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# Cloning and sequence analysis of the StsI restriction-modification gene: presence of homology to FokI restriction-modification enzymes

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## ABSTRACT

**StsI endonuclease (R.StsI), a type IIs restriction endonuclease found in *Streptococcus sanguis* 54, recognizes the same sequence as FokI but cleaves at different positions. A DNA fragment that carried the genes for R.StsI and StsI methylase (M.StsI) was cloned from the chromosomal DNA of *S.sanguis* 54, and its nucleotide sequence was analyzed. The endonuclease gene was 1,806 bp long, corresponding to a protein of 602 amino acid residues ($M_r$ = 68,388), and the methylase gene was 1,959 bp long, corresponding to a protein of 653 amino acid residues ($M_r$ = 76,064). The assignment of the endonuclease gene was confirmed by analysis of the N-terminal amino acid sequence. Genes for the two proteins were in a tail-to-tail orientation, separated by a 131-nucleotide intercistronic region. The predicted amino acid sequences between the StsI system and the FokI system showed a 49% identity between the methylases and a 30% identity between the endonucleases. The sequence comparison of M.StsI with various methylases showed that the N-terminal half of M.StsI matches M.NlaIII, and the C-terminal half matches adenine methylases that recognize GATC and GATATC.**

## INTRODUCTION

Restriction-modification enzymes are attractive systems for structural studies of the sequence-specific recognition of double-stranded DNA. More than 1,000 restriction endonuclease (ENases) have been isolated, and about 60 genes were cloned and their nucleotide sequence were analyzed (1). However, only EcoRI has been studied in detail. The crystal structure of the complex between EcoRI and the cognate oligonucleotide provide a detailed structural basis of sequence-specific DNA-protein interactions (2). As for EcoRI, BamHI, and EcoRV, amino acids which interacted with nucleotides in the recognition site and were responsible for cleavage were analyzed by site-directed or random mutagenesis (3–6).

The amino acid sequences were compared among various restriction ENases (1). No similarities have been reported between ENases that recognize different sequences, nor between ENases that recognize the same sequences but cleave in different positions. ENases that catalyze identical reactions sometimes have similar amino acid sequences, EcoRI and RsrI (7–9), TaqI and TthHB8I (10,11), Cfr9I and NgoPII (12,1); sometimes dissimilar, HhaII and HinfI (13,14). Recently, it was pointed out that EcoRI and RsrI did not recognize nor cleave identically (15,16).

We have already isolated the R-M gene of FokI and analyzed the complete nucleotide sequence (17). X-ray diffraction analysis is in progress for crystallized R.FokI protein (18). In addition to the structural analysis, comparison of properties and amino acid sequence of enzymes that recognize the same sequence will provide useful information about the amino acid residues involved in cleavage and recognition of the sequence. As type IIs restriction ENases recognize asymmetric nucleotides and cleave out of the recognition site, they probably have separate domains for recognition and cleavage. We isolated a new type IIs restriction ENase, StsI, from *Streptococcus sanguis* 54 (19), which is a heteroschizomer of FokI (20). Here, we report the cloning and sequence analysis of the restriction and modification gene of *S.sanguis* 54, and a comparison of StsI system with FokI and other systems.

## MATERIALS AND METHODS

### Bacterial strains, phage, and plasmid

*Streptococcus sanguis* 54 was from the laboratory culture collection of H. Ohta (Okayama University, Japan). *Escherichia coli* strains HB101 (21) and JM109 (22), and plasmid pBR322 (23,24), pUC119, were the host-vector system used for cloning. M13mp18 and mp19 were used for DNA sequencing (22). λgtλc was used for assay of *in vivo* restriction activity. Plasmid and phage DNA were prepared by the procedure described by Maniatis *et al.* (25).

### Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase, T4 DNA ploymerase, and a M13 sequencing kit and kilo-base deletion kit were obtained from Takara Shuzo Co., Ltd. and used according to the manufacturer's instructions. Sequenase and a Tth polymerase sequencing kit were donated by Toyobo. [$\gamma$-$^{32}$P] ATP and [$\alpha$-$^{35}$S] dCTP was purchased from Amersham Inc.

### Growth of *S.sanguis* 54

*S.sanguis* 54 was grown at 37°C under static conditions for 18 hr in 1 l of APT broth (Difco) contained in a 1-l bottle. Approximately 8 g wet weight of cells were obtained.

### Purification and analysis of N-terminal amino acid sequence of R.StsI

The R.StsI in the final purification step described before (19) was desalted and the N-terminal amino acid sequence analyzed. The protein was degraded sequentially with a protein analyzer (Applied Biosystems model 470A) with an on-line HPLC apparatus (model 120A).

### Enzyme assay

The R.StsI and M.StsI activities *in vivo* and *in vitro* were measured using the method described before (17). R.StsI activity *in vitro* was measured in 50 μl of the reaction mixture containing 10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 60 mM NaCl, 0.01% BSA, and 1 μg of λ-DNA.

### Construction and screening of *S.sanguis* 54 library

*S.sanguis* 54 chromosomal DNA was isolated and purified by the procedure of Thomas *et al.* (26). Partial MboI digests were fractionated by agarose gel electrophoresis. Fractions predominantly containing the fragments of 3−10 kb were recovered from gel, and inserted into BamHI-digested, dephosphorylated pBR322, with T4 DNA ligase. The ligated DNA was used to transform *E.coli* HB101 and approximately $1.5\times10^5$ $Amp^r$ transformants were obtained. Transformants were screened for M.StsI activity by the procedure suggested by Mann *et al.* (27) and R.StsI activity by the procedure described before (17).

### Subcloning and deletion analysis of the StsI-encoding gene

Plasmid pSts2 was isolated from a restriction-modification positive clone and a 7.9-kb insert DNA was recovered after digestion with ClaI and NaeI. The 5′ end was filled in by T4 DNA polymerase, then the fragment was ligated to SmaI-digested, dephosphorylated pUC119. The products were introduced into *E.coli* JM109 cells. Two plasmids in which insert DNA was carried in reverse orientation were selected and digested at the SphI and XbaI sites in the multicloning site. Sequential deletion was constructed by Exonuclease III according to the manufacturer's instruction.

### DNA sequencing

The dideoxynucleotide chain-termination method (28,29) was used for analysis of the 5.2-kb insert of the plasmid (pSts627). The subfragments generated were cloned into M13mp18 and mp19 to prepare template, and the sequences were deduced from the data for both strands. Sequence information is available in DDBJ through accession number D11101.

### Computer analysis of nucleotide and amino acid sequences

The nucleotide and amino acid sequences were analyzed using the software package DNASIS (Hitachi, Japan). The program PROTES was used to generated dot-matrix comparison with a window of 20 amino acid with 8 residues matching. Data bases were searched using the TFASTA program on a VAX computer system.

## RESULTS AND DISCUSSION

### N-terminal amino acid sequence of R.StsI

The first 21 amino acids of StsI were X-Ile-Ser-Ile-Asn-Glu-Tyr-Ser-Asp-Leu-Asn-Asn-Leu-Ala-Phe-Gly-Leu-Gly-Gln-Asp-Val (X; unidentified). There was no homology in the N-terminal amino acid sequence between R.StsI and R.FokI.

### Cloning of StsI gene

As the genes encoding ENase and cognate MTase are close together, the recombinants carrying the M.StsI gene was isolated

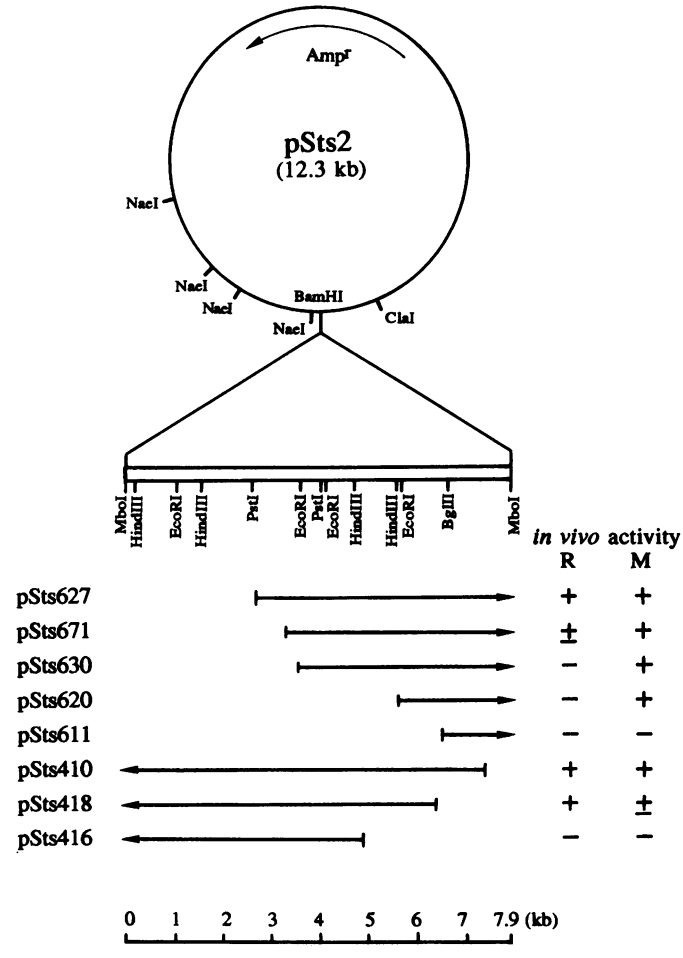



**Figure 1.** Restriction map of pSts2 and deletion derivatives. Deletion derivatives of 7.9-kb insert were produced from both ends (Materials and Methods). Below the restriction map, remaining portions of the insert are indicated by lines. Activities were assayed *in vivo* and presented as the approximate level to those of pSts2: +, equivalent level; ±, levels about one-tenth; −, no detectable activity. The direction of transcription from the *lac* promoter is shown by arrows.

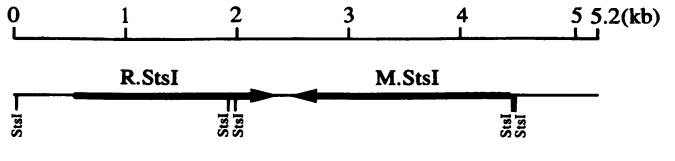



**Figure 2.** Gene organization of the StsI system. The orientation of the StsI restriction-modification genes and the StsI sites in the 5183 bp region are shown.

from a chromosomal DNA library. When chromosomal DNA of *S.sanguis* 54 was incubated with an excess of R.FokI, no cleavage was observed. Modification of GG$^{m6}$ATG and C$^{m6}$ATCC inhibit DNA cleavage by R.FokI, but modification of CAT$^{m5}$CC, CATC$^{m5}$C, and CATC$^{m4}$C do not (30). It was expected the adenine residues in the recognition sequence of R.StsI were modified by M.StsI in a similar manner as M.FokI did. So R.FokI was used for selection of the MTase gene of StsI. Plasmids from $1.5 \times 10^5$ colonies were intensively digested with R.FokI, and 20 clones randomly picked up was found to be resistant to R.FokI digestion. The R.StsI activity was analyzed by *in vivo* assay procedure and among these clones, pSts2, carrying the 7.9-kb insert, showed restriction endonuclease activity (Fig. 1).

## Gene organization and nucleotide sequence

To analyze the organization of the two genes encoded in the 7.9-kb fragment, series of deletion mutants were constructed, and their abilities to confer restriction and modification phenotypes on *E.coli* cells were examined. The results are summarized in Fig. 1. pSts671 was the smallest one that confers both phenotypes. By introduction of a 3.5-kb deletion from the left end of the 7.9-kb insert, restriction activity was lost completely, but MTase activity was at the same level as pSts2.

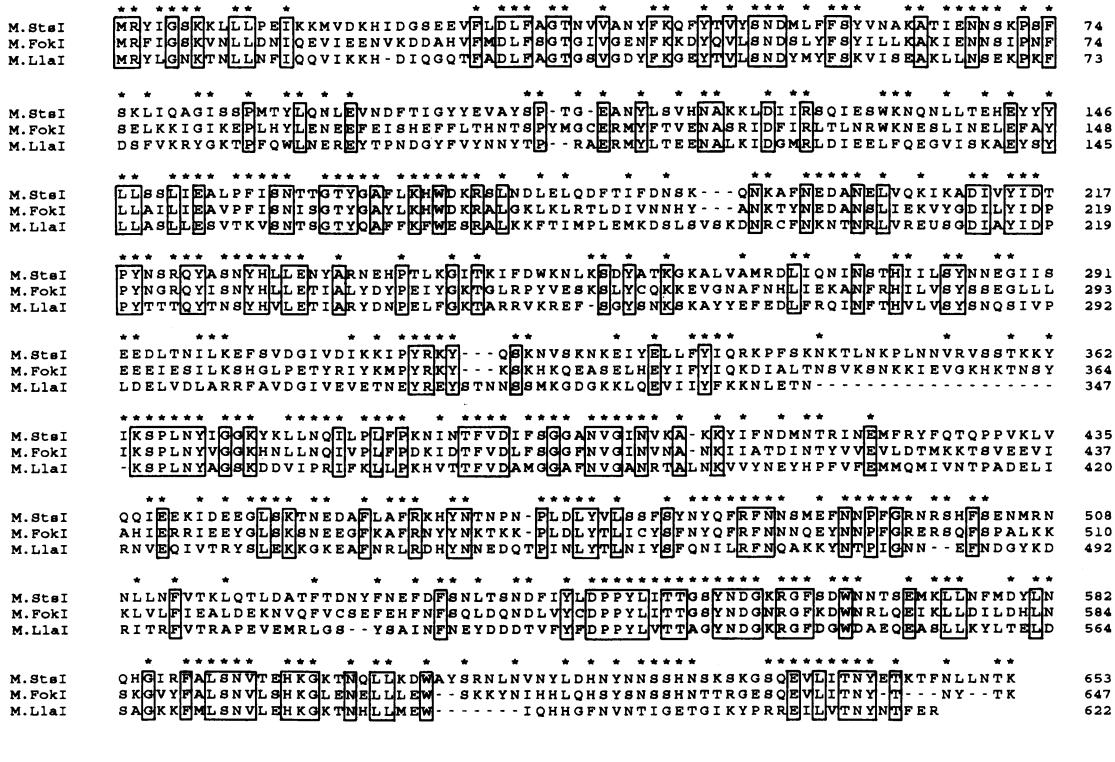



**Figure 3.** Alignment of M.StsI, M.FokI and M.LlaI. Identical amino acids in three MTases are boxed and those in M.StsI and M.FokI are indicated by asterisks. Gaps in the aligned sequences are also indicated (−).

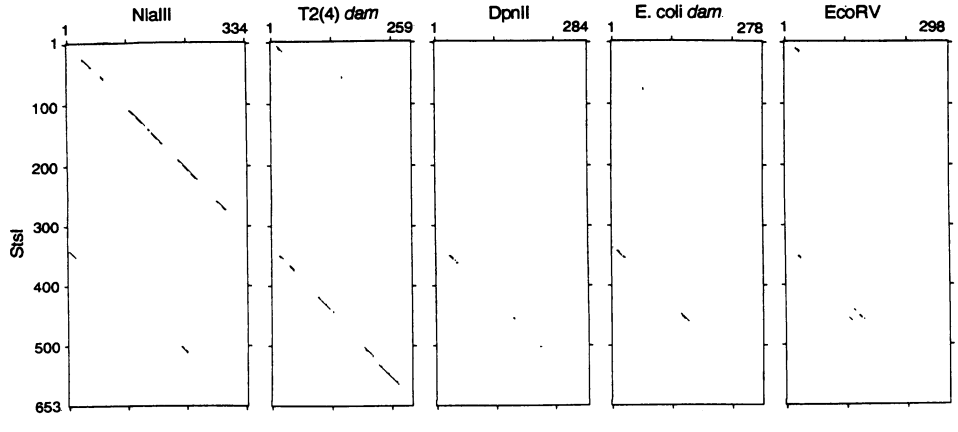
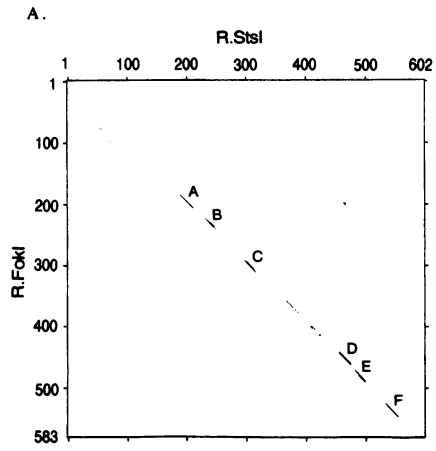



**Figure 4.** Dot-matrix comparison between StsI and various adenine MTases. The DNASIS program PROTES was used (Materials and Methods).




B.

(A) R.StsI 185 KFDLGEHFGFIDELGFETAPIEIFLNGLAQA
R.FokI 181 KFDLGKNLGFSGESGFTSLPEGILLDTLANA

(B) R.StsI 227 IKSNFESTSDKYNRWLAGVLVTAGL
R.FokI 219 IRNNWEGSSDKYARMIGGWLDKLGL

(C) R.StsI 297 RSRKRVMWEFLAT
R.FokI 290 RVPKRVYWEMLAT

(D) R.StsI 451 GFSGKHLGGSNKPDG
R.FokI 438 GYRGKHLGGSRKPDG

(E) R.StsI 474 DCAIILDSKAYSEGFPL
R.FokI 465 DYGVIVDTKAYSGGYNL

(F) R.StsI 529 FAYVSGSFSGNYKEQL
R.FokI 521 FLFVSGHFKGNYKAQL

**Figure 5.** Dot-matrix comparison and alignment of homologous blocks between R.StsI and R.FokI. The DNASIS program PROTES was used (Materials and Methods). Asterisks indicate identical amino acids between R.StsI and R.FokI. Panel A shows dot-matrix comparison. Panel B shows amino acid sequences of homologous blocks (A to F in panel A) together with the numerical positions of each block.

MTase activity was lost either by introduction of a 6.5-kb deletion from the left end or a 1.5-kb deletion from the right end of the 7.9-kb insert. These results indicate that the 5.2-kb region on the right side of the 7.9-kb insert is essential for encoding both restriction and methylase with R.StsI on the left side and M.StsI on the right side.

The DNA for the 5.2 kb insert in pSts627 was analyzed. Analysis of open reading frames (ORF) indicated that there were two large enough to encode enzymes. The two ORF were aligned tail to tail, and there was a 131-bp spacer region between them (Fig. 2). A putative palindromic sequence was seen within the spacer region. This could be the transcriptional termination site for both genes. The gene organization of the StsI system was distinct from that of FokI. In the FokI system, two genes aligned, MTase comes first and then ENase. In the StsI system the two genes were convergent. ORF from 555 bp to 2360 bp coded for ENase and it consists of 1806 bp and encoded a 602-residue polypeptide. The first 21 amino acids of R.StsI exactly corresponded to that predicted from the nucleotide sequence at position 561. The predicted molecular mass, 68,388 Da, was close enough to the value that estimated by SDS-PAGE (19). A potential SD sequence, AGGAG, is 10 bp upstream of the start codon.

The other open reading frame from ATG at 4450 bp to AAA at 2492 bp was supposed to encode the MTase gene. In this ORF, there were two amino acid segments, DTPY (from 216 to 219) and DPPY (from 546 to 549), which were characteristic of adenine MTases (12,31,32). The latter was homologous to the consensus sequence (DPPY), the former was different from the consensus sequence at the 2nd position. The presence of two copies of a segment of tetra amino acids that is characteristic to adenine MTases supports the idea that this ORF encodes the M.StsI gene. Although there were two other ATG near 4450 bp in the same frame, there was no SD sequence at an appropriate position for these two start codons. So it is reasonable to think that MTase gene starts from ATG at 4450 bp.

Since *E.coli* harboring the plasmid in which the 7.9-kb fragment was inserted in the opposite orientation express both ENase and MTase activities, the sequence from the coding region was searched for sequence similarities to the *E.coli* promoter-like sequence (33). There were *E.coli*-like promoter sequences upstream of both genes.

**Table I.** MTases which showed homology to M.StsI

| Enzyme | Recognition sequence |
|---|---|
| NlaIII | CATG |
| FokI, StsI | GGATG/CATCC |
| dam | GATC |
| DpnII | GATC |
| EcoRV | GATATC |
| LlaI | unknown |

Recognition sequences are written from 5′ to 3′.

There were five StsI recognition sequences in the 5.2-kb region (Fig. 2). One site occurred upstream of the initiation codon of R.StsI, two sites within the R.StsI gene, and the other two sites immediately upstream of the initiation codon of M.StsI. The location of the two StsI sites upstream of MTase gene was very similar to that of M.FokI gene. This arrangement might influence expression of the genes.

## Comparison of M.StsI, M.FokI, and other MTases

*Nucleotide sequences.* The nucleotide and deduced amino acid sequences of M.StsI and M.FokI were compared. The M.StsI gene was encoded by 1959 bp, and the M.FokI gene by 1941 bp. There was no significant nucleotide sequence similarity between these two genes. G+C content of M.StsI and M.FokI genes were 28% and 30%, respectively. Both amino acid composition and the codon usage of M.StsI were similar to those of M.FokI (data not shown).

*Amino acid sequences.* The M.StsI protein sequence was compared with sequences in the GenBank and EMBL data bases. A high level of identity was found between M.StsI and M.FokI (49%) and M.StsI and M.LlaI (35%). Alignment of the amino acid sequences of FokI, LlaI, and StsI MTases are shown in Fig. 3. While the recognition sequence of LlaI remained to be identified, it was suggested that the specificity of LlaI was different from that of FokI (34). When the recognition specificity

of LlaI MTase is identified, the responsibility of the amino acid residues which are identical in all three MTases and those identical between M.StsI and M.FokI is expected to be clarified. In addition, a high level of identity was found in several adenine MTases. The amino terminus of M.StsI showed significant identity (35%) to the entire M.NlaIII (35), and the carboxy terminus of M.StsI also showed significant identity to T4 *dam* (33%) (36), T2 *dam* (28%) (37), and M.DpnII (22%) (38). Figure 4 shows a dot-matrix comparison of M.StsI with several adenine MTases. Though *E.coli dam* (39) and EcoRV (40) MTases showed no significant identity to M.StsI, they were homologous to the carboxy terminus of M.StsI. These similarities were also found between M.FokI and adenine MTases as described above (41). Recognition sequences of these MTases are shown in Table I. The sequence 5'-ATG-3' overlaps the recognition sequence of StsI, 5'-GGATG-3', and that of NlaIII. The sequence of 5'-ATC-3' overlaps the recognition sequence of StsI, 5'-CATCC-3', and that of another five MTases.

We have shown that in M.FokI the amino terminal DPPY motif participated in the methylation of 5'-GGATG-3', and the carboxy terminal DPPY motif in 5'-CATCC-3' (42). Truncated M.FokI was created by Looney *et al.* (41), and they demonstrated that the amino terminal two-thirds of M.FokI methylated 5'-GGATG-3', and the carboxy terminal two-thirds did 5'-CATCC-3'. These results suggest that both M.StsI and M.FokI have arisen from different proteins; the amino terminal half of the protein, which is responsible for the recognition and methylation of 5'-GGATG-3', and the carboxy terminal half protein which is responsible for the recognition and methylation of 5'-CATCC-3'. M.StsI and M.FokI showed significant contrast to M.HgaI, which were independent proteins encoded by adjacent genes (43). The M.HgaI proteins closely resemble one another, and probably diverged from a common ancestor.

The DPPY motif is widely conserved among m6A and m4C MTases, and is thought to be involved in the modification of the exocyclic amino group (12). Though there are several differences in this motif, the first proline had been completely conserved as far as we know. This is the first example that the first proline is replaced by threonine. Amino acid change from proline to threonine is a C-A transition on DNA. The possibility of cloning artifact can not be eliminated.

### Comparison of R.FokI and R.StsI

*Nucleotide sequences.* The nucleotide and deduced amino acid sequences of R.StsI and R.FokI were compared. The StsI gene was encoded by 1806 bp and the FokI gene by 1741 bp. There was no similarity between the two nucleotide sequences. Both G+C content of R.StsI and R.FokI genes are 34%. R.StsI consisted of 602 amino acids and R.FokI 583 amino acids. Both amino acid composition and the codon usage of R.StsI were similar to those of R.FokI (data not shown).

*Amino acid sequences.* The R.StsI protein sequence was compared with sequences in the GenBank and EMBL data bases. As expected, no significant homology was detected between R.StsI and other ENases except for R.FokI (30% identity). There were six regions that were highly homologous to each other, and they are aligned in Fig. 5. As shown in Figure 5, another homologous region was present from amino acid sequence 350 to 420. However, the extent of homology of this region was not as high as that of the other six regions (data not shown). As these two enzymes recognize the same sequence but cleave at different positions, we suppose that these homologous regions are potential domains involved in the recognition of the asymmetric sequence and the amino acid residues which are involved in the cleavage of phosphodiester bonds are located in the region that showed no significant homology. Now we plan to exchange the amino acid domains between FokI and StsI. The analysis of the mutants will give us interesting information about the reaction mechanism of type IIs restriction-modification enzymes.

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