JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 768–775
0021-9193/83/080768-08$02.00/0

Vol. 155, No. 2

hipA, a Newly Recognized Gene of *Escherichia coli* K-12 That Affects Frequency of Persistence After Inhibition of Murein Synthesis

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Received 21 March 1983/Accepted 24 May 1983

Except for a small fraction of persisters, 10^{-6} to 10^{-5}, *Escherichia coli* K-12 is killed by prolonged inhibition of murein synthesis. The progeny of persisters are neither more resistant to inhibition of murein synthesis nor more likely to persist than normal cells. Mutants have been isolated in which a larger fraction, 10^{-2}, persists. The persistent response of the mutants, Hip (high persistence), is to inhibition of murein synthesis at early or late steps by antibiotics (phosphomycin, cycloserine, and ampicillin) or by metabolic block (starvation for diaminopimelic acid). Killing of the parent strain by each of the four inhibitors has two phases: The first is rapid and lasts about 30 min; the second is slower, but still substantial, and lasts 3 to 4 h. The first phase also occurs in the Hip mutants, but then viability of the mutants remains constant after about 30 min. Neither tolerance, resistance, impaired growth, nor reversion of spheroplasts accounts for high-frequency persistence. Two of the mutations map at 33.8 min in a region containing few other recognized functions. This position and the phenotypes define *hipA* as a newly recognized gene. Transposons Tn*5* and Tn*10* have been inserted close to *hipA* making it possible to explore the molecular genetics of persistence, a long recognized but poorly understood phenomenon.

Persistence was recognized as a result of an early inquiry into the occasional failure of penicillin in the treatment of infections by penicillin-sensitive staphylococci (3). Small fractions, about 1 in 10^6 cells, of staphylococcal cultures were found to remain viable despite prolonged exposure to bactericidal doses of penicillin. The progeny of such persisters are neither more resistant to penicillin than normal organisms nor more likely to survive exposure to penicillin. Thus, persistence cannot be attributed to selection of mutants from among a heterogeneous population. The phenomenon is widespread among penicillin- (16) and ampicillin-sensitive bacteria including *Escherichia coli* (11). The discoverer of persistence suggested that persisters are briefly in a nondividing phase and survive because penicillin kills only bacteria which are able to divide; it was necessary to also propose that penicillin prevents nondividing bacteria from resuming growth (3). A refinement of that explanation relates persistence to periodicity of murein synthesis and inhibitory effects of penicillin on murein hydrolases (10). Involvement of tolerant mutants, which are susceptible to the static but not the bactericidal effects of penicillin (19), and spheroplasts (14) in persistence has been considered, but neither is an entirely satisfactory explanation (10, 11). The study of persistence has not progressed beyond such observations and the finding that persistent staphylococci exhibit aberrant septation (8). Analysis of the problem has undoubtedly been thwarted by the low frequency of persistence, the absence of genetic markers affecting it, and the lack of insights into its physiological role.

Bacteria have, for the most part, precisely regulated metabolism and might be expected to have evolved a regulatory mechanism for responding to an inadequate rate of murein biosynthesis and thereby avoiding its lethal consequence; persistence could be an indicator of such a mechanism. The small fraction of the population protected might reflect the rarity of differential inhibition of murein synthesis throughout evolutionary time before 1942 and the beginning of large-scale manufacture of β-lactam antibiotics. Thus, persistence might be viewed as either a vestigial regulatory system or one designed for such a rare event that preservation of representatives rather than an entire population is a satisfactory solution. In either case it should be possible to obtain mutants with increased frequency of persistence.

This study describes the isolation of mutants of *E. coli* K-12 with vastly increased frequencies

of persistence (Hip mutants), a convenient method for scoring the Hip phenotype, the mapping of *hipA* by conjugation and transduction, and the initial characterization of isogenic *hipA* and *hipA*$^+$ strains which establishes that high and low frequency persistence are responses to any impairment of murein synthesis, not just that caused by β-lactam antibiotics.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this work are listed in Table 1. The rich 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. When necessary, the medium was supplemented with 30 mg of diaminopimelic acid (DAP) per liter. M9 (17) was the minimal medium; required vitamins were added at 1 mg per liter, amino acids at 40 mg per liter, and purines at 20 mg per liter.

Antibiotics, other than murein inhibitors, were employed in the following amounts per liter: 50 mg of chloramphenicol (Cm), 75 mg of kanamycin sulfate, 100 mg of neomycin sulfate, and 20 mg of tetracycline HCl.

Isolation of Hip mutants. Each of the mutants used in this study was isolated independently. After mutagenesis with ethylmethane sulfate (EMS) and phenotypic expression (17), log-phase cultures were treated with 100 μg of ampicillin per ml until lysis was complete, 1.5 to 2 h. Ampicillin was then removed by centrifugation and washing. When the survivors had resumed exponential growth, the cultures were again lysed by the addition of ampicillin and were spread on plates containing 100 μg of ampicillin per ml. After 20 h, the plates were sprayed with a solution of 10,000 U of penicillinase per ml. Each plate received about 2,000 to 3,000 U. Colonies appearing after another 20 h of incubation were purified and scored as Hip (mutant) or Hip$^+$ (parental).

Scoring Hip. Colonies were picked up with the broad end of a flat toothpick and streaked on rich medium containing 100 μg of ampicillin per ml or, in the case of *dapA* strains, on rich medium. Fifty diagonal streaks, each about 0.8-cm long, were made on each plate. After 24 h, the plates were sprayed with penicillinase or with DAP and incubated for an additional 10 to 20 h. The mutant character, Hip, was recorded if growth in the streak was confluent, or the parental character, Hip$^+$, was recorded if there were five or fewer colonies in the streak. To score the transfer of Hip$^+$ to a Hip population, colonies had to be purified, but for transfer of Hip to Hip$^+$ populations, purification was unnecessary.

Genetic analysis. Standard methods were employed for conjugation and transduction (17). For calculation of map distances from cotransduction frequencies, the length of transposons was substracted from the length of the transducing particle (9).

Drug sensitivities. Minimal inhibitory concentrations (MICs) were determined by inoculating plates containing antibiotics with log-phase cultures. For each determination, four samples, each containing about 10^4 cells in 0.003 ml, were used. Efficiencies of plating, calculated as the ratios of CFUs on antibiotic-containing media to CFUs on antibiotic-free media, were determined by inoculating plates with about 10, 100, and 1,000 cells in 0.003 ml for each concentration of antibiotic.

RESULTS

Choice of Hip mutants for study. Approximately 10% of colonies surviving the enrichment method proved to be Hip. Mutants with unimpaired growth rates would have been preferred; however, all had some reduction; those with substantially reduced growth rates were discarded. Mutants with increased resistance to ampicillin were also discarded as were mutants which failed to grow on the minimal, defined media for the parents. Of 24 attempts to obtain independent Hip mutants after mutagenesis, 4 yielded strains acceptable for further study. These were HM3 and HM33 from a *dapA* strain, AT984, and HM7 and HM9 from HM6, a multiply marked F$^-$ strain. No spontaneous Hip mutants were detected.

The effects of varying the conditions for scor-

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

Strain	Relevant genotype or description	Source and reference
AT984	F$^+$ *dapA*	Bukhari and Taylor (6)
K612	F$^-$ *aroA ilv metB his purE proC cycA rpsL*	M. Russell
KF1270	*zdd-262*::IS*10* Cm^r IS*10* *zdd-263*::Tn*5* *zde-264*::Tn*10*	Fouts and Barbour (9)
KF1366	*zda-268*::IS*10* Cm^r IS*10* *zdc-261*::Tn*5* *zde-264*::Tn*10*	Fouts and Barbour (9)
HM3	Same as AT984 except Hip	EMS mutagenesis of AT984
HM33	Same as AT984 except Hip	EMS mutagenesis of AT984
HM6	Same as K612 except *guaB*	EMS mutagenesis of K612
HM7	Same as HM6 except *hipA7*	EMS mutagenesis of HM6
HM9	Same as HM6 except *hipA9*	EMS mutagenesis of HM6
HM11	Same as HM7 except *zde-264*::Tn*10*	(P1 · KF1366 → HM7)
HM12	Same as HM9 except *zde-264*::Tn*10*	(P1 · KF1366 → HM9)
HM21	Same as AT984 except *zde-264*::Tn*10*	(P1 · HM11 → AT984)
HM22	Same as AT984 except *zde-264*::Tn*10* *hipA7*	(P1 · HM11 → AT984)
HM23	Same as AT984 except *zde-264*::Tn*10* *hipA9*	(P1 · HM12 → AT984)
HM24	Same as HM6 except *zdd-262*::IS*10* Cm^r IS*10* *zdd-263*::Tn*5* *zde-264*::Tn*10*	(P1 · KF1270 → HM6)

ing Hip were assessed with strains HM6, HM7, and HM9. The phenotype of each was the same with 25 or 400 μg of ampicillin per ml as with 100 μg/ml, the standard amount, and after 12 or 48 h of exposure to ampicillin as after 24 h, the standard duration of exposure. Addition of 10^{-2} M $MgSO_4$, which has been reported to inhibit autolysis in *E. coli* (15), did not alter the scoring of phenotypes, nor did omission of NaCl.

Before further analysis was undertaken, it was important to assess the possibility that high persistence was a secondary effect of reduced growth rates however slight. Fortuitously, assessment was possible because in minimal media one mutant, HM7, grew as rapidly as its parent, and another mutant, HM9, grew more rapidly. The characteristic frequencies of persistence of the mutants and the parent in rich media were also observed in minimal media; thus, the 1,000-fold-higher persistence of the mutants was observed at growth rates both higher and lower than those of the parent.

Further characterization of HM7 and HM9 was not undertaken until the mutations could be transferred to isogenic backgrounds, a process that required preliminary genetic analysis.

Approximate mapping by conjugation. In these determinations, the parental character, Hip^+, was transferred from standard Hfr strains of several points of origin and directions of transfer to the Hip mutants, HM7 and HM9. Exconjugants were selected for nutritional markers proximal to the origin; distal nutritional markers and Hip or Hip^+ were unselected. Colonies were purified before scoring for Hip^+. The results of the key crosses are shown in Table 2. Analysis of the crosses described in Table 2 establishes that the Hip characteristic depends on at least one mutation that maps in the region between about 32 and 42 min. HM7 and HM9 appear to have mutations in the same region. HM3 and HM33 are Hip derivatives of AT984, which is not suitable for similar analysis by conjugation.

Analysis by transduction. A transduction analysis was made with P1 under the assumption that the phenotypes of HM7 and HM9 result from a mutation in the region 32 to 42 min.

The region 35 to 42 min was found to be an unlikely location for these mutations as the Hip character does not cotransduce with either *aroD* at 37 min or *fadD* at 40 min. The region between 30 and 35 min was examined next by using as donors strains containing transposons covering the entire region, KF1366, or a narrow segment, HM24 and KF1270 (Table 1). The transductional analysis of the Hip character in HM7 and HM9 made with these transposon resistance markers is summarized in Table 3. The high persistence mutation of HM7, designated *hipA7*, maps close to and probably counterclockwise from *zde-264*::Tn10 (Table 3, experiments 1a, b, and c). The similar mutation in HM9, designated *hipA9*, is also closely linked to *zde-264*::Tn*10* (Table 3, experiment 2). The linkage of both *hipA7* and *hipA9* to *zde-264*::Tn*10* is confirmed by transductions in the opposite direction (Table 3, experiments 3, 4, and 5). *hipA7* is about 0.2 min from *zdd-262*::IS*10* Cm^r IS*10* (Table 3, experiment 6b) and about 0.1 min from *zdd-263*::Tn*5* (Table 3, experiment 7). *hipA9* is also about 0.1 min from *zdd-263*::Tn*5* (Table 3, experiment 8). These linkages and the weighted average of the cotransduction frequencies place *hipA7* and *hipA9* at 33.8 min. Additional transduction analysis suggests that *hipA7* and *hipA9* are different mutations.

Characterization of *hipA7* and *hipA9* in an isogenic background. The background organism, AT984 (6), chosen for the next series of experiments has these desirable properties. It is sensitive to phosphomycin and cycloserine and is rapidly killed by starvation for DAP, presumably because of the nature of its defect in *dapA* and other characters that permit a rapid depletion of the DAP pool.

The strains used for further characterization

TABLE 2. Mapping of $hipA^+$ by conjugation

Mating pair	Direction of transfer	Nutritional markers		% Hip^+ (no. positive/no. tested)
		Selected	Unselected	
HM7 × HfrH	Origin, 97 min; *proC*, 9 min; *aroA*, 20 min; *his*, 44 min	Pro^+	—	5 (2/40)
		Pro^+	Aro^+	9 (3/32)
		Pro^+	His^+	57 (17/30)
HM7 × KL96	Origin, 45 min; *his*, 44 min; *aroA*, 20 min	His^+	—	23 (52/204)
		His^+	Aro^+	55 (22/40)
HM7 × PK191	Origin, 42 min; *his*, 44 min; *guaB*, 53 min	His^+	—	0 (0/220)
		His^+	Gua^+	0 (0/9)
HM7 × KL208	Origin, 33 min; *aroA*, 20 min; *proC*, 9 min	Aro^+	—	0 (0/175)
		Aro^+	Pro^+	0 (0/16)
HM9 × KL96	Origin, 45 min; *his*, 44 min; *aroA*, 20 min	His^+	—	18 (14/77)
HM9 × KL208	Origin, 33 min; *aroA*, 20 min; *proC*, 9 min	Pro^+	—	0 (0/77)

TABLE 3. Mapping of *hipA* by transduction

Donor	Recipient	Expt no.	Nutritional marker		Cotransduction frequency (no. positive/no. tested)	Calculated position of Hip$^+$ or Hip (min)
			Selected (position in min)[a]	Unselected		
KF1366 *zde-264*::Tn*10 hipA*$^+$	HM7 *hipA7*	1a	Tc^r (34.2)	Hip$^+$	49/96	33.9
zdc-261::Tn*5 zda-268*::IS*10*		1b	Nm^r (32.6)	Hip$^+$	3/96	33.7
Cm^r IS*10*		1c	Cm^r (30.3)	Hip$^+$	0/96	—
KF1366 *zde-264*::Tn*10 hipA*$^+$	HM9 *hipA9*	2	Tc^r (34.2)	Hip$^+$	26/48	33.9
HM11 *zde-264*::Tn*10 hipA7*	HM6 *hipA*$^+$	3	Tc^r (34.2)	Hip	23/48	33.8
HM11 *zde-264*::Tn*10 hipA7*	AT984 *hipA*$^+$	4	Tc^r (34.2)	Hip	43/96	33.8
HM12 *zde-264*::Tn*10 hipA9*	AT984 *hipA*$^+$	5	Tc^r (34.2)	Hip	45/96	33.8
KF1270 *zde-264*::Tn*10 hipA*$^+$	HM7 *hipA7*	6a	Tc^r (34.2)	Hip$^+$	44/104	33.8
zdd-262::IS*10* Cm^r IS*10*		6b	Cm^r (33.6)	Hip$^+$	39/56	33.8
HM24 *zdd-263*::Tn*5 hipA*$^+$	HM7 *hipA7*	7	Km^r (33.9)	Hip$^+$	86/96	33.8
HM24 *zdd-263*::Tn*5 hipA*$^+$	HM9 *hipA9*	8	Km^r (33.9)	Hip$^+$	45/56	33.8

[a] Abbreviations: Tc, tetracycline HCl; Nm, neomycin sulfate; Km, kanamycin sulfate.

of the Hip phenotype were HM21 (AT984 *zde-264*::Tn*10 hipA*$^+$), HM22 (AT984 *zde-264*::Tn*10 hipA7*), and HM23 (AT984 *zde-264*::Tn*10 hipA9*) (Table 1).

Growth rates. The three strains, HM21, HM22, and HM23, have indistinguishable growth rates; therefore, the phenotypes of *hipA7* and *hipA9* are neither causes of nor caused by reduced growth rates. The small differences observed in the original strains, HM7 and HM9, most have had extraneous causes.

Lysis and killing by inhibitors of murein synthesis. Neither *hipA7* nor *hipA9* alters susceptibility to lysis by phosphomycin, cycloserine, starvation for DAP, and ampicillin as determined by turbidity measurements (Fig. 1). The effects of these inhibitors on viability, however, are vastly different in *hipA* and *hipA*$^+$ strains (Fig. 2). Over the first 30 min, killing of the three strains was equally rapid, but after 30 min, there was a striking difference. The viability of HM21, the *hipA*$^+$ strain, continued to decline at a lower but still substantial rate over the next few hours; the viability of HM22 and HM23, *hipA7* and *hipA9*, remained stable. The second phase of the biphasic killing was absent from the *hipA* strains. Because the vast majority of cells which are killed subsequently lyse, the approximately 100-fold decline in viability of the *hipA* strains which occurs before the killing stops accounts for the failure of turbidity measurements to distinguish *hipA* from *hipA*$^+$ strains (Fig. 1). The lytic and bactericidal responses were similar with phosphomycin, which inhibits the first step of murein synthesis; with cycloserine and starvation for DAP, which inhibit different steps in the middle of the process; and with ampicillin, which affects several late steps in murein synthesis (18). High persistence of *hipA* strains is therefore neither site nor drug specific and probably occurs with any impairment of murein synthesis.

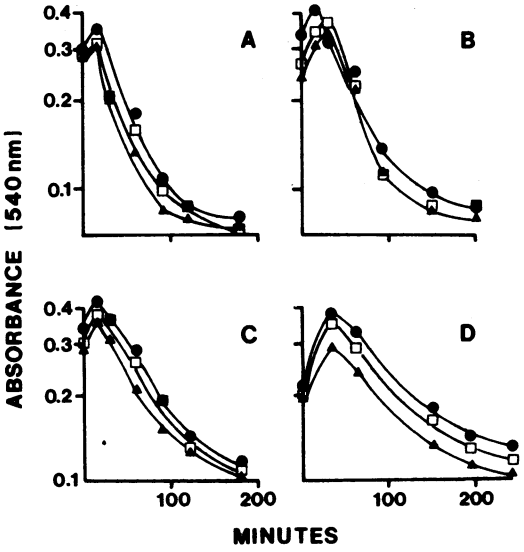

FIG. 1. Lysis of *hipA* and *hipA*$^+$ strains by inhibitors of murein synthesis. (A) Ampicillin, 100 μg/ml. (B) Phosphomycin, 50 μg/ml. (C) Cycloserine, 50 μg/ml. (D) Starvation for DAP. Symbols: ●, HM21 (*hipA*$^+$); ▲, HM22 (*hipA7*); □, HM23 (*hipA9*).

Reversion of spheroplasts does not account for persistence. The phenotypes, as observed in the screening procedure, of neither the *hipA* nor the *hipA*$^+$ strains are modified by the ionic strength of the medium. The frequency of persistence of the *hipA* strains is the same after 4 or 24 h. The most compelling evidence against involvement of spheroplasts was obtained by phase microscopy of cultures which had been exposed to 100 μg of ampicillin per ml for 4 h. The *hipA* and *hipA*$^+$ strains could not be distinguished on the basis of amounts of spheroplasts or other aberrant forms and debris; however, the *hipA* strains

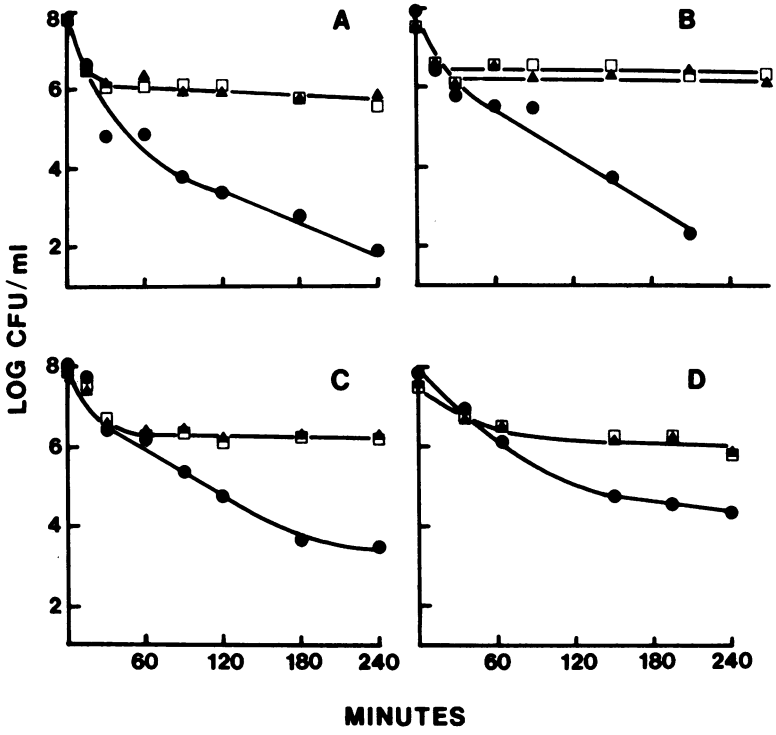

FIG. 2. Killing of *hipA* and *hipA*$^+$ strains by inhibitors of murein synthesis. (A) Ampicillin, 100 μg/ml. (B) Phosphomycin, 50 μg/ml. (C) Cycloserine, 50 μg/ml. (D) Starvation for DAP. Symbols: ●, HM21 (*hipA*$^+$); ▲, HM22 (*hipA7*); □, HM23 (*hipA9*).

contained many rods of normal appearance, whereas the *hipA*$^+$ strain contained no rods or too few to be detected. If, as seems likely, these rods are the persisters, involvement of spheroplasts would require reversion to rods in the absence of murein synthesis.

Selection of tolerant mutants does not account for persistence. The frequency of persistence, whether of *hipA* or *hipA*$^+$ strains, was the same for progeny of survivors of impaired murein synthesis and for cultures from stock which have not been inhibited previously (Table 4). These data establish that the persistent response is not selection of tolerant mutants from among a heterogeneous population.

Selection of resistant mutants does not account for persistence. The MICs of the *hipA* and *hipA*$^+$ strains for ampicillin, cycloserine, and phosphomycin were determined with plates containing a narrow range of drug concentrations. The MICs for HM21 (*hipA*$^+$), HM22 (*hipA7*), and HM23 (*hipA9*) are shown in Table 5. These results establish that the *hipA* mutants are not resistant to inhibitors of murein synthesis, an observation confirmed by efficiency of plating which is a more sensitive method for detecting increments of resistance (Table 5). The latter method also shows that neither *hipA7* nor *hipA9* skews the distribution of resistance upward; there is, in fact, a small but consistent decrease in resistance to phosphomycin in the *hipA* strains.

Other high-persistence mutants. Strains HM3 and HM33 had frequencies of persistence to ampicillin of about 10^{-2}, whereas the frequency for the parent organism, AT984, was 10^{-5}. The increased persistence of HM3 and HM33 was also observed upon starvation for DAP. Elevated temperature (42°C) eliminated the high persistence of HM3 and HM33. In contrast, persistence of *hipA7* and *hipA9* strains was not temperature sensitive.

The mutations of HM3 and HM33 are not linked by transduction to *hipA7* and *hipA9* and may, therefore, identify one or two additional loci for the control of persistence. Further studies of HM3 and HM33 will be made after the mutations are mapped and transferred to isogenic backgrounds. In particular, it will be interesting to observe the consequences of combining either of these mutations with *hipA7* or *hipA9*.

DISCUSSION

The position of *hipA7* and *hipA9* at 33.8′ (Fig. 3) places these mutations in the cotransduction

TABLE 4. Frequency of persistence

Strain and source	CFU per ml in:		Persistence frequency[a]
	− Ampicillin	+ Ampicillin	
HM21 stock culture[b]	5.0×10^8	1.5×10^3	3.0×10^{-6}
HM21 Ap 100 rescue[c]	4.6×10^8	2.3×10^3	5.0×10^{-6}
HM21 DAP⁻ rescue[c]	4.8×10^8	3.0×10^3	6.2×10^{-6}
HM21 DAP⁻ survivors[d]	5.2×10^8	3.8×10^3	7.3×10^{-6}
HM22 stock culture	4.1×10^8	2.0×10^6	4.9×10^{-3}
HM22 Ap 100 rescue	3.7×10^8	2.6×10^6	7.0×10^{-3}
HM22 DAP⁻ rescue	3.4×10^8	2.0×10^6	5.9×10^{-3}
HM22 DAP⁻ survivors	5.0×10^8	1.4×10^6	2.8×10^{-3}
HM23 stock culture	3.8×10^8	3.6×10^6	9.5×10^{-3}
HM23 Ap 100 rescue	3.7×10^8	2.2×10^6	5.9×10^{-3}
HM23 DAP⁻ rescue	2.9×10^8	1.8×10^6	6.2×10^{-3}
HM23 DAP⁻ survivors	3.7×10^8	2.9×10^6	7.8×10^{-3}

[a] Appropriate dilutions of log-phase cultures were plated on media with and without 100 μg of ampicillin per ml. In the former case, persisters were rescued after 22 h by spraying with penicillinase.

[b] Stock cultures were not previously exposed to inhibitors of murein biosynthesis.

[c] The inocula were cells which had been rescued by destroying ampicillin (Ap 100 rescue) or restoring DAP to DAP-starved cultures as in the screening method for *hipA*. The source of the culture in the case of HM21 was five pooled colonies; for HM22 and HM23, the numbers were undetermined but undoubtedly larger as the inocula were from confluent streaks.

[d] The cultures were from pools of at least 10 colonies recovered from populations starved for DAP (Fig. 2D).

gap of the *E. coli* chromosome extending from 31 to 34 min (1) in which only a few functions have been recognized and only two have been mapped (4, 5). Furthermore, the *hipA* locus is distinct from the loci of other genes known to be involved with murein biosynthesis (1). This unique position and the new phenotypes for which *hipA7* and *hipA9* are responsible define *hipA*$^+$ as a newly recognized gene. The normal function of *hipA*$^+$ is a potential key to understanding persistence and perhaps the lethality of impaired murein synthesis; it awaits elucidation. The phenotypes of *hipA* mutants provide the only clues so far. The most striking feature of the phenotypes of both *hipA7* and *hipA9* is the rapid rate of killing over the first 30 min of inhibition of murein synthesis followed by an abrupt and complete cessation of killing. In contrast, the *hipA*$^+$ strain continues to lose viability for several hours at a lower but still substantial rate after the initial period of rapid killing (Fig. 2).

The Hip phenotype is not an indirect consequence of impaired growth as the growth rates of *hipA*$^+$ and *hipA* strains are the same in otherwise isogenic backgrounds. Selection of tolerant mutants or of resistant mutants from among a heterogeneous population has also been eliminated as the basis for high-frequency persistence (Tables 4 and 5). The presence of rods of normal appearance in cultures of *hipA* but not *hipA*$^+$ strains after prolonged inhibition by ampicillin eliminates a role for spheroplasts; such a role would require reversion to the bacillary form in the absence of murein biosynthesis. These observations and the work of others (10, 11) have also eliminated such mechanisms as explanations for the low-frequency persistence characteristic of many species (11, 16). However, the earliest explanation of persisters as cells that

TABLE 5. Drug sensitivities of *hipA*$^+$ and *hipA* strains

Strain	Growth[a]								Efficiency of plating[b]					
	Ampicillin		Cycloserine		Phosphomycin				Ampicillin		Cycloserine		Phosphomycin	
	8.0[c]	10.0	15.0	17.5	1.0	1.5	2.0	2.5	8.0	10.0	15.0	17.5	0.25	2.50
HM21 (*hipA*$^+$)	+	−	+	−	+	+	+	−	1.0	0.0	1.0	0.0	0.7	0.0
HM22 (*hipA7*)	+	−	+	−	+	−	−	−	1.0	0.0	1.0	0.0	0.4	0.0
HM23 (*hipA9*)	+	−	+	−	+	−	−	−	1.0	0.0	1.0	0.0	0.3	0.0

[a] +, Detectable growth; −, no detectable growth.

[b] Ratio of CFU on antibiotic-containing media to CFU on control media.

[c] MIC (micrograms per milliliter).

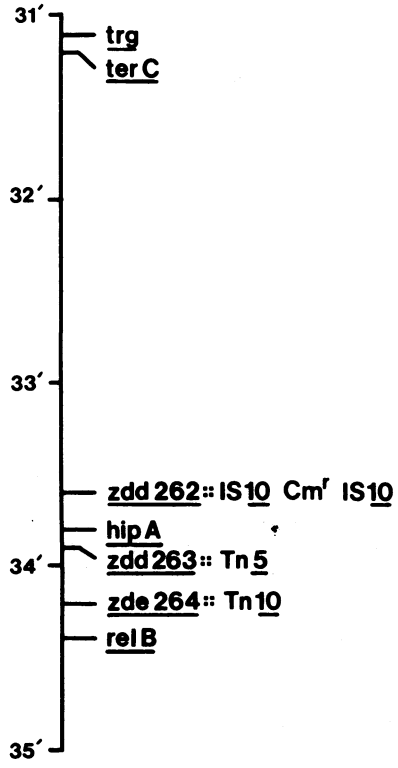

FIG. 3. Genetic map of the *E. coli* K-12 chromosome in the vicinity of the *hipA* gene (4, 5, 9).

had been in a nondividing state at the time of exposure to penicillin (3) remains a useful concept. It could also account for the Hip phenotype if it is assumed that the mutations increase the frequency of temporarily incapacitated cells. But evidence relating impairment of murein synthesis and the death of *E. coli* to the division cycle is more compelling.

Susceptibility to killing by penicillin (13), ampicillin (7), and presumably other impairment of murein synthesis reaches a sharp peak shortly after completion of a round of DNA synthesis as do rate of incorporation of precursors into murein (13) and levels of several murein hydrolases (2, 12). Inhibition of murein synthesis at the time of accelerated hydrolysis of murein is believed to cause irreversible damage to the cell wall, death, and eventually lysis (13). A modest extension of this reasoning could account for persistence. Persisters would be those few cells that are neither in the stage of susceptibility nor capable of proceeding to that stage. Increased frequency of persistence, the Hip phenotype, would result from mutations that decrease the duration of susceptibility in relation to other stages of division or reduce the ability of cells to proceed to the susceptible state in the absence of murein synthesis. The last interpretation could account for the increased sensitivity of the *hipA* strains to the static (Table 5) but not the bactericidal (Fig. 2) effects of phosphomycin.

If the cell cycle is involved, the frequency of persistence might be altered when growth rates are reduced sufficiently to produce differential effects on the duration of the phases of the cycle. If, as proposed, persisters have been arrested in a particular stage of division, they might be expected to resume growth in a cycle or two of synchronous division upon resumption of murein synthesis. The increased frequency of persistence of the *hipA* strains, the availability of isogenic *hipA*$^+$ and *hipA* strains, and the ability to elicit the persistent response without resorting to antibiotics makes it feasible to perform and assess experiments on these matters.

The close proximity of easily selected markers, *zdd-263*::Tn*5* and *zdd-262*::IS*10* Cm^r IS*10*, to the wild-type *hipA* genes, as well as the *hipA7* and *hipA9* mutant alleles, will facilitate further genetic analysis of *hipA*. In turn, such analysis is highly likely to sharpen choices among the biochemical approaches to persistence. For example, it will be important to learn whether *hipA* or *hipA*$^+$ is dominant; to determine whether *E. coli* can tolerate multiple copies of either the mutant or parental genes and, if so, whether multiple copies enhance the phenotypes; and to identify the *hipA*$^+$ product, establish its cellular location, and its response to perturbations of murein synthesis. With such data in hand, biochemical and enzymological comparisons of *hipA*$^+$ and *hipA* strains will be more revealing.

ACKNOWLEDGMENTS

We gratefully acknowledge Alexander Tomasz who generously made his laboratory available to H.S.M. during a sabbatical leave. We thank Thomas Dougherty, Marjory Russel, and Kathleen Postle for helpful discussions.

This work was supported by Public Health Service grant AI 16735 from the National Institute of Allergy and Infectious Diseases. K.P.B. is a recipient of Research Career Development Award AI 00470 from the National Institute of Allergy and Infectious Diseases.

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