The site-specific recombinase encoded by *pinD* in *Shigella dysenteriae* is due to the presence of a defective Mu prophage

Akira Tominaga

Tel: +81 086 251 7864. Fax: +81 086 254 4648. e-mail: atomi@cc.okayama-u.ac.jp

Department of Biology,
Faculty of Science,
Okayama University
Okayama 700, Japan

The DNA inversion systems are made up of an invertible DNA segment and a site-specific recombinase gene. Five systems are known in prokaryotes: the *Salmonella typhimurium* H segment and *hin* gene (H-*hin*), phage Mu G-*gin*, phage P1 C-*cin*, *Escherichia coli* e14 P-*pin*, and *Shigella sonnei* B-*pinB* systems. In this report a site-specific recombinase (*pinD*) gene of *Shigella dysenteriae* was cloned and sequenced. *pinD* mediated inversion of five known segments at the same extent in *E. coli*. Although one *inv* sequence was identified, no invertible region was detected in a cloned fragment. The predicted amino acid sequences of PinD and three ORFs showed high homology to those of Gin and its flanking gene products. An ORF homologous to Mom of Mu conserved a functional activity to modify intracellular plasmid DNA. Southern analysis showed that the cloned fragment contains two homologous regions corresponding to the left and right ends of the Mu genome. Together these results indicated that the *pinD* gene in *S. dysenteriae* is derived from a Mu-like prophage.

Keywords: *Shigella sonnei*, *Shigella dysenteriae*, *pinD* nucleotide sequence, B-*pinB*, phage Mu

INTRODUCTION

Inversional switching systems in prokaryotes, which control alternative expression of two sets of genes, are made up of an invertible DNA segment, its flanking two crossover sites (*inv*) and a site-specific recombinase gene. Four systems have previously been characterized in detail: the *Salmonella typhimurium* H segment-*hin* gene (H-*hin*) (Silverman *et al.*, 1979; Szekely & Simon, 1981; Zieg & Simon, 1980), phage Mu G-*gin* (Koch *et al.*, 1987), phage P1 C-*cin* (Hiestand-Nauer & Iida, 1983; Iida, 1984; Iida *et al.*, 1982) and *Escherichia coli* e14 P-*pin* (Plasterk *et al.*, 1983; Plasterk & van de Putte, 1985). In *Salm. typhimurium*, H inversion concerns flagellar phase variation, and in both phages inversion of the segments mediates reciprocal expression of two sets of tail fibre genes. Phenotypic change caused by P inversion is unknown. In contrast to the difference in biological function, these recombinases are functionally

interchangeable with one another (Kamp & Kahmann, 1981; Kutsukake & Iino, 1980; Momota & Enomoto, 1986; van de Putte *et al.*, 1984).

Previously, I reported two recombinase genes from strains of the genus *Shigella* (Tominaga *et al.*, 1991). These, *pinB* and *pinD*, are derived from *Shigella boydii*, and *Shigella dysenteriae*, respectively. Afterwards, the strain of *S. boydii* used (Tominaga *et al.*, 1991) was proved to be *Shigella sonnei*. Although phenotypic changes in *S. sonnei* and *S. dysenteriae* are unknown, the difference of inversional activity between *pinB* and *pinD* for known invertible segments suggests that *pinB* and *pinD* are different. The invertible B segment and *pinB* gene analysed in nucleotide level are highly homologous to phage Mu G-*gin* system (Tominaga *et al.*, 1991). On the other hand, it is unclear whether *pinD* is different from known recombinase genes, since the switching system of *S. dysenteriae* has not been identified.

In this report, to elucidate the differences in function and structure between *pinD* and known recombinase genes, the nucleotide sequences of the *pinD* gene and its flanking regions from *S. dysenteriae* were determined. The result indicates that the genetic organization of

Abbreviation: IRR, inverted right repeat.

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pinD flanking regions is identical to that of the G segment and β region of Mu. Moreover, Southern analysis and functional analysis indicate that the *pinD* system is the remnant of a Mu-like prophage.

METHODS

Bacterial strains, phage and plasmids. *S. dysenteriae* Sh16 (Scott, 1968) was used as DNA donor for Southern hybridization and cloning the *pinD* gene (Tominaga *et al.*, 1991). *E. coli* K-12 strains used were as follows. EJ2282 (Δ *fliC purB51* Δ *pinE recA56 hsdR*) (Tominaga *et al.*, 1991) and MV1184 (Vieira & Messing, 1987), which was found to be defective in *pinE* function, were used as the hosts for recombinant plasmids. Mucls (Tominaga *et al.*, 1986) was used as probe for hybridization.

Plasmid pTY501 carrying a 6.75 kb *Sall* fragment containing the *H-fljB* region fixed at the 'off' state was used for the detection of H inversion as described below. Plasmids containing the invertible segment fixed in the (–) orientation were constructed as follows. A 3.1 kb *HincII* fragment containing the P segment of ϕ 14 was subcloned from the plasmid pHA201 (Tominaga *et al.*, 1991) into the *SmaI* site of pUC118 (Vieira & Messing, 1987), yielding pPSM62. To construct plasmid pCSM103, a 6.6 kb *BamHI*–*MluI* fragment containing the C segment of phage P1 was subcloned from the plasmid pAT101 (Tominaga *et al.*, 1986) into pUC118. To construct plasmid pHSM64, a 3.3 kb *PvuII* fragment from the pTY109 containing the H segment (Okazaki *et al.*, 1993) was subcloned into the *SmaI* site of pUC118. A 4.2 kb *BamHI*–*Sall* fragment from pTSB918 containing the B segment (Tominaga *et al.*, 1991) was subcloned into the *BamHI* and *Sall* sites of pUC118, to give pBSM73. A 6.6 kb *PstI*–*AvaI* fragment containing the G segment of phage Mu (Koch *et al.*, 1987) was cloned into the *PstI* and *AvaI* sites of pUC119 (Vieira & Messing, 1987), to give pGSM97. Plasmid pTSD23 containing *pinD* is a pACYC177 (Chang & Cohen, 1978) -derivative that carries a 1.6 kb *PvuII* fragment from a deletion plasmid for sequencing. pTSD29 is a pUC119-derivative that carries a 1.3 kb fragment containing a *lacZ'*–ORF3 fusion (composed of the 5' 30 bp of *lacZ* and the 3' 723 bp of ORF3) at the polylinker site.

Media. TLY broth, nutrient agar and nutrient semisolid agar (NSS) were as described by Enomoto *et al.* (1983). Chloramphenicol (Sigma), kanamycin (Meiji), tetracycline (Sigma) and ampicillin (Sigma) were used at concentrations of 12.5, 50, 25 and 50 μ g ml^{–1}, respectively. IPTG (Sigma) was added to a final concentration of 1 mM.

Detection of recombinase gene activity in recombinant plasmids. EJ2282 (Δ *fliC* Δ *pinE recA56*) bearing plasmid pTY501 (EJ2286) was used as the host for transformation. Since the H segment of this plasmid is fixed in the (–) orientation by the *hin* mutation, this strain is non-motile. The presence of recombinase genes capable of complementing the *hin* mutation is detected by restoration of *fljB* expression. Recombinant plasmids were transformed into EJ2286 and the motility of transformants was examined on NSS containing chloramphenicol and ampicillin.

DNA manipulations and sequencing strategy. Total host DNA was prepared by the method described by Tominaga *et al.* (1991). Plasmid isolation, agarose gel electrophoresis, digestion with restriction enzymes, and ligation were done by standard methods (Maniatis *et al.*, 1982). Restriction enzymes, T4 DNA ligase, Klenow fragment, exonuclease III and mung

bean nuclease were purchased from Takara Shuzo and used as recommended. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) with a sequencing kit (Takara) and [³⁵S]dCTP (Amersham, 37 TBq mmol^{–1}). Fragments subcloned from plasmid pTSD608 into pUC118 in both directions were deleted stepwise using exonuclease III and mung bean nuclease (Takara), and a series of deletion plasmids was used for sequencing. Nucleotide and amino acid sequences were analysed using DNASIS sequence analysis software (Hitachi Software Engineering).

Construction of probes and Southern assay. Mu probes that comprise almost all of the Mu genome (Howe, 1987) were prepared as follows. A 5.1 kb left-hand end to the *EcoRI* fragment containing transposase gene (probe MU1) was recovered directly from agarose gel after *EcoRI* digestion of total Mu DNA and electrophoresis. Five fragments of the Mu genome were subcloned into pUC118 or pUC119; a 9.4 kb *EcoRI*–*BamHI* fragment containing the semi-essential region (probe MU2) was ligated into the *EcoRI* and *BamHI* sites of pUC119, a 2.7 kb *BamHI* fragment containing the head genes (probe MU3) was ligated into the *BamHI* site of pUC118, a 6.5 kb *BamHI*–*EcoRI* fragment containing head and tail genes (probe MU4) was ligated into the *BamHI* and *EcoRI* sites of pUC118, a 5.1 kb *EcoRI*–*PstI* fragment containing tail genes (probe MU5) was ligated into the *EcoRI* and *PstI* sites of pUC119, and a 6.6 kb *PstI*–*AvaI* fragment of pGSM97 containing the G segment was used as probe MU6. A 1.3 kb *PstI* fragment containing *pinE* and a part of P segment fixed in the (–) orientation (probe PINE) was subcloned from pHA201 into the *PstI* site of pUC118. A 1.0 kb (Fig. 1a, position 490–1485) fragment containing *pinD* was used as probe PIND. Southern analysis was performed by blotting agarose gels onto nylon membrane filters (Amersham Hybond-N) using a vacuum blotter. The restriction fragments used for probes were prepared as described before (Tominaga *et al.*, 1991) with digoxigenin-labelled dUTP with a non-radioactive DNA-labelling kit (Boehringer). Hybridization was done by standard methods (Maniatis *et al.*, 1982), and hybrid bands were detected by the digoxigenin-ELISA method using the Boehringer detection kit.

RESULTS AND DISCUSSION

Cloning and expression of the *S. dysenteriae pinD* gene in *E. coli*

A site-specific recombinase gene *pinD* in *S. dysenteriae* strain Sh16 was found previously through its ability to mediate H segment inversion (Tominaga *et al.*, 1991). Digests with *BamHI*, *HindIII* or *Sall* of total Sh16 DNA were hybridized with the probe containing the B segment and *pinB* gene (B-*pinB*) from *S. sonnei*. *BamHI* or *Sall* digest showed one hybrid band of approximately 21 kb and *HindIII* digest showed two bands of 2.1 and 12.5 kb (data not shown). The size of hybrid bands with *BamHI* or *Sall* digest suggests that a region containing the *pinD* gene is different in restriction sites from the corresponding region of *S. sonnei* since the 5.9 kb *BamHI* fragment (B-*pinB*) contains no *HindIII* sites and a unique *Sall* site (Tominaga *et al.*, 1991).

For cloning the *pinD* gene, *Sall* fragments were inserted into pBR322 and introduced into the non-motile strain EJ2286 [Δ *fliC*/pTY501(H[–] *fljB*⁺)] (Tominaga *et al.*, 1991). One of the recombinant plasmids, pTSD608,

which conferred motility on the host by causing inversion of the H segment, was isolated and contained a 10.1 kb insert (Fig. 1b). To localize the *pinD* gene, this 10.1 kb *SalI* fragment, whose size was different from that expected from Southern hybridization, was subdivided into several smaller plasmids. Using the inversion activity for the H segment as an assay, a 3.0 kb *HindIII*–*PvuII* fragment (pTSD611) was found to be the smallest fragment which carried the *pinD* gene (Fig. 1b). An invertible segment was expected to lie in either side of *pinD* by analogy with other switching systems. However, no inversion region was detected in this fragment when pTSD608 prepared from successive cultures was digested with restriction enzymes (Fig. 1b). In a previous report (Tominaga *et al.*, 1991), *pinD* mediated inversion of C, H and P segments with different frequencies to those for genes intrinsic to each segment. To compare the inversional activity of *pinD* for different segments, pTSD23 containing *pinD* was introduced into MV1184 along with compatible plasmids carrying one of five segments fixed in the (–) orientation: B, C, G, H or P. After approximately 30 generations, all plasmids digested with appropriate restriction enzymes produced two sets of fragments specific to both orientations resulting from the inversion of segments (data not shown). In all cases, the inversion was equilibrated in both orientations, but no inversion was detected even after longer incubation when pTSD23 was absent. This result indicates that PinD has qualitatively the same activity for the recombination of all invertible segments examined in *E. coli* K-12. The quantitative differences in activities of recombinases observed previously (Tominaga *et al.*, 1991) may have been due to different levels of expression of *pinD* or availability of other accessory factors (Glasgow *et al.*, 1989; Johnson *et al.*, 1987; Kahmann *et al.*, 1985).

Nucleotide sequences of the *pinD* gene and its flanking regions

The nucleotide sequence of the 3.0 kb *HindIII*–*PvuII* fragment bearing the *pinD* gene was determined (Fig. 1a). Three complete and two incomplete ORFs were inferred (Fig. 1b). A complete ORF, starting with GTG instead of ATG as in *gin* and *pinB* (Plasterk *et al.*, 1983; Tominaga *et al.*, 1991) and preceded by the Shine–Dalgarno (SD) sequence and Pribnow box, was identified as the *pinD* gene. It consists of 591 bp, encoding a 197 amino acid polypeptide with a molecular mass of 22 307 Da. The predicted amino acid sequence of PinD, except for four C-terminal amino acids, is highly homologous to that of Gin (193 amino acids); 98.9% (191 of 193) amino acids are identical. Two amino acid residues are different in their polarity (Asp-48 to Ala and Ala-184 to Thr), but they are in the non-conserved region among known recombinases (Glasgow *et al.*, 1989). In the upstream region of the *pinD* gene, a 38 bp sequence, which contains a 26 bp *inv* sequence for invertible recombination, was identical to the inverted right repeat (IRR) of the B segment, and the incomplete ORF1 (598 bp, 199 amino acids) including part of the IRR

sequence was inferred to be part of the invertible D segment, highly similar to that of the variable region S'v (199 of 318 amino acids) in the G segment (Kahmann & Kamp, 1987).

A short sequence (positions 1–227) in the 5' end of the fragment was identical to the 3' end sequence of the insertion element IS600 (Matsutani *et al.*, 1987). Another putative IS600 element (Fig. 1b) was discovered by sequencing (data not shown) and so the D segment is disrupted by insertion of two IS600 elements. Two complete ORFs, ORF2 and 3, with the same transcriptional direction as *pinD*, are highly homologous to the *com* and *mom* genes, respectively, on the β region of Mu. ORF2, partially overlapping with ORF3, was preceded by a perfect SD sequence and a possible promoter (Fig. 1a). Amino acid sequence homologies between ORFs and three corresponding genes are shown in Fig. 1(b). Although the *mom* gene is followed by the *attR* sequence that is required for transposition, a sequence corresponding to *attR* was not found in the downstream region of ORF3. ORF4 was found by database searching to be 64.1% (66 of 103) and 76.8% (43 of 56) homologous to the incomplete hypothetical ORFs *Aorf3* and *orfx*, which are present at flanking regions of plastid ATP synthase operons of the red alga *Antithamnion* sp. (Kostrzewa & Zetsche, 1992) and *Galdieria sulphuraria* (Kostrzewa & Zetsche, 1993), respectively.

Since D-*pinD* is very similar to G-*gin* and B-*pinB*, a comparison of nucleotide sequences among three systems was carried out. Although *pinD* also showed highest homology for *gin* (567 of 591 identical bases), a 50 bp sequence located between the IRR and the *pinD* gene was most homologous to the corresponding region of B-*pinB* (44 of 50 identical bases). Among upstream regions of the IRR sequence the highest homology was observed between B and G segments (558 of 578 identical bases). This result suggests that these three systems are closely related to one another and had exchanged horizontally during evolution to generate chimeras.

Southern analysis

Many IS sequences are detected in *S. dysenteriae* and are shown to be responsible for deletions or rearrangements of flanking DNA (Galas & Chandler, 1989). Two copies of IS600 were contained in the cloned *SalI* fragment, and no invertible region was detected in this fragment, though the IRR sequence was present near *pinD*. To discover whether the region containing the invertible D segment and *pinD* has undergone rearrangements and whether a Mu-like genome besides the G segment and β region is present in the chromosome, hybridization analyses were carried out (Fig. 2). *S. dysenteriae* total DNA was digested with *PvuII* and hybridized with two kinds of Pin probes and six kinds of Mu probes that comprise almost all of the Mu genome (see Methods). Probe PIND containing *pinD* showed a 4.2 kb main band and a 7.2 kb weak band (Fig. 2, lane 4). The size of this main band was consistent with that of the *PvuII* fragment containing the D-*pinD* in pTSD608. In ad-

Fig. 2. Southern hybridization analysis of *S. dysenteriae* DNA with probes specific for site-specific recombinase genes and Mu genome. Genome (lanes 1, 2, 4 and 5) or plasmid pTSD608 (lane 3) DNA was digested with *Pvu*II, and processed as described in Methods and hybridized with probes: lane 1, probe MU6 containing the G segment of Mu; lanes 2 and 3, probe MU1 containing the 5.1 kb left end of Mu; lane 4, probe PIND; lane 5, probe PINE.

dition, the existence of the weak 7.2 kb band suggests the presence of an inversion in the genome or another recombinase gene. To identify the source of the 7.2 kb band, probe PINE containing *pinE* of *E. coli*, which is most closely related to *Shigella* in phylogeny (Ochman & Wilson, 1987), was hybridized with the same *Pvu*II digest and showed a 7.2 kb main band in addition to a 4.2 kb weak band (Fig. 2, lane 5). This result indicates that the 7.2 kb fragment contains a recombinase gene homologous to *pinE* and that the 4.2 kb fragment contains the *pinD* gene relatively less similar to *pinE* and suggests no detectable inversion of a chromosomal segment.

Four kinds of Mu probes, MU2, 3, 4 and 5, which cover the central 24 kb region encoding most of structural genes, showed no hybrid band (data not shown). Two kinds of Mu probes, MU1 and MU6, showed positive bands. Probe MU1 containing the left end to *kil* gene region showed a 4.2 kb band (Fig. 2, lane 2), indicating the presence of a homologous region corresponding to the left end region of Mu. Probe MU6 containing tail genes, the G segment, and a part of *gin* gene showed a 4.2 kb main band and three weak bands, of 3.5, 6.5 and 7.2 kb (Fig. 2, lane 1), suggesting that the 4.2 and 7.2 kb fragments are the same fragments detected by probes PIND and PINE, respectively, and that G-*gin* is more similar to D-*pinD* than to *pinE*. Other weak bands might have been derived from sequences homologous to some tail genes. Since the size of the genomic fragment detected by the probe MU1 is the same as that of fragment containing D-*pinD*, it was predicted that a region homologous to the left end of Mu was present in the cloned *Sall* fragment. To confirm this possibility, probe MU1 was hybridized with a *Pvu*II digest of pTSD608, and the 4.2 kb *Pvu*II fragment containing *pinD* was indeed detected (Fig. 2, lane 3). This Mu-homologous region is inferred in a 0.2 kb left end of the 4.2 kb *Pvu*II fragment (Fig. 1b), since the region to the left of this fragment was not detected by MU1. These results indicate that regions homologous to the right and

Fig. 3. Assay for the DNA modification. DNA samples of pTSD29 carrying ORF3 were prepared before (lanes 1 and 2) and after (lanes 3–5) induction of the ORF3 product. Three micrograms of DNA was used for *Pvu*II digestion, which divided the plasmid into two fragments, 1.1 and 2.8 kb, and electrophoresed in a 1.0% agarose gel: lane 1, sample prepared before induction without *Pvu*II digestion; lane 2, before induction; lanes 3, 4 and 5: 40, 80 and 120 min after induction, respectively.

left ends of the Mu genome are close in the 4.2 kb *Pvu*II fragment from *S. dysenteriae*, suggesting that regions homologous to both ends of Mu are remnants generated by deletion or rearrangement of an internal region of Mu-like prophage. The structure of a remnant containing a recombinase gene and one inverted repeat sequence could be an intermediate to create inversion systems in host bacteria by the appearance of secondary inverted repeat sequences (Rozsa *et al.*, 1995). The discrepancy of the size between the cloned fragment and that detected by Southern analysis is unknown, but the strain used for the Southern analysis might have contained a minor subpopulation of cells with the DNA in the inverted orientation and it may have been this DNA which was isolated in pTSD608.

ORF3 encodes a *mom* homologue

Since ORF3 was significantly homologous to *mom* and contains only four altered amino acids apart from four extra amino acids, the function of ORF3 was examined. Mom acts to modify intracellular DNA including host DNA and co-resident plasmid DNA besides Mu DNA (Toussaint, 1976), and *mom*-specific modification affects digestion with *Hga*I, *Pvu*II and *Sal*I, but not *Bam*HI and *Hind*III (Kahmann & Hattman, 1987). To control the expression of ORF3, plasmid pTSD29 carrying a *lacZ'*-ORF3 fusion was constructed. To determine whether the ORF3 product modifies DNA or not, pTSD29 DNA was prepared before and after induction of ORF3 and cleaved with *Pvu*II. Before induction, the *Pvu*II digestion was not restricted, whereas, at least 40 min after induction the digestion was severely restricted (Fig. 3). In contrast, the cleavage with *Hind*III was complete in spite of ORF3 induction (data not shown),

suggesting that ORF3 has the same sequence specificity to modify DNA as Mu *mom*.

Two inversion systems, B-*pinB* and D-*pinD*, found as a part of a defective prophage in *Shigella* spp. are highly homologous to that of Mu. Moreover, Southern analysis with probe PINE suggested that an $\epsilon 14$ -like element is present in *S. dysenteriae*. Although the biological function of these systems has not been determined as in P-*pinE* on *E. coli* $\epsilon 14$, reproductive fitness has been reported in *E. coli* lysogenized with various phage (Edlin *et al.*, 1975, 1977). Thus, the cryptic prophage containing the inversion system in *Shigella* spp. may produce a phenotypic change in undetectable ways under the laboratory conditions and confer a selective advantage to bacteria under some growth conditions.

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