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Control of cell division in *Escherichia coli*. DNA sequence of *dicA* and of a second gene complementing mutation *dicA1, dicC*

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ABSTRACT

A mutation in a gene dicA of Escherichia coli leads to temperature-sensitive cell division, by allowing expression of a nearby division inhibition gene dicB (1). We have now established the sequence of the DicA region and identified DicA as a 15.5 KD protein. A second gene dicC transcribed divergently from dicA and coding for an 8.5 KD protein can also complement mutation dicA1 when provided on a multicopy plasmid.

INTRODUCTION

In Escherichia coli, cell division and its control remain poorly understood. Several genes lying in the 2 min region of the chromosome are involved in initiation of septum formation, septum biosynthesis and separation of polar caps (2). Various pieces of evidence point toward a key role of gene ftsZ (3,4) as a gene regulating the earliest steps of division (5,6). Interaction of the SOS-induced SfiA protein with ftsZ has been demonstrated (7,8), and ftsZ seems to be also under partial control of the CRP-cAMP complex (9). However, interactions between essential division genes and effector proteins or small molecules are likely to be far more numerous to account for the tight spacial and temporal regulation of division to cell growth and DNA replication. The mapping of a number of division loci whose function remains obscure (10) suggests that other levels of interaction will be found. We have previously reported the isolation and mapping at 34.9 min of one such mutation, dicA1 (1). This preliminary analysis indicated that dicA behaves as a repressor of a second gene dicB coding for a cell division inhibitor and located near dicA, and showed that the dicAB gene cluster is dispensible. Identification of the factor(s) which elicit dicB activity should provide a new insight on how division is regulated. In addition, the identification of the target of dicB among essential division genes may prove helpful in establishing its exact role. As a step in these directions, we have characterized gene dicA by DNA sequencing and complementation analysis. In

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this paper, we report that a defect in gene dicA leading to temperature-sensitive division can be complemented by two genes, dicA and dicC, located close to each other.

MATERIALS AND METHODS

Strains

Bacterial strains of Escherichia coli used in this study were: JS1 (1) argE3 lacY1 galK2 mtl1 rpsL700 dicA1; JS23 (1) leu thyA thi (ϕ80 immλ ind) ΔtrpE63 dicA1; JS54 (1) JS23 dicB1::Tn5; JS97 (this work) JS23 rpsL srlC::Tn10 recA1; JA200 (11) F^+ recA. Strain JM101 Δ(proB-lac) F'lacIq lacZΔM15 (12) was used for cloning into pUC plasmids and isolation of M13mp-derived phages. Strain MC1061 Δ(lacIPOZ-YA) (13) was used as a background for beta-galactosidase assays and CSR603 (recA1 uvrA6 phr-1) (14) for analysis of plasmid-coded proteins.

Cloning and sequencing methods

Procedures for the construction and analysis of recombinant DNA were essentially those described by Maniatis et al (15). DNA fragments were purified from agarose gels by the method of Vogelstein and Gillespie (16) and further treated by endonucleases and DNA polymerase large fragment in the presence of 5 mM spermidine (17) when required. Sequencing by primer extension was carried out either on derivatives of phage M13mp11 (18) or on irreversibly denatured plasmid DNA (19) previously purified by cesium chloride -ethidium bromide centrifugation. DNA, labelled with [α^{35}S]dATP, was run on thermostated 8% acrylamide - 8M urea gels with varying width (0.2 to 0.6 mm from top to bottom).

Plasmids

Plasmids pUC8, pUC9 (20), pMC1403 (21) pGB2 (22) and pGEM1 (derived from pSP64, 23) were used as cloning vectors. Plasmids pJPB36 and pBS44 were previously described (1). pBS39 is a subclone of a plasmid obtained by the same method as pBS28Ω4 (1). The construction of other plasmids used in this study is briefly described hereafter. Restriction sites are indicated by a one letter code: B = BamH1, D = Dde1, H = Hpa1, M = Sma1, N = HindIII, P = Pst1, R = EcoR1, S = Sal1, U = Sau3A, X = Xho1. Sites destroyed during constructions are in bracket. Numbers refer to position on the sequence. The initial plasmid pJPB36 (1) is lacPO-NPS1-U -NPSBMR-Z'. The H313-U938 fragment of this plasmid was cloned between the S and B sites of pUC9 to yield pBS60 lacPO-NP-(S/H313)-(U938/B)MR-Z'. pBS60 was cleaved at the X690 and R sites, and filled-in ends were ligated to give pBS71 lacPO-NP-(S/H313)-(X69O/R)-Z'. The S1-X690 fragment of pJPB36 was cloned into the Sal1 site of pUC9 to yield pBS44 lacPO-NPS1-(X690/S)PNPSBMR-Z'. Deletion of this plasmid between the H313 and the M site gave pBS49 lacPO-NP-S1-(H313/M)R-

Z', from which the NR fragment was substituted to the pUC8 linker to yield pBS53. The two U106-P fragments of pBS44 were cloned into pUC9 to yield pBS47 and pBS48, respectively lacPO-NP-S1-(U106/B)MR-Z' and lacPO-NP-(S/X690)-(U106/B)MR-Z'. Transfer of a H313-P fragment of pBS44 into pUC9 gave pBS45 lacPO-NP-(S:-X690)-(H313/S)BMR-Z'. The U106-B fragment of pBS44 was used to construct pBS64 and subsequent derivatives (see text). pBS61 lacPO-NP-(S/N)P-S1-(D515/S)BMR-Z' was constructed by inserting the N-D515 filled-in fragment of pBS44 into the HincII site of pUC9. Deletion of pBS61 between the S1 and the H313 sites gave pBS65, and deletion between the S1 and AccI (position 141) sites yielded pBS91.

Plasmid pBS75 was fragment H313-X690 from pBS63 (see text) cloned between the SalI and SmaI sites of vector pUC8. Fragment H413-RsaI of pBS63 was inserted at the HincII site of pUC8 to give pBS84.

Plates contained either 75 µg/ml ampicillin or 100 µg/ml spectinomycin and 25 µg/ml streptomycin for plasmid selection. After restriction analysis, plasmids were transferred into JS97 pregrown at 27°C, and selection of transformants was at room temperature. Complementation tests were usually done on L-agar plates at room temperature, 30°C, 37°C and 42°C. However pUC8 and pUC9 impaired growth at 42°C, therefore this temperature was omitted for testing their derivatives.

Other methods

Measurements of beta-galactosidase activity was carried out according to Miller (24). Restriction endonucleases site assignments and open reading frame analysis were carried out using Genofit COMPSEQ software package on an Olivetti M24 microcomputer. Program TARGSEARCH (25), based on the compilation of Hawley and McClure (26), was used to estimate possible promoter locations.

RESULTS

Insertional mutagenesis of $dicA^+$ and cloning of dicA1

Cloning of the wild-type DicAB region (1) allowed the construction of the restriction map of the dicA region shown in Figure 1. Previous analysis had shown that insertion of Tn5 on the chromosome at approximately 1800 bp clockwise to the SalI site suppressed the mutation. Therefore plasmid pJPB36 (1), which carries the SalI-$Sau3A_3$ fragment inserted into vector pUC9 (20) and complements the mutation, was presumed to be $dicA^+$ and $dicB^-$. This plasmid was chosen for a search of the location of gene dicA by γδ mutagenesis (27). Strain JA200 carrying pJPB36 was crossed with strain JS1 (dicA1, rpsL) and Str^r Amp^r colonies were selected at room temperature. Out of 50 colonies, none had retained temperature-sensitivity. Analysis of the plasmids in exconjugants showed that γδ had inserted at various positions in the vector or the insert (not shown). This negative result could be explained by

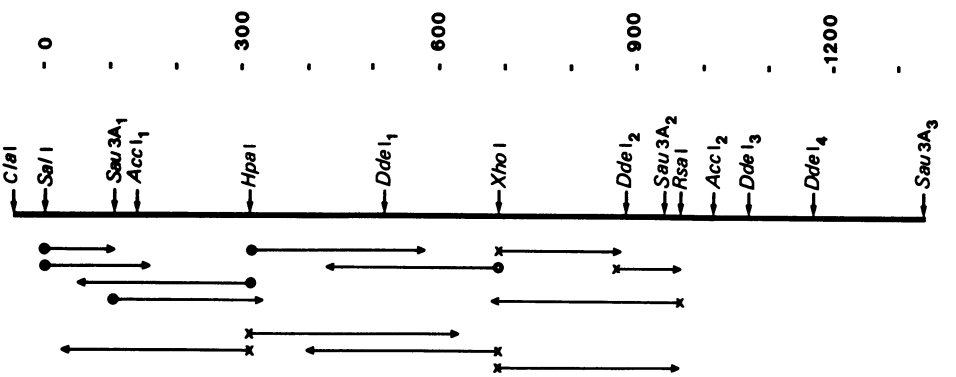

Figure 1. Restriction map of the dicA-dicC region and sequencing strategy. Arrows indicate the direction and extent of each sequence reading. Open circles show the sequences obtained by the method of Chen and Seeburg (19), and crosses indicate those obtained by single strand DNA sequencing. The lowest four arrows refer to sequencing of dicA1 DNA.

supposing that the Sal1-Sau3A$_3$ fragment carries more than one complementing function.

In addition, the dicA1 allele was cloned from the DNA of strain JS54 (dicA1 dicB::Tn5) cleaved with endonucleases EcoR1 and Cla1 and ligated to the unique sites of vector pBR322. The resulting plasmid, pBS63, complemented the dicA1 mutation at temperatures up to 42°C. Although residual activity of the dicA1 gene product at 42°C could not be ruled out, this result again pointed toward the existence of two complementing products in the dicA region.

Sequence of the dicA locus

As an aid in analyzing the complementation data, we determined the sequence of the dicA DNA. Plasmid pBS44 (1, and Materials and Methods) and four of its derivatives, pBS 47, pBS48, pBS49 and pBS45, which carry respectively fragments Sal1-Sau3A$_1$, Sau3A$_1$-Xho1, Sal1-Hpa1 and Hpa1-Xho1 inserted into pUC9, were used to determine the sequence of the dicA region by the supercoiled DNA sequencing method of Chen and Seeburg (19). Additional sequencing of the Xho1-Rsa1 fragment was obtained from an insert into phage M13mp11 (18), and was confirmed independently by sequencing of the dicB region (to be published). 72% of the sequence was deduced from sequencing on both strands (Figure 1) , and confirmation came from the sequencing of the mutant DNA (see below).

The sequence from the Sal1 to the Rsa1 site is shown in Figure 2. Two main reading frames, extending from bp 354 to bp 757 on the upper (clockwise) strand and from bp 269 to bp 42 on the lower (counterclockwise) strand appeared from sequence analysis. The first of these ORFs, designated ORF353, scores as "no opinion" (P=0.77)

Figure 2. Sequence of the Sal1-Rsa1 fragment. Numbering starts from the first base of the Sal1 recognition sequence. Sequence of both strands is shown along the first 800 base pairs. Possible -35 and -10 promoter sequences are under and overlined. Inverted repeats are shown by arrows. Sequence is discontinued at position 674 to indicate the change in the dicA1 mutant.

according to Fickett's coding probability analysis (28), and the predicted molecular weight of the protein is 15656 daltons. The second, ORF269, scores as "coding" (P = 0.98) for a 8578 daltons protein. In addition to these ORFs, a possible ribosome binding site GAGCUG (position 632-637 in Figure 2) at 6 bases from an AUG is found within the sequence of ORF353. This third ORF would code for an 18 aminoacids

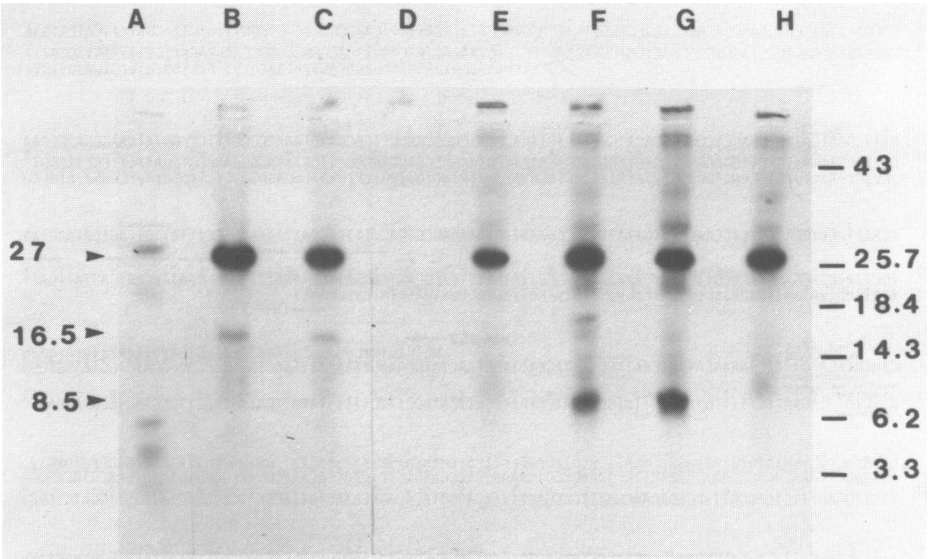

Figure 3. Analysis of proteins labelled in UV-irradiated recA cells. Sizes of markers (prestained proteins from Bethesda Research Laboratories), in kilodaltons, are indicated to the right. Proteins were resolved on 15% acrylamide gels in the presence of 6M urea as specified by the manufacturer (Focus 6:3, 5) A: pBS43; B: pJPB36; C: pBS60; D: no plasmid; E: pUC9; F: pBS44; G: pKC20 and H: pGEM1.

peptide. In phase fusion of this short ORF to lacZ' in pBS44 leads to a blue color, enhanced by addition of 10^{-4} M IPTG, on JM101 selective plates, but there is no evidence for any function of this ORF in vivo. Direct evidence for synthesis of ORF353 and ORF269 proteins was provided by the analysis of plasmid-coded proteins labelled in vivo in UV-irradiated recA cells (29). Plasmid pBS37 (1), which carries the 2.3 Kb ClaI-EcoRI fragment into pBR322, showed only one labelled protein in addition to vector encoded proteins, with an apparent molecular weight of 16.5 KD (not shown). Plasmid pJPB36 (fragment SalI-$Sau3A_3$ into pUC9) codes for a protein with the same size (Figure 3, lane B). This protein is not found in a derivative of pJPB36 carrying γδ inserted approximately at position 675 bp of the sequence (Figure 3, lane A), indicating that the 16.5 KD protein is the product of ORF353. Plasmid pBS44 carries a fusion at the XhoI site between ORF353 and a non-coding phase of the Z' fragment of pUC9, with a predicted mass of 15578 KD for the fusion protein. A protein with a slightly larger size was actually observed (Figure 3, lane F). Interestingly, pBS44 showed the presence of a second protein of 8.5 KD, not detected in plasmids carrying larger inserts, suggesting synthesis of the product of ORF269. Plasmid pBS61 (SalI-$DdeI_1$, Figure 4) carries a functional ORF269 but was

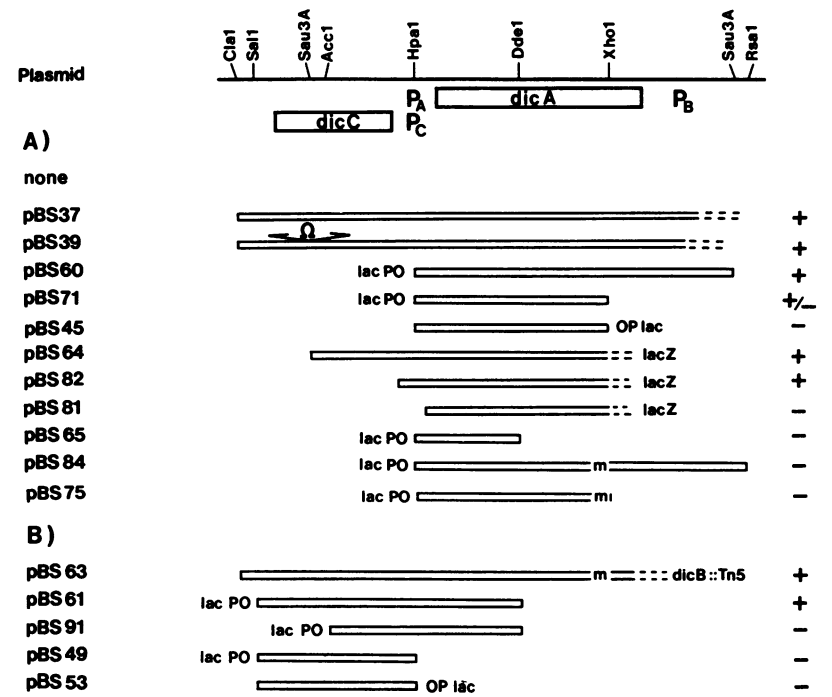

Figure 4. Plasmids used for complementation analysis. The top line indicates the position of restriction sites referred to in the text, and the main features of the sequence are drawn underneath. For each plasmid is indicated, from left to right: its designation, the vector used, the extend of the insert DNA (m indicates dicA1 DNA), the effect of the plasmid on temperature sensitivity (+: complementation, -: no complementation) in strain JS97 at 37°C. A and B: Plasmids used to demonstrate complementation by dicA and dicC respectively.

not used in these experiments because two fusion proteins of approximately 8 KD, under control from the lac promoter, were predicted from the sequence. Proteins synthesized by plasmid pKC20 are shown in figure 3, lane G. This plasmid also carries the Sal1-Dde1$_1$ fragment, but this time inserted in vector pGEM1 in which only insert-coded proteins can be detected in addition to beta-lactamase. Again, an 8.5 KD protein was detected, demonstrating the existence of the product of ORF269. Additional evidence for translation of the ORFs into proteins came from the construction of ORF353-lacZ (see below) and ORF269-lacZ (data not presented) gene fusions.

ORF353 lacks a recognizable Shine-Dalgarno sequence (30). On the other hand, six different possible promoters with scores ranging from 40 to 50 were found by a program written according to Mulligan et al (25) within the 72% AT-rich region

spanning 130 nucleotides before the translation start. In order to detect promoter activity in this region, a 618 base pairs long Sau3A fragment was excised from plasmid pBS44 and inserted at the BamH1 site of the promoter probe vector pMC1403 (21). According to the sequence of the Sau3A fragment, either one of the two fusion proteins were expected depending upon the insertion orientation. One of the colonies yielding a blue color on X-gal indicator plates carried the ORF353-lacZ fusion in plasmid pBS64, and was retained for further analysis. pBS64 was digested with endonuclease EcoR1 (upstream from the inserted $Sau3A_1$ site) and treated for limited exonucleolytic degradation with nuclease Bal31. The ends were repaired and ligated to the spectinomycin-resistance fragment Ω (31) cut by Sma1. Spectinomycin-resistant transformants were analyzed for beta-galactosidase activity and for the size of the remaining HindIII fragment extending from within the Ω fragment to the pBS44 linker. pBS64 (no deletion) and pBS80 (deletion down to 205 ± 10 bp) gave respectively 29 and 25 beta-galactosidase units. Plasmid pBS82 (deletion down to 275 ± 10 bp) gave 6.5 units while deletions to position 335 ± 10 bp or longer had less than 0.3 unit of beta-galactosidase. These results indicated the presence of a promoter beyond position 275 but did not distinguish between the presence of two promoters or of a single promoter subjected to positive control. Two possible promoters shown in Figure 2, TTGTTA - 16bp - TAGATG and ATGTTA - 16bp - TACGAT, gave a score of 42 according to the program of Mulligan et al, suggesting low (if any) promoter function. A third possibility, not shown in Figure 3, makes use of a -10 sequence TAGCAT (position 338-343) but lacks a properly spaced -35 sequence matching the consensus TTGACA. The region between 260 and 310 bp carries two inverted repeats of 7 and 18 bp respectively (Figure 2). The second of these repeats overlaps the promoter of ORF 354.

No rho-independent terminator appears to be present between the end of ORF353 and the promoter of the dicB region, located between bp 821 and bp 849 (to be published). However a potential rho-dependent terminator is suggested by the presence of an 11 bp inverted repeat (position 751-761 and 773-783).

Contrary to ORF353, ORF269 is preceeded by a sequence, AGGTG, matching the canonical Shine-Dalgarno sequence and centered at 9 bp from the ATG. A good candidate for promoter activity (-35 sequence: TTGACT, -10 sequence: TATCTT, spacing 17 bp, homology score 60) is present between positions 306 and 330 of the sequence, and a "-44 signal" TAAAT (32) is also found. This spacial arrangement suggests that ORF353 and ORF269 may be subjected, at least in part, to a common regulation.

DicA is the product of ORF353

Identification of DicA as the product of ORF353 rests on both sequencing and

genetic evidence. The mutant SalI - RsaI fragment was sequenced from derivatives of plasmid pBS63 cloned into phage M13mp11 (Figure 1). A single difference was found at position 674 of the sequence (Figure 2). The change was a C to T transition, in agreement with the mutagenic specificity of nitrosoguanidine used for dicA mutagenesis (33). This change leads to a replacement of a leucine by a phenylalanine at the 108th residue of the ORF353 protein. Sequencing therefore suggested that dicA is the gene coding for the 15.5 KD protein. However, since nitrosoguanidine favours the introduction of multiple linked mutations, genetic evidence was necessary to conclude that this mutation is necessary and sufficient to confer the dicA1 phenotype.

To analyze complementation by the 15.5 KD protein, plasmids carrying ORF353 under control of the lac promoter were constructed by inserting various fragments starting from the HpaI site in front of the lac promoter of plasmid pUC9. Their ability to complement mutation dicA1 was then tested in strain JS97 (Figure 4). Plasmid pBS60, which carries both ORF353 and the dicB promoter, complemented the mutation, while pBS84, which differs from pBS60 by the presence of the C-T transition, had no complementing activity at all. Using a standard streak test, plasmid pBS71, which lacks 21 aminoacids of ORF353, showed partial complementation since, contrary to JS97 which grows well at 27°C and poorly at 30°C, JS97/pBS71 grew normally at 34°C. At 37°C, however, growth was dependent on initial cell density. Complementation was entirely dependent on the presence of the upstream lac promoter, as shown by the results obtained with pBS45. The mutant counterpart of pBS71, pBS75, had no complementing effect. It is therefore clear that complementation is achieved by wild-type but not by mutated ORF353. From these data, we conclude that DicA is the product of ORF353.

Plasmid pBS65, which carries only 40% of the dicA coding sequence, did not complement the mutation. The dicA-lacZ fusion plasmid pBS64, on the other hand, complemented the mutation up to 42°C despite its probable lower copy number compared to the rom$^-$ plasmid pBS71 (34). Complementation by the dicA-lacZ fusion was abolished in plasmids deleted for dicA promoter activity.

A second dicA1-complementing gene, dicC

The results reported in the previous section, showing that the dicA gene product is inactive in the dicA1 mutant, were in apparent contradiction with the fact that plasmid pBS63 (dicA1) complements the mutation. Subcloning of the dicA region resolved this problem by showing that the product of ORF269 can also complement the dicA1 mutation.

In addition to pBS63, complementation was exhibited by pBS43 (Figure 3) which carries a $\gamma\delta$ insertion in gene dicA. To demonstrate complementation by

Table 1
Sequences of DicA and DicC homologous to known operator-binding domains. Sequences taken from Ref. 35 and those found in DicA and DicC are shown using the one-letter code and underlined at the most conserved positions.

Protein	← Helix 2 →←turn →← Helix3 →
Lambda Rep	Q-E-S-V-A-D-K-M--G-M-G--Q-S-G-V-G-A-L-F-N
Lambda cro	Q-T-K-T-A-K-D-L--G-V-Y--Q-S-A-I-N-K-A-I-H
Lambda cII	T-E-K-T-A-E-A-V--G-V-D--K-S-Q-I-S-R-W-K-R
CRP	R-Q-E-I-G-Q-I-V--G-C-S--R-E-T-V-G-R-I-L-K
LacR	L-Y-D-V-A-E-Y-A--G-V-S--Y-G-T-V-S-R-V-V-N
DicA	Q-R-S-L-A-K-A-L--K-I-S--H-V-S-V-S-Q-W-E-R
DicC	K-T-K-L-A-Q-A-A--G-I-R--L-A-S-L-Y-S-W-K-G

ORF269, we constructed pBS61, in which only the short, non-complementing fragment of pBS65 is left in addition to ORF269. This plasmid also complements the mutation. Removal of part of the ORF269 coding region (pBS 91) or of part of the promoter (pBS49) abolished complementation. From these results, we conclude that the product of ORF269 also complements the mutation, and for this reason we propose to name this gene dicC. The case of plasmid pBS53 (Figure 4) is peculiar and will be discussed later.

DicA and DicC have potential DNA recognition sequences

DicA and DicC are basic proteins with calculated isoelectric points of 10.2 and 9.8 respectively. To assess the possibility that dicA and dicC are actually repressors, we wrote a computer program that tests sliding blocks of 20 aminoacids. The program sums, within each block, the number of times each aminoacid is found at the same position in the compilation of DNA recognition sequences of Pabo and Sauer (35) (Tn3 resolvase was omitted). While sequences from the compilation gave scores between 89 and 141, scores above 75 were seldom found among non DNA-binding proteins. Two sequences with scores of 125 and 117 were detected in DicA and DicC respectively. They are indicated in Table 1 together with those of a few other DNA binding proteins. These sequences start at the 23rd and at the 6th aminoacid of DicA and DicC respectively and the most notable difference from consensus is the presence in DicA of Lys instead of Gly at the beginning of the turn between the putative alpha-helices.

DISCUSSION

The dicA1 mutant was discovered during the search of mutations residing in the region of termination of replication in Escherichia coli and conferring a conditional lethal division-less phenotype. In W1485 derivatives, dicA1 mutants stop dividing in broth above 31°C. Preliminary analysis had indicated that gene dicA is

not essential for cell division since the mutation could be suppressed either by transposon insertion in a nearby dicB locus or by deletion of the dicA-dicB sequences (1). We concluded that dicA1 encodes a temperature-sensitive repressor of the division inhibition gene dicB. We have now determined the nucleotide sequence of a 968 bp fragment that complements the mutation. This fragment codes for two proteins of 15.5 and 8.5 KD that can be detected after labelling of UV-irradiated recA cells carrying appropriate plasmids. Sequencing of dicA1 DNA showed that dicA is the gene coding for the 15.5 KD protein. This protein possesses a sequence closely related to that of other DNA-binding proteins and located, as in most repressors, at the N-terminal end of the protein. We have now measured the activity of Tn5-lac insertions in dicB (to be published) and confirmed directly the repressor function of DicA (and DicC). Since mutation dicA1 leads to an aminoacid replacement in the C-terminal part of the protein, it is likely to alter some other function than operator recognition. Interestingly, removal of the last 21 aminoacids abolished almost entirely DicA activity, but fusion of the remaining segment to beta-galactosidase restored it. The ability of repressor-beta-galactosidase fusions to retain or even restore efficient repression in vivo has already been reported (36, 37); the dicA-lacZ fusion of pBS64 may stabilize the tertiary structure of the C-terminal part of the molecule.

In addition to dicA, the gene coding for the 8.5 KD protein, dicC, can also complement the dicA1 mutation when produced from a multicopy plasmid. Plasmids deleted either for part of dicC coding sequence or for part of the dicC promoter failed to complement, but plasmid pBS53 (Figure 4), which has a complete dicC coding sequence in front of the lac promoter failed to complement dicA1, even in the presence of 10^{-3} M IPTG. The inverted repeat (position 274-313 of the sequence) may promote a stable secondary structure of the mRNA (calculated free energy: -9.8 Kcal/mol) acting as a transcription terminator and/or impeding access to the Shine-Dalgarno sequence (position 276-281) of dicC. Further analysis will be necessary to clarify this point.

DicC, like DicA, contains in its N-terminal part a sequence related to that of other DNA-binding proteins. The reason why the chromosomal copy of DicC does not repress dicB in the dicA1 mutant is not fully understood. That dicC is under negative control by dicA is suggested by the fact that the DicC protein is detected in maxicell experiments only when the plasmid does not carry gene dicA. However, since labelling of proteins in maxicells was carried out at 37°C, this posibility would contradict the original hypothesis (1) stating that dicA1 codes for a temperature-sensitive repressor. In order to know the exact defect in the dicA1 mutant, we are now trying to determine how dicA, dicB and dicC are regulated by the wild type and

mutant DicA protein. Our preliminary results indicate that regulation by dicAC region somewhat resembles that exhibited by the repressors of the immunity region of lambdoïd phages.

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