JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 1150–1156
0021-9193/94/$04.00+0

Vol. 176, No. 4

Division Inhibition Gene *dicF* of *Escherichia coli* Reveals a Widespread Group of Prophage Sequences in Bacterial Genomes

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Received 30 July 1993/Accepted 14 December 1993

The genomes of various eubacteria were analyzed by Southern blot hybridization to detect sequences related to the segment of the defective lambdoid prophage Kim which encodes DicF RNA, an antisense inhibitor of cell division gene *ftsZ* in *Escherichia coli* K-12. Among the homologous sequences found, one fragment from *E. coli* B, similar to a piece of Rac prophage, and two fragments from *Shigella flexneri* were cloned and sequenced. *dicF*-like elements similar to transcriptional terminators were found in each sequence, but unlike *dicF* these had no effect on division in *E. coli* K-12. Like *dicF*, these sequences are flanked by secondary structures which form potential sites for RNase III recognition. Coding sequences located upstream from the *dicF*-like feature in *E. coli* B are related to gene *sieB* of bacteriophage λ, while sequences downstream of the *S. flexneri* elements are similar to the immunity region of satellite bacteriophage P4. Under hybridization conditions in which only strong sequence homologies were detected in *E. coli* B and *S. flexneri*, the genomes of a large variety of microorganisms, including some gram-positive bacteria, hybridized to the *dicF* probe. Our results suggest that *dicF* and its flanking regions are markers of a widespread family of prophage-like elements of different origins.

Three defective lambdoid prophages, Rac, Qsr', and Kim (=Qin), have been identified in the genome of *Escherichia coli* K-12 (5, 21). Prophage Kim maps to 35 min in the replication terminus region of the chromosome (6). The first genetic element identified in Kim was the *QSR'* region, which can rescue a λ *Q* mutant by homologous recombination (11). Later, repressor-like gene *dicA* and *cro*-like gene *dicC* (see Fig. 1), resembling the immunity region of bacteriophage P22, were identified and mapped to a position 10 kb from the *QSR'* region (2, 3). Genetic analysis showed that inactivation of the *dicA* gene product leads to high-level expression of the neighboring operon, which is under control of a p_L-like promoter (2). This operon (here referred to as the *dicBF* operon) consists of two groups of three open reading frames (ORFs) separated by a 520-bp untranslated region (see Fig. 1).

The *dicBF* operon encodes two types of division inhibitors. One, DicB, is a small protein specified by the first ORF of the second cluster (12). DicB activates the *E. coli* MinC protein to inhibit cell division protein FtsZ (9). The second inhibitor, DicF, is a 53-nucleotide (nt) RNA molecule generated by transcription termination and by RNase III and E cleavages within the untranslated region (12). DicF RNA prevents the translation of *ftsZ* mRNA by an antisense mechanism (24). The orientation of the *dic* genes with respect to the *QSR'* cluster and the observation that *dicF* hybridizes to Rac DNA suggested that *dicAC* and the *dicBF* operon, respectively, correspond to the immunity region and a part of the early left region of prophage Kim (5).

The existence of an untranslated region from a lambdoid sequence, specifying a biologically active RNA, prompted us to look for similar elements in other genomes. No other phage of the λ family was known to carry such sequences. The finding of a Rac prophage fragment hybridizing to a *dicF* probe (5) led us to extend this study to the genomes of natural hosts of lambdoid phages, members of the family *Enterobacteriaceae*, as possible reservoirs of homologous sequences.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used for hybridization experiments were MC1061 (Rac$^+$ *dicABCF*$^+$); JS216, derived from MC1061 by replacement of the *dicABCF* locus by spectinomycin resistance gene *aadA*; GC2892, a *zcf289*::Tn*5* derivative of AB1157 (ΔRac); W3110 IN (*rrnC-rrnD*); and strains from the ECOR collection (16). *E. coli* B was strain BB (4). Other strains are indicated elsewhere in the text.

cRNA-DNA hybridization. A *SalI-HpaI dicF* cRNA probe labelled with [α-^{35}S]UTP was prepared by runoff transcription with RNA polymerase SP6 as described by Faubladier et al. (12). Digests of bacterial DNA (1 to 2 μg) were fractionated on 0.5% agarose gels run at a voltage gradient of 1.5 V/cm and transferred onto Hybond N$^+$ membranes (Amersham). Hybridization was carried out at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solution containing 50 mM Na phosphate buffer (pH 6.5), 1% sodium dodecyl sulfate, 1 mg of Ficoll per ml, 1 mg of polyvinylpyrolidone per ml, 250 μg of denatured salmon sperm DNA per ml, and 20% (vol/vol) formamide. Markers consisted of different plasmids carrying gene *dicF* that were digested to give a range of fragment sizes and mixed in an approximately equimolar ratio. The ten markers had sizes ranging from 1.1 to 28.2 kb.

Cloning procedures. DicF-like sequences from *E. coli* B and *Shigella flexneri* were cloned by the double-sizing procedure of Nicholls et al. (18). Preliminary experiments established that the minor signal of *E. coli* B belonged to a 3.9-kb *Bam*HI or a 1.8-kb *Bam*HI-*Hin*dIII fragment. Four micrograms of *E. coli* B DNA was digested with *Bam*HI and fractionated on an agarose

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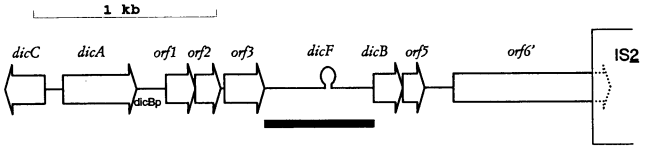

FIG. 1. Genetic organization of the *dicABCF* region of defective prophage Kim. Genes *dicA* and *dicC* are expressed under their own promoter. *orf-6* (dotted arrow) ends within insertion sequence IS2, which interrupts the *dicBF* operon (1). The untranslated region of the operon containing *dicF* is shown by the bar.

gel. DNA approximately 4 kb long was recovered and cleaved with *Hin*dIII, and then fragments of approximately 1.8 kb were recovered after gel electrophoresis and inserted in the *tet* gene of plasmid pBR322. The plasmid of interest, pMF3, was identified by dot blot hybridization of extracted plasmid DNA to the *dicF Sal*I-*Hpa*I cRNA probe. The same basic procedure was used for recovery of 2.5- and 1.6-kb *Hin*dIII-*Eco*RI fragments containing *dicF*-like sequences from *S. flexneri* cloned into vector pBR325. The plasmids obtained were pMF5 (1.6-kb insert; minor signal) and pMF6 (2.5-kb insert; major signal).

DNA sequencing. To locate *dicF*-homologous sequences precisely, deletion mutants were isolated and subjected to sequencing. The *Hin*dIII-*Bam*HI fragment of pMF3 and the *Eco*RI-*Bam*HI fragments of pMF5 and pMF6 were inserted into vector pUC19, yielding pMF7, pMF9, and pMF10, respectively. Plasmid pMF7 was then cleaved by *Sac*I and *Bam*HI, and pMF9 and pMF10 were cleaved by *Pst*I and *Bam*HI endonucleases. The linearized molecules were trimmed by exonuclease III and nuclease S1 treatment, end repaired, and circularized. Hybridization of the deleted plasmids to the *dicF* cRNA probe was used as a guide for selection of the plasmids to be sequenced. Sequencing was carried out on double-stranded DNA in accordance with standard procedures.

Methods for sequence analysis. The PC/Gene sequence analysis package (distributed by IntelliGenetics Inc.) and the promoter analysis program TAGSEARCH (17) were used for primary sequence analysis. The program of Zucker (25) was used to analyze RNA secondary structures. Searches for sequences related to those determined in this work were carried out at the National Center for Biotechnology Information (Bethesda, Md.) by using the BLAST network service.

Nucleotide sequence accession numbers. The nucleotide sequences of *E. coli* B Rac (1,007 bp) and *S. flexneri* SFS (1,596 bp) and SFW (1,494 bp) have been deposited in the GenBank/EMBL data bank and assigned accession numbers Z23096, Z23100, and Z23101, respectively.

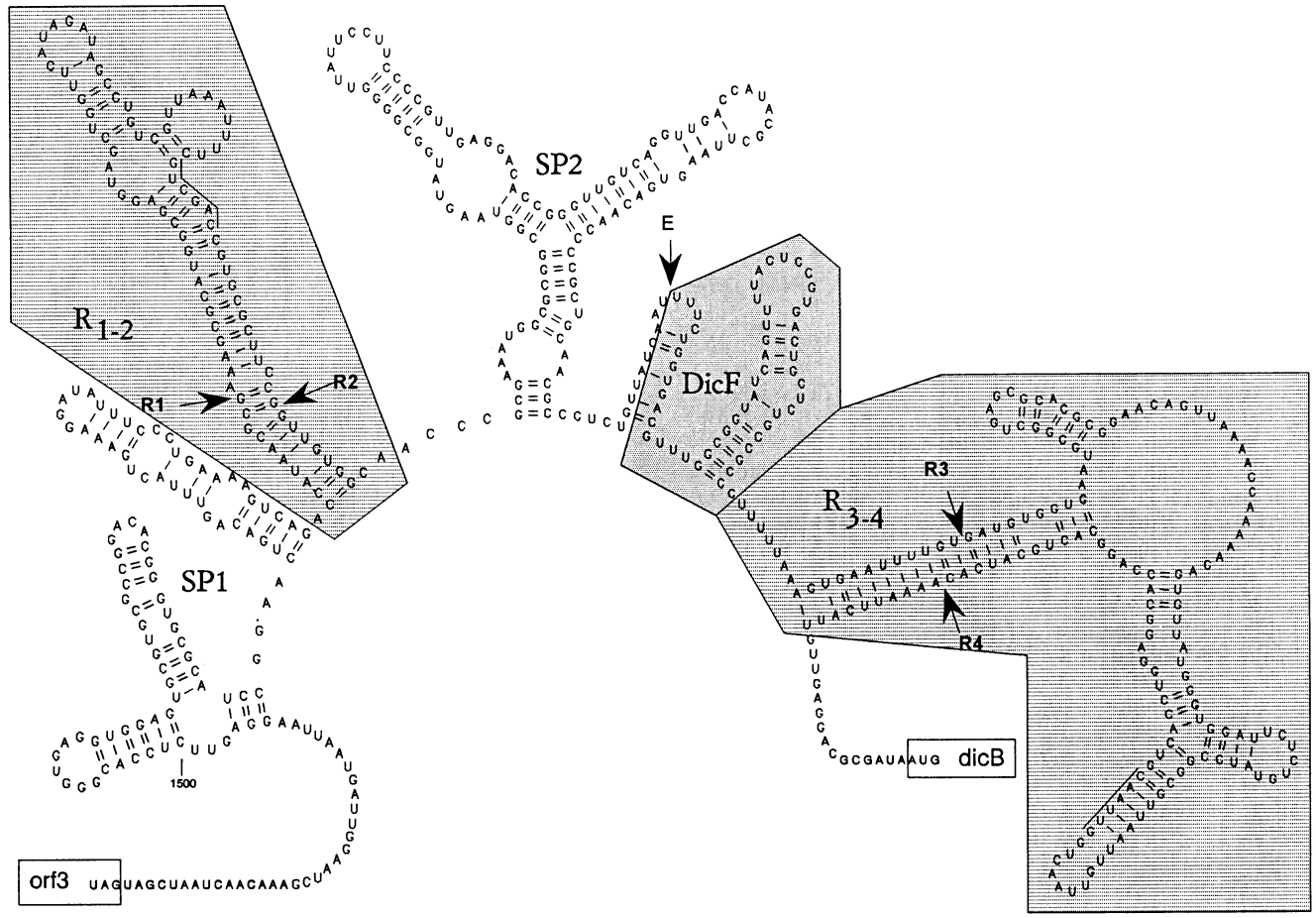

FIG. 2. Secondary structure and domains of the untranslated region of the *dicBF* operon. The sequence extends from the stop codon of *orf-3* to the start codon of gene *dicB*. The arrows indicate the sites of cleavage by RNases III (R1 to R4) and E (E). The sequences at the limits of the *Sal*I-*Hpa*I probe are overlined.

RESULTS AND DISCUSSION

Hybridization of *Escherichia* and *Shigella* DNAs with sequences of the *dicF* region. The untranslated region of the *dicBF* operon (Fig. 1) may be divided into five segments, as shown in Fig. 2: an upstream spacer (designated SP1) located immediately after the third ORF of the operon; the R_{1-2} segment, which forms an RNase III-processing site; a 120-nt spacer (designated SP2) with an unknown function; the transcription terminator (*dicF*), whose processed product inhibits cell division; and the R_{3-4} segment, which forms a second RNase III site (12). The secondary structure of this untranslated region was built from the requirements for transcription termination in *dicF* and for RNase III processing in R_{1-2} and R_{3-4} (12) and from computations of the most stable secondary structures in SP1 and SP2.

In a preliminary search for related sequences (5), a 308-bp *Sal*I-*Hpa*I DNA probe containing *dicF* was hybridized under stringent conditions to digested *E. coli* K-12 DNA. These experiments identified a homologous fragment in prophage Rac with significant sequence similarity to the probe (see below). A cRNA probe corresponding to the same 308-nt fragment was used in all subsequent experiments. This probe is complementary to the sequence between GUCGAC (within R_{1-2}) and GUUAAC (within R_{3-4}) (positions are indicated in Fig. 2). Therefore, it contains sequences from four of the five elements of the untranslated region.

Figure 3 shows the result of hybridizations to the DNAs of various *E. coli* and *Shigella* strains carried out under conditions in which only *dicF* and Rac fragments were detected in *E. coli* K-12. A strong band and a weak band were revealed with MC1061 (*dicF*$^+$ Rac$^+$). The strong band was absent from JS216 (Δ*dicF*), and the weak band was absent from GC2892 (ΔRac), indicating that they correspond to *dicF* and Rac, respectively. Under these conditions, the number of bands hybridizing varied among different strains: 1 in *E. coli* W and ECOR15, *S. dysenteriae*, *S. boydii*, and *S. sonnei*; 2 in *E. coli* B; and at least 10 bands in *E. coli* ECOR39 and ECOR44. Three bands of 28, 16, and 1.9 (not visible in Fig. 3) kb, were revealed in *S. flexneri*.

DNA fragments from *E. coli* B and *S. flexneri* related to *dicF* were cloned and sequenced as indicated in Materials and Methods. The sequence from *E. coli* B (1,007 nt) differed by less than 1% from the equivalent Rac sequence of *E. coli* K-12 (7). This indicated that *E. coli* B contains part of the Rac prophage, and we have designated this sequence Rac_B. Sequences in *S. flexneri* corresponding to strong (1,586 nt) and weak (1,494 nt) signals were designated SFS and SFW, respectively. Figure 4, which is discussed below, shows a comparison of the genetic maps of the four chromosomal sequences and of the relevant regions of bacteriophages λ and P4.

Sequences related to *dicF* in *Escherichia coli* B. The Rac_B fragment had four regions of at least 15 nt with greater than 90% sequence identity to the probe which together extend over 37% of the probe length. The sequence showed elements related to Kim R_{1-2}, *dicF*, and R_{3-4} (Fig. 5), flanked by divergently expressed ORFs. The incomplete ORF upstream from R_{1-2} is similar to λ *sieB* (39% amino acid identity; Fig. 6). Gene *sieB* encodes a function responsible for excluding superinfection by phage L in λ and P22 lysogens of *S. typhimurium* (23). Ranade and Poteete (20) found two conserved boxes in the sequences of the promoter regions of the bacteriophage λ and P22 *sieB* genes and two long segments of hydrophobic amino acids in the protein sequence. These features were also found in the gene from Rac_B (Fig. 6). These data suggest that

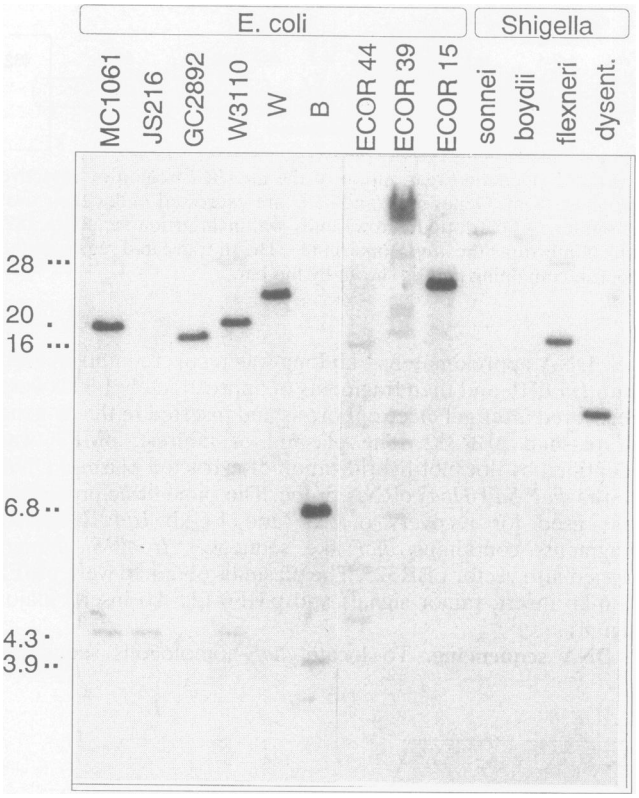

FIG. 3. Hybridization of a *dicF* probe to *E. coli* and *Shigella* genomic DNAs. A *Sal*I-*Hpa*I cRNA probe was hybridized to *Bam*HI-digested chromosomal DNA as indicated in Materials and Methods. Lanes, from left to right, correspond to *E. coli* K-12 strains MC1061, JS216 (Δ*dicF*), GC2892 (ΔRac), and W3110, *E. coli* W, B, ECOR44, ECOR39, and ECOR15; *S. sonnei*, *S. boydii*, *S. flexneri*, and *S. dysenteriae*, respectively. The sizes of fragments hybridizing in *E. coli* MC1061 and B and *S. flexneri* are indicated by one, two, and three dots, respectively.

Rac contains a gene that confers superinfection exclusion properties on the host.

The ORF located downstream from R_{3-4} was identical to *orfE*, which was identified by Clark and coworkers in *E. coli* K-12 (7). *orfE* codes for a putative protein of 73 amino acids. This protein, although apparently unrelated to DicB, is also a division inhibitor (8).

SFW sequence of *S. flexneri*. The weakly hybridizing fragment SFW had four sequences of at least 15 nt with greater than 90% sequence identity to the probe which together extend over 37% of the probe length. Six elements could be distinguished within the 1,494-bp sequence. The first 328 bp corresponded to the segment extending from the *Hin*dIII site to the right end of insertion element IS2. The second segment (118 bp) had no resemblance to known sequences, except for the last 38 bp, which show homology to Rac. It was followed by three elements resembling R_{1-2}, *dicF*, and R_{3-4}, respectively (Fig. 4 and 5).

Beginning immediately after R_{3-4}, an ORF that encodes a protein of 179 amino acids was detected. The *orf-179* sequence showed no homology to the corresponding region of Kim or Rac but resembled the immunity region of satellite bacteriophage P4 (Fig. 4).

Recently, Ghisotti et al. described three important features of the immunity region of this bacteriophage (10, 13, 14). First,

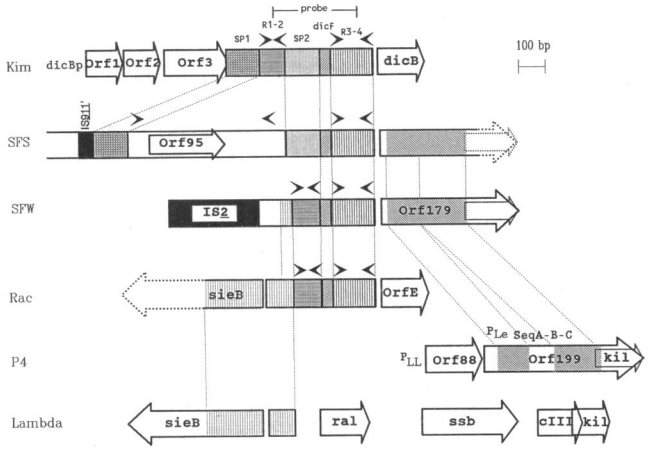

FIG. 4. Comparison of the genetic organization of the *dicBF* operon with the those of SFS, SFW, and Rac_B and of the regions of the bacteriophage λ and P4 genomes discussed in the text. Inverted repeats constituting proven or putative recognition structures for RNase III are shown by inverted black arrows. Regions of sequence homology are indicated by different shades and are linked by dotted lines. Dotted arrows indicate the 3′ end of incompletely sequenced ORFs. The extent of the probe used to detect related sequences is shown at the top.

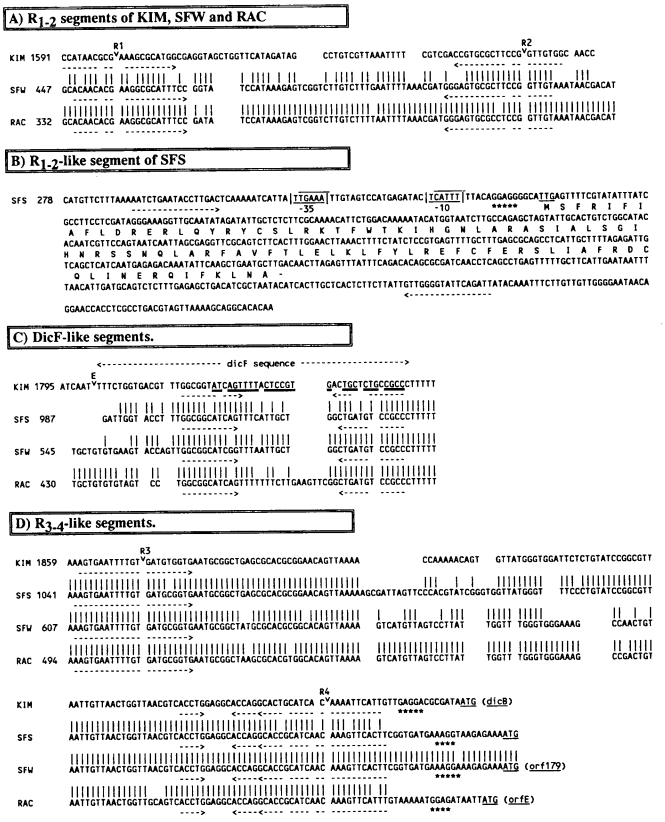

FIG. 5. Alignment of the sequences of the R_{1-2}-like, *dicF*-like, and R_{3-4}-like regions of Rac_B, Kim, SFW, and SFS. Putative promoter signals are boxed, and Shine-Dalgarno sequences are indicated by asterisks. Inverted arrows indicate regions of RNA pairing. The sequences are interrupted at the positions of RNase cleavage in Kim. Vertical bars indicate sequence identities between the most closely related sequences, shown in the order Kim, SFS, SFW, and Rac, as in Fig. 4. The sequence of DicF RNA that is complementary to the *ftsZ* mRNA sequence is doubly underlined.

transcripts initiated at the immunity promoter p_{LE} (Fig. 7) are not translated and terminate within ca. 300 bp. In this transcribed region, three sequences, *seqA*, *seqB*, and *seqC* (Fig. 7), can form *seqA-seqB* or *seqB-seqC* secondary structures and a stable *seqB* RNA (CI RNA) is generated in P4 lysogens. It was suggested that two sequences in regions of CI RNA not engaged in secondary structures may interfere with nascent transcripts in the *seqA* region and bring about premature transcription termination (10, 14; these sequences are doubly underlined in Fig. 7). Second, the whole immunity region may be translated as *orf-199* (13). This ORF, whose 5′ end is located upstream of p_{LE} (Fig. 7), would be expressed from a different promoter (p_{LL}) not involved in lysogeny. Third, the 3′ end of *orf-199* can be expressed as an internal, in-frame *kil* gene whose product inhibits cell division (13). Since transcripts initiated at p_{LE} terminate before the ribosome-binding site of the *kil* gene, this gene, like *orf-199*, is expressed from p_{LL} only.

Most of the above-described features were found in SFW (Fig. 7). First, a candidate for a strong promoter was present at the same position as p_{LE} (−35, TTGAAA; −10, TATAGT; spacing, 18 nt). This promoter has a computed score of 68, according to Mulligan et al. (17), placing it among the strongest. In addition, RNA sequences of SFW corresponding roughly to *seqA*, *seqB*, and *seqC* can form *seqA-seqB* or *seqB-seqC* pairs according to secondary-structure predictions obtained with the program of Zucker (25), despite considerable divergence (47%) from P4 sequences. Inverted repeats in the *seqB* region, which are supposed to play an important role in SeqB RNA stability, are also conserved (not shown in Fig. 7). More remarkably, the complementarity between the *seqB* and *seqA* sequences proposed for the interaction in *trans* is perfectly conserved in SFW (double underlines in Fig. 7), despite poor sequence similarity to P4. Second, most of the N-terminal part of the putative *orf-199* product can be aligned with the amino acid sequence of the product of *orf-179* located downstream from R_{3-4} in SFW (44 of 98 amino acids identical). This alignment shows a higher level of drift at the third versus the first and second codon positions (average, 50% versus 34 and 39%, respectively). These data support the notion that both *orf-199* of P4 and *orf-179* of SFW are actually expressed. Third, the sequence suggests that *orf-179* encodes an internal in-frame protein at its 3′ end. This protein, although unrelated to the *kil*-encoded protein of bacteriophage P4, would also be a basic protein (pI = 9.9). However, when two derivatives of pMF9 (see Materials and Methods) containing this part of the sequence under control of *lacZp* were analyzed, induction of the *lac* promoter was not found to affect cell growth or division.

Taken together, these characteristics of the SFW sequence strongly suggest that it is derived from a bacteriophage related to P4 or to a composite of bacteriophages P4 and λ. SFW contains all of the elements of the immunity region of a phage of the P4 family but is different from P4 itself. This immunity region may be functional in *S. flexneri*. By contrast, the presence of a piece of the IS*2* sequence suggests that other elements of the prophage are defective.

SFS sequence of *S. flexneri*. The fragment hybridizing most strongly, SFS, had four segments of at least 15 nt with greater than 90% sequence identity to the probe that together extend over 76% of the probe length. Eight functional elements were identified within the 1,586-bp sequence. Starting from the genomic *Eco*RI site used for cloning (to the left in Fig. 4) was

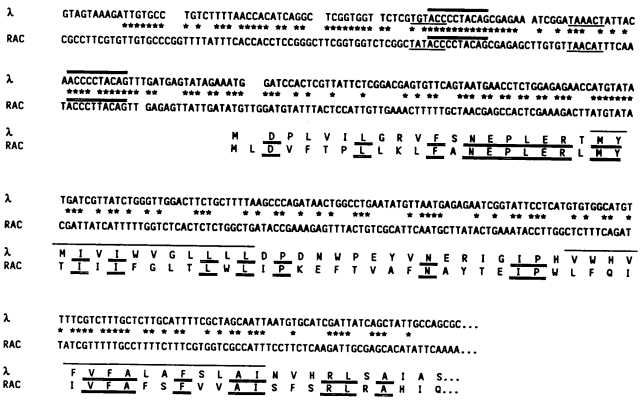

FIG. 6. Similarities between Rac_B and bacteriophage λ in the 5′ region of *sieB*. Identical bases and amino acids are indicated by stars and underlines, respectively. Sequence repeats in the control region of *sieB* and putative transmembrane domains are overlined. The Shine-Dalgarno sequence and candidate −35 and −10 promoter sequences for Rac *sieB* are underlined.

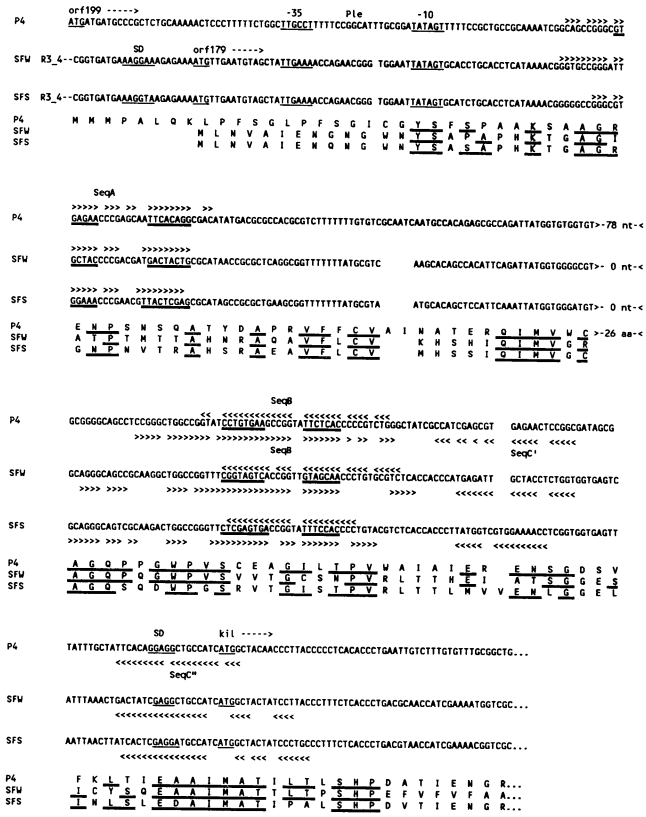

FIG. 7. Similarity of SFS and SFW to the repressor region of bacteriophage P4. Start codons, Shine-Dalgarno (SD) sequences, and −35 and −10 promoter boxes are underlined. Matching sequences of *seqA* and *seqB* or of *seqB* and *seqC* (consisting of *seqC′* and *seqC″*) are shown as inverted arrowheads above and below the sequence, respectively. Sequences proposed to be responsible for the interaction of CI RNA with nascent transcripts, defining immunity specificity, are doubly underlined. The partial amino acid sequences of *orf-199* and *orf-179* are shown under the nucleic acid sequences. Amino acids (aa) of P4 *orf-199* that are identical to those in SFS and/or SFW *orf-179* are doubly underlined.

a 91-bp sequence nearly identical to the beginning of the *pap* operon sequence of *E. coli* J96 (15). It was followed by a piece of insertion sequence IS*911* (bases 61 to 1, as oriented in Fig. 4). In contrast to Rac and SFW, segments nearly identical to SP1 (including the C-terminal part of Kim *orf-3*) and SP2 were present. They were separated by a 596-nt sequence (Fig. 5B) whose RNA ends can form a 17-bp perfect double-stranded structure ($\Delta G = -23$ kcal/mol [1 cal = 4.184 J]). Between these inverted repeats, an ORF that codes for a putative 95-amino-acid protein with a UUG start, preceded by a Shine-Dalgarno sequence (GGAGG) and a candidate promoter (score of 53, according to Mulligan et al. [17]), was also detected. Segment SP2 was followed by *dicF*-like and R_{3-4}-like sequences. The region next to R_{3-4} closely resembles SFW and P4 (Fig. 7). This includes the beginning of an ORF strongly related to *orf-179* of SFW (87 of 117 amino acids identical), an in-phase translation start within this ORF at the same position as in SFW and P4 *kil*, and sequence characteristics of a P4-like immunity region. It is possible that SFS expresses immunity to a P4-related bacteriophage, as proposed for SFW. The presence of a piece of IS*911* suggests that upstream regions were inactivated in two steps: insertion of IS*911* first and then deletion of most of IS*911* with adjacent prophage sequences.

DicF-like sequences do not inhibit cell division. When examined in detail, the sequences spanning the SP1-R_{3-4} interval presented a number of remarkable similarities and differences (Fig. 4 and 5). The first similarity was the conservation of DicF-like sequences. The three cloned sequences shared with DicF RNA the same distal stem-loop, followed by a run of U residues, suggesting a similar role in transcription termination. Second, sequences further downstream could all form a long double-stranded region, which in Kim is a substrate for RNase III. Third, a long double-stranded region upstream from the *dicF* sequence, similar to a known RNase III site, was found in two cases, and in the third a similar structure formed by a different sequence occupied the same position (Fig. 5B). It is therefore likely that a mechanism identical to that used in Kim (12)—involving, at the 3′ end, either termination or RNase III processing followed by retrodegradation and, at the 5′ end, RNase III processing—operates in each case to generate a short DicF-like RNA. To determine whether these *dicF*-like elements cause cell division inhibition, sequences from 263 to 597 of Rac, 523 to 717 of SFW, and 953 to 1062 of SFS were cloned under control of *lacZp* in high-copy-number plasmids. None of the resulting plasmids caused division inhibition when *lacZp* was induced. This finding is consistent with the absence of complementarity between these sequences and *E. coli ftsZ* mRNA (Fig. 5). The possibility that the SFS or SFW sequence is complementary to *S. flexneri ftsZ* mRNA cannot be ruled out.

Hybridization of sequences of the *dicF* region with DNAs from distant bacterial species. The results presented above indicated that under our hybridization conditions, genetic elements related to the probe are specifically detected in *E. coli* B and *S. flexneri*. Since lambdoid phages in which the Kim and Rac sequences are found infect a variety of members of the family *Enterobacteriaceae*, DNAs from various enteric strains were probed under the same experimental conditions, for the presence of *dicF*-related sequences. The results (Fig. 8A) revealed multiple hybridizing sequences, ranging from 3 for *Proteus mirabilis* to at least 12 for *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Morganella morganii*. Thus, it appears that some enterobacterial species contain many *dicF*-related elements that presumably originate from temperate bacterio-

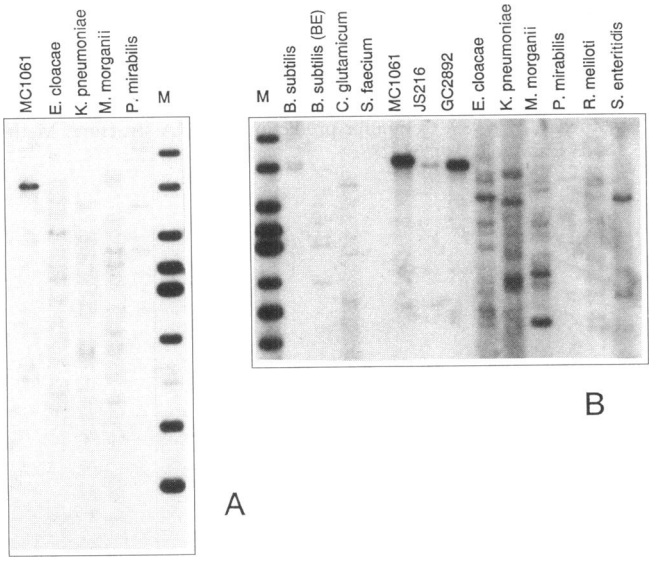

FIG. 8. Hybridization of a *dicF* probe to the DNAs of various bacterial strains. (A) Enterobacterial species. (B) Gram-negative and gram-positive species. Bacterial DNA was digested by *Bam*HI, except for the third lane of panel B (BE), where DNA was digested with *Bam*HI and *Eco*RI. Lanes M contained molecular size markers.

phages. The wide variation in the number of these elements is reminiscent of the situation observed with insertion sequences (19, 22). It suggests that unknown genetic factors influence the permissiveness of a given enterobacterial species or clone for lysogeny (or for the generation of defective derivatives).

In another experiment, the analysis was extended to more remote species (Fig. 8B). At least seven bands could be detected in the genome of *Rhizobium meliloti*. Surprisingly, at least two fragments from the chromosomes of the gram-positive organisms *Bacillus subtilis* and *Corynebacterium glutamicum* gave a signal with an intensity similar to that of Rac. The DNA from *Streptococcus pneumoniae* also yielded a positive signal (data not shown). Only *Streptococcus faecium* DNA did not hybridize to the probe (Fig. 8B). We are confident that the positive signals are significant, since the hybridizations were carried out under conditions in which only a few segments, each with extensive homology, responded positively in *E. coli* K-12 and B and *S. flexneri*. It can be speculated that the R_{1-2}–*dicF*-like–R_{3-4} region has some essential and well-conserved function in bacteriophages. However, verification of this hypothesis awaits the characterization of bacteriophages carrying similar sequences.

ACKNOWLEDGMENTS

We thank the following colleagues from Toulouse, France, for gifts of strains or bacterial DNA: R. Alazard (*E. coli* BB), M. F. Frère (members of the family *Enterobacteriaceae* other than *E. coli*), J. P. Claverys (*S. pneumoniae* and *S. faecium*), S. Bonassie (*C. glutamicum*), P. Boistard (*R. meliloti*), and P. Stragier (*B. subtilis* DNA). We are grateful to A. J. Clark (Berkeley, Calif.) and D. Ghisotti (Turin, Italy) for communicating data from their laboratories prior to publication and to D. Lane and H. M. Krisch (Toulouse, France) for improving the manuscript.

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