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Molecular Cloning and Expression of *hipA*, a Gene of *Escherichia coli* K-12 That Affects Frequency of Persistence after Inhibition of Murein Synthesis

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The *hipA* gene at 33.8 min on the *Escherichia coli* chromosome controls the frequency of persistence upon inhibition of murein synthesis; for strains bearing *hipA*$^+$ the frequency is 10^{-6}, and for *hipA*$^-$ strains the frequency is 10^{-2}. *hip*$^+$ has been cloned by selection for a kanamycin resistance determinant at 33.9 min. *hipA*$^+$ is dominant over *hipA*$^-$ in both *recA*$^+$ and *recA*$^-$ backgrounds. The smallest DNA insert which contains *hipA*$^+$, as determined by the ability of the plasmids to complement *hipA*$^-$ strains, is 1,885 base pairs. Both orientations of *hipA*$^+$ are obtained when the cloning site of vector is remote from strong promoters; both orientations complement *hipA*$^-$, and both encode a unique peptide of 50,000 M_r. The probable direction of transcription has been deduced from the pattern of peptides encoded by plasmids from which either end of the insert and adjacent vector sequences have been deleted. This information and the recovery of only one orientation of *hipA*$^+$ when the cloning site is close to a strong promoter suggest that a high level of expression of the gene is not tolerated by *E. coli*.

The frequency of persistence during prolonged inhibition of murein synthesis has been shown to be under the control of a gene, *hipA*, located at min 33.8 of the *Escherichia coli* chromosome (20). This places *hipA* in the recently bridged transduction gap (4, 11) of the chromosome in which only a few functions have been mapped (5, 6). The *hipA* locus is remote from other loci affecting murein metabolism (2). This unique position and the phenotypes for which *hipA*$^+$ and its mutant alleles, *hipA7* and *hipA9*, are responsible define *hipA* as a newly recognized gene (20).

The normal function of *hipA* and the regulation of its expression are potential keys to understanding the lethality of impaired murein synthesis and the mechanism which permits small fractions of genetically homogeneous populations to survive that lethality. The lethal consequence of impaired murein synthesis is of special interest because of its strong temporal linkage to events of the division cycle (13). This study describes the further genetic analysis of *hipA*, which addresses the following questions. Is *hipA*$^+$ a regulatory region or does it encode a protein? Which genotype, *hipA*$^+$ or *hipA*$^-$, is dominant? Can *E. coli* tolerate multiple copies of *hipA*? If so, do multiple copies enhance the Hip phenotype?

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains used in this study and their construction are described in Table 1. The plasmids used as vectors are pACYC177 and pACYC184 (8). The complex medium contained, per liter, 10 g of tryptone, 5 g of yeast extract, and 8 g of NaCl. For plates, 15 g of agar was added. The medium was supplemented with 30 mg of diaminopimelic acid (DAP) per liter as indicated.

Antibiotics, when used, were added in the following amounts per liter: 100 mg of ampicillin, 25 mg of chloramphenicol, 75 mg of kanamycin sulfate, and 20 mg of tetracycline hydrochloride.

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Tests for Hip. The Hip phenotype cannot be selected directly, but can be scored by either of two previously published methods (20). Colonies less than 24 h old were picked with flat toothpicks and applied to plates in streaks of about 1 cm in length. The media either contained ampicillin or, in the case of *dapA* strains, omitted DAP. After 24 h of incubation the plates were sprayed with penicillinase or DAP and incubated for an additional 12 to 24 h. There is, at least, a 1,000-fold difference between the death of Hip$^+$ and Hip$^-$ strains upon inhibition of murein synthesis; thus, streaks of Hip$^+$ strains contained fewer than five and most often no colonies, whereas streaks of Hip$^-$ strains were confluent. This assay for Hip, the screening assay, was used to monitor cloning and subcloning of *hipA*.

Quantitative evaluation of the Hip character was made by determining the surviving fraction of CFU after intervals of inhibition of murein synthesis. Appropriate dilutions of cultures were plated on medium containing ampicillin or, in the case of *dapA* strains, medium lacking DAP. After designated intervals the plates were sprayed with either penicillinase or DAP and incubated for an additional 12 to 24 h, and the numbers of CFU were recorded. Determinations of the Hip character of plasmid-bearing strains were made on media containing an antibiotic for which the plasmid encodes a resistance determinant.

Genetic analysis. Standard methods were employed for transduction (18) and transformation (17).

Preparation, digestion, and gel electrophoresis of DNA. Chromosomal DNA from strain HM25 was prepared by the method of Berman et al. (3). Plasmid DNA was prepared by the method of Humphreys et al. (15). Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Bethesda Research Laboratories or from Boehringer Mannheim and used according to the manufacturer's recommendations. Restriction digests were fractionated by sucrose gradient centrifugation or by electrophoresis in agarose (12) or polyacrylamide (19) slab gels.

Plasmid copy number. Plasmid copy number was deter-

TABLE 1. *E. coli* K-12 strains

Strain	Relevant genotype	Source or reference
AT984	*hipA*$^+$ *dapA*	(7)
JC10240	*srlC300*::Tn*10 recA56*	(10)
HM6	*hipA*$^+$	(20)
HM7	*hipA7*	(20)
HM9	*hipA9*	(20)
HM21	*hipA*$^+$ *dapA zde-264*::Tn*10*	(20)
HM22	*hipA7 dapA zde-264*::Tn*10*	(20)
HM23	*hipA9 dapA zde-264*::Tn*10*	(20)
HM24	*hipA*$^+$ *dapA zdd-262*::IS*10* Cam^r IS*10 zdd-263*::Tn*5 zde-264*::Tn*10*	(20)
HM25	*hipA*$^+$ *dapA zdd-262*::IS*10* Cam^r IS*10 zdd-263*::Tn*5*	P1 · HM24 → AT984
HM222	*hipA7 zdd-262*::IS*10* Cam^r IS*10*	P1 · HM24 → HM7
HM223	*hipA9 zdd-262*::IS*10* Cam^r IS*10*	P1 · HM24 → HM9
HM70	*hipA*$^+$ *dapA zdd-262*::IS*10* Cam^r IS*10*	P1 · HM222 → AT984
HM72	*hipA7 dapA zdd-262*::IS*10* Cam^r IS*10*	P1 · HM222 → AT984
HM73	*hipA9 dapA zdd-262*::IS*10* Cam^r IS*10*	P1 · HM223 → AT984
HM701	*hipA*$^+$ *dapA srlC300*::Tn*10* Cam^r IS*10*	P1 · JC10240 → HM70
HM721	*hipA7 dapA srlC300*::Tn*10* Cam^r IS*10*	P1 · JC10240 → HM72
HM731	*hipA9 dapA srlC300*::Tn*10* Cam^r IS*10*	P1 · JC10240 → HM73

mined by the hybridization procedure described by Adams and Hatfield (1).

DNA-directed translation. The methods of Zubay (21) and Collins (9) were used to detect synthesis in vitro of the protein product of *hipA*$^+$ cloned into plasmids. The extracts, substrates, and reagents were purchased in kit form from Amersham. Electrophoresis employed the method of Laemmli (16).

RESULTS

Cloning and subcloning of *hipA*$^+$. The construction of the hybrid plasmids used in this study and their important restriction sites are summarized in Fig. 1. Chromosomal DNA was isolated from strain HM25 (Table 1), in which Tn*5* maps within 0.1 min or about 4 kilobases from *hipA*$^+$. This small distance made it reasonable to expect that both *hipA*$^+$ and the kanamycin resistance determinant of Tn*5* could be obtained on a fragment of suitable length for cloning into the standard vectors. Chromosomal DNA was partially digested with *Sau*3A, and the digest was fractionated by sucrose density gradient centrifugation. DNA was purified from the fraction in which the median fragment length was 15 to 20 kilobases and ligated into the *Bam*HI site within the tetracycline resistance determinant of pACYC184. The vector had been previously digested with *Bam*HI and alkaline phosphatase. The ligation mixture was used to transform strain JA221. Kanamycin resistant, tetracycline-sensitive transformants of strain JA221 were examined by a screening procedure (14) for plasmids of the expected size range. One plasmid of about 25 kilobases pHM4, transformed both strains HM22 and HM23 (Table 1) to kanamycin resistance and changed the Hip$^-$ phenotypes of these strains to Hip$^+$.

The *hipA*$^+$-containing fragment was reduced from approximately 20 kilobases to a 1,885-base-pair (bp), *Bam*HI-*Bgl*II fragment by the following steps. A fragment containing part of Tn*5* and adjacent chromosomal sequences was removed from pHM4 by digestion with *Sal*I and religation to yield pHM41. A *Bam*HI fragment from pHM41 was ligated into the *Bam*HI site of pACYC 184, producing pHM418. A *Bam*HI-*Bgl*II fragment of pHM418 was ligated into the *Bam*HI sites of pACYC177 and pACYC184. Only one orientation, pHM419, could be obtained with pACYC184 as the vector. Both orientations, pHM519 and pHM520, were obtained with pACYC177.

In further attempts to reduce the size of the insert, pHM519 was digested with *Hin*dIII and religated, producing pHM519d; pHM520 was digested with *Cla*I and religated, producing pHM520d. In the case of pHM519d, approximately 690 bp of the insert was removed along with part of the vector, including the terminus of the kanamycin resistance determinant. In the case of pHM520d, removal of the *Cla*I fragment from pHM520 resulted in the loss of approximately 535 bp from the other end of the insert and, again, part of the vector, including an even larger portion of the terminus of the kanamycin resistance determinant. Neither pHM519d nor pHM520d was able to complement *hipA*$^-$ strains.

The inference that pHM4 and the plasmids derived from it contain all or part of *hipA*$^+$ rather than an extragenic suppressor of both *hipA7* and *hipA9* is based on the close

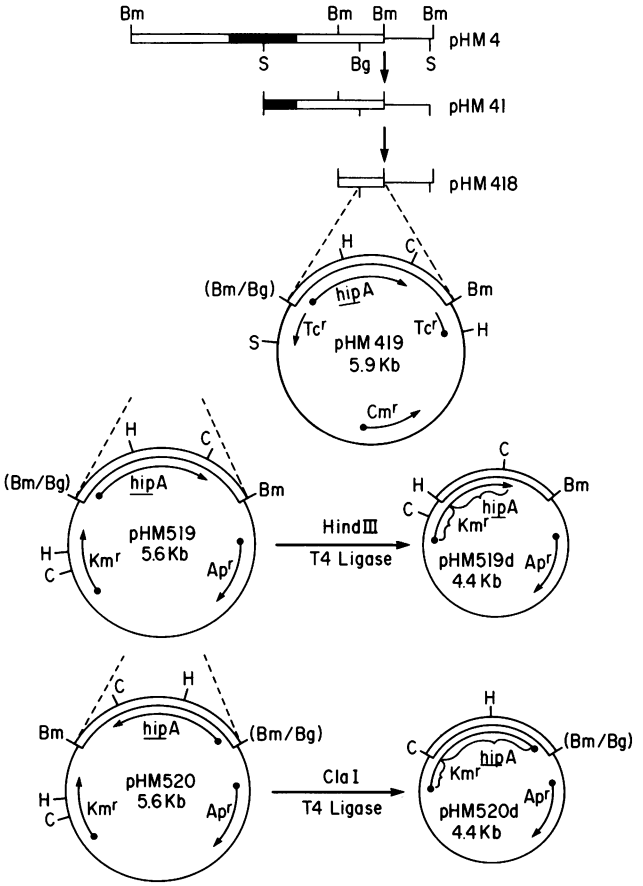

FIG. 1. Cloning and subcloning of *hipA*$^+$. The open boxes represent chromosomal DNA, and the solid boxes indicate Tn*5*. Vector DNA, represented by the lines, was from pACYC184 (pHM4, pHM41, pHM418, and pHM419) or from pACYC177 (pHM519, pHM519d, pHM520, and pHM520d). Abbreviations: Bg, *Bgl*II; Bm, *Bam*HI; C, *Cla*I; H, *Hin*dIII; and S, *Sal*I.

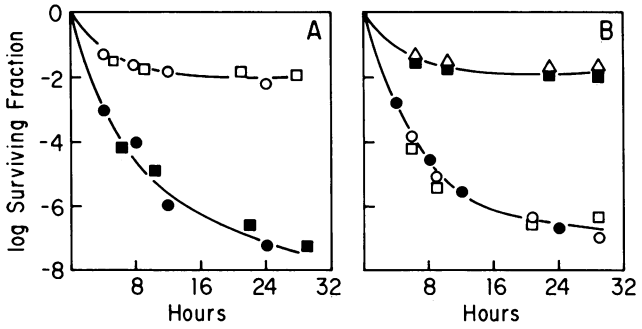

FIG. 2. Complementation of *hipA7*. The log of the surviving fraction equals log CFU after the designated interval of starvation for DAP minus log CFU without starvation. (A) Effects of vectors: ●, HM21(pACYC177)+ampicillin; ○, HM22(pACYC177)+ampicillin; ■, HM21(pACYC184)+chloramphenicol; □, HM22(pACYC184)+chloramphenicol. (B) Effects of hybrid plasmids: ●, HM22(pHM4)+chloramphenicol; ○, HM22(pHM519)+ampicillin; □, HM22(pHM520)+ampicillin; ■, HM22(pHM519d)+ampicillin; △, HM22(pHM520d)+ampicillin.

agreement of the physical (Fig. 1) and genetic (19) mapping of *hipA* with respect to *zdd-263*::Tn*5*. This inference is confirmed by the observations that a *recA*$^+$ *hipA7* strain, HM22, bearing pHM520d exhibited a high rate of reversion (10^{-2}) to the Hip$^+$ phenotype, whereas the reversion rate was less than 10^{-4} with either the vector alone or pHM519d and in a *recA*$^-$ *hipA7* strain (HM721), bearing pHM520d. The reversion rate of HM23 *recA*$^+$ *hipA9* was also increased to 10^{-2} by pHM520d.

These observations on the effects of pHM520d on reversion also establish the loci of *hipA7* and *hipA9* to be in the proximal portion of the gene.

Complementation of *hipA*$^-$ by *hipA*$^+$ plasmids. A quantitative evaluation was made of the Hip phenotype of plasmid-bearing strains. The issues were as follows: do any of the *hipA*$^+$ plasmids produce an enhanced Hip$^+$ phenotype, and, second, is either pHM519d or pHM520d, which were found to be noncomplementing using the screening method for Hip, capable of partial complementation as would be detectable in the more precise quantitative test? The results of quantitative evaluation (Fig. 2) revealed nearly identical phenotypes for a *hipA*$^-$ strain bearing either pHM4, pHM519, or pHM520 and a *hipA*$^+$ strain bearing the corresponding vector. Neither pHM519d nor pHM520d could be distinguished from the vector itself and, therefore, showed, no evidence of even partial complementation.

Complementation of *hipA*$^-$ in a *recA*$^-$ background. To assess whether recombination rather than complementation might account for the apparent dominance of *hipA*$^+$ over *hipA*$^-$, the effect of *hipA*$^+$ plasmids was examined in *hipA*$^-$ *recA*$^-$ backgrounds. Complementation of both mutant alleles of *hipA*$^-$ by *hipA*$^+$ cloned in either orientation was *recA* independent (Table 2).

TABLE 2. Hip phenotypes of *hipA recA* strains bearing *hipA*$^+$ plasmids

Strain	Genotype	Plasmid	Hip phenotype
HM721	*hipA7 recA*	pACYC177	−
HM731	*hipA9 recA*	pACYC177	−
HM721	*hipA7 recA*	pHM519	+
HM731	*hipA9 recA*	pHM519	+
HM721	*hipA7 recA*	pHM520	+
HM731	*hipA9 recA*	pHM520	+

TABLE 3. Copy numbers of *hipA*$^+$ plasmids and corresponding deletions

Bacterial strain	Plasmid	Copies per cell
HM22	pHM519	4
HM22	pHM520	5
HM22	pHM519d	2
HM22	pHM520d	3
HM22	pACYC177	14
JA221	pHM519	3
JA221	pHM520	5

Copy number of *hipA*$^+$ plasmids. Insertion of chromosomal DNA into the vector pACYC177 reduced the copy number by a little more than half as compared with pHM519 and pHM520; removal of a portion of the vector as in pHM519d and pHM520d caused a further reduction in copy number to 2 and 3, respectively (Table 3). The latter reduction is an unlikely explanation for the failure of these plasmids to cause even partial complementation of *hipA*$^-$.

Expression of *hipA*$^+$ in vitro. Figure 3 is an autoradiograph of DNA-dependent translation reactions comparing the template activity of the vector, pACYC177, with that of the *hipA*$^+$ plasmids pHM519 and pHM520 and the deleted plasmids pHM519d and pHM520d. A unique protein was encoded by both *hipA*$^+$ plasmids. pHM519d, which was constructed by deleting portions of both the insert and the vector, encoded a protein larger in size than that encoded by the *hipA*$^+$ plasmids; the vector-determined protein corresponding in size to the kanamycin resistance protein was not produced. pHM520d, which was constructed by deleting part of the opposite end of the insert and a somewhat larger segment from the same end of the vector, encoded a protein smaller in size than that encoded by the *hipA*$^+$ plasmids; again, the vector-encoded protein for kanamycin resistance was not produced.

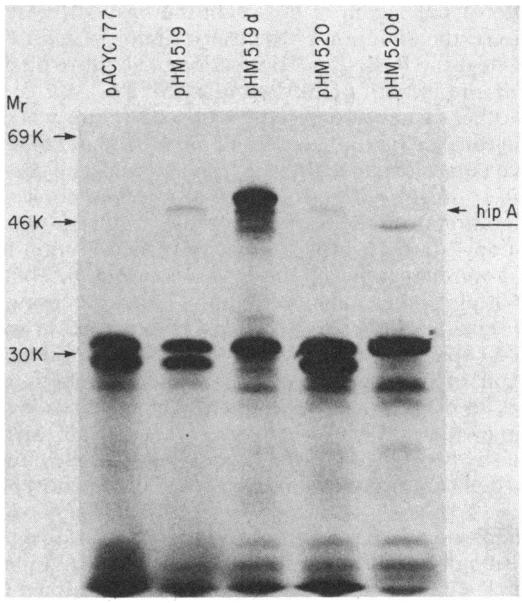

FIG. 3. In vitro protein synthesis directed by plasmids containing *hipA*$^+$.

DISCUSSION

The introduction of easily selected markers in close proximity to *hipA*$^+$ was essential for mapping this gene as none of the alleles currently available expresses a phenotype which can be selected directly (20). One of these nearby markers, kanamycin resistance, has also made it feasible to clone *hipA*$^+$ (Fig. 1). The *Bgl*II-*Bam*HI fragment from pHM418 is the smallest fragment still containing functional *hipA*$^+$. It is 1,885 bp in length. Only a single orientation of this fragment, as in pHM419, was obtained by cloning into pACYC184; both orientations, pHM519 and pHM520, were obtained with pACYC177 as the vector (Fig. 1). Because *hipA*$^+$ is dominant over both of its mutant alleles in *recA*$^-$ as well as *recA*$^+$ backgrounds (Table 2), it is probable that *hipA*$^+$ as cloned contains a coding region which is expressed. Because *hipA*$^+$ is functional in both orientations (Table 2), it is likely that the promoter as well as the coding region has been cloned.

The preceding conclusions were substantiated by observations using an in vitro coupled transcription-translation system. Plasmids containing *hipA*$^+$ in either orientation, pHM519 and pHM520, were templates for a unique protein of 50,000 M_r (Fig. 3). Assuming an average M_r of 110 per amino acid residue, the coding region of *hipA*$^+$ is calculated to be approximately 1,365 bp. The promoter, other regulatory regions, and unrelated DNA would account for the remainder of the insert, about 520 bp. Removal of a fragment of similar length, 535 bp, from one end of the insert as in pHM520d, or removal of 690 bp from the other end as in pHM519d (Fig. 1), rendered the plasmids incapable of complementing mutant strains containing *hipA*$^-$ (Fig. 2). Plasmid pHM519d produced substantial amounts of a peptide larger than any seen with the vector, pHM519, or pHM520; pHM520d produced a unique peptide of 47,000 M_r (Fig. 3). These observations suggest that the peptide produced by pHM520d is an inactive product of a *hipA* gene from which a small portion of the promoter distal end has been removed. The larger protein made with pHM519d as a template is probably encoded by a fusion of the promoter-proximal region of the kanamycin resistance determinant and the promoter-distal region of *hipA*. On the basis of these interpretations, the direction of the transcription of *hipA* (Fig. 1) is from near the *Bgl*II-*Bam*HI junction to about 90 bp beyond the *Cla*I site in both pHM519 and pHM520.

The other issues addressed by this study are whether *E. coli* can tolerate multiple copies of *hipA*$^+$ and, if so, whether multiple copies enhance the Hip$^+$ phenotype. The possibility that *hipA*$^+$ might not be tolerated in multiple copies was a consideration from the start of this study. Since *hipA*$^+$ in the single-copy state governs frequency of death upon impairment of murein synthesis, there was reason to be concerned that multiple copies might initiate the lethal process without such a signal. Another possibility of lesser concern was that multiple copies might increase the frequency of death after inhibition of cell wall synthesis. The fact that it proved possible to clone *hipA*$^+$ in multicopy plasmids relieved the most immediate of these concerns. *E. coli* can tolerate as many as five copies of *hipA*$^+$ (Table 3), and the elevated gene dosage does not produce an enhanced Hip$^+$ phenotype (Fig. 2).

An additional issue, whether *E. coli* can tolerate a level of expression of *hipA*$^+$ greater than that determined by its own promoter, emerges from the following observations. Only a single orientation of *hipA*$^+$ could be obtained when insertion was near a strong promoter, as in the tetracycline resistance determinant of pACYC184; that orientation was in opposition to the tetracycline resistance promoter. The orientation in the same direction as the tetracycline resistance promoter might well have resulted in high-level expression of *hipA*$^+$ which if not tolerated by *E. coli*, would account for recovery of only the opposite orientation. Both orientations of *hipA*$^+$ were obtained when the insertion was relatively remote from other promoters, as in pACYC177 (Fig. 1). This issue involves the regulation of *hipA*$^+$ expression, a subject which is being examined through an analysis of the structure of the gene, particularly its sequence and the identification of its promoter and termination regions. Elucidation of the physiological signals which regulate expression of *hipA*$^+$ will provide valuable clues as to its function and biochemical mechanism.

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