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Minicell-Forming Mutants of *Escherichia coli*: Suppression of Both DicB- and MinD-Dependent Division Inhibition by Inactivation of the *minC* Gene Product

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We have determined the nucleotide sequence of the *minB* operon of 10 *min* mutants of *Escherichia coli*, characterized by impaired inhibition of polar divisions. These mutants were either sensitive or resistant to the division inhibitor DicB. All the mutations were found to lie in *minC* or *minD*, confirming the requirement of both gene products in the process of inhibition of polar sites. Mutations conferring resistance to inhibitor DicB were found exclusively in *minC*. In agreement with de Boer et al. (P. A. J. de Boer, R. E. Crossley, and L. I. Rothfield, Proc. Natl. Acad. Sci. USA 87:1129–1133, 1990), these results provide evidence that, in addition to promoting division inhibition with MinD, protein MinC acts in concert with DicB to inhibit division by a second, MinD-independent process.

The initiation of septation requires proper control of its frequency, its timing relative to other events of the cell cycle, and the location of the septum inside the cell (see reference 9 for a review). The isolation by Adler et al. (1) of a mutant of *Escherichia coli* producing DNA-less cells as by-products of polar divisions indicated that the cell poles conserve division sites with the capacity for septation and that some cellular functions are normally involved in inhibiting these sites while keeping normally used division sites unaffected (17). The single mutation carried by their strain, *min-1*, is within a locus, *minB*, located at 26 min on the standard genetic map (4, 16). The wild-type *minB* locus was cloned and found to correct the *min-1* defect when provided by a low-copy-number plasmid (6). A detailed analysis of *minB* indicated that it consists of an operon coding for three proteins, MinC, MinD, and MinE. From a study of the effects of different combinations of *min* genes on division, de Boer et al. (7) concluded that MinC and MinD are required together to inactivate division nonspecifically, while MinE appears to relieve this inhibition at mid-cell sites (or channels it to the polar sites).

Other evidence also points towards a role of the *minB* gene products in inhibition of septation. In the course of a search for mutants resistant to DicB, a division inhibitor coded for by the *dicB* operon (2), some of the mutations obtained mapped at, or very near to, the *minB* locus (14). de Boer et al. (8) found that among the *minB* genes, *minC* was the only gene whole activity is essential for the action of inhibitor DicB. In this article, we have determined the nucleotide sequence of a number of *minB* mutations, defined as yielding a minicell-forming phenotype, some conferring resistance to DicB and others not. Our data provide an independent support for the above models.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains GC7240 (*min-1*), GC7245 (*min-2*), GC7246 (*min-3*), GC7247 (*min-4*) (12), and GC7277 (*min-5*) are derivatives of GC7237 (*met hsdR gal supE sfiA85 sfiC*). To test their resistance to DicB, these strains were transformed by plasmids pAM1 (*lacI*^{q}) and pKC17 (*lacP-dicB*) (2), and transformants were tested for sensitivity to DicB produced after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Strains JS427 (*min-16*), JS429 (*min-17*), JS430 (*min-18*), JS505 (*min-19*), and JS428 (*min-20*) are derivatives of JS219 [*araD* Δ(*ara-leu*) *galU galK supE* Δ(*lac*)*X74 hsdR rpsL malPp*::*lacI*^{q}] (2). The mutations were introduced into JS219 by cotransduction of the original mutations with *fadR*::Tn*10* (14). Strain JS513 carries the *min-5* allele of GC7277, introduced by cotransduction with *zcf117*::Tn*10* into JS219. These derivatives of JS219 also carry plasmid pCL11. Plasmid pCL11 is a derivative of pBR322 in which a 540-base-pair (bp) *Pvu*II fragment, containing the *lacPo-dicB* fusion of pKC17 (2), was cloned at the *Eco*RV site in the orientation opposite to that of the *tet* gene.

Cloning of mutant alleles. (i) Method 1. Plasmid pHGB2, a derivative of vector pGB2 (3) temperature-sensitive for replication, was constructed by substitution of the wild-type *repA* by a mutant gene (11). This plasmid expresses gene *aadA*, which specifies resistance to streptomycin and spectinomycin. Fragments of the *minB* operon, containing the complete 3′ region but varying at their 5′ end and lacking the P1 promoter (7), were cloned into pHGB2. Derivatives of strain JS219(pCL11) carrying various DicB-resistant *min* mutations were transformed with these plasmids, and spectinomycin-resistant colonies were selected at 42°C. Some of these colonies had regained sensitivity to DicB (expressed from plasmid pCL11 in the presence of IPTG), indicating a reconstitution of the wild-type operon after recombination of the plasmid into the chromosome. The promoterless, mutant version of the integrated operon was recovered (see Results) together with pHGB2 *ori* and *aadA* by appropriate digestion of the chromosomal DNA and religation.

(ii) Method 2. Total DNA from mutant strains (3 to 5 μg) was digested by *Eco*RI and fractionated on an agarose gel, and the region of the gel containing the *minB* operon was excised. The regions to excise from the different lanes were determined from a preliminary Southern blot analysis (not shown) indicating that *minB* is within a 9-kilobase (kb)

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*Eco*RI fragment in all strains except GC7246, where it is carried by a 15-kb fragment. Purified DNA was further digested by *Pvu*II and fractionated on a gel, and the DNA of approximately 4.8 kb (5, 13) was extracted. The final material (approximately 5 ng) was inserted between the *Eco*RI and *Hin*cII sites of plasmid pUC19 or pGB2 and used to transform strain DH5α (*lacZΔM15*). Alleles *min-3*, *min-5*, *min-16*, *min-17*, *min-19*, and *min-20* (all DicB resistant) and *min-2* were cloned into pUC19, while DicB-sensitive alleles *min-1* and *min-4* were found in pGB2 only. In all cases, the plasmids conferred a mixed filamentation and minicell formation phenotype which facilitated colony screening.

DNA sequencing. The sequence of the *minB* operon of the mutants was established in the 5′ to 3′ direction only, with ClCs-purified plasmid DNA and five 17-mer primers covering intervals 153 to 169, 548 to 563, 945 to 962, 1345 to 1362, and 1745 to 1762 of the *minB* sequence (7). The location of the primers on the sequence is given in Fig. 1.

Measurement of polar divisions. To estimate the percentage of polar divisions, cultures growing exponentially in L broth at 26 or at 37°C were concentrated 10 to 100 times by centrifugation and resuspension at 45°C in medium containing 0.5% agarose. One microliter of this suspension was spread under pressure between a slide and a cover slip, and septa (either internal or polar) were scored by using a phase-contrast Leitz Ortholux II microscope at 1,200× magnification. A total of 210 to 250 septa were scored for each measurement.

RESULTS

Cloning of *minB* mutant alleles. Two different methods were used to clone the *minB* mutant operons. The first made use of the same principles as recently described by Hamilton et al. (10) for generating gene replacements and deletions, by use of homologous recombination of a derivative of pSC101 temperature sensitive for replication. Nevertheless, of the plasmids recovered from seven different mutants, only one carried a mutation (*minC18*), as deduced from sequence determination. This unexpected result may be due to some growth inhibition by spectinomycin in cells carrying the integrated plasmid, favoring integration of oligomers of the plasmid carrying the wild-type sequence. All the other mutations were cloned by the method of Nicholls *et al.* (15) after double fractionation on agarose gels of fragments containing the *minB* operon. Mutations *min-1* and *min-4* could only be cloned into a low-copy-number plasmid and conferred a mixed filamentation-polar division phenotype on their *min*$^+$ host. The other mutations conferred the same phenotype when cloned into high-copy-number vectors. The appearance of minicells due to these *minE*$^+$ (see below) plasmids agrees with the observations of de Boer et al. (7) that *minE* overexpression by plasmids (with or without *minC*$^+$ and/or *minD*$^+$) leads to minicell formation in strains having the *minB*$^+$ chromosomal locus.

Sequence of the *minB* mutations. The sequence of the *minB* mutations was established by use of five oligonucleotide primers permitting sequencing of the entire operon with the exception of the P1 promoter region (7). The results of this analysis are shown in Fig. 1. Mutations *min-16* to *min-20* belong to the class of DicB-resistant mutations, called *minB*^{sup}, which mapped in or very close to the *minB* operon (14). The sequence of these mutations indicates that they all reside in gene *minC*. The two other *minB* mutants independently isolated and subsequently found to be DicB-resistant, *min-3* (12, 14) and *min-5* (isolated by A. Jaffé), also have mutations in *minC*. The nonsense mutations they carry are not suppressed in the *supE* background of the strains used here.

Mutants carrying alleles *min-1* (1), *min-2*, and *min-4* (12) were fully sensitive to division inhibition by DicB (14). In contrast to the *minB*^{sup} mutants, sequencing indicates that their mutations lay in *minD*. Mutations *min-1* (*minD1*) and *min-4* were identical. Mutant GC7245 carried two mutations, one being in *minD* (*minD2*) and the second in an inverted repeat sequence at the end of the operon which has been proposed to act as a rho-independent terminator (7).

With the exception of JS429 and GC7245, the strains studied yielded between 22 and 30% polar divisions when examined for the minicell phenotype. These figures are comparable to those reported previously (12, 14). Since mutations *minC3*, *minC5*, and *minC16* specified incomplete proteins, we took ca. 30% as indicative of a complete defect in the control of polar divisions. Strain GC7245 *minD2 mint2* yielded approximately 3% polar divisions, in agreement with previous measurements (12). Strain JS429 (*minC17*) yielded only 10% (22 of 212) and 13% (30 of 226) polar divisions at 26 and 37°C, respectively. It should be pointed out that *minC17* is the only *minC* mutant allele that did not confer complete resistance to DicB.

DISCUSSION

We have determined the location of the mutations in 10 different *minB* mutants, including the original *min-1* (*minD1*) mutation of Adler et al. (1). Our results indicate that all the mutations leading to the formation of polar divisions are located either in *minC* or in *minD*. The results of de Boer et al. (7) indicated that proteins MinC and MinD must be present together to inhibit division at polar and at nonpolar division sites, while moderate amounts of MinE relieve this inhibition specifically at nonpolar sites. This model, which predicts that mutations inactivating either MinC or MinD should result in a lack of division inhibition at the poles and in minicell formation, is supported by our results.

While none of the *minD* mutations confer resistance to DicB division inhibition, the seven *minC* mutations examined lead to such resistance. This result suggests that DicB and MinC proteins cooperate to inhibit division and that this process is indifferent to the state of MinD activity. This conclusion agrees with that drawn by de Boer et al. (8) from the effects of different combinations of gene expression in a strain carrying a deletion of the wild-type *minB* operon. Thus, MinC appears to be a common coinhibitor of DicB- and of MinD-mediated division inhibition.

The simplest hypothesis would be that DicB or MinD interacts with MinC to turn it into an active division inhibitor. However, DicB and MinD have little resemblance: DicB is a small (7 kilodalton) basic protein (pI 9.7), while MinD is 30 kilodaltons and acidic (pI 5). The only sequence similarities found, shown in Fig. 2, are poor, and they do not encompass the regions where *minD* mutations have been obtained (Fig. 1). All the *minC*, DicB-resistant mutants examined in this study produced minicells, indicating that they are also insensitive to MinD-dependent division inhibition. Nevertheless, this cannot be taken as any indication of a common interaction. First, three *minC* mutations are nonsense mutations leading necessarily to both phenotypes. Second, all the remaining mutations (*minC17*, *minC18*, *minC19*, and *minC20*) were chosen among DicB-resistant mutants for their minicell formation phenotype (14). A direct examination of the ability of the different peptides to interact

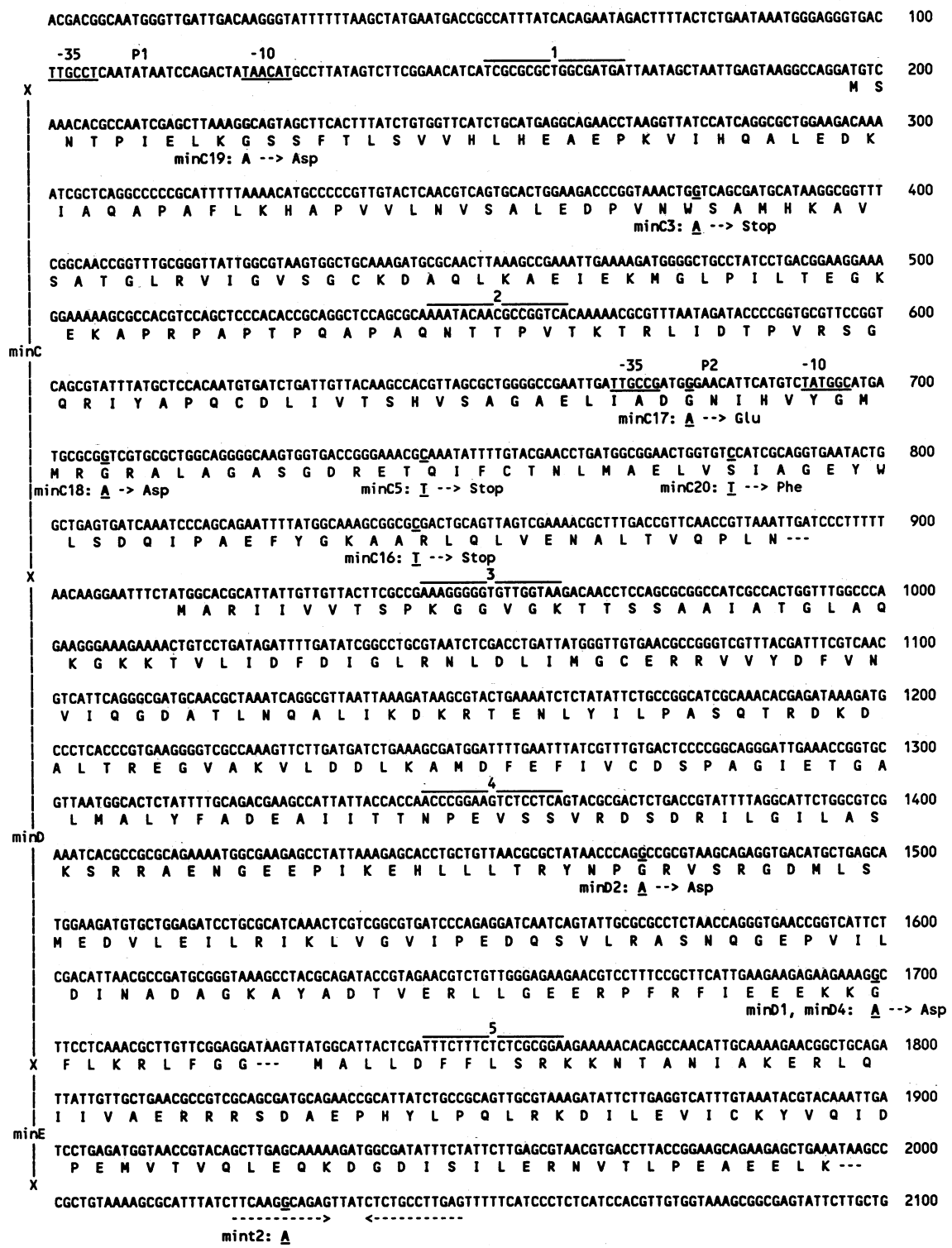

FIG. 1. Location of *minB* mutations. The sequence of the *minB* operon and the translation of the genes (in the one-letter code) are represented. The −35 and −10 sequences of promoters P1 and P2 and the mutated bases are underlined. The inverted arrows indicate the stem of putative terminator T1 (7). The sequences of the oligonucleotide primers, numbered from 1 to 5, are indicated by overlines.

physically will show whether MinC activity as a division inhibitor rests on a common mechanism of protein-protein interaction with MinD or DicB. Alternatively, the isolation of *minC* mutants exhibiting resistance to either of the coactivators (i.e., showing only resistance to DicB or only a minicell-forming phenotype) would be in favor of a direct interaction. We are currently attempting to isolate such mutants.

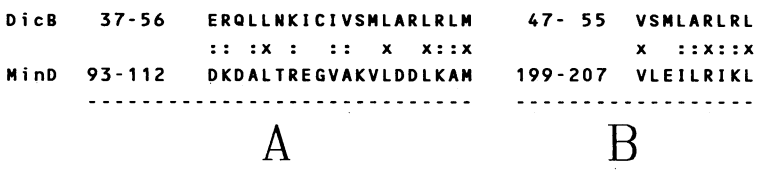

FIG. 2. Similarities between the sequences of proteins DicB (62 amino acids) and MinD (270 amino acids). A and B indicate the best matches found by aligning 10-amino-acid overlapping windows by use of the Dayhoff MDM-78 matrix (5). Note that these matches are mutually exclusive. Crosses and double dots indicate identical and related amino acids, respectively.

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