Cloning and Complete Nucleotide Sequences of the Type II Restriction-Modification Genes of *Salmonella infantis*

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The complete type II restriction-modification system of *Salmonella infantis* was cloned in *Escherichia coli* as an R · *Sau*3AI fragment of 3,430 base pairs. The clone was shown to express the restriction endonuclease as well as the modification methylase. The nucleotide sequence of the above fragment showed two open reading frames of 461 and 230 codons in tail-to-tail orientation. These were shown to represent the modification methylase M · *Sin*I and the restriction endonuclease R · *Sin*I, respectively. The methylase M · *Sin*I amino acid sequence revealed a considerable similarity to those of other deoxycytidylate methylases. In contrast, endonuclease R · *Sin*I did not exhibit such a similarity to other restriction enzymes.

Restriction-modification systems arouse interest not only as powerful biochemical tools but also as a convenient model of protein-DNA interaction. The specific recognition of only a few bases in combination with the large number of evolutionarily distant isoschizomers could give information about the exact requirements of this interaction. In 1981 Lupker and Dekker (11) described a restriction endonuclease from a strain of *Salmonella infantis*, which was an isoschizomer of R · *Ava*II (G⁺G[A/T]CC). The large number of *Sin*I isoschizomers in the distant group of cyanobacteria (22) and the evolutionary closeness of *S. infantis* to *Escherichia coli* make the *Sin*I system a perfect object for this type of research.

We cloned and sequenced the *Sin*I restriction-modification system and compared its predicted amino acid sequences with those of other type II systems.

MATERIALS AND METHODS

Strains and media. *E. coli* MC1061 (3), which is an *mcr*B mutant (17), was used in all cloning experiments with plasmids containing the M · *Sin*I gene. MC1061 was grown in brain heart infusion (BHI) medium or in Luria-Bertani (LB) medium (Oxoid Ltd., Basinstoke, United Kingdom). The antibiotic ampicillin was used at 70 µg/ml. A wild-type *S. infantis* strain isolated in our laboratory (11) was the source of chromosomal DNA; it was grown in BHI.

Enzymes and chemicals. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.), Promega-Biotec (Leiden, The Netherlands), and Pharmacia (Uppsala, Sweden). Calf intestine phosphatase was from Boehringer GmbH (Mannheim, Federal Republic of Germany). All were used according to the manufacturers' specifications. Hydrazine (pure) was from Serva (Heidelberg, Federal Republic of Germany), and piperidine was from E. Merck AG (Darmstadt, Federal Republic of Germany). Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer.

Construction of the colony bank. DNA from *S. infantis* isolated as described previously (10) was digested partially with R · *Sau*3AI (12), and DNAs from the various incubations were pooled and fractionated by agarose gel electrophoresis.

Fragments ranging in size from 2 to 9 kilobases were extracted from the gel (27) and ligated into plasmid pUC19 (26) that had been cut with R · *Bam*HI and dephosphorylated with calf intestine phosphatase. A clone bank of 25,000 colonies was constructed by transforming *E. coli* MC1061 with the resulting recombinant plasmids. The pooled transformants were transferred from plates into 750 ml of BHI medium and grown for 6 h at 37°C before being harvested by centrifugation. Plasmids were isolated as described previously (12).

Selection. Since the product from a successfully cloned M · *Sin*I gene should render the two *Sin*I sites of pUC19 resistant to cleavage by R · *Sin*I, a 5-µl sample of the pooled plasmid bank (representing approximately 2.5% of the total yield) was incubated with 50 U of endonuclease R · *Sin*I for 8 h, after which the DNA was extracted with phenol and precipitated with ethanol. The cleavage procedure was repeated overnight. The resulting DNA was purified and introduced into *E. coli* MC1061. The resulting transformants were plated onto agar containing ampicillin and gave rise to approximately 150 colonies.

Eleven randomly chosen colonies were further investigated, of which nine contained a plasmid that could no longer be cut with R · *Sin*I in vitro. To ensure that we had not isolated mutants lacking both *Sin*I sites in pUC19 but genuine clones containing the M · *Sin*I gene, we removed the total insert by excising a *Pvu*II fragment (Fig. 1) from all nine recombinants. The resulting deletion clones were again sensitive to R · *Sin*I, which proved that a reversible modification rather than some mutation had caused them to become R · *Sin*I resistant. The R · *Sin*I-resistant clone with the largest insert, pSI4, was used in all subsequent experiments.

DNA sequencing. The DNA sequence was determined mainly by the chemical degradation method of Maxam and Gilbert (14). Parts of the insert were sequenced as a denatured double-stranded plasmid with specific oligonucleotides with the dideoxynucleotide protocol of Sanger et al. (18).

Isolation of endonuclease R · *Sin*I. *E. coli* MC1061 harboring plasmid pSI4 was grown overnight in BHI and harvested by centrifugation. Isolation of R · *Sin*I from these cells was performed as described earlier (11).

Primer extension. RNA was isolated from *E. coli* cells containing plasmid pSI4 with the hot phenol method (24) and then hybridized to terminally ³²P-labeled synthetic oligonu-

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FIG. 1. Linear representation of the inserts of the various plasmids mentioned in the text. Plasmid pSI4 contains the whole *Sau3AI* fragment and consequently both the ORFs coding for the restriction and the methylation proteins ($r^+ m^+$). The insert is represented as a thin line, the DNA from the vector pUC19 is shown as the thicker lines flanking the insert. On both sides two restriction sites of the polylinker of pUC19 are shown. For the pSI4 derivatives the deletions are shown as well as the phenotypes of the cells harboring these plasmids. In plasmid p Δ HH2 an extra *NheI* site was introduced by destroying the old *HindIII* site. In this way the *HindIII* site in the coding region of *R* · *SinI* became the only one in this plasmid. The A+T percentages for the various regions of the insert are given at the bottom of the figure.

cleotides that were complementary to putative stretches of either *R* · *SinI* or *M* · *SinI* mRNA (Fig. 2). These probes were extended by avian myeloblastosis virus reverse transcriptase (Pharmacia) as described previously (6).

After the elongated primers were boiled in 90% formamide–10 mM NaOH, they were loaded onto denaturing polyacrylamide gel next to sequence ladders generated with the same oligonucleotides on plasmid pSI4 DNA. Total RNA isolated from cells transformed by pUC19 was used for a reference.

Computer programs. For the analysis of sequence data we used the software package (GCG) distributed by the University of Wisconsin (5). Protein files generated with the aid of the GCG programs from published DNA sequences were compared on an Atari ST microcomputer. To this end a program was developed to score for similarity with a preset window size and maximum number of mismatches. Although scoring tables for comparing proteins suggested by the GCG programs and by Schwarz and Dayhoff (20) were also used, the best pictures, i.e., those with the best signal/noise ratio, were obtained by utilization of the original amino acid sequences.

RESULTS

Cloning. Plasmids containing *S. infantis* DNA were constructed and selected for *R* · *SinI* resistance, i.e., the presence of a fully functional *M* · *SinI* gene. Subsequent analysis showed that one of these plasmids, pSI4, also coded for the *R* · *SinI* restriction enzyme, since cells transformed by this plasmid produced the *SinI* endonuclease. The *R* · *SinI* enzyme could be isolated from these cells by the same method that had been used to isolate it from its natural source *S. infantis* (11). Ten grams of *E. coli* MC1061 transformed with pSI4 yielded about 500,000 U of exonuclease-free *R* · *Sin*, as determined by cleavage assay on phage lambda DNA (data not shown).

Localization of genes. The sequences of both strands of

plasmid pSI4 were determined, revealing two nonoverlapping large open reading frames (ORFs) of 1,383 and 690 nucleotides (nt) in opposite strands. The ORFs are positioned tail to tail. To determine which of the ORFs in pSI4 codes for either gene, deletion mutants were made (Fig. 1).

A plasmid (p Δ HH2) missing the small *HindIII* fragment (nt 2728 polylinker) still directed the synthesis of *M* · *SinI*, as indicated by its resistance to *R* · *SinI* in vitro when isolated. This indicates that the large ORF is coding for *M* · *SinI* and the smaller ORF is coding for *R* · *SinI*. Accordingly, an *XbaI* deletion (nt 1402 polylinker) of pSI4 which had lost the 3' end of the large ORF (plasmid p Δ XX3), no longer gave rise to any detectable *M* · *SinI* activity. To rule out the possibility that the detected *R* · *SinI* activity was due to a vector-directed fusion protein and to prove at the same time that the shorter ORF is indeed coding for *R* · *SinI*, a plasmid (pHp $\rightarrow$ N Δ BH6) missing the internal *BglII*–*HindIII* fragment (nt 2647 through 2728) was constructed. Cells harboring this plasmid did not produce any *R* · *SinI* capable of cutting bacteriophage lambda DNA in vitro after standard isolation procedures.

Transcriptional start and stop signals. The transcriptional start points of the two genes were determined in primer extension experiments (Fig. 3). Oligonucleotides with the sequence 5'-ATGCATCAATGCTCCCCTGC-3' (presumably complementary to the *M* · *SinI* mRNA) and 5'-CGAGCGCGGATTTGAACAAC-3' (complementary to the *R* · *SinI* mRNA) were hybridized to total RNA isolated from cells harboring plasmid pSI4 and extended by reverse transcriptase toward the mRNA 5' termini.

The resulting autoradiograms (Fig. 3) show that transcription of the *R* · *SinI* gene starts at nt 3033 through 3036. Upstream one finds the sequence 5'-TTATACT-3' at position –10 and 5'-TTGACT-3' at –35 (Fig. 2). Just upstream of the putative translation start there is a ribosome-binding site at nt 3022 through 3027.

Transcription of the *M* · *SinI* gene starts near U-883 (Fig. 2; see Discussion). The area directly upstream of this position contains easily recognizable promoter sequences (very similar to those of *E. coli* [23]): 5'-CATAAT-3' (nt 872 through 877) at position –10 and 5'-TTGACT-3' (nt 849 through 854) at positions –35. A possible but rather weak Shine-Dalgarno sequence is at nt 891 through 893, further upstream from the ATG than in the case of the *R* · *SinI* gene.

Since the genes are situated tail to tail and are separated only by 31 bases, they most likely have a common terminator. A candidate structure for that function is indeed to be found at nucleotides 2301 through 2320: it is formed by a stem of 8 base pairs (containing 6 G · C pairs) and a loop of 4 bases (see underlined bases in Fig. 1). This structure is flanked on both sides by stretches rich in A+T, although not purely poly(dT) as is usual for a "classical" terminator.

Similarity between *M* · *SinI* and other deoxycytidylate methylases. The predicted amino acid sequence of the *M* · *SinI* enzyme was compared with those of some modification deoxycytidylate methylases: *M* · *BspRI*-GGCC (16), *M* · *BsuRI*-GGCC (9), *M* · *EcoRII*-CCWGG (21), *M* · *HhaI*-GC GC (4), *M* · *MspI*-CCGG (R. J. Roberts, personal communication), *M* · *Rho11s*-GGCC/GAGCTC (1), and *M* · *SPR*-GGCC/CCGG/CCWGG (8, 15). The last two are encoded by *Bacillus* phages.

These comparisons are represented as dot plots in Fig. 4. There is a striking similarity between the amino acid sequences of *M* · *SinI* and the methylases from the *Bacillus* group, whether they are coded by the bacteria (*M* · *BspRI* and *M* · *BsuRI*) or by the phages (*M* · *Rho11s* and *M* · *SPR*).

FIG. 3. mRNA start sites of the R·*SinI* and M·*SinI* genes. (I) Autoradiogram of a primer-extension experiment with a synthetic oligonucleotide (antisense to R·*SinI* mRNA). This primer was elongated with avian myeloblastosis virus reverse transcriptase on RNA as the template. The resulting extension products were fractionated in lane P. The arrows indicate the only bands found in this lane. They were run next to a dideoxynucleotide sequence ladder made with the same primer on plasmid DNA. The corresponding sequence is given on the left of the picture. In lane C an extra band is seen comigrating with the A; this band did not once appear in the sequence ladders according to either Maxam and Gilbert or Sanger et al. and must be considered an artifact of the elongation reaction under these precise conditions. (II) Corresponding experiment for M·*SinI* mRNA. Here the complementary sequence is presented for easy comparison with the sequence in Fig. 2.

(2, 7, 9, 19, 25), no similarity was found between these two components of the restriction-modification system.

DISCUSSION

The overall A+T content (58%) of the *SinI* restriction-modification system is fairly high. The distribution of A+T along the insert is shown in Fig. 1. In this respect the *SinI* restriction-modification system resembles those of the *EcoRI*, *EcoRV*, and *BsuRI* genes (2, 7, 9). This phenomenon might play a regulatory role. In line with this observation

there is a clear preference for A or T (Table 1) at the third or wobble position.

Transcriptional starts were identified experimentally, and promoter-like sequences were found for both genes. The start of the M·*SinI* gene as determined by primer extension shows that the mRNA for this protein starts with U-883. Whether this unexpected result is due to mRNA synthesis actually starting with UTP or to mRNA processing remains uncertain.

Promoterlike sequences, however, at nt 849 through 854 and 872 through 877 do not exclude the former possibility: since RNA polymerase can only span a defined number of nucleotides the new mRNA must start very close to nt 883. The *SinI* M and R genes are situated tail to tail in the *Salmonella* genome (M→←R), which differs from the tandem organization R→M→ in *BsuRI* (9) and M→R→ in *HhaII* (19). A head-to-head organization of the R and M genes has been described for *PstI* (25). Only the *EcoRII* (21) system appears to have a tail-to-tail conformation like that described here for *SinI*. The tail-to-tail organization of the two genes could be an additional regulatory element.

The difference in intensity between the bands shown in the primer extension experiments might be explained as an indication of a different level of expression of the two genes. However, since the isolation of mRNA represents only the level of the two messengers at a particular moment and the nucleotide sequence of the primer might influence the elongation reaction, such conclusions would be premature.

When the M·*SinI* amino acid sequence is compared with those of other dCMP methylases a good degree of similarity is found in a small but definite number of regions conserved in a large range of methylases from evolutionarily distant species. There even is a significant degree of similarity with the predicted amino acid sequence of M·*AquI*, a methylase isolated from the cyanobacterium *Agmenellum quadruplicatum* (C. Karreman and A. de Waard, manuscript in preparation).

FIG. 4. Similarities between methylase M·*SinI* and other modification methylases: dot-plot representation. The predicted amino acid sequence of M·*SinI* was compared with those of other deoxycytidine methylases. The program was set to score five perfect matches in a window of eight. M·*SinI* is plotted on the horizontal axis against the various other methylase proteins on the vertical axis. The unit on both axes in the various panels is 100 amino acids. M·*SinI* was compared with the following: I, M·*BspRI*; II, M·*BsuI*; III, M·*Rho113*; IV, M·*SPR*; V, M·*EcoRII*; VI, M·*HhaI*; VII, M·*MspI*.

TABLE 1. Codon usage of the R · *SinI* and M · *SinI* genes^a

Amino acid	Codon	R · <i>SinI</i>		M · <i>SinI</i>	
		No.	Fraction	No.	Fraction
Ala	GCA	4		8	
	GCT	6	1.00	8	0.62
	GCC	0		4	
	GCG	0	0.00	6	0.38
Arg	CGA	2		3	
	CGT	0		7	
	AGA	8	0.77	13	0.82
	CGC	2		0	
Asn	CGG	1		1	
	AGG	0	0.23	4	0.18
	AAT	16	0.84	13	0.65
	AAC	3	0.16	7	0.35
Asp	GAT	10	0.71	17	0.77
	GAC	4	0.29	5	0.23
Cys	TGT	3	1.00	2	0.67
	TGC	0	0.00	1	0.33
Gln	CAA	5	0.71	13	0.81
	CAG	2	0.29	3	0.19
Glu	GAA	18	0.72	21	0.68
	GAG	7	0.28	10	0.32
Gly	GGA	3		9	
	GGT	2	1.00	15	0.65
	GGC	0		6	
	GGG	0	0.00	7	0.35
His	CAT	3	1.00	8	0.80
	CAC	0	0.00	2	0.20
Ile	ATA	3		12	
	ATT	8	0.92	13	0.74
	ATC	1	0.08	9	0.26
Leu	CTA	1		8	
	CTT	8		10	
	TTA	5	0.74	21	0.73
	CTC	3		0	
	CTG	1		1	
Lys	TTG	1	0.26	13	0.27
	AAA	19	0.95	26	0.84
	AAG	1	0.05	5	0.16
Met	ATG	3	1.00	11	1.00
Phe	TTT	9	0.64	8	0.50
	TTC	5	0.36	8	0.50
Pro	CCA	4		14	
	CCT	5	0.75	7	0.78
	CCC	0		4	
	CCG	3	0.25	2	0.22
Ser	TCA	7		4	
	TCT	4		11	
	AGT	5	0.76	8	0.72
	TCC	1		1	
	TCG	1		3	
Thr	AGC	3	0.24	5	0.28
	ACA	3		7	
	ACT	3	0.75	7	0.70
	ACC	0		3	
Trp	ACG	2	0.25	3	0.30
	TGG	5	1.00	5	1.00
Tyr	TAT	3	0.75	10	0.63
	TAC	1	0.25	6	0.37
Val	GTA	4		5	
	GTT	5	0.69	12	0.74
	GTC	3		2	
	GTG	1	0.31	4	0.26

^a Shown are the numbers of the different codons used in both genes and the fractions of codons with A or T or with G or C at the wobble position.

The adenosine methylases are far less similar either among themselves or with the cytidine methylases (only *dam* methylase has a marked resemblance to M · *DpnII* G^{Me} ATC [13] and to M · *EcoRV* GATATC; our own observation).

Since some of the type II systems are coded on transferable elements, it is conceivable that these systems are migrating through the bacterial kingdom and that the uniformity of the deoxycytidylate methylases might imply that they represent a relatively young set of genes not yet completely integrated into their hosts.

It should therefore be very interesting to compare isoschizomers of distantly related bacteria to see how much similarity there still (?) is between them. The codon usage of the methylation genes might also give some clarification on this point.

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