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Genetics

Inversions of specific DNA segments in flagellar phase variation of *Salmonella* and inversion systems of bacteriophages P1 and Mu

(*din* mutants/phase determinant/G segment/C region/*trans*-acting factor)

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ABSTRACT Prophages P1 and Mu produce a *trans*-acting factor possessing the din$^+$ activity which catalyzes the inversion of the specific DNA segment responsible for flagellar phase variation of *Salmonella*. *din* mutants were isolated from P1CM*clr100* phage by selecting phages that did not suppress the *vh2* mutation of *Salmonella* in prophage state. No inversion loop structure was detected among DNA forms arising after denaturation and rehybridization of DNAs extracted from the *din* mutants. The DNA fragment containing C region of P1 was cloned on a plasmid vector, pCR1. The resulting hybrid plasmid, pKK2, was shown to possess the din$^+$ activity: the *vh2* mutant of *Salmonella* harboring the plasmid changed the flagellar phase. From analysis of the plasmid by use of *Bam*HI and *Bgl* II, the *din* gene specifying the din$^+$ activity was located near or within the C region of P1. It is highly plausible that the *din* gene of P1 was also involved in the inversion of the C region. Similarly, the DNA fragment containing the G and β segments of Mu was cloned on pCR1. The resulting hybrid plasmid, pKK101, also possessed the din$^+$ activity.

Diphasic strains of *Salmonella* possess two nonallelic structural genes, *H1* and *H2*, for flagellin, the component protein of flagellar filaments. The *H1* and *H2* genes specify phase 1 and phase 2 flagellin, respectively. They are expressed alternatively in a bacterial clone. This phenomenon is called "flagellar phase variation." Lederberg and Iino (1) analyzed the mechanism of this phenomenon by P22-mediated transduction. They concluded that the ability to switch from the expression of one gene to that of the other is controlled by a genetic element linked to the *H2* gene and that the state of the *H2* gene determines the flagellar phase of the bacterium. This genetic element is called "phase determinant (*PD*)" (2).

The *H2* gene constitutes an operon with the *rh1* gene which specifies a repressor of the expression of the *H1* gene (3–5). When the *H2* operon is active (*H2*-on), phase 2 flagellin is synthesized and the *H1* gene is repressed by the product of the *rh1* gene. When the *H2* operon is inactive (*H2*-off), the *H2* and *rh1* genes are not transcribed and thus phase 1 flagellin is synthesized. From genetic studies of a stable phase strain of *Salmonella*, Iino (6) showed that the ability to manifest flagellar phase variation is controlled by the *vh2* gene which is closely linked to the *H2* gene. Diphasic strains, such as *S. typhimurium* LT2, carry the *vh2*$^+$ allele and change their flagellar phase at frequencies of 10^{-3} to 10^{-5} per bacterial division (7). Stable phase strains, such as *S. abortusequi* SL23, carry the *vh2*$^-$ allele and change their flagellar phase at frequencies less than 10^{-7} per bacterial division (6).

It was shown that the expression of the *H2* gene is controlled by a recombinational event which inverts a region of DNA containing elements necessary for the transcription of the *H2* operon and this invertible DNA region corresponds to *PD* (8, 9). We showed that the *vh2* gene specifies the cytoplasmic factor that catalyzes the inversion of *PD* (10).

Meanwhile, it was found that bacteriophage Mu DNA contains a 3000-base-pair sequence which undergoes inversion (11). This region is called "G segment." G segment contains at least two genes essential for the phage growth (12). The specific inversion of G segment appears to be correlated with the formation of infectious phage particles (13, 14). The gene that specifies a *trans*-acting factor involved in the site-specific inversion of G segment is called *gin* (15). It is located in the β segment of Mu DNA. An identical inversion region was reported in bacteriophage P1 DNA (16, 17) and is called "C region" (18).

We showed that prophages P1 and Mu produce a *trans*-acting factor which catalyzes the inversion of *PD* in the *vh2*$^-$ strains of *Salmonella* (10). This phage-specifying activity which suppresses the *vh2* mutation of *Salmonella* was termed "din." In this paper, we present evidence that the din$^+$ activity is also involved in the inversion of the G segment of Mu or the C region of P1.

MATERIALS AND METHODS

Bacterial Strains, Plasmid Vector, and Bacteriophages. Tester strains for the din$^+$ activity were KK1251 (*galE*), KK1252 (*strA*), and KK1253 (*galE*), all derived from the *S. typhimurium* strain SJW1250 (*vh2*$^-$ *H2*$^-$ *H1*-gt) (10). KK1251 and KK1252 were fixed in phase 2 (*H2*-on) by the *vh2* mutation and were nonmotile owing to the *H2* mutation. KK1253 was fixed in phase 1 (*H2*-off) and thus was motile (Fig. 1).

The recipient strain for transformation by hybrid plasmids was a *recA* derivative of an *Escherichia coli* K-12 strain, KH802 (F$^-$ *galK lacY met su*II$^+$ *r*K$^-$ *m*K$^+$) (19). An F′ factor used for mobilization of hybrid plasmids was from an *E. coli* strain, LC169 (Δ*proB-lac leu thy*/F′*lac*$^+$), provided by Y. Takeda. *E. coli* strain Bu8072 (SuIII$^+$ Mu^r) was used for the propagation of λ–Mu hybrid phages (20).

Plasmid vector pCR1 (21) was prepared from C600 (pCR1). The P1 phage used was P1CM*clr100* (22). Hybrid λ phage containing the G and β segments of Mu (23) was provided by A. I. Bukhari.

Isolation of P1 Lysogens. P1CM*clr100* lysogens were prepared from KK1251 or KK1253 according to Rosner (22).

Preparation of Phage and Plasmid DNAs. Phage DNA was prepared from purified phage particles obtained after thermal induction of a corresponding lysogen or lytic infection. All preparations involved extraction with phenol and precipitation with ethanol. Plasmid DNA was prepared according to Nishimura *et al.* (24).

Restriction Endonuclease Digestion and Agarose Gel

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Abbreviations: *PD*, phase determinant; *H2*-on and *H2*-off, active and inactive states of the *H2*-operon.

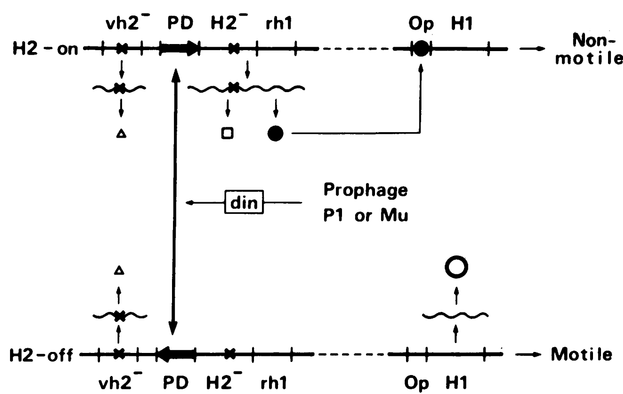

FIG. 1. Regulatory mechanism of flagellar phase variation of the tester strains for the din+ activity. Horizontal heavy and wavy lines, chromosomes of *Salmonella* and mRNA molecules, respectively; heavy arrows, orientation of *PD*, which is expressed as the direction of transcription from a promoter residing in *PD*; △ and □, defective gene products of *vh2* and *H2*, respectively; ● and ○, active gene products of *rh1* and *H1*, respectively; Op, binding site of the gene product of *rh1*. KK1251 and KK1252 were fixed in *H2*-on, and thus were nonmotile. KK1253 was fixed in *H2*-off and thus was motile.

Electrophoresis. Restriction endonucleases *Eco*RI, *Bam*HI, and *Bgl* II were purchased from Bethesda Research Laboratories (Rockville, MD). They were used according to Bächi and Arber (25). Gel electrophoresis of DNA fragments was carried out on slab gels containing 0.8% agarose. After electrophoresis, the gels were stained with aqueous ethidium bromide solution and the DNA bands were visualized over an ultraviolet transilluminator. The gels were photographed on Polaroid P/N 665.

Construction of Hybrid Plasmids. *Eco*RI-digested DNA fragments were excised after agarose gel electrophoresis. The DNA was eluted from the agarose and ligated to *Eco*RI-digested pCR1 according to Sugiura and Kusuda (26). Transformation was done by the method of Lederberg and Cohen (27). The transformants were selected by plating on L-agar plates containing 25 μg of kanamycin per ml.

All experiments involving the construction and propagation of recombinant molecules were performed in the P2-EK1 condition according to the "Guidelines for Recombinant DNA Experiments in Research Institutes such as Universities" (Ministry of Education, Science, and Culture, Japan, 31 March 1979).

Introduction of Hybrid Plasmids into Salmonella. An F′*lac*+ factor was transferred from LC169 into KH802 *recA* strains harboring hybrid plasmids. Then, the hybrid plasmids were introduced from them into the *Salmonella* strains by F-mediated conjugational crosses.

Mutagenesis of Phages. Mutagenized P1CM*clr100* phages were obtained from mutagen-treated cultures of KK1251 harboring P1CM*clr100*. Mutagenesis was carried out with 100 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine per ml.

Electron Microscopy of P1 Phage DNA. P1 phage DNA was denatured and hybridized by the formamide technique according to Yamagishi *et al.* (28). The DNA was observed with a JEM100C electron microscope.

RESULTS

Isolation of the *din* mutants from P1CM*clr100*. To investigate the relationship of the din+ activity of prophage P1 to its own inversion system of C region, *din* mutants which did not suppress the *vh2* mutation of *Salmonella* were isolated from P1CM*clr100*. Cells of KK1251 were infected with mutagenized phages obtained from mutagen-treated P1CM*clr100* lysogen, and lysogenic clones were isolated by selecting chloramphenicol-resistant ones at 30°C. Among them, 15 clones failed to produce motile subclones. Thus, they did not change their flagellar phase from *H2*-on to *H2*-off. This indicates that the phages existing in these lysogens failed to catalyze the inversion of *PD* from *H2*-on to *H2*-off.

Phages prepared from these lysogens then were applied to the cells of KK1253, and lysogenic clones were selected. Then, clones cured of phages were isolated from them by selecting temperature-resistant P1-sensitive ones. The resulting clones cured of phages were all motile; by contrast, the individual clones cured of wild-type phages were fixed in either a motile or nonmotile state (Table 1). Thus, these mutant phages also failed to catalyze the inversion of *PD* from *H2*-off to *H2*-on. Therefore, we concluded that the phages isolated above possessed the defects in the gene that specified the din+ activity.

Phage DNAs prepared from two of the *din* mutants (P1 17 and P1 28) were denatured, rehybridized by the formamide technique, and observed by electron microscopy. No inversion loop structure was detected in the preparations from the *din* mutant phages, although inversion loop structures of about 3000 bases were observed in about 20% of molecules prepared from wild-type phages (Table 2). This fact indicates that the *din* mutants of P1 also failed to invert the C region of their own DNA.

Isolation of DNA Fragment Carrying the din+ Activity. Bächi and Arber (25) reported that, among the DNA fragments generated by *Eco*RI digestion of P1 DNA, the largest one

Table 1. Ability of mutant P1 phages to catalyze inversion of *PD* from *H2*-off to *H2*-on

Phage	Colonies tested, no.	Colonies fixed, no. in Motile	Colonies fixed, no. in Nonmotile
P1CM*clr100*	300	178	122
P1 17	500	500	0
P1 28	500	500	0

KK1253 harboring one of the phages was grown in L broth at 30°C for 30 generations. Then, the culture was diluted, spread on L-agar plates, and incubated at 42°C. For each colony developed from a survival cell, which was expected to be cured of phages and fixed in a phase at the time of the curing, the flagellar phase of the component cells was examined by cultivating them on a motility agar plate after confirming their P1-sensitivity as described (10). If the flagellar phase variation occurred in an original liquid culture, *H2*-on (nonmotile) as well as *H2*-off (motile) ones must be detected among the colonies derived from the liquid culture. On the contrary, when the flagellar phase was fixed, an *H2*-on colony should not be detected. Only data from representative experiments are shown. Phages P1 17 and P1 28 were isolated from P1CM*clr100* as the mutants that could not catalyze the inversion of *PD* of *Salmonella* from *H2*-on to *H2*-off. The results from the remaining 13 mutants were essentially the same as those shown. The experiments were repeated on each phage mutant at least three times on a scale similar to that in the table, and segregation of nonmotile colonies was not observed in any of them.

Table 2. Inversion of C region in the *din* mutants of P1

Phage	Molecules observed	Molecules carrying inversion loop
P1CM*clr100*	51	10
P1 17	58	0
P1 28	56	0

Phage DNA was prepared from phage particles obtained after thermal induction of a P1 lysogen of KK1251. DNA was denatured, hybridized and observed by electron microscopy.

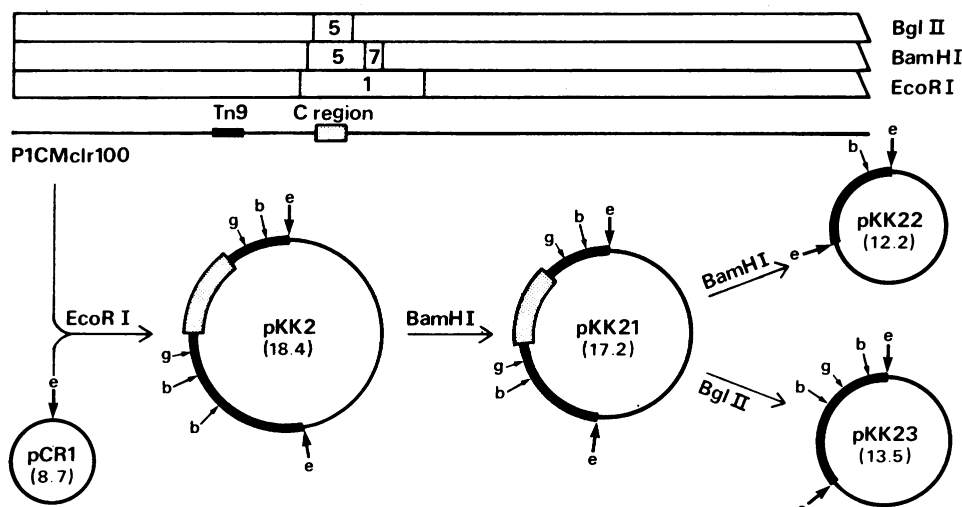

FIG. 2. Construction of hybrid plasmids carrying the *din* gene of P1. The restriction endonuclease cleavage map of P1CM*clr100* DNA is drawn according to Bächi and Arber (25) taking into account the data from De Bruijn and Bukhari (29). Only the DNA fragments concerned in the present study are indicated. Stippled areas, invertible region (C region) of P1 DNA; heavy and thin lines, cloned DNA fragment and the vector DNA, respectively; arrows, target sites of restriction endonucleases (e, *Eco*RI; b, *Bam*HI; g, *Bgl* II); numerals in parentheses, molecular weights ($\times 10^{-6}$) of the plasmids. Plasmids pKK2 and pKK21 showed the din$^+$ activity; pKK22 and pKK23 did not.

(*Eco*RI 1) contains the C region. We isolated this DNA fragment after *Eco*RI digestion of P1CM*clr100* DNA and agarose gel electrophoresis and inserted it into the *Eco*RI cleavage site of pCR1 (Figs. 2 and 3). The resulting hybrid plasmid, pKK2, showed the din$^+$ activity because strain KK1252 carrying the plasmid manifested flagellar phase variation. The cloned DNA fragment contained three *Bam*HI cleavage sites, and its *Bam*HI digest yielded the *Bam*HI 5 and *Bam*HI 7 fragments of P1 DNA.

We first isolated the plasmid lacking the *Bam*HI 7 DNA fragment after *Bam*HI digestion and ligation of pKK2. The resulting plasmid, pKK21, still showed the din$^+$ activity. Next, the plasmid lacking the *Bam*HI 5 fragment was isolated from pKK21. The resulting plasmid, pKK22, did not show the din$^+$ activity. Thus, at least a part of the din$^+$ activity was coded from the *Bam*HI 5 DNA fragment of P1 DNA. This DNA fragment contained the *Bgl* II 5 fragment of P1 DNA (Figs. 2 and 3). The plasmid lacking the *Bgl* II 5 fragment was isolated from pKK21 after *Bgl* II digestion and ligation. The resulting plasmid, pKK23, did not show the din$^+$ activity.

Similarly, we constructed the hybrid plasmid containing the G and β segments of phage Mu DNA (Figs. 4 and 5). Because linear Mu DNA carried no *Eco*RI cleavage site in the β segment, λ–Mu hybrid phage containing G and β segments of Mu was used for this purpose. This hybrid phage DNA is cleaved four times by *Eco*RI, resulting in five fragments; the second largest of them contained the G and β segments. This fragment was isolated and ligated with *Eco*RI-digested pCR1. The resulting hybrid plasmid, pKK101, also showed the din$^+$ activity.

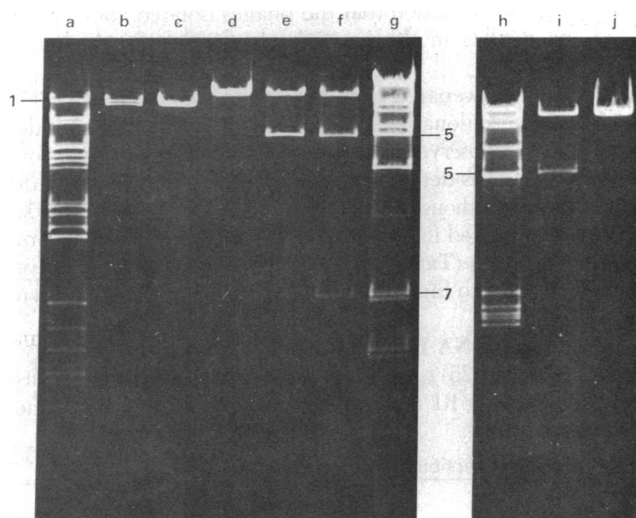

FIG. 3. Electrophoretic analysis of restriction endonuclease digests of hybrid plasmids carrying the *din* gene of P1. Only the DNA fragments concerned in the present study are indicated; they are numbered according to Bächi and Arber (25). Restriction endonucleases used were lanes a through c, *Eco*RI in lanes a–c, *Bam*HI in lanes d-g, and *Bgl* II in lanes h–j. DNAs were: P1CM*clr100* in lanes a, g, and h; pCR1 in lane c; pKK2 in lanes b and f; pKK21 in lanes e and i; pKK22 in lane d; and pKK23 in lane j.

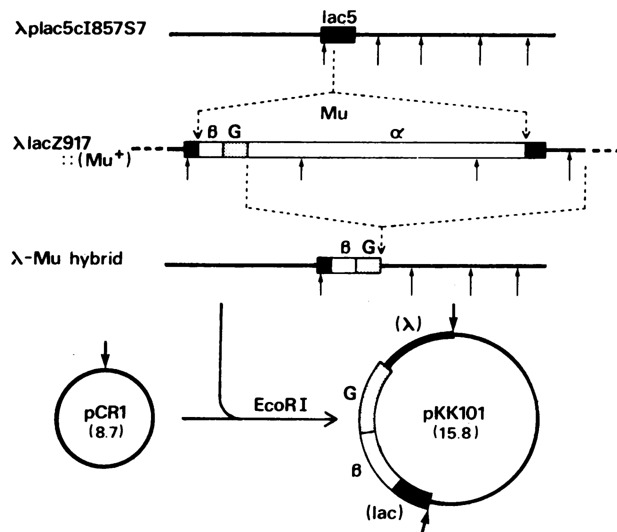

FIG. 4. Construction of a hybrid plasmid carrying the *din* gene of Mu. λ–Mu hybrid phage containing β and G segments of Mu was used as the DNA donor. The *Eco*RI restriction map of phages is drawn according to Allet and Bukhari (23). Arrows, target sites of *Eco*RI; solid and open areas, substitution of p*lac*5 and the β segment of Mu DNA; stippled areas, invertible region (G segment) of Mu; numerals in parentheses, molecular weights ($\times 10^{-6}$) of the plasmids.

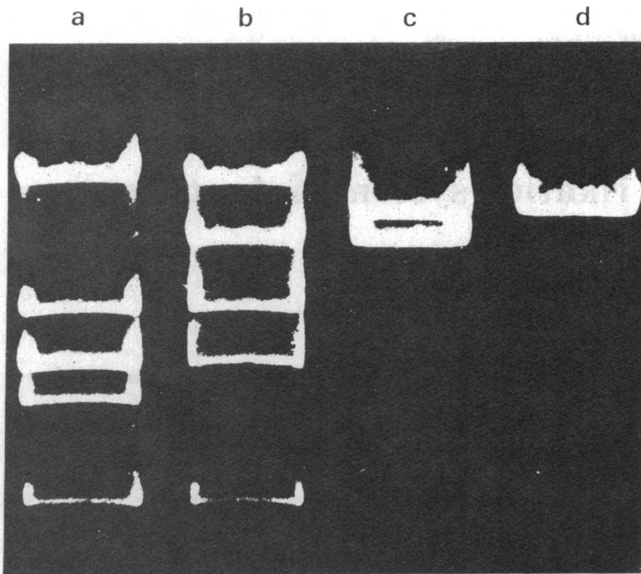

FIG. 5. Electrophoretic analysis of *Eco*RI digests of the plasmid carrying the *din* gene of Mu. DNAs were: λ*cI857S7*, lane a; λ-Mu hybrid phage, lane b; pKK101, lane e; and pCR1, lane d. All the DNAs were digested by *Eco*RI.

DISCUSSION

In our earlier work (10), we showed that prophages P1 and Mu produced a cytoplasmic factor that catalyzed the inversion of *PD* of *Salmonella*. The gene specifying this factor was termed *din*. The data presented in this paper make possible a refinement in our understanding of the relationship between the *din* gene activity and the inversion event of the G segment of Mu or of the C region of P1. The *din* mutants isolated from P1 failed to invert their C region. Thus, it is highly plausible that the product of the *din* gene is also involved in the inversion of the C region in bacteriophage P1. The *din* gene of P1 was shown to reside near or within the C region according to the results from DNA cloning experiments (Fig. 2). Similarly, the *din* gene of Mu was shown to reside in the G or β segment. In bacteriophage Mu, the *gin* gene has been reported to be involved in the inversion of the G segment and to be located in the β segment (15). Therefore, the *gin* gene of Mu may specify the din$^+$ activity.

The G segment of a *gin* mutant of Mu has been shown to be inverted by the inversion system of P1 (14). Furthermore, the DNA sequences of the invertible regions of the C region of the P1 and G segments of Mu have been shown to be identical with each other (17). On the contrary, the size of the invertible region of *PD* of *Salmonella* is quite different from the sizes of P1 and Mu. The *PD* of *Salmonella* consists of only about 800 base-pair sequence (30). On the other hand, the invertible regions of P1 and Mu were DNA segments of about 3000 base pairs (17). Nevertheless, the inversion systems of P1 and Mu can invert the *PD* of *Salmonella*. This fact is best explained if we assume that the specific site involved in the inversion of a DNA segment is relatively short and consists of only a part of the invertible region.

Two factors are prerequisite for the inversion of a specific DNA segment. One is a *cis*-acting factor—i.e., a specific DNA sequence mentioned above. The other is a *trans*-acting one—i.e., a cytoplasmic factor that recognizes the specific DNA sequence and inverts the DNA segment. We propose that the latter factor be called, in general, "DNA invertase." The *vh2* gene of *Salmonella*, the *gin* gene of Mu, and the *din* gene of P1 specify the DNA invertases. The regulatory mechanisms of gene expression by inversion of a specific DNA segment may be widespread in both eukaryotic and prokaryotic cell systems. The phase variation of *Salmonella* and the inversion systems of bacteriophages P1 and Mu may be taken as models for the analysis of the molecular mechanisms of such phenomena.

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