthesis system (Protein Research Kit (S), CellFree Science) (4). The resulting solutions were centrifuged at 12 krpm for 10 min at 4°C and soluble fractions were recovered.

To test DNA cleavage activity, 1 uL of the soluble fraction was incubated with 150 ng of lambda DNA (TaKaRa) in 1x NEB1 buffer (New England Biolabs) at 37°C for 1 h, which was followed by 0.8% agarose gel electrophoresis and detection with ethidium bromide or Gel Red (Biotium Inc.) by ultraviolet light irradiation.

Restriction enzyme expression *in vivo*

pET28a::*pabIR*, pET28a::*ccoLIR*, or pET28a::*hpyAXIIR* was introduced into *E. coli* T7 Express *lysY/I*^{q} harboring pBAD30_*cviQIM* (11). Methyltransferase M.CviQI was always induced in *E. coli* T7 Express *lysY/I*^{q} with 0.5% arabinose. If necessary, 0.5 mM IPTG and 0.5% glucose were added to control the expression level of the restriction enzyme.

Purification of restriction enzymes

E. coli T7 Express *lysY/I*^{q} harboring pBAD30_*cviQIM* and one of the restriction enzyme expression plasmids, pET28a::*pabIR*, pET28a::*ccoLIR*, or pET28a::*hpyAXIIR*, was streaked on LB agar with 50 ug/mL Ap, 30 ug/mL Km, and 0.5 % arabinose. After overnight incubation, a single colony was picked up and transferred to 5 mL LB medium containing the same concentrations of reagents as above. After overnight incubation, the 5-mL culture was transferred to 1 L LB medium containing the same

concentrations of reagents. After 3 h of incubation with shaking, IPTG was added to a final concentration of 0.5 mM, and the culture was further incubated for 6 h. The cells were collected by centrifugation at 5 krpm for 10 min at 4ºC and resuspended in 15 mL of 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. Then they were sonicated (Ultrasonic Disruptor UD-200, TOMY) and centrifuged at 7 krpm for 20 min. The supernatant containing R.PabI or R.PabI(D214A) was heated at 75ºC in a water bath for 120 min while the supernatant containing R.HpyAXII or R.CcoLI was left on ice. The supernatants were centrifuged at 7 krpm for 20 min again and filtered using a 0.45-um PDVF filter.

The filtrates were bound to 4 mL of Ni-NTA Agarose resin (QIAGEN, NO.1018244) equilibrated with a binding buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl), and washed with the same buffer, and eluted in 2 mL of elution buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 300 mM imidazole) using a gravity flow column. The fused His-tag was removed by thrombin digestion using a Thrombin Cleavage Capture Kit (Novagen, No.69022-3FRZ) following the manufacturer's protocol. The eluate was reacted with 1 uL (1 U) thrombin at 25ºC overnight. The agarose resin and His-tag were removed using a centrifuge column (Pierce Centrifuge Columns, Thermo, 0.5 mL). The flowthrough containing the restriction enzyme was directly loaded onto a Heparin HP column (HiTrap Heparin HP, GE healthcare Life Sciences, No. 17-0406-01) equilibrated with a Heparin binding buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl), and then the proteins were eluted in 2 mL elution buffer for heparin (20 mM Tris-HCl pH 7.5, 2 M NaCl). The proteins were concentrated using a centrifugal filter (Amicon Ultra-0.5mL, Ultracel-10K, UFC501096).

The buffer in the protein solution was replaced with 2x stock buffer (20 mM MES pH 6.0, 200 mM NaCl) using a centrifugal filter (Amicon Ultra-0.5mL, Ultracel-10K, UFC501096) and the protein was concentrated. Concentrated protein solution was mixed with appropriate volumes of glycerol, 100 mM EDTA, and 100 mM DTT to generate a final protein stock solution in 10 mM MES pH 6.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol.

DNA cleavage assay

An 861-bp linear double-stranded DNA fragment was used as a substrate for the cleavage assay (Figure 3). This substrate was generated by PCR-amplification of the ampicillin resistance gene (containing one GTAC site) of pBAD30 using primers pBAD30F and pBAD30R (Table S2). The cleavage reaction was performed in 10 uL with 0.1 M sodium phosphate buffer (pH 6.5), 2.66 pmol (266 nM) of purified enzyme, and 0.38 pmol (0.38 nM) of substrate at 85ºC (R.PabI / R.PabI(D214A)) or 37ºC (R.HpyAXII / R.CcoLI) for 1 h. DNA was separated by agarose gel electrophoresis and visualized with ethidium bromide and ultraviolet light.

DNA glycosylase assay

Each of the top and bottom strands of the 40-mer oligonucleotide with a single "GTAC" (Table S2) was labeled with γ^{32}P-ATP (Perkin-Elmer) using T4 polynucleotide kinase (New England Biolabs), and annealed with its complementary oligonucleotide. 0.2 pmol of the 40-bp substrate was incubated with 0-1.4 pmol of purified enzyme (that is, with 4-fold dilution) in 20 uL of 0.1 M phosphate buffer (pH 6.5) at 60°C (R.PabI) or 37°C (R.HpyAXII, R.CcoLI) for 1 h. For R.CcoLI, we also incubated the 0.2 pmol 40-bp substrate with 0-0.2 pmol enzyme in 20 uL of 20 mM MOPS-KOH (pH 7.0) for 1 h. Half the reaction mixture was treated with 0.1 M NaOH at 70°C for 10 min to cleave DNA at the generated AP sites, and then neutralized with HCl. The products were separated by 18% denaturing PAGE. Gels were exposed to an imaging plate, and the plate image was detected using an FLA 5100 scanner (Fujifilm).

AP lyase assay

To construct the AP site-containing 40-bp substrate, uracil-containing (5'-GTUC/3'-CUTG) double-stranded oligonucleotides (Table S2) were labeled and generated as described above, and then incubated with uracil N-glycosylase (UNG) (New England Biolabs) (11). Complete conversion to AP sites was confirmed by NaOH cleavage. An AP site is present instead of an adenine nucleotide in the recognition sequence (5'-GTAC/3'-CATG) in this substrate.

The reaction was performed in 20 uL of 0.1 M phosphate buffer (pH 6.5) or 20 mM MOPS buffer (20 mM MOPS-KOH, pH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT), containing 0.2 pmol of AP site-containing substrate (^{32}P-labeled top or bottom strand) and 0-2 pmol of purified R.CcoLI at 37°C for 1h. Samples were separated by 18% denaturing PAGE.

$NaBH_4$ trapping

The method described (11) was modified as follows. Briefly, R.CcoLI (0-6 pmol, 0-300 nM) and a 40-bp substrate DNA (0.2 pmol, 10 nM), containing 5'-GT#C (# = AP site)/3'-C#TG and a 5'-^{32}P label on the top strand, were incubated in 20 uL of 20 mM MOPS buffer (20 mM MOPS-KOH, pH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) at 37°C for 20 min and then with 100 mM $NaBH_4$ at 25°C for 30 min. DNA-R.CcoLI complexes were denatured in gel loading buffer containing 3% SDS at 90°C for 10 min and separated through 10% SDS-PAGE.

For the control, a single-strand labeled oligonucleotide with a single "GTUC" was reacted with uracil N-glycosylase to generate a single AP site and annealed with a bottom strand with "GTAC". 20 units of Endo III (New England Biolabs) and 0.2 pmol (10 nM) of this P^{32}-5'-GT#C/3'-CATG substrate were incubated in 20 mM Tris-HCl (pH 8), 1 mM EDTA , 1 mM DTT (total 20 uL) at 37°C for 20 min followed by incubation with 100 mM $NaBH_4$, as above. DNA-Endo III complexes were analyzed as

described for R.CcoLI.

Restriction of phage propagation

The wild-type or mutant *E. coli* strain harboring (or not harboring) an RM expression plasmid was grown at 37°C overnight in LB medium with 50 ug/mL Ap, 30 ug/mL Km, and 0.5% arabinose. Then the culture was diluted 100-fold and grown in tryptone broth (1% tryptone and 0.5% NaCl), supplemented with 0.2% maltose and 10 mM $MgSO_4$, at 37°C for 1 h. IPTG was added to 0.1 mM, and incubated at 37°C for 3 h. 2 mL of culture was mixed with 2 mL top agar (1% polypeptone, 0.5% NaCl, 0.6% agar), vortexed for several seconds, and then poured onto a dried bottom agar plate (1% polypeptone, 0.5% NaCl, 1% agar) and allowed to solidify at room temperature for 10 min. Bacteriophage lambda *vir* (plaque forming units / mL $\approx 1.2\times10^7$) was serially diluted and 5 uL of each dilution was spotted on the plates. The plates were incubated at 37°C overnight for plaque counting and estimating plaque-forming units/mL.

Restriction of the chromosome *in vivo*

The wild-type or the mutant strain harboring (or not harboring) an RM expression plasmid was grown overnight at 37°C in LB medium with 50 ug/mL Ap, 30 ug/mL Km, and 0.5% arabinose, and the culture was diluted 100-fold in LB medium with 50 ug/mL Ap, 30 ug/mL Km, and 0.5% arabinose and grown at 37°C for 2-3 h until OD_{600nm} = 0.2 by a plate reader. IPTG was added to a final concentration of 0.1 mM prior to incubation at 37°C for 0.5 h. The culture was chilled on ice. 1 mL of the culture was centrifuged at 5 krpm for 10 min at 4°C, and the cells were resuspended and adjusted to OD_{600nm} = 0.2 with chilled saline. The suspension was diluted 10-fold with chilled saline and immediately spotted on the following plates: (i) LB agar with 0.5% arabinose, 50 ug/mL Ap, and 30 ug/mL Km; (ii) LB agar with 0.5% glucose, 50 ug/mL Ap, and 30 ug/mL Km. The plates were incubated overnight at 37°C before colony counting and calculation of the colony-forming units/mL.

Restriction in transformation

Plasmid pBAD30_*cviQIM* (0.84 pmol) was treated with purified R.PabI (8.4 pmol) in 50 uL of 0.1 M sodium phosphate buffer (pH 6.5) at 37°C for 1 h as previously described (11) to generate AP sites, and the product was purified with a Gen-Elute PCR Clean-Up Kit (Sigma). A 200-ng equivalent of the AP site containing pBAD30_*cviQIM* was transferred into the original and the mutant T7 Express *lysY/I^q E. coli* strains by electroporation to count the colony-forming units/mL.

To evaluate the re-circularization efficiency of restriction glycosylase-treated plasmids, plasmid pHSG398m (2.1 pmol) was first treated with purified R.PabI (10.2 pmol), R.CcoLI (10.2 pmol), or KpnI

(20U) (Fermentas) in 50 uL at 37ºC for 1 h. R.PabI and R.CcoLI reactions were conducted in 0.1 M sodium phosphate buffer (pH 6.5), while the KpnI reaction was in 1× KpnI buffer (Fermentas). The R.PabI products were further purified using a Gen-Elute PCR Clean-Up Kit and treated with Endonuclease IV (New England Biolabs) at 37ºC for 1 h to cleave the DNA at an AP site. All linearized products were purified by agarose gel electrophoresis and with a NucleoSpin Extract II Kit (Macherey-Nagel). Then, the three types of linearized plasmid products were treated with T4 DNA ligase (New England Biolabs) at 16ºC for 2 h. The products (100 ng each) were dissolved in 0.1 M $CaCl_2$ and transferred to *E. coli* HST08 competent cells by heat-shock transformation.

Motif frequency analysis

Expected motif frequency (E_{GTAC}) was defined following the Markov maximum order model (15): $E_{GTAC} = (N_{TAC} \text{ x } N_{GTA})/N_{TA}$, where N_{GTA}, N_{TAC}, and N_{TA} are the numbers of motifs GTA, TAC, and TA, respectively. Motif frequency R_{GTAC} (observed count/expected count) was defined as N_{GTAC}/E_{GTAC}. This calculation was performed using Bioconductor package Biostrings (36). Motif frequency was determined for complete chromosome sequences (Figure 9) of 22 prokaryotic species and complete sequences of *H. pylori* plasmids (Figure S5) retrieved from NCBI RefSeq database.

***Helicobacter pylori* strains for transcriptome analysis**

H. pylori strains were grown in Brucella Broth (BB) [BD Bioscience] supplemented with 10% fetal bovine serum (FBS) [Cell Culture Laboratories] with shaking at 37ºC in a multi-gas CO_2 incubator in the presence of 10% CO_2 and 5% O_2. Primers used for this experiment are listed in Table S3. *Helicobacter pylori* strain P12 derivatives carrying a deletion in the *pabIR* homolog (=HPP12_0511) alone (strains PIK65, PIK70) or both the *pabIR* homolog and the *pabIM* homolog (=HPP12_0510) (strain PIK69) were constructed using homologous recombination. The approximately 800-bp regions flanking HPP12_0511, and the chloramphenicol resistance gene on plasmid pHEL2, were PCR-amplified using primer pairs HPP12_0511_Lf and HPP12_0511_Lr (for HPP12_0511 upstream), HPP12_0511_Rf and HPP12_0511_Rr (for HPP12_0511 downstream), and catf and catr for the chloramphenicol resistance gene with KOD FX Neo DNA polymerase (Toyobo). These three fragments were combined using PCR. Then the combined fragment was inserted into vector pUC19 using an In-Fusion HD Cloning Kit (TaKaRa). The resulting construct was designated pMZA3. Next, the insert in pMZA3 was PCR-amplified using the primer pair HPP12_0511_Lf and HPP12_0511_Rr and then electroporated into P12 cells washed with 300 mM sucrose. After overnight incubation on blood agar plates [BBL™ Trypticase™ Soy Agar with 5% Sheep Blood, BD Bioscience], the cell mixture was diluted in 0.85% NaCl and plated on Brucella broth containing 10% fetal bovine serum (FBS), 1.5% agar (BB-FBS agar),

and 10 ug/mL chloramphenicol. The candidate transformant colonies were streaked on a selective plate, and then one of the clones that appeared was designated as PIK65 (ΔHPP12_0511).

The chloramphenicol resistance gene region of PIK65 or the chloramphenicol resistance gene region together with its upstream gene HPP12_0510 (*pabIM* homolog) was further deleted from PIK65 using the same method. The plasmid constructs used in this experiment were pHY1278 and pHY1279. For construction of pHY1278, approximately 800-bp regions of HPP12_0510 upstream and HPP12_0511 downstream and the kanamycin resistance gene (*aphA-3*) on plasmid pHEL3 were PCR-amplified using primer pairs, HPP12_0510_Lf and HPP12_0510_Lf, HPP12_0511_Rf4 and HPP12_0511_Rr, and AphA3f and AphA3r, respectively. Then, the two fragments were combined by PCR using primers HPP12_0510_Lf and HPP12_0511_Rr. The combined fragment was inserted into pUC19. This gave rise to pHY1278. Similarly, pHY1279, which carries approximately 800-bp regions of HPP12_0511 upstream and HPP12_0511 downstream and the kanamycin resistance gene on pUC19, was constructed. The inserts on pHY1278 and pHY1279 were PCR-amplified, and then electroporated into competent cells of PIK65. Kanamycin-resistant clones were selected and purified on a BB-FBS ager plate with 5 ug/mL kanamycin. The obtained clones were designated as PIK70 (ΔHPP12_0511; $r_{HpyPXII}^{-}$ $m_{HpyPXII}^{+}$) and PIK69 (Δ(HPP12_0510-HPP12_0511; $r_{HpyPXII}^{-}$ $m_{HpyPXII}^{-}$).

Transcriptome analysis

Total RNA was extracted from two replicate exponential-phase cultures (OD_{600nm} = 0.4-0.5) of PIK69 and PIK70 using a PureLink RNA-min Kit (Thermo Fisher Scientific). Then, ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit for Gram-negative bacteria (Epicentre). The rRNA-depleted samples were used for cDNA library construction using a SureSelect Strand Specific RNA Seq Library Construction Kit (Agilent Technologies). The cDNA library was sequenced on the HiSeq2500 platform. 100 base-long reads were mapped onto the strain P12 chromosome and plasmid pHPP12 using BWA software (16), and then the number of reads mapped to the coding regions were counted using HTseq software (17). To detect differentially expressed genes, read counts were normalized and compared between PIK70 ($r^{-}m^{+}$) and PIK69 ($r^{-}m^{-}$) using Bioconductor TCC, following TMM-normalization and the edgeR iteration protocol (18). Read depth per strand was calculated using BEDtool software (19).

Accession number

NGS data was submitted under DRA accession no. DRA004356.

Results

1 Mesophilic R.PabI homologs has endonuclease (AP lyase) activity

1) Expression and purification of R.PabI, R.HpyAXII and R.CcoLI

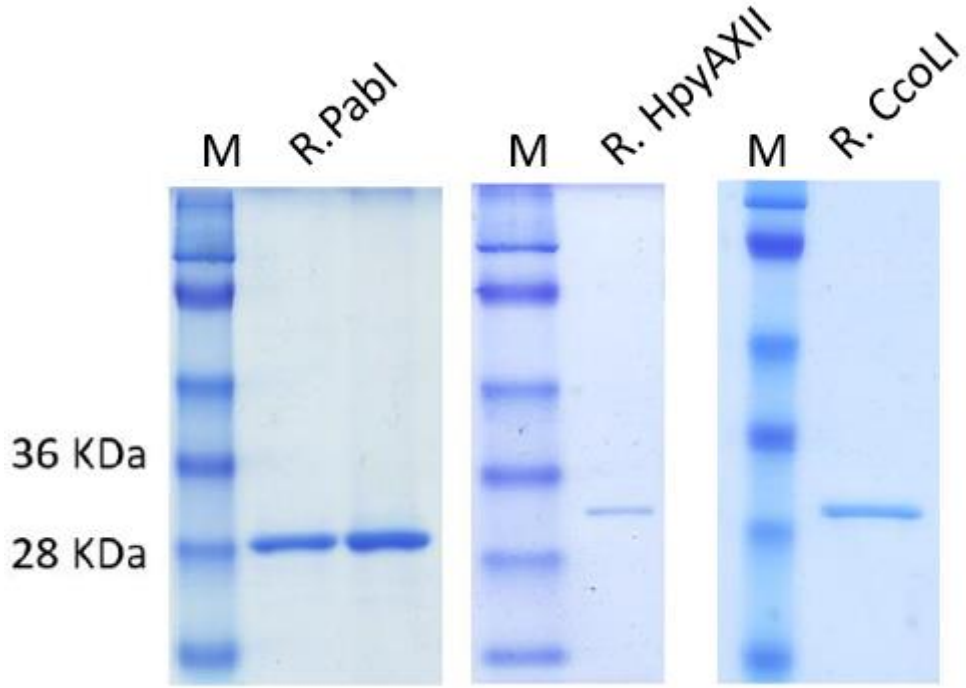

Figure 2. Purification of R.PabI, R.PabID214A, R.HpyAXII and R.CcoLI

Purified R.PabI, R.PabID214A, R.HpyAXII and R.CcoLI were visualized follwing 12% SDS-PAGE. The R.PabI/R.PabID214A, R.HpyAXII and R.CcoLI are expected to be 26 kDa (observed size ≈ 28 kDa), 29.3 kDa (observed size ≈ 33 kDa) and 27.8 kDa (observed size ≈ 29 kDa), respectively.

R.PabI is from a hyperthermophilic archaeon and shows no detectable AP lyase activity at 37°C, at which *in vivo* restriction can be studied with *E. coli*. We therefore turned to its homologs in mesophilic bacteria, R.CcoLI and R.HpyAXII from *Campylobacter coli* and *Helicobacter pylori*. They were expressed first *in vitro* with a wheat-germ-based cell-free protein synthesis system, and R.CcoLI was shown to have cleavage activity with lambda DNA (data not shown).

Then the two homologs were expressed with a His-tag in *E. coli* T7 Express *lysY/I*^{q} in the presence of a Chlorella virus DNA methyltransferase recognizing the same sequence. I purified them by Ni-NTA Agarose and Heparin HP column, and removed the His-tag. The purified enzymes were visualized by electrophoresis with 12% SDS-PAGE as a single band (Figure 2). The R.PabI/R.PabID214A, R.HpyAXII and R.CcoLI are expected to be 26 kDa, 29.3 kDa and 27.8 kDa.

2) Cleavage activity of R.PabI, R.PabID214A, R.HpyAXII and R.CcoLI

A 861-bp PCR product with a single “GTAC” site was reacted with R.PabI, R.PabID214A(20), R.HpyAXII and R.CcoLI. The DNA substrate can be cleaved by R.PabI by two products (309 bp, 551 bp)

The R.PabID214A did not show the cleavage activity. R.CcoLI and R.HpyAXII showed the same level of cleavage at 37ºC as R.PabI at 85 ºC (Figure 3). The R.PabI was demonstrated that the cleavage activity can be inhibited by m^6A methylation on "GTAC" site, we treated the plasmid pBAD30_*cviQIM* (which carrying a methyltransferase gene M. cviQI and the expression under the control of arabinose) with R.PabI and the mesophilic homologs, the result showed that the cleavage activity of the mesophilic homologs R.CcoLI and R.HpyAXII can also be inhibited by methylation on "GTAC" site (Figure S1).

3) Glycosylase and AP lyase activities of PabI homologs

Treatment with R.PabI and its homologs, R.HpyAXII and R.CcoLI, of DNA carrying a single recognition site (5'GTAC) gave the same product (by β-elimination) in phosphate buffer. When NaOH was added, another product was formed (by δ-elimination). These results show that the homologs have glycosylase activity at 37ºC (Figure 4A). An increase in the products with addition of NaOH suggests that the glycosylase and AP lyase are not coupled. The cleavage occurred with the same efficiency at the top strand and the bottom strand (Figure 4B). The specific glycosylase activity was estimated to be 0.11 pmol/pmol R.CcoLI/h (by calculation of the cleavage percentage, average of the top and bottom, from 0-0.2 pmol R.CcoLI when NaOH was added).

The AP lyase activity of CcoLI was confirmed by an oligonucleotide substrate with AP sites, which was generated by excision of uracil from 5'GTUC. The results show that R.CcoLI cleaves the AP site substrate and gives the same product at 37ºC as R.PabI did at a higher temperature (Figure 4C). The efficiency was the same with the top and bottom strands. It was estimated to be 0.072 pmol/pmol R.CcoLI/h in 0.1 M phosphate buffer (by calculation of the cleavage percentage, average of the top and bottom, from 0-1 pmol R.CcoLI). The ratio of the specific activities of glycosylase to AP lyase was 1.5:1(0.11 / 0.072, pmol/pmol R.CcoLI/h), which was much lower than the ratio (3.7:1) in R.PabI at 70ºC (11). In other words, R.CcoLI displays more coupling of AP lyase with glycosylase.

We found that the optimal NaCl concentration is 50 mM and the optimal pH is 7.0 for R.CcoLI glycosylase in Tris-HCl buffer. Under these conditions, the glycosylase and AP lyase activities of R.CcoLI were approximately 8 times higher than with 0.1 M phosphate buffer (Figure S2). However, the glycosylase/AP lyase ratio remained the same (1.4:1).

The presence of AP lyase in R.CcoLI was confirmed by trapping its Schiff-base intermediate by reduction with NaBH4 (Figure 4D), as with R.PabI (11). We do not know the nature of Complex 1 or Complex 2 or why their formation depended on the amount of enzyme. They could be related to monomer-dimer equilibrium.

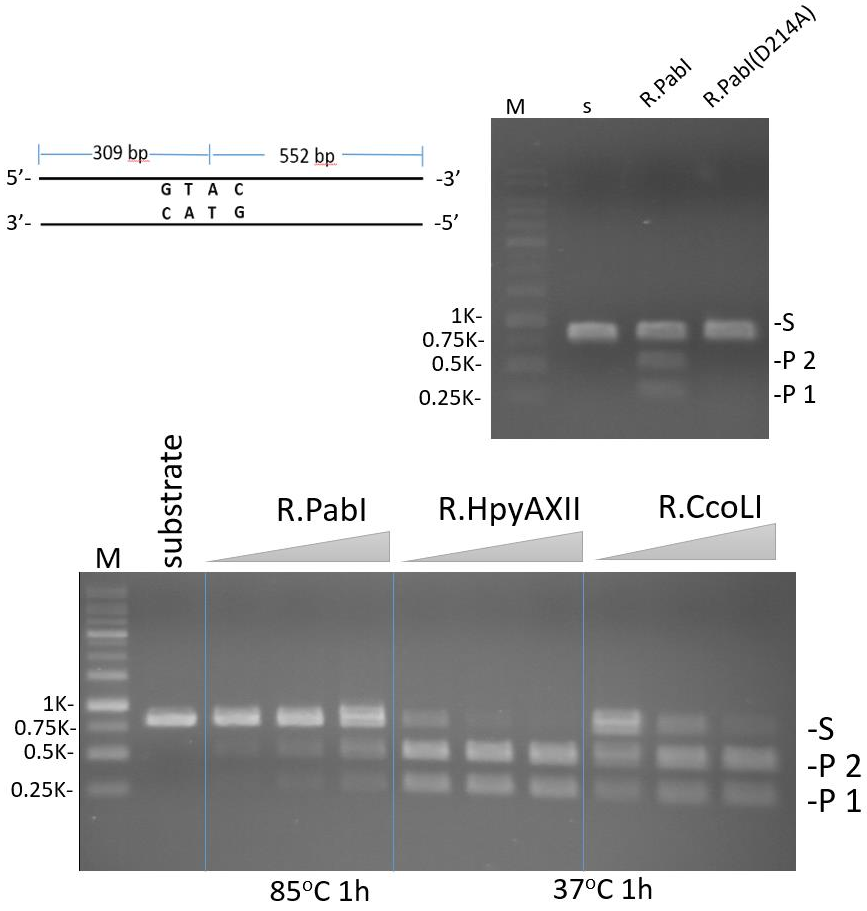

Figure 3 Cleavage activity of R.PabI, R.PabID214A, R.HpyAXII and R.CcoLI

Cleavage reactions were 10ul with 2.66 pmol of purified enzymes(3 fold dilution) and 0.38 pmol of PCR product DNA in 0.1 M sodium phosphate buffer (pH 6.5) at 85 ºC(R.PabI / R.PabID214A) or 37ºC (R.HpyAXII / R.CcoLI) for 1 h. DNA was separated by agarose gel electrophoresis

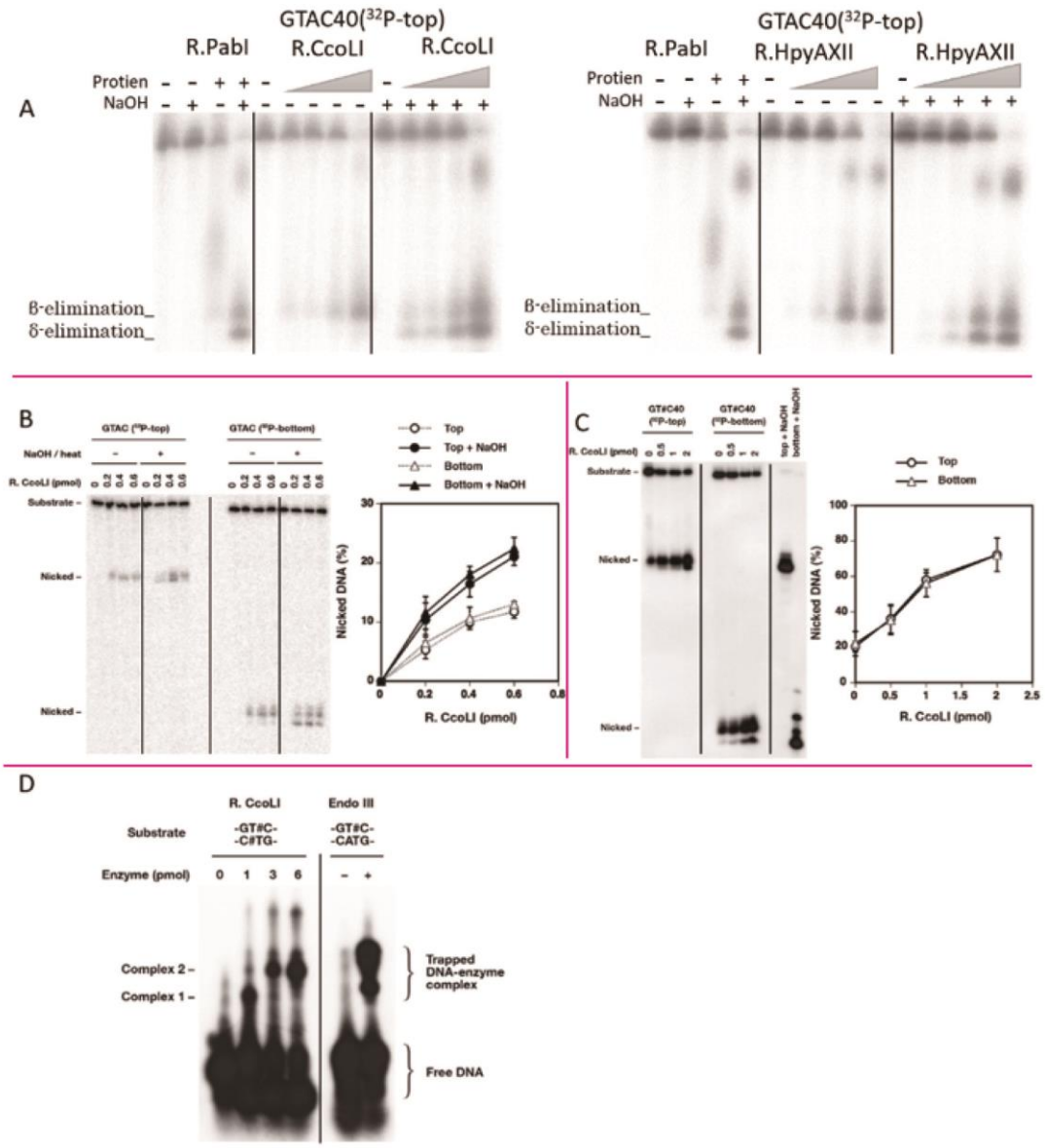

Figure 4. Biochemical activities of mesophilic homologs

A. 40-mer oligonucleotide with single "GTAC" (0.2pmol) (Top strand P^{32} labeled at 5') was treated with R.PabI, R.HpyAXII or R.CcoLI (1.4pmol, 4 fold dilution) in 100 mM phosphate buffer, pH6.5 for 1 h and then treated with 0.1M NaOH at 70°C for 10min and neutralized with HCl. Electrophoresis was with 18% denaturing PAGE. B. 0.1 pmol oligonucleotide (5'-GTAC/3'-CATG, P^{32}-labeled top or bottom strand) in 20 ul reaction mixture with 0-0.6 pmol purified R.CcoLI in 100 mM phosphate buffer, pH 6.5 at 60 °C or 37°C for 1h. Samples were separated by 18% denaturing PAGE. C. Oligos (5'-GTUC/3'-CUTG) treatment with UNG to generate the double AP sites. 20 ul reaction mixture with 0.1 pmol of the AP site substrate (P^{32}-labeled top or bottom strand) and 0-2 pmol purified R.CcoLI in100 mM phosphate buffer, pH 6.5 at 60 °C or 37°C for 1h. Samples were separated by 18% denaturing PAGE. D. The double or

single AP site substrates were generated by the UNG treatment, with the top strand P^{32}-labelled at 5' end. 0.2 pmol substrates were reacted with 0-6 pmol R.CcoLI or 20 units of Endo III in a buffer (20 mM MOPS-KOH, PH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) or another buffer (20 mM Tris pH 8.0, 1 mM EDTA , 1 mM DTT) at 37 °C for 20 min, and then incubated in 100 mM $NaBH_4$ at 25 °C for 30 min. DNA-R.CcoLI complexes were denatured in gel loading buffer containing 3% SDS at 90 °C for 10 min and separated by 10% SDS-PAGE. (B-D were carried out by Toshiaki Nakano, Tomoyuki Matsuzaka and Hiroshi Ide in the University of Hiroshima.)

2 Restriction of phage and chromosome *in vivo*

1) Restriction of phage propagation

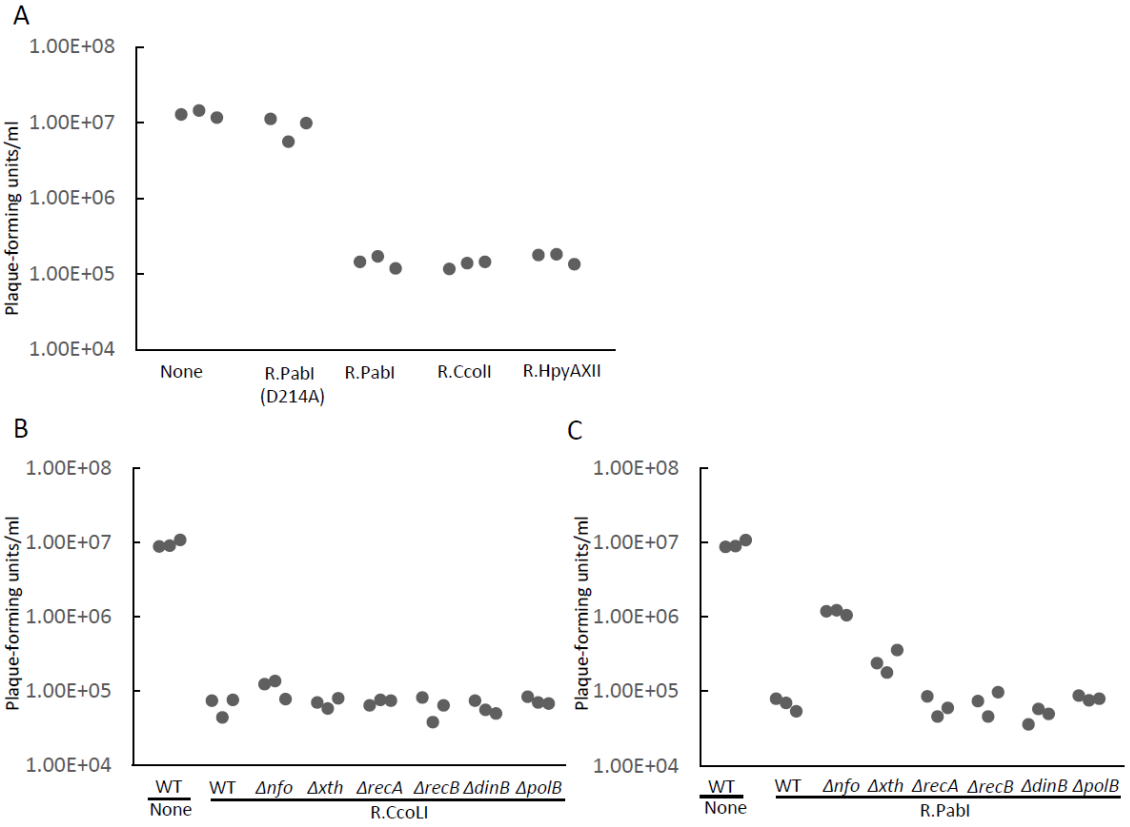

Figure 5. Restriction *in vivo* of bacteriophage λ propagation

Infection with bacteriophage λ of *E. coli* and mutant strains expressing a PabI family restriction enzyme. M.CviQI and restriction enzymes expression were induced by arabinose and IPTG, respectively. Plaques on the phage on the cells were counted. **A.** Restriction by PabI family. R-, with methyltransferase CviQI but without a restriction enzyme; R.PabI(D214A), with M.CviQI and R.PabI(D214A) with a mutation inactivating the glycosylase. **B.** Restriction by R.CcoLI in *E. coli* with deletion mutation in various genes. **C.** Restriction by R.PabI in *E. coli* mutants.

Whether or not R.PabI family restriction glycosylases can block phage propagation has not yet been tested. We expressed the restriction enzymes together with the chlorella methyltransferase, which methylates 5'GTAC, in *E. coli* cells and measured their ability to restrict infection with bacteriophage lambda *vir*. The wild-type lambda carries 113 "GTAC" sites. R.PabI decreased plaque formation by two orders of magnitude (Figure 5A). The restriction was not seen with R.PabI mutant D214A. As shown in previous studies (11) (29), the mutant could not cleave DNA with "GTAC" site (Figure 3). R.CcoLI and R.HpyAXII displayed the same level of restriction as wild-type R.PabI.

Because DNA glycosylases initiate base excision repair, we examined whether cellular DNA repair systems could enhance or alleviate the restriction mediated by R.PabI family enzymes. *E. coli* carry two major AP endonucleases, endonuclease IV encoded by *nfo* (21) and exonunuclease III encoded by *xth* (22)(23). For R.CcoLI (Figure 5B), the restriction level was not affected by deletion of these genes for AP endonucleases or by deletion of *dinB* or *polB* implicated in repair DNA synthesis (24) (25). The recA and recB deletions had no detectable effect. Lack of homologous recombination repair is expected because of the condition of single infection.

R.PabI, however, showed a different response to the mutations. Deletion of either of the two AP endonuclease genes weakened the restriction (Figure 5C). This indicates that the AP endonuclease promotes, as opposed to diminishes, the restriction, possibly by introducing a DNA strand break. The rec mutations or DNA polymerase mutations did not have a detectable effect.

2) Restriction of endogenous bacterial chromosomes

Restriction enzymes attack endogenous bacterial chromosomes under specific conditions (26). For Type II RM, such an attack takes place in cells that have lost the RM genes and is called post-segregational killing (2). The chromosome breakage is repaired by homologous recombination involving RecA and RecBCD proteins (27). We tried to replicate chromosome attack by suppressing expression of the chlorella methyltransferase gene under arabinose inducible promoter by the addition of glucose. As shown in Figure 6A, the suppression led to a decrease in viable cell counts by three orders of magnitude. In *recA* mutant and *recB* mutant strains, the decrease was larger. We did not detect such a decrease in the control with expressed methyltransferase (Figure 6B). This indicates that the chromosomal damage was repaired by homologous recombination, presumably between sister chromosomes as with Type II restriction phosphodiesterases (27).

With R.PabI, we observed a comparable decrease in viable cell counts, which was eliminated by the D214A mutation (Figure 6C). The decrease was again more severe in the *recA* and *recB* mutants, which suggests recombination repair of the damage. The mutations in two AP endonuclease genes diminished the cell killing. The effect was stronger with *nfo* mutant (Figure 6C, D). This indicates that AP

endonucleases promote cell killing likely through chromosome cleavage at AP sites.

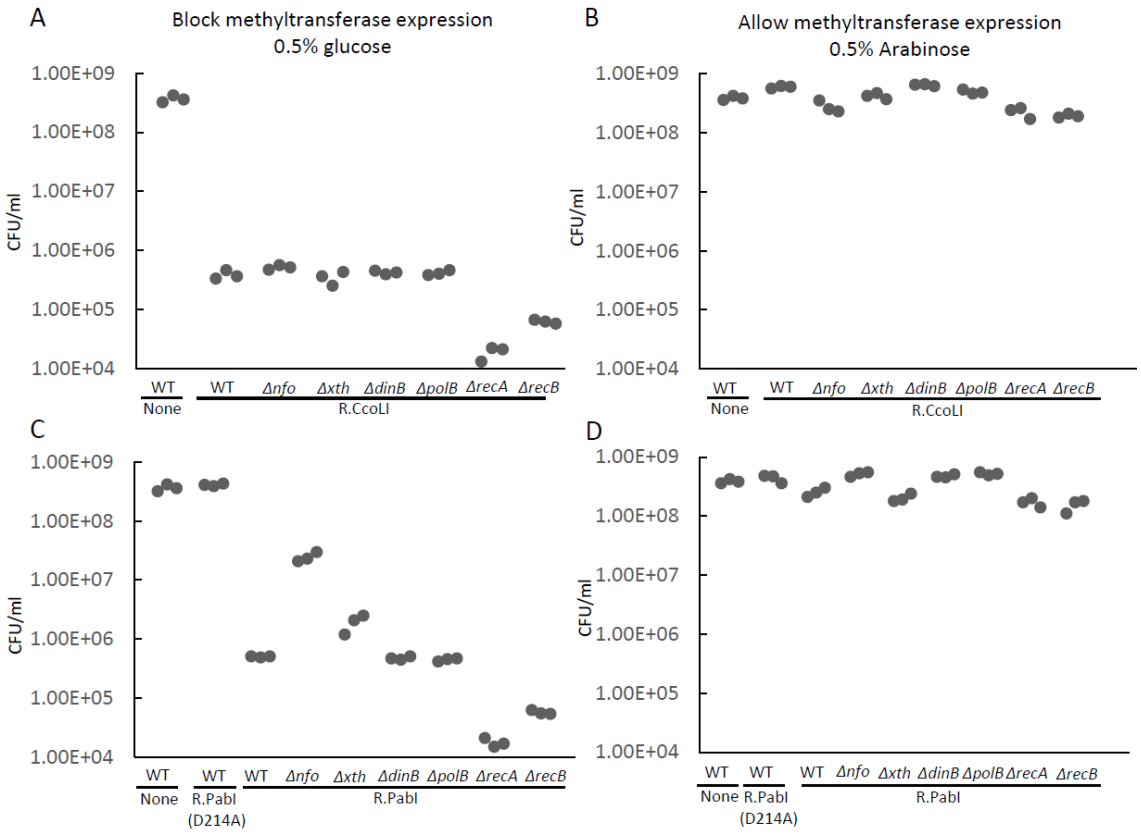

Figure 6. Restriction of bacterial endogenous chromosome *in vivo*

Survival of *E. coli* wild type and mutant strains expressing a PabI family restriction enzyme and a DNA methyltransferase after block of methyltransferase expression. Expression of M.CviQI and PabI family enzymes in *E. coli* T7 Express *lysY/Iq* was induced by arabinose and IPTG, respectively. After removal of the inducers, the methyltransferase was decreased and exposed newly replicated chromosomal sites to lethal attack by the remaining restriction enzyme molecules.

3）Restriction of transforming plasmid

An earlier study demonstrated the HpyAXII RM system restricts incoming plasmid and chromosomal DNA in *H. pylori* (20). When a plasmid preparation was treated with R.PabI at 37°C, it remained supercoiled, which means that it contained no strand breaks at all, but it had lost the ability to transform into *E. coli* (11). This suggests that base excision is responsible for the restriction. In order to see what gene function is involved in this restriction, we repeated this experiment with *E. coli* mutated in the above genes (Figure 7A, B). We found that the mutations in the two AP endonuclease genes increased transformation efficiency (Figure 7B). This suggests again that the AP endonucleases promote restriction

likely by introducing strand cleavage at the AP sites on the incoming plasmid.

3 Difficulty in repair by rejoining of the cleavage ends

As expected from the atypical end structure, the AP lyase-generated ends are not rejoined by DNA ligase (28). Indeed, DNA treated with R.PabI was not easily rejoined by DNA ligase *in vitro* (6). We examined whether this is also the case *in vivo*. We prepared a plasmid carrying a single site for PabI homologs and KpnI (5'GGTACC) (Figure S3). When we treated the plasmid with KpnI or CcoLI (Figure 7C), the transformation efficiency decreased by four orders of magnitude (Figure 7D). Treatment with DNA ligase moderated this decrease for KpnI but not for CcoLI (Figure 7D).

When the plasmid was treated with R.PabI and then with AP endonuclease (endonuclease IV), we expected the end structure illustrated in Figure 1 vi. We found the same decrease in transformation efficiency. This decrease was not moderated by treatment with DNA ligase, as expected (Figure 7C, D).

This and the above results suggest that the biological significance of the PabI family of restriction glycosylases may lie in the difficulty of repair by end joining, especially for incoming, un-methylated DNA. We carried out relevant analyses as discussed in the two following sections.

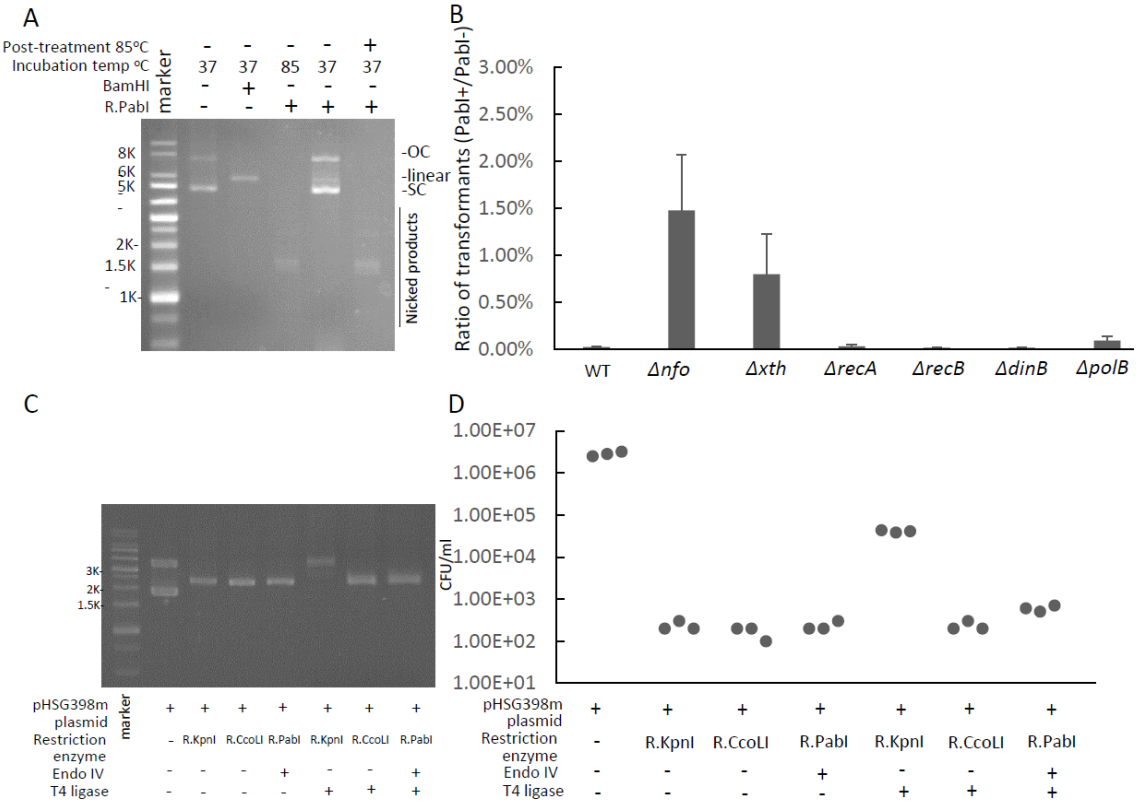

Figure 7. Restriction of transforming plasmid

A. An un-methylated plasmid (pBAD30_*cviQIM* prepared under the condition of suppression of methyltransferase expression) was treated with purified R.PabI or BamHI at 37ºC for 1h. **B.** The supercoiled plasmid treated with R.PabI at 37ºC in A was purified and electroporated into T7 Express *lysY/I^q E. coli* wild type and its mutant derivatives. **C**. A plasmid with a single site for PabI family (= a site for KpnI) (pHSG398m) was treated purified R.PabI, R.CcoLI or KpnI at 37ºC for 1h. The product with R.PabI was further treated with endonuclease IV (an AP endonuclease) for strand cleavage. The three linearized plasmids were purified and treated with T4 DNA liagase. **D**. The products in Figure C were used for transformation into *E. coli* HST08. The colony forming units were counted.

Discussion

These results indicate that, in the restriction action by the PabI family, endonucleases play a crucial role in the restriction action by the PabI family. Tighter coupling of AP lyase to glycosylase activity in a mesophilic enzyme demonstrates their close collaboration *in vitro* and suggests that *in vivo*. It is of interest which amino acid is involved in the trapped Schiff intermediate in the lyase reaction (Figure 4D). We did not detect a decrease in AP lyase activity in R.PabI mutants at either of the two conserved Lys residues (K73A, K 202A) (29).

The *E. coli* AP endonucleases assisted restriction in *E. coli* by R.PabI, which shows undetectable AP lyase activity at 37ºC (11). AP endonucleases are involved in DNA damage repair (30) but also in DNA destruction in mammalian apoptosis (31).

Our results suggest that the AP lyase and AP endonucleases promote restriction by introducing a DNA strand break. However, we cannot exclude the contrasting, but not necessarily exclusive, possibility that the endonucleases release the restriction glycosylase from DNA after base excision and allow its reaction on another substrate

In AP endonuclease mutants, R.PabI-mediated restriction of the incoming DNA or chromosomal DNA is modulated. This suggests that AP sites are repaired or tolerated by other mechanisms such as translesion DNA synthesis. In relation to this, we do not know whether the PabI family acts as a mutator, an anti-mutator, or neither.

What could be the biological significance of these restriction glycosylases? Type II RM systems attack the host bacterial chromosome in post-segregational killing. When their genes are lost from a cell, its descendant will carry fewer methylase molecules to protect hemimethylated sites generated after chromosome replication. The remaining restriction enzyme will cleave exposed sites. Bacterial homologous recombination systems (RecA, RecBC) assisted repair of restriction cleavage of chromosomal DNA likely through recombination between sister chromosomes but did not help repair of a single invading DNA (27) (33). As in type IIP systems, this represents the interaction of three selfish elements: the bacterium, the RM system, and the invading DNA. The bacterial genome protects its

chromosomes but not the invading DNA.

These glycosylases may discriminate between incoming DNA and endogenous chromosomal DNA in another way. R.PabI and R.CcoLI can cleave hemi-methylated DNA, present on newly-replicated chromosomal DNA, to generate a single-strand break as type IIP restriction phosphodiesterases (11) (Figure S4). A single-strand break of hemi-methylated DNA will be readily repaired by the base excision repair machinery (34) (35) as well as by homologous recombination. On the other hand, incoming DNAs are likely to be either fully methylated or un-methylated. The latter will suffer a double-strand break, which cannot be repaired by base excision repair or by recombination repair in single infection (33). The unique feature of these restriction glycosylases is the generation of ends with atypical structures lacking bases via AP lyase or AP endonucleases (Figure 1), which are not readily repaired by end joining (Figure 7). . RM systems consisting of a restriction glycosylase and methylase are, therefore, very effective means of combating invading non-self DNAs but are helpful in repairing endogenous bacterial chromosomes, once restriction avoidance has prevailed in the chromosomes (Figure 8). Our results are consistent with such a concept of distinguishing endogenous and invading DNAs. The small effect of the *H. pylori* homolog on gene expression (Table S4, Figure 9), possibly in part due to the low number of GTACs in the genome, highlights their role as 'a strong poison to invaders' as opposed to a gene regulator. There may be many other factors that can affect efficiency of restriction. Clearly, we need more experiments to test this hypothesis.

The difficulty in repair by end joining may be useful in applications. For example, in treating with a mixture of an R.PabI family enzyme and a type II restriction phosphodiesterase *in vitro*, only ends created by the latter will be easily rejoined by a ligase. *In vivo*, a specific target DNA may be effectively destroyed by these restriction glycosylases. These effects may be useful in genome manipulation.

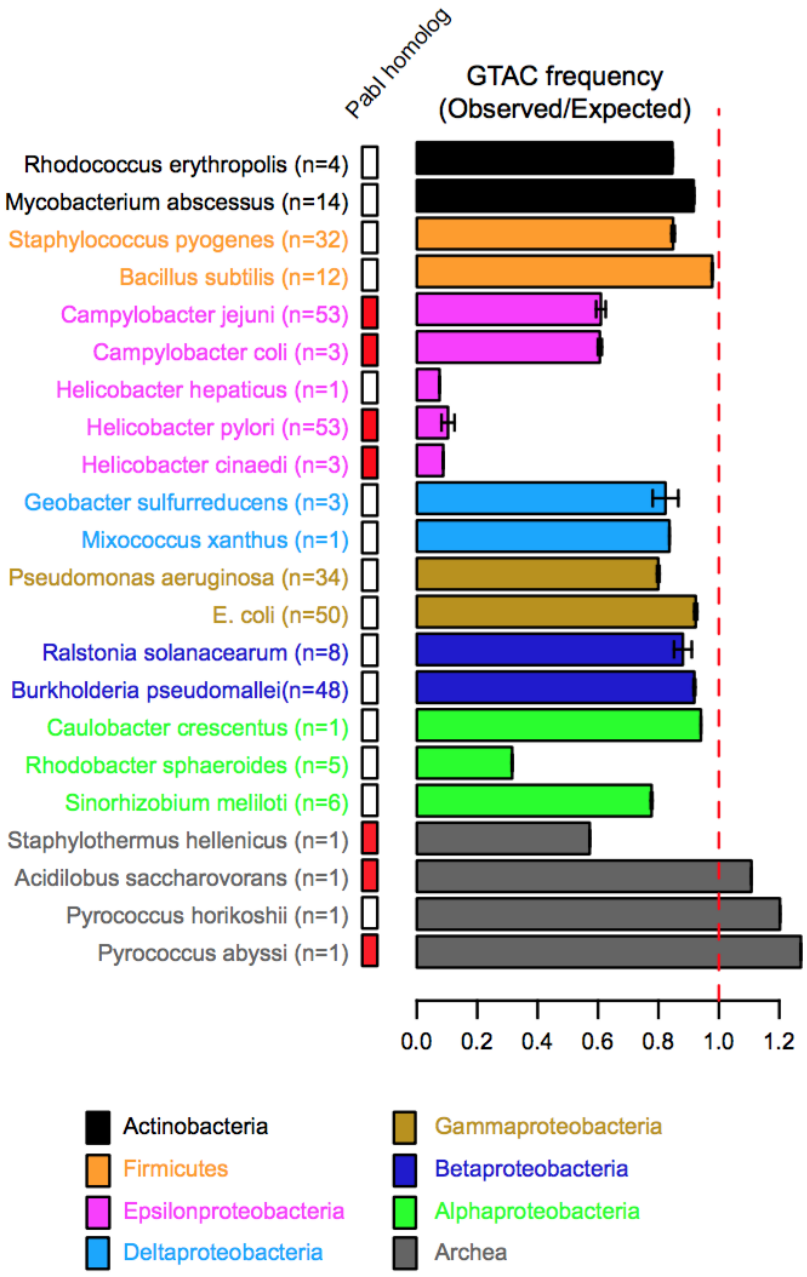

Figure 8. Avoidance of GTAC motif on the chromosome of Campylobacter and Helicobacter. Species belonging to different taxa are shown in distinct colors. Barplot indicate the mean and SD. Sidebar indicates presence (red) or absence (white) of PabI family RM system. By Dr. Hirokazu Yano.

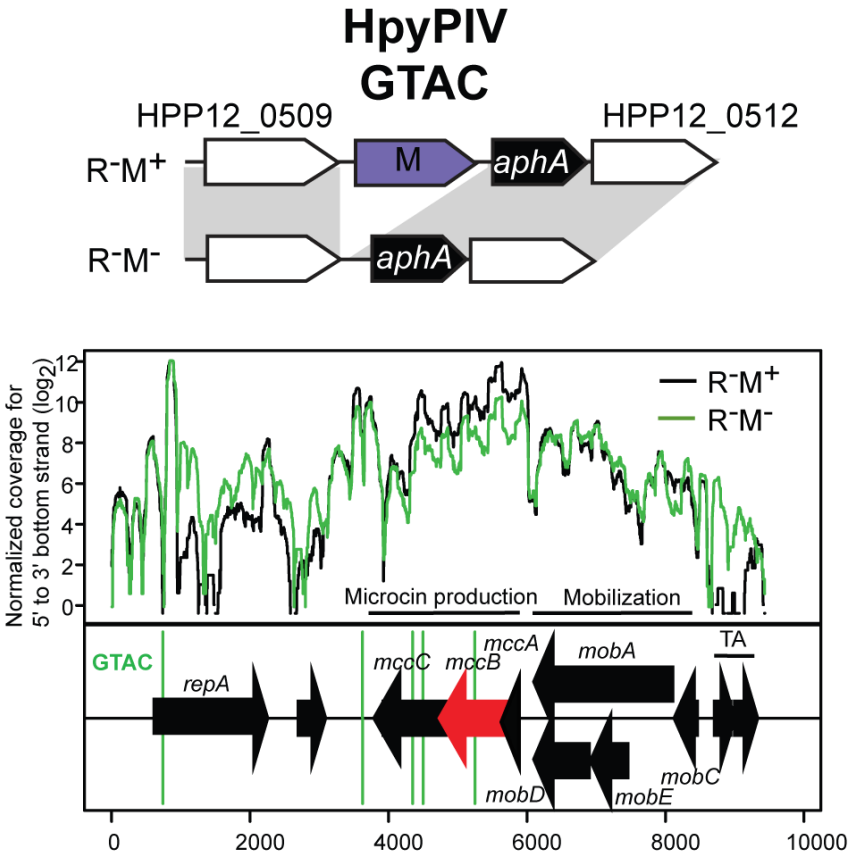

Figure 9. Effect of *hpyPIVM* knockout on *H.pylori* gene expression.

Upper panel: genetic map of the HpyPVI locus in the two isogenic strains PIK70 (up) and PIK69 (bottom). Lower panel: RNA-seq read coverage on the plasmid pHel12 indicating the reduced expression of the *mcc* operon in the R^-M^- strain PIK69. The *mccB* gene (red) differentially expressed. Vertical lines on the lower gene map indicate the position of GTAC site，aphA: a kanamycin resistance gene. Experiment carried out by Dr. Hirokazu Yano.

Contribution

In this thesis, I carried out most of the experiments. The trapping of R.CcoLI-DNA intermediate was carried out by Dr. Toshiaki Nakano, Tomoyuki Matsuzaka and Hiroshi Ide in in Hiroshima University. Dr. Ken Ishikawa expressed R.CcoLI *in vitro* and detected its DNA cleavage activity. Dr. Hirokazu Yano analyzed restriction site avoidance. Dr. Yano with the members of labs of Sumio Sugano and Yokata Suzuki carried out transcriptome analysis. Masaki Fukuyo, Noriko Takahashi guided me in the experiment. pEU3-NIIb plasmid was kindly provided by Dr. Yaeta Endo, Ehime University, Japan. This work was conducted under the supervision and project design by Professor Ichizo Kobayashi. Ichizo Kobayashi.

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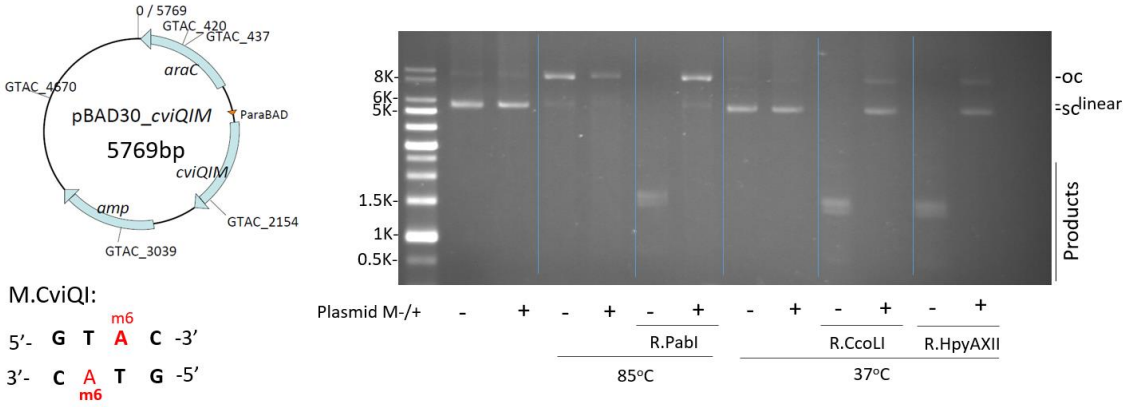

Figure S1. Cleavage activity inhibition by "GTAC" methylation

The pBAD30_*cviQIM* plasmid was modified or un-modified under the control of arabinose in medium culture. The modified and un-modified plasmid were treated with R.PabI (85°C), R.CcoLI and R.HpyAXII (37°C) for 1h.

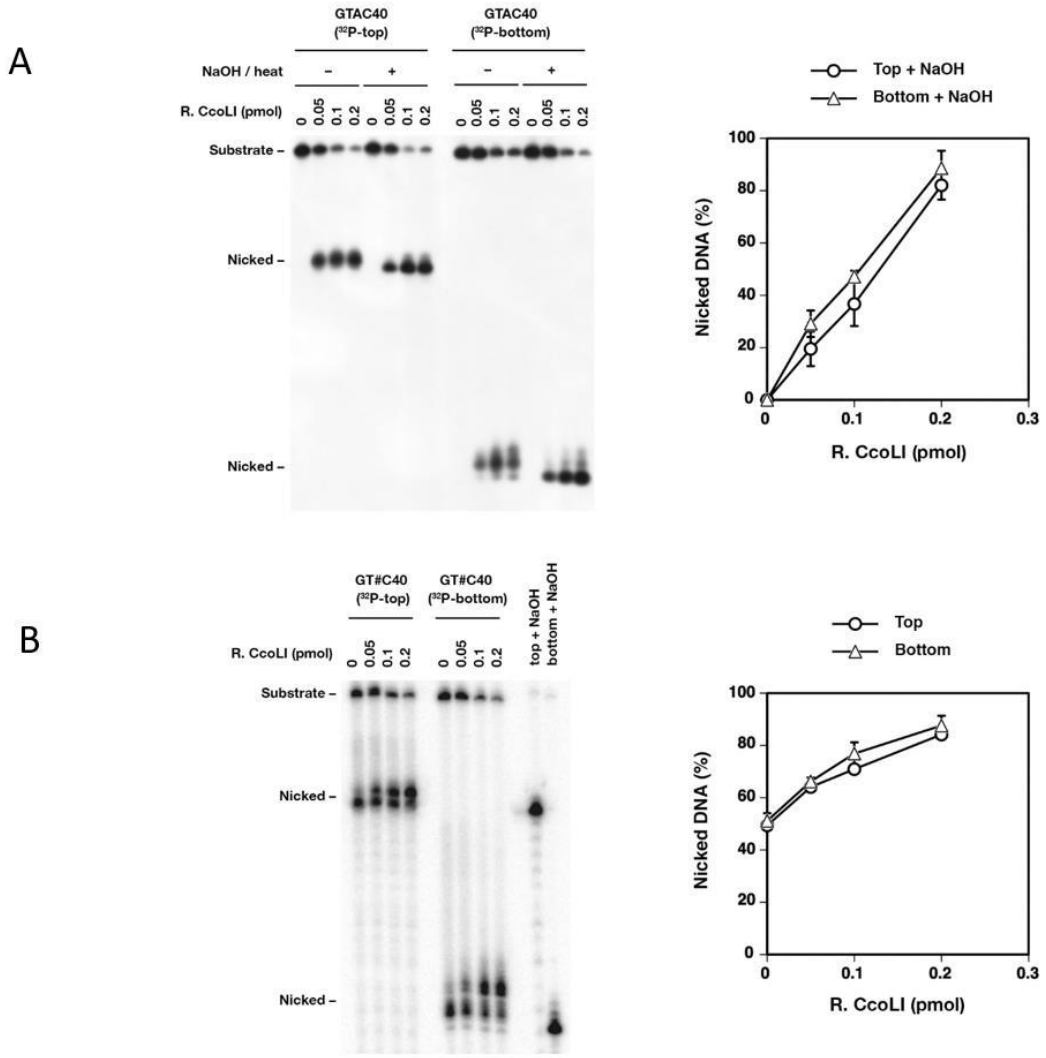

Figure S2. Glycosylase and AP lyase activities of R.CcoLI under an optimized condition

The reaction condition was optimized for glycosylase activity in 20 mM MOPS buffer (50 mM NaCl, pH 7.0) at 37 ^{o}C. **A**. Glycosylase. A 40 mer oligo with single "GTAC" (0.2 pmol) (Top or bottom strand P^{32} labeled at 5') was treated with 0 - 0.2 pmol R.CcoLI for 1h. The mixture was further treated with 0.1 M NaOH at 70 ^{o}C for 10min, neutralized with HCl and electrophoresd through 18% denaturing PAGE. **B**. AP lyase. An oligo with 5'-GTUC/3'-CUTG was treated with Uracil DNA Glycosylase to generate the double AP sites. A 20 ul reaction mixture with 0.1 pmol of the AP-site substrate (P^{32}-labeled in the top or bottom strand at 5' end) and 0 - 0.2 pmol R.CcoLI for 1 h. Samples were separated through 18% denaturing PAGE.

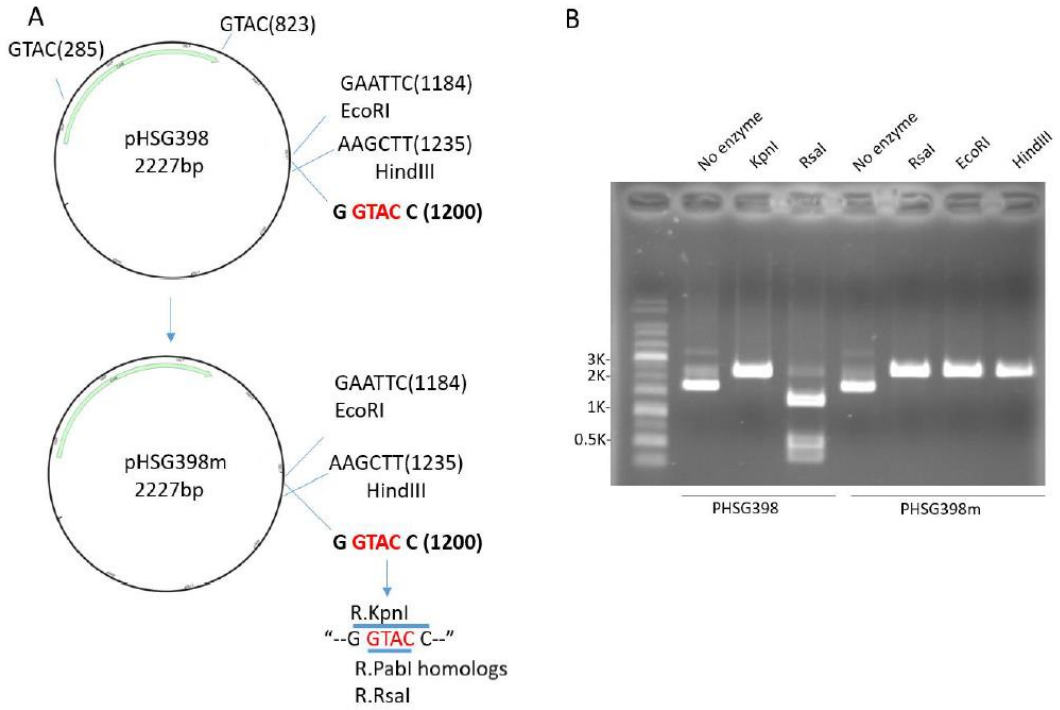

Figure S3. Construction of a plasmid with a single R.PabI site (= KpnI site)

A. Plasmids. **B.** Confirmation of structure of pHSG398m by cleavage with restriction enzymes. R.RsaI recognizes 5'GTAC as R.PabI.

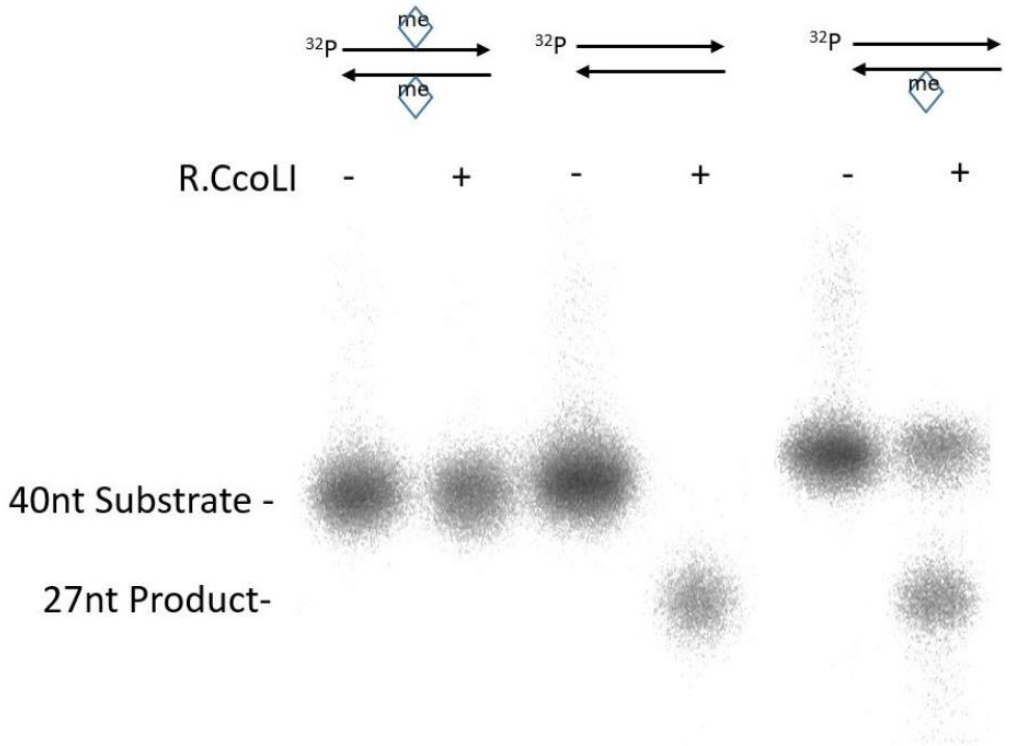

Figure S4. Cleavage of hemi-methylated DNA by R.CcoLI

Hemi-methylated substrates were generated by annealing with different paired strand. The substrates was with P^{32}labelled at 5' of top strand, 10 ul reaction mixture with 0.1 pmol substrate and 0.7 pmol R.CcoLI in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 37^{o}C for 1h, samples were separated by 10% denaturing PAGE.

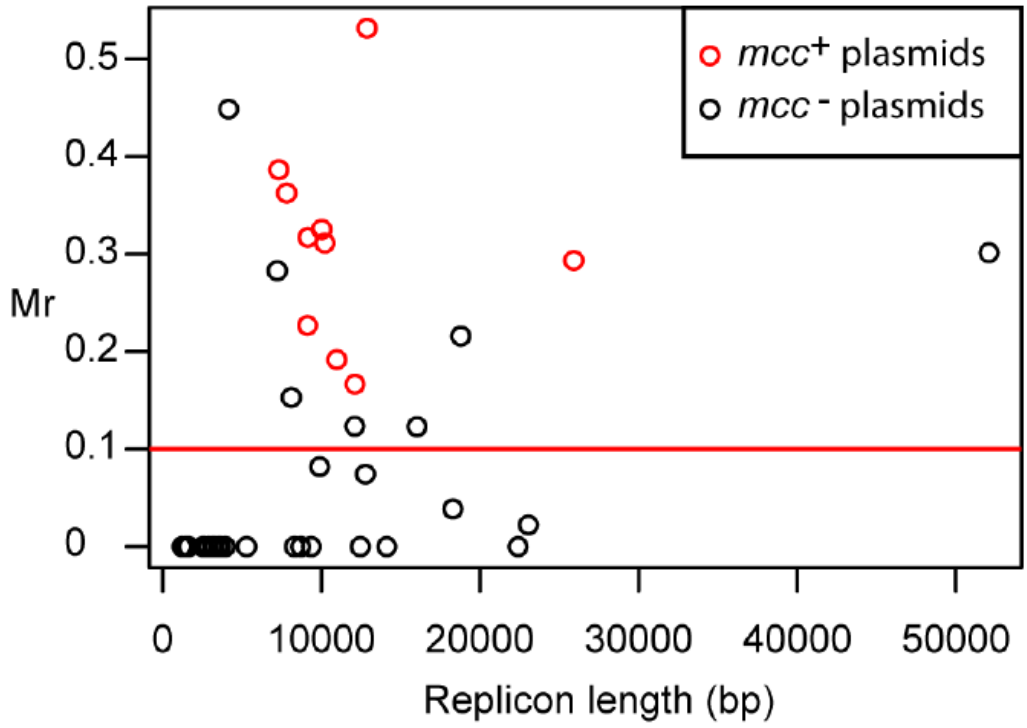

Figure S5. GTAC motif acoidance in Helicobacter pylori plasmids (N=41)

Some plasmids show GTAC motif frequency higher than chromosome average (0.10, red line). Note that some plasmids without mycrocin operon (*mcc*-) also show relatively high GTAC motif frequency.

Table S1. *E. coli* strains and plasmids used

	Strains NO.	Strains name	Relevant Properties	source
E. coli Strains	BIK35001	*E. coli* HST08	*F⁻, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ⁻*	TaKaRa
	BIK35004	*E. coli* T7 Express *lysY/I^q*	*MiniF lysY lacI^q(CamR) / fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10--Tet^S) endA1 Δ(mcrC-mrr) 114::IS10*	New England Biolabs
	BIK35016	*E. coli* JW2146	*Δnfo*-786::kan	KEIO-collection
	BIK35019	*E. coli* JW1738	*Δxth*::kan	KEIO-collection
	BIK35020	*E. coli* JW0221	*dinB*749(del)::*aph*	KEIO-collection
	BIK35022	*E. coli* JW0059	*polB*770(del)::*aph*	KEIO-collection
	BIK35028	*E. coli* JW2669	*recA*774(del)::*aph*	KEIO-collection
	BIK35029	*E. coli* JW2788	*recB*745(del)::*aph*	this work
	BIK35006	*E. coli* T7 Express *lysY/I^q* (M+PabI+)	BIK35004 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK35009	*E. coli* T7 Express *lysY/I^q* (M+PabID214A+)	BIK35004 (pBAD30_*cviQIM*)(pET28a_*pabIRD214A*)	this work
	BIK35010	*E. coli* T7 Express *lysY/I^q* (M+HpyAXII+)	BIK35004 (pBAD30_*cviQIM*)(pET28a_*hpyAXIIR*)	this work
	BIK35011	*E. coli* T7 Express *lysY/I^q* (M+CcoLI+)	BIK35004 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK35015	*E. coli* T7 Express *lysY/I^q* (M+)	BIK35004(pBAD30_*cviQIM*)(pET28a)	this work
	BIK35036	*nfo-E. coli* T7 Express *lysY/I^q*	*Δnfo*-786, P1 Transduction from BIK35016 to BIK35004	this work

	BIK3503 7	*polB-E. coli* T7 Express *lysY/I*^{q}	*Δxth*, P1 Transduction from BIK35019 to BIK35004	this work
	BIK3503 8	*Xth-E. coli* T7 Express *lysY/I*^{q}	*ΔdinB*749, P1 Transduction from BIK35020 to BIK35004	this work
	BIK3503 9	*dinB-E. coli* T7 Express *lysY/I*^{q}	*ΔpolB*770*,* P1 Transduction from BIK35022 to BIK35004	this work
	BIK3504 0	*recA-E. coli* T7 Express *lysY/I*^{q}	*ΔrecA*774, P1 Transduction from BIK35028 to BIK35004	this work
	BIK3504 1	*recB-E. coli* T7 Express *lysY/I*^{q}	*ΔrecB*745*,*P1 Transduction from BIK35029 to BIK35004	this work
	BIK3504 8	*nfo-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35036 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3504 9	*polB-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35037 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3505 0	*Xth-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35038 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3505 1	*dinB-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35039 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3505 2	*recA-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35040 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3505 3	*recB-E. coli* T7 Express *lysY/I*^{q} (M+PabI+)	BIK35041 (pBAD30_*cviQIM*)(pET28a_*pabIR*)	this work
	BIK3505 4	*nfo-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35036 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK3505 5	*polB-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35037 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK3505 6	*Xth-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35038 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK3505 7	*dinB-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35039 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK3505 8	*recA-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35040 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
	BIK3505 9	*recB-E. coli* T7 Express *lysY/I*^{q} (M+CcoLI+)	BIK35041 (pBAD30_*cviQIM*)(pET28a_*ccoLIR*)	this work
Plasmids		pBAD30	P_{BAD},Cml^{R}	2015,Fukuyo,NAR

pBAD30_*cviQIM*	pBAD30 CviQIM+	2015,Fukuyo,NAR
pET28a	P_{T7}, Kan^R, $lacI^Q$	2015,Fukuyo,NAR
pET28a_*pabIR*	pET28a::*pabIR*	2015,Fukuyo,NAR
pET28a_*pabIRD214A*	pET28a::*pabIR(D214A)*	this work[27]
pET28a_*ccoLIR*	pET28a::*ccoLIR*	this work
pET28a_*hpyAXIIR*	pET28a::*hpyAXIIR*	this work
pTAKN-2-*ccoLI*	pTAKN-2::*ccoLI*	Funakoshi Co., Ltd
pTAKN-2-*hpyAXII*	pTAKN-2::*hpyAXII*	Funakoshi Co., Ltd
pEU3-NIIb_*ccoLIR*	pEU3-NIIb::ccoLIR	this work
pEU3-NIIb_*hpyAXII*	pEU3-NIIb::*hpyAXII*	this work
PFLP3	Ap^R, Tc^R; Source of Flp recombinase	Addgene

Table S2 Oligos and primers used in *E. coli* experiment

Oligonucleotide name	Lengthnt /bp	sequence	Source
GTAC40T	40	5'GGGGAGGCGCCGGCAGTGCGTCAGGTACTCCGCCACGTCC3'	Hokkaido System Science
GTAC40B	40	5'GGACGTGGCGGAGTACCTGACGCACTGCCGGCGCCTCCCC3'	Hokkaido System Science
GTAC40Tme	40	5'GGGGAGGCGCCGGCAGTGCGTCAGGTmACTCCGCCACGTCC3'	Hokkaido System Science
GTAC40Bme	40	5'GGACGTGGCGGAGTmACCTGACGCACTGCCGGCGCCTCCCC3'	Hokkaido System Science
GTUC40T	40	5‘GGGGAGGCGCCGGCAGTGCGTCAGGTUCTCCGCCACGTCC3’	Hokkaido System Science
GTUC40B	40	5‘GGACGTGGCGGAGTUCCTGACGCACTGCCGGCGCCTCCCC3’	Hokkaido System Science
GT#C40	40	5‘GGGGAGGCGCCGGCAGTGCGTCAGGT#CTCCGCCACGTCC3’ 5‘GGACGTGGCGGAGT#CCTGACGCACTGCCGGCGCCTCCCC3’	Treat GTUC40T/GTUC40B with UNG, # = AP site
R.pabI_R	44	5‘TGGTGGTGGTGGTGCTCGAGTTATGAAGTGCCGATAATACTCCT3’	Hokkaido System Science
R.pabI_F	36	5‘CGCGCGGCAGCCATATGATTCATTTGACTAGTGTAG3’	Hokkaido System Science
HpyAXII-F	39	5‘GTGCCGCGCGGCAGCCATATGTCCCTCATTCGCATCGAC3’	Hokkaido System Science
HpyAXII-R	45	5‘GTGGTGGTGGTGGTGCTCGAGTTAATTCTGCAGAATTTTCTCCAG3’	Hokkaido System Science
CcoLI-F	39	5‘GTGCCGCGCGGCAGCCATATGAAATTCAAAATTGACTAT3’	Hokkaido System Science
CcoLI-R	44	5‘GTGGTGGTGGTGGTGCTCGAGTTACTTACTGTTCAGAATGAACT3’	Hokkaido System Science
P-D214A-R	71	5'TGGTGGTGGTGGTGCTCGAGTTATGAAGTGCCGATAATACTCCTCAAAAATTTAACAATAGCATTCTTGTG3’	Hokkaido System Science

P-D214A-F	36	5‘CGCGCGGCAGCCATATGATTCATTTGACTAGTGTAG3’	Hokkaido System Science
m-phsg398-F	24	5‘TCAGTTGCTCAATGCACCTATAAC3’	Hokkaido System Science
m-phsg398-R	24	5‘CTCATCGCAGTATTGTTGTAATTC3’	Hokkaido System Science
m-phsg398-F-2	20	5‘GAATTACAACAATACTGCGATGAG3’	Hokkaido System Science
m-phsg398-R-2	20	5‘GTCTGGTTATAGGTGCATTGAG3’	Hokkaido System Science
pBAD30F	22	5‘ATGAGTATTCAACATTTCCGTG3’	Hokkaido System Science
pBAD30R	23	5‘TTACCAATGCTTAATCAGTGAGG3’	Hokkaido System Science
CF-CcoLI-F	37	5'ATCCATATGAAATTCAAAATTGACTATGAACTGCCGT3'	Hokkaido System Science
CF-CcoLI-R	44	5'CGGGATCCCGTTACTTACTGTTCAGAATGAACTCAATAATCTGA3'	Hokkaido System Science
CF-HpyAXII-F	28	5'ATCCATATGTCCCTCATTCGCATCGACA3'	Hokkaido System Science
CF-HpyAXII-R	43	5'CGGGATCCCGTTAATTCTGCAGAATTTTCTCCAGAATTTTGAG3'	Hokkaido System Science
nfo-F	20	CACTACATCTTGCTCCTGTT	Hokkaido System Science
nfo-R	20	CAATTTCGTTCTGCTGAATC	Hokkaido System Science
xthA-F	20	GACATCATTAACAACCATCG	Hokkaido System Science
xthA-R	20	CAAGGTTAATTCTCCTGACC	Hokkaido System Science
polB-F	19	TTACGGGCAGTAATGACTG	Hokkaido System Science
polB-R	22	CCGTGCTTATGAGGTAGTGGTG	Hokkaido System Science
dinB-f	19	GTGGTGCAGCCGCTGGTGC	Hokkaido System Science

dinB-r	20	CAGCGAGAATTCGATGCATG	Hokkaido System Science
recA-F	20	TGCTTCAACAGAACATATTG	Hokkaido System Science
recA-R	20	AGGCGAGCATATGCCGGGCG	Hokkaido System Science
recB-F	20	AGTGTGGGAGAACGTCAGCG	Hokkaido System Science
recB-R	20	AATTGCACATCCAGCGGGCG	Hokkaido System Science

Table S3 Primers used in *Helicobacter pylori* transcriptome analysis

Primers	Sequence	Use	Source
HPP12_0511_ Lf	CTCTAGAGGATCCCCTTTAGTCAATCCTA AAAGAGAAAA	Construction of pMZA3, pHY1279	This study
HPP12_0511_ Lr	ACACAATATGGCGGATTAATCAAATTCAC AAGGGTGTC	Construction of pMZA3,	This study
HPP12_0511_ Lr2	CTCAAATGGTTCGCTGGGTTTTAATCAAA TTCACAAGGGTGTCC	Construction of pHY1279	This study
catf	TCCGCCATATTGTGTTGAAAC	Construction of pMZA3	This study
catr	GGGCACCAATAACTGCCTTA	Construction of pMZA3	This study
HPP12_0511_ Rf	CAGTTATTGGTGCCCTAATGATACGAATT GTTAGAAAGG	Construction of pMZA3	This study
HPP12_0511_ Rr	TCGAGCTCGGTACCCTTTTGATAGTCGGC TGGCCAA	Construction of pMZA3, pHY1279	This study
HPP12_0510_ Lf	CTCTAGAGGATCCCCTGACTTTTTTCTATC GTTATTTACGCC	Construct ion of pHY1278	This study
HPP12_0510_ Lr	CTCAAATGGTTCGCTGGGTTTTACACATC TAGCTGTTTCACGTCTTT	Construction of pHY1278	This study
HPP12_0511_ Rf4	ATGAATTGTTTTAGTACCTAGATTTAGATG TCTAAAAATAATGATA CGAATTGTTAGAAAGGGAATATC	Construction of pHY1278, pHY1279	This study
AphA3f	TTTTTAGACATCTAAATCTAGGTACTAAA AC	Construction of pHY1278, pHY1279	This study
AphA3r	AACCCAGCGAACCATTTGAG	Construction of pHY1278, pHY1279	This study

Table S4. Genes were differentially expressed upon the knockout of the methyltransferase gene

temporal gene ID	product-name *	position (left)*	position (right)*	strand	refseq_locus_tag	old_locus-tag	GTAC motif around the gene	M+R-rep1	M+R-rep2	M-R-rep1	M-R-rep2	a.value	m.value	p.value	q.value
gene_91	Uncharacterized protein	96046	96222	-	HPP12_RS00485	HPP12_0093	N	208.56	187.82	22505.97	21837.98	11.03	6.81	2.14E-97	3.46E-94
gene_517	M. HpyPIV	53543	53643	+	HPP12_RS02620	HPP12_0510	N	1160.39	1395.52	0.00	2.05	5.18	-10.29	2.04E-77	1.65E-74
gene_954	Uncharacterized protein	10026	10028	-	NA	NA	N	14.05	13.62	818.43	641.15	6.65	5.72	2.22E-45	1.19E-42
gene_1552	hypothetical protein	16244	16245	+	NA	NA	N	12.57	6.81	924.03	562.29	6.41	6.26	2.72E-40	1.10E-37
gene_136	(S)-2-hydroxy-acid oxidase domain protein	14590	14610	+	NA	NA	N	7.40	8.76	180.15	161.82	5.22	4.40	1.92E-23	6.20E-21
gene_890	hypothetical protein	92924	92955	-	NA	NA	N	11.83	10.70	184.81	171.04	5.48	3.98	1.32E-21	3.55E-19
gene_1526	Type I RM system S protein (GRAN7TAYC)	15980	15992	-	HPP12_RS07730	HPP12_1508	N	149.39	146.95	841.72	1398.03	8.67	2.92	5.01E-19	1.16E-16
gene_1170	Uncharacterized protein	12396	12399	+	NA	NA	N	14.79	18.49	225.18	161.82	5.83	3.54	5.08E-18	1.02E-15
gene_318	membrane protein	32815	33028	-	NA	NA	N	4749.53	3850.82	19712.14	21870.75	13.21	2.27	2.13E-17	3.82E-15
gene_231	Uncharacterized protein	23545	23563	+	NA	NA	N	8.14	10.70	88.52	100.37	4.90	3.33	1.64E-13	2.64E-11
gene_479	hypothetical protein	49192	49270	-	HPP12_RS02425	HPP12_0473	N	27.36	14.60	267.11	147.48	6.04	3.30	5.20E-13	7.63E-11
gene_11	hypothetical	11826	11830	+	HPP12_RS05	HPP12_	N	6.66	2.92	23.29	48.14	3.71	2.90	7.12	9.59

23	protein	13	44		650	1109								E-07	E-05
gene_883	alkylphosphonate uptake protein	92125 5	92158 4	+	HPP12_RS04430	HPP12_0872	N	485.90	464.20	978.39	1401.10	9.55	1.32	2.88 E-06	3.41 E-04
gene_1608	Microcin C biosynthesis protein	(plasmid pHel12)	(plasmid pHel12)	-	NA	NA	Y	4968.44	9146.78	2253.39	2797.09	12.04	-1.48	2.96 E-06	3.41 E-04
gene_185	hypothetical protein	4851 19031 5	5903 19068 6	-	NA	NA	N	19.97	9.73	80.76	46.09	4.94	2.09	2.06 E-05	2.22 E-03

* Product name and gene positions are based on revised annotations for our resquenced strain P12

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