Cofactor Requirement of HpyAV Restriction Endonuclease

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Abstract

**Background:** Helicobacter pylori is the etiologic agent of common gastritis and a risk factor for gastric cancer. It is also one of the richest sources of Type II restriction-modification (R-M) systems in microorganisms.

**Principal Findings:** We have cloned, expressed and purified a new restriction endonuclease HpyAV from *H. pylori* strain 26695. We determined the HpyAV DNA recognition sequence and cleavage site as CCTTC 6/5. In addition, we found that HpyAV has a unique metal ion requirement: its cleavage activity is higher with transition metal ions than in Mg²⁺. The special metal ion requirement of HpyAV can be attributed to the presence of a HNH catalytic site similar to ColE9 nuclease instead of the canonical PD-X-D/EXK catalytic site found in many other REases. Site-directed mutagenesis was carried out to verify the catalytic residues of HpyAV. Mutation of the conserved metal-binding Asn311 and His320 to alanine eliminated cleavage activity. HpyAV variant H295A displayed approximately 1% of wt activity.

**Conclusions/Significance:** Some HNH-type endonucleases have unique metal ion cofactor requirement for optimal activities. Homology modeling and site-directed mutagenesis confirmed that HpyAV is a member of the HNH nuclelease family. The identification of catalytic residues in HpyAV paved the way for further engineering of the metal binding site. A survey of sequenced microbial genomes uncovered 10 putative R-M systems that show high sequence similarity to the HpyAV system, suggesting lateral transfer of a prototypic HpyAV-like R-M system among these microorganisms.

Introduction

Restriction-modification (R-M) systems that recognize and cleave DNA in a highly specific manner are ubiquitous in prokaryotic microorganisms (and their viruses) [1]. Helicobacter pylori, the etiologic agent of common gastritis and a risk factor for gastric cancer [2], curiously is one of the richest sources of Type II restriction-modification (R-M) systems in any living organisms [3,4]. The extraordinary number of Type II R-M systems appears to be the result of *H. pylori’s* natural competency of transformation by exogenous DNA [4–6]. In addition to defense against invading phages, evidence has suggested that the MTases (within active R-M system [7] or orphan MTases [8,9]) are involved in transcriptional regulation of other genes akin to the epigenetics of mammalian cells.

Genome mining of sequenced microbial genomes has resulted in a wealth of restriction enzymes with new specificities or unique properties (ApeKI (G°CWGC), PhoI (GG°CC), CviKI-1 (RG°CY), NseAIII (GCCGAG 20–21/18–19) [10,11], Nt.CviPII (°CCGD) [12]; NEB catalog 2009/10) [1]. The goal of this work was to clone, express, purify and characterize HpyAV restriction endonuclease (REase), which is one of the putative R-M systems from *H. pylori* 26695 [3]. During the purification process, we found that Ni²⁺ has a stimulatory effect on HpyAV activity. Bioinformatics analysis showed that HpyAV contains a HNH catalytic site highly similar to that of colicin E9 (ColE9). Sequence alignment of HpyAV and ColE9 and other HNH nucleases identified four highly conserved catalytic residues. By site-directed mutagenesis we confirmed that these residues are important for DNA cleavage. In addition to Ni²⁺, we found that HpyAV is also active in Mn²⁺ and Co²⁺. We therefore surveyed a few other HNH REases and found that KpnI is also active in a multitude of transition metals. Finally, a BLASTP search in sequenced bacterial genomes revealed ten putative HpyAV R-M systems. These microorganisms reside within human bodies or in mammals that are closely associated with humans, suggesting a possible lateral transfer mechanism.

Results

**The HpyAV R-M System**

Restriction mapping and run-off sequencing results indicated that the native HpyAV REase isolated from *Helicobacter pylori* strain 26695 recognizes the asymmetric target sequence CCTTC
and cleaves 6 nt and 5 nt downstream of the top strand and the bottom strand, respectively (CCTTC 6/5; data not shown). Enzymes that recognize asymmetric sequences frequently require two methyltransferases (MTases) to modify the two strands of DNA. In the case of HpyAV the MTase(s) must modify a C of the top strand and an A of the bottom strand in the target sequence. From the genomic sequence of *H. pylori* 26695 (Genbank nucleotide accession NC_000915), the R gene (hp_0053) of the HpyAV R-M system is located downstream of the M gene (hp_0054) and runs in the same direction as the M gene (Fig. 1A). In addition, the M gene of the HpyAV R-M system is a fusion of a C5 cytosine MTase and an N6 adenine MTase highly homologous to M1.Hin4II and M2.Hin4II, respectively (Fig. 1A). We re-sequenced the junction of the two MTase domains from the cloned M gene and from a PCR product derived from the genomic DNA and found no stop codon.

**Figure 1.** Gene organization of the HpyAV/Hin4II R-M systems and the structural model of the HpyAV catalytic site. A. Organization of the HpyAV and Hin4II R-M systems. The Hin4II R-M system consists of separate M1 and M2 genes for C5 cytosine (dark grey) and N6 adenine (light grey) methylation, respectively, preceding the ORF of Hin4II [80]. M.HpyAV is a fusion of C5 and N6A MTase domains with high sequence similarity to M1.Hin4II and M2.Hin4II, respectively. B. The structural model of HpyAV catalytic site and structural alignment to HNH endonucleases. Modeling of amino acid residues 281 to 360 of HpyAV to the \(b\)-Me motif of ColE9 and the structural alignment to ColE9, I-HmuI, Hpy99I and KpnI were done as described in Materials and Methods. The blue dots underneath the alignment indicate metal-binding residues; the red dots indicate the general base His and the green dot indicates the Asn implied to decrease the pKa of the general base His in ColE9 and I-HmuI. Amino acid residues that are assigned to \(b\)-strands and the helix of the \(b\)-Me motif are indicated by red arrows and blue rods, respectively. The conserved residues implicated in catalysis are colored in black or grey.

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between the two domains, confirming that M.HpyAV is a true fusion of C5 cytosine MTase and N6 adenine MTase, although the size of the translation product has not been confirmed biochemically. An over-expression E. coli strain was constructed by transforming E. coli ER3081 (NEB) with pSYX20-hpyAVM and pAII17-hpyAVR by sequential transformation (See Materials and Methods).

Metal Ion Requirement for DNA Cleavage

The purified recombinant HpyAV exhibited very low cleavage activity on λ DNA in the standard reaction condition containing 4 mM MgSO4 (Fig. 2A). However, when 2 mM NiSO4 was added into the cleavage reaction in the presence or absence of 4 mM MgSO4, HpyAV exhibited equally high cleavage activity (Fig. 2A). This shows that HpyAV prefers Ni²⁺ for cleavage activity. This discovery prompted us to examine HpyAV against other divalent metal ions including Ca²⁺, an alkaline earth metal ion that is inhibitory to REases containing the canonical PD-(D/E)XK catalytic motif, and ions of other transition metals in the same period (Mn²⁺, Co²⁺, Cu²⁺ and Zn²⁺). We found that HpyAV showed a different degree of cleavage activity with divalent metal ions. It was most active with MnCl₂, NiSO₄ or CoSO₄ - complete cleavage of λ DNA was achieved with 0.5 to 4 mM of these three metal ions (Fig. 2B). For Cu(OAc)₂ and Zn(OAc)₂, concentrations higher than 2 mM were inhibitory to HpyAV endonuclease activity (data not shown), and complete cleavage was not obtained under the assay conditions. HpyAV showed much lower activity in the presence of CaCl₂ or MgSO₄ (Fig. 2B). Table 1 summarizes the specific activity of HpyAV with various metal ions. HpyAV is equally active in MnCl₂, CoSO₄ and NiSO₄ (specific activities are within a 2-fold margin for a 2-fold dilution series of the enzyme), and complete cleavage of λ DNA was not achieved in the presence of MgSO₄, CaCl₂, Cu(OAc)₂ or Zn(OAc)₂ at the highest enzyme concentration available (40 μmol of HpyAV on 0.3 pmol (1 μg) of λ DNA). By comparing the cleavage patterns, HpyAV is estimated to exhibit less than 0.4% of cleavage activity in buffers with MgSO₄ and CaCl₂, less than 6% with Cu(OAc)₂ and less than 0.8% with Zn(OAc)₂.

**Figure 2.** HpyAV endonuclease activity in buffers with various divalent cations. A. Cleavage activity of HpyAV in MgSO₄ and NiSO₄. Decreasing concentrations of HpyAV were added to reactions containing 1 μg of λ DNA, 20 mM Tris-HCl, pH 7.9, 200 mM NaCl supplemented with 2 mM of NiSO₄, 4 mM of MgSO₄, 2 mM of NiSO₄ and 4 mM of MgSO₄ or no divalent metal ions. The reactions were carried out as described in Materials and Methods. The reactions that exhibit 1 U of HpyAV activity (complete cleavage) are marked with a dot. B. DNA cleavage activity of HpyAV in buffers supplemented with the indicated concentration of metal ion solutions. Eight units of HpyAV were incubated with 1 μg of λ DNA in 20 mM Tris-HCl, 200 mM NaCl as described in Materials and Methods. doi:10.1371/journal.pone.0009071.g002
Homology Modeling of Catalytic Residues

The strong stimulation effect of Ni$$^{2+}$$ on endonuclease activity is unique to HpyAV. It led us to examine the HpyAV amino acid sequence in more details. HpyAV is not homologous to any known REases except its isoschizomer Hin4II (data not shown). Manual examination of the amino acid sequence of HpyAV revealed a HNH catalytic motif highly homologous to that of colicin E9. Homology modeling of amino acid (aa) residues 287–325 of HpyAV to the βα-Me motif of ColE9 (aa 95–131) resulted in a model free of clashes and with all the conserved catalytic residues (His102, His103, Asn118 and His127 in ColE9; H294, H295, N311, and H320 in HpyAV) structurally aligned to the HNH endonucleases including I-HmuI and Hpy99I (Fig. 1B).

Site-Directed Mutagenesis of the HNH Catalytic Site

From biochemical and structural studies of colicin E9, His103 acts as the general base to deprotonate a water molecule for the hydrolysis of the scissile phosphodiester bond. His102 and His127 coordinate the single divalent metal ion for transition state stabilization. Asn87 of I-HmuI and Asn118 of ColE9 are proposed to form a hydrogen bond to the general base His and increase its pKa for the activation of the nucleophilic water [13,14]. In this study, the corresponding residues of HpyAV (His294, His295, Asn311 and His320) were mutated to verify their role in catalysis. Mutants H294D, H295A, and H320A were constructed and purified. H294D and H320A did not show any cleavage activity at up to 7.5 mg of protein (Fig. 3 and data not shown) in the presence of 2 mM NiSO4 or MgSO4, indicating that (i) the removal of the imidazole group at position 320 eliminated cleavage activity; (ii) the negatively charged Asp (as found in I-HmuI, Hpy99I and KpnI at the same aa position; Fig. 1B) cannot replace the histidine residue at position 294 for metal coordination in HpyAV. It is somewhat unexpected that substitution of the general base His295 by Ala did not completely eliminate the cleavage activity (Fig. 3); H295A still retains approximately 1% of wt activity, suggesting that an alternative weaker general base exists in the catalytic site when the general base His295 is absent. To explore the consequence of other amino acid substitutions, we also mutated His295 to Lys, Asn or acidic resides Asp/Glu. IPTG-induced cell extracts expressing these four mutants (H295K, H295N, H295D, and H295E) did not show any cleavage activity (data not shown), indicating that Lys, Asn, Asp, or Glu residues cannot replace His295 in the catalytic site. Cell extract with

| Table 1. Specific activity of HpyAV and KpnI. |
|---------------------------------------------|
| HpyAV | Sp. Activity (U/mg) | % | KpnI | Sp. Activity (U/mg) | % |
| Mg$$^{2+}$$ | <200 | <0.4 | 282000 | 100 |
| Ca$$^{2+}$$ | <200 | <0.4 | 7000 | 2.5 |
| Mn$$^{2+}$$ | 2000 | 200 | N/D | N/D |
| Co$$^{2+}$$ | 830 | 83 | 42000 | 15 |
| Ni$$^{2+}$$ | 1000 | 100 | 32000 | 10 |
| Cu$$^{2+}$$ | <200 | <0.6 | N/D | N/D |
| Zn$$^{2+}$$ | <200 | <0.8 | 28000 | 10 |

$^{a}$Specific activity are average values of triplicate experiments for HpyAV and duplicate experiments for KpnI.

$^{b}$Complete cleavage of substrate DNA was not achieved with the highest available concentration of HpyAV. Percentage activity was estimated by comparing the cleavage pattern of the highest concentration of HpyAV with the indicated metal ion to the matching pattern with Ni$$^{2+}$$ (data not shown).

$^{c}$The specific activity of KpnI was not determined in Mn$$^{2+}$$ because star activity appeared before complete cleavage of the substrate DNA was achieved.

$^{d}$Specific activity of KpnI was not determined in Cu$$^{2+}$$ because the same incomplete cleavage pattern was observed over a 120-fold difference in enzyme concentration.

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Figure 3. DNA cleavage activity of the catalytic residue mutants. Purified protein of WT, H294D, H295A and H320A were assayed as described in Materials and Methods in the presence of 2 mM NiSO4. Five μl of undiluted, three-fold and nine-fold dilutions of 1.5 mg/ml of enzyme solution were assayed on 1 μg of λ DNA.

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N311A variant failed to show any detectable cleavage activity (data not shown). It is concluded that His294, H295, Asn311 and His320 are important residues for HpyAV endonuclease activity.

KpnI Endonuclease Activity with Different Divalent Metal Ions

KpnI is an HNH Type IIP REase that has been shown to be active with Mg" or Ca" [15,16]. The high activity of HpyAV with transition metal ions prompted us to investigate if it is also true for KpnI. Fig. 4 shows that KpnI is active in all of the transition metals tested. Table 1 shows that the specific activity of KpnI is highest with MgSO4, followed by CoSO4 (15% of MgSO4), NiSO4 and Zn(OAc)2 (both 15% of MgSO4) and CaCl2 (2.5% of MgSO4). The specific activity could not be determined with MnCl2 because cleavage at non-cognate sites (star activity) was observed before complete cleavage of the cognate sites was achieved (data not shown). The specific activity in Cu(OAc)2 was also not determined because the same incomplete cleavage pattern was observed over a 120-fold difference in enzyme concentration (data not shown). As a control, 10 U of EcoRI, a canonical PD-(D/E)XK Type IIP REase, was also tested. Under the assay conditions, EcoRI was most active in MgSO4, with very low level of activity in MnCl2 and CoSO4 and no activity in CaCl2, NiSO4, Cu(OAc)2 or Zn(OAc)2. For all three enzymes, no cleavage activity was observed without the added divalent metal ions, indicating that all the activities observed were caused by the presence of the metal ion cofactors.

Homologous HpyAV Systems from Sequenced Microbial Genomes

HpyAV is an isoschizomer of Hin4II. M.HpyAV contains a C5 cytosine and a N6 adenine MTase domain highly homologous to M1 and M2.Hin4II (Fig. 1A). Sequence analysis showed that the corresponding regions of M.HpyAV are 57% and 56% identical to M1 and M2.Hin4II, respectively (Table 2). The REases HpyAV and Hin4II also share 48% sequence identity (Table 2). A BLASTP search of the GenBank genomes discovered 10 putative R-M systems that are highly homologous to the HpyAV system. The MTases and REases of these homologous systems, along with those of HpyAV and Hin4II, are shown in Table 2. Except for the Yersinia kristensenii and Vibrionales bacterium SWAT-3 systems, the M gene precedes the R gene with both of them oriented in the same direction. Also, like the HpyAV system, all of these R-M systems contain single MTase that are fusions of C5 cytosine and N6 adenine MTases. Their high sequence similarity suggests that these putative R-M systems may share the same recognition
An HNH-Type Endonuclease HpyAV

Table 2. Homologous HpyAV R-M systems.

| REase*     | Length (aa) | % ID | MTase† | Length (aa) | % ID | Organism          |
|------------|-------------|------|--------|-------------|------|------------------|
| HpyAV      | 419         | 100  | M. HpyAV | 823         | 100  | Helicobacter pylori 26659 |
| HpyPORF48P | 423         | 92   | M.HpyPORF48P | 822         | 98   | Helicobacter pylori P12 |
| HpyGORF49P | 419         | 96   | M:HpyGORF49P | 823         | 97   | Helicobacter pylori G27 |
| CupORF1468P | 339       | 50   | M.CupORF1468P | 817         | 64   | Campylobacter upsaliensis RM3195 |
| SeqZORF1536P | 417        | 50   | M. SeqZORF1536P | 810         | 52   | Streptococcus equi subsp. zooepidemicus MGCS10565 |
| Hin4II     | 418         | 48   | M1.Hin4II | 387         | 57†  | Haemophilus influenzae RFL4 |
| BhyWAORF699P | 386       | 43   | M.BhyWAORF699P | 812         | 43   | Brachyspira hydysenteriae WA1 |
| Nme180ORF295P | 296       | 31   | M.Nme180ORF295P | 862         | 42   | Neisseria meningitidis FAM18 |
| MmyLCORF8BP | 330        | 40   | M. MmyLCORF8BP | 834         | 55   | Mycoplasma mycoides subsp. mycoides LC str. GM12 |
| YkoORF13790P | 426       | 39   | M.YkoORF13790P | 826         | 44   | Yersinia kristensenii ATCC 33638 |
| Bst43183ORF2897P | 390     | 33   | M.Bst43183ORF2897P | 856         | 43   | Bacteroides stercoris ATCC 43183 |
| VbsORF22060P | 326        | 30   | M.VbsORF22060P | 820         | 43   | Vibriionales bacterium SWAT-3 |

*Names as in REBASE. All REases, except HpyAV and Hin4II, are putative (names end with P).
†In the genomic sequence, HpyGORF49P contains a deletion that introduces a stop codon within the HNH catalytic site. The reported length is a theoretical number based on the re-introduction of the deleted nucleotide to the genome sequence.
‡Sequence identity for M1 and M2.Hin4II calculated based on pair-wise alignment of segments of M.HpyAV that can be aligned to M1 and M2.Hin4II, respectively as shown in Figure 1.

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sequence (CCTTC). It is also noticeable that these homologous R-M systems are mostly carried by infectious microorganisms of human or mammalian hosts closely associated with humans. However, there are two putative endonucleases (EsaSS23P = 393 aa; EsaSS44P = 385 aa) without companion MTases from shotgun-sequenced environmental samples that share significant amino acid sequence identity with HpyAV and Hin4II (EsaSS23P vs HpyAV = 34% aa sequence identity; EsaSS44P vs Hin4II = 29% aa sequence identity). They may recognize CCTTC or similar target site with 1-bp difference.

Discussion

Metal Ion Cofactor Preference of HNH Endonucleases

It has been well-documented that for restriction endonucleases (REases) with the canonical PD-X-D/E/KX catalytic site, Mg** and Mn** support catalysis but Ca++ only supports DNA binding. One to two coordinated metal ions have been observed in the catalytic site in the crystal structures of REases in the presence of substrate DNA. Catalytic mechanisms for one- and two-ion-induced cleavage have been proposed [17–20]. It has been generally accepted that most Type IIP REases use a two-metal ion mechanism where metal ion A deprotonates the nucleophile water molecule and metal ion B stabilizes the pentavalent phosphate transition state and activates a water molecule for protonation of the leaving 3’-phosphate oxygen. Some REases [EcoRI and BglII], however, appear to use a one-metal ion mechanism where the metal ion occupies site 1 and coordinates a nucleophile water molecule for the attack of the scissile phosphate, although it has been noted that the second metal ions might have escaped detection because the second metal ions tend to have low occupancy in other structures [21–23]. Recently, Pingoud and colleagues presented experimental evidence and MD simulation results to support a generalized one-metal ion catalytic mechanism where site 1 has a higher affinity to Mg** and site 2 plays a modulating role in the cleavage activity [23].

Endonucleases that contain the β²ξ-Me motif, on the other hand, are only observed with one coordinated divalent metal ion in their catalytic sites. The β²ξ-Me motif adopts a V-shape conformation consisting of two beta-strands connected by a loop in one arm followed by a helix that constitutes the other arm. The β²ξ-Me motif is present in non-specific endonucleases such as Serratia nuclease [24,25], E. coli defense nucleases colicin E7 [26,27] and E9 [28,29], Holliday junction resolving T4 endonuclease VII [30] and homing endonucleases I-PpoI of the His-Cys family [31,32]. HNH enzymes are a sub-group of the β²ξ-Me family where the metal ion is coordinated by two negatively charged amino acid residues (two histidines in ColE9 [29,33,34]; a glutamate and a asparagine in I-HmuI [13,14] and Hpy99I [35]) and the non-bridging oxygen of the scissile phosphodiester bond of the transition state. In His-Cys homing endonucleases and Serratia nuclease, only one asparagine is involved in metal ion coordination. The coordinated metal ion is believed to stabilize the transition state by neutralizing the negatively charged pentavalent phosphoanion transition state. In ColE9, it has been proposed that the water molecule coordinated by the metal ion and His131 acts as the general acid that protonates the leaving group [29]. The conserved asparagine lowers the pKa of the invariable histidine which in turn activates the nucleophilic water molecule for in-line attack of the scissile phosphate. Recently, more Type II restriction endonucleases, namely, KpnI [36], MnlI [37], Hpy99I [35], Eco31I [38,39], HphI [40], SpI [41], PaeI and others [42] are identified as containing this HNH motif through X-ray crystallography or sequence alignment/structural prediction. GIY-YIG
endonucleases (including homing endonucleases I-TevI [43,44], nucleotide excise repair enzyme UvrC and Type IIP REases Hpy188I [45] Eco29kl [46,47] and Cfr42I [47,48]) is proposed to adapt a similar catalytic mechanisms as HNH/His-Cys endonuclease except for the use of Tyr as the general base based on the structure of UvrC [49].

In this study, we demonstrated the importance of conserved catalytic residues H294, H295, N311, and H320 by site-directed mutagenesis. HpyAV variants H294D, N311A, and H320A lack any detectable catalytic activity (less than 0.1% activity). Substitution of the general base His295 by Ala displays impaired cleavage activity only (H295A retains approximately 1% of wt activity), suggesting that an alternative weaker general base exists in the catalytic site when the general base His295 is mutated. In addition, positively charged aa substitution by Lys in H295K did not rescue the catalytic activity, indicating Lys cannot replace His as the general base in HpyAV. Other charged aa residue substitutions at the 295 position (HpyAV variants H295N, H295D, and H295E) failed to show any detectable cleavage activity. The observation that deletion of the proposed histidine general base in the HpyAV active site yields an endonuclease with reduced activity, rather than completely inactivating the enzyme, is unusual for the HNH superfamily, but not unprecedented. Mutation of the active histidine general base in the I-Ppol homing endonuclease also results in reduced activity [50]; this result is attributed to the ability of a neighboring histidine to participate in a less-efficient long-range proton transfer reaction and activation of the nucleophilic water with reduced activity as compared to the wild-type enzyme.

MnII and I-Ppol are the only ββα-2-Mc endonuclease whose metal ion preference has been systematically studied. In the presence of 1 mM Mn++, the order of MnII activity was shown to be Mg++ > Ni++ = Co++ > Mn++ > Ca++ > Zn++ [51]. I-Ppol activity follows the order of Mg++ > Mn++ > Ca++ = Co++ > Ni++ > Zn++ (10 mM) [52]. Hpy99I is active in Mg++ and Mn++ but not in Ca++ or Zn++ [35]. It has also been reported that ColE9 prefers Mg++ and Ca++ for dsDNA and Ni++ for ssDNA substrates [18,34,53]. Our preliminary results showed that PacI and SphiI are active with Ni++ but less so than in Mg++, whereas HphI showed comparable activity with Ni++ and with Mg++ (data not shown).

Non-specific HNH endonucleases colicin E9 has also been reported to have distinct metal ion preference: Mg++ prefers Mg++ over Co++, but less so than in Mg++, for Yersinia pestis and Vibrio cholerae. Our preliminary results showed that PacI and SphiI are active with Ni++ but less so than in Mg++, whereas HphI showed comparable activity with Ni++ and with Mg++ (data not shown).

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sented in sequence databases. It is possible that HpyAV homologous R-M systems exist in non-mammalian-associated microorganisms. Shotgun sequencing of marine samples has revealed two ORFs EsaSS23P and EsaSS44P that are also homologous to HpyAV and HimH aa sequences.

Materials and Methods

Strains, DNA Sequences and Mutagenesis

The HpyAV R-M system was identified in Helicobacter pylori 26695 (Genbank nucleotide accession NC_000915). ORF hp_0053 is the M gene which was amplified in PCR and inserted into pSYX20 at the EcoRV and SphI sites with a GGAGGT ribosome-hinding site and upstream stop codons in all three ORFs of pSYX20 carries pSC101 replication origin, KmR, and TcR. Expression of the M gene is under the control of the TcR promoter. ORF hp_0053 is the R gene which was amplified in PCR and inserted into pAII7 (NEB) at NdeI and BamHI sites, under the control of the T7 promoter. The over-expression strain was constructed by sequential transformation of E. coli ER3081 (NEB) by pSYX20-hpyAV1M and then pAII7-hpyAV1R. 0.381 (hSA22 8 lacZ-gf;T7 gene1 [lon] ompT gal attP;CD13/Pet-gfx; lacA) [Spec’]; sulA11 T7(mcr-73::miniTn10—Tet’ [den] R(Zhgb-210:Tex10—Tet’) endAI Δ(mcr-con-mvr114::IS10) is a derivative of ER2566 (T7 Express, NEB). This strain contains the T7 RNA polymerase gene at the chromosomal lac operator, replacing much of lacZr; the K128Y mutant of T7 lysozyme (lytI) and the lacP gene are expressed from the chromosomal attb site. Stable integration of the lytI and lacP genes was accomplished using the pCD13PKS plasmid described by Platt et al. [73, 74]. Site-directed mutagenesis was carried out using a modified inverse PCR procedure [75] using pAII7-hpyAV1R isolated from the over-expression strain as template. Primers designed to construct mutants (H295A, H320A and N311A) were synthesized by Integrated DNA Technologies. All DNA sequences were verified by DNA sequencing.

Protein Expression and Purification

The over-expression strain of HpyAV was cultured in LB medium containing 100 μg/ml ampicillin and 30 μg/ml kanamycin at 30°C and 200 rpm overnight (~15 h). Ten milliliters of the overnight culture was inoculated into 1 L of LB medium containing the same antibiotics and cultured at 30°C and 200 rpm to log phase. The culture was cooled down to 25°C before IPTG was added to a final concentration of 0.25 mM. Growth was then continued at 25°C until the cultures were harvested by centrifugation. The cell pellet was resuspended in 100 ml of 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM EDTA (Buffer A) supplemented with 1% PMSF and sonicated on ice. After centrifugation, the supernatant was loaded onto a Heparin HiTrap column (5 ml; GE Life Sciences). Peak fractions from a linear elution gradient of 0.05–1 M NaCl in Buffer A was diluted 4-fold in Buffer A and loaded onto a HiTrap SP HP column (5 ml; GE Life Sciences). Peak fractions from a 0.05–1 M NaCl gradient were pooled and concentrated by Vivaspin 15 (10 kDa MWCO; Sartorius). An equal volume of 60% glycerol was added to the concentrated protein for storage at ~20°C.

DNA Cleavage Activity Assays

The DNA cleavage activity of the crude extract or purified HpyAV was assayed in 50 μl reactions containing 20 mM Tris-HCl, pH 7.9, 200 mM NaCl supplemented with the indicated concentrations of MgSO4, CaCl2, MnCl2, CoSO4, NiSO4, Cu(OAc)2 or Zn(OAc)2 and 1 μg of A DNA at 37°C for 1 h. KpnI was assayed in 20 mM Tris-HCl, 50 mM NaCl, pH 7.9 with the same battery of salts using 1 μg of pXba DNA (a 10 kb Xbal fragment of adenovirus DNA inserted into pUC19; NEB). EcoRI activity assay was carried out in the same buffer using 1 μg of A DNA. The cleavage reactions were then analyzed by 1.2% agarose gel electrophoresis in 1x TBE. One enzyme unit is defined as the amount of enzyme needed to cleave the 1 μg of the designated DNA completely at 37°C in 1 h. Specific activity is defined as the number of units per mg of enzyme. Specific activity was determined in duplicate (KpnI) or triplicate (HpyAV) by titrating the enzymes (in steps of 2-fold dilution) in their respective reaction buffer in the presence of 2 mM MgSO4, CaCl2, MnCl2, CoSO4 or NiSO4, or 0.1 mM Cu(OAc)2 or Zn(OAc)2. KpnI and EcoRI were from NEB. All reaction buffers and metal ion solutions were prepared using MilliQ water.

Homology Modeling and Structural Alignment

Amino acid residues 281 to 360 of HpyAV were modeled to the ββ2-Me motif of ColE9 (mutant H103A; PDB: 1V14) by homology modeling using SWISS-MODEL [76, 77]. The structural model of the HpyAV ββ2-Me motif was aligned pair-wise with the crystal structures of ColE9 (PDB: 1V14), E-HmuI (PDB: 1U3E), Hpy911 (PDB: 3GOX) and the KpnI model built by Nagaraja and colleagues [36] using the TM-Align module [78] of STRAP [79].

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Author Contributions

Conceived and designed the experiments: SHC SYX. Performed the experiments: SHC LO LH SYX. Analyzed the data: SHC LO LH SYX. Contributed reagents/materials/analysis tools: SHC DO SYX. Wrote the paper: SHC SYX.

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