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Joint Tolerance to β-Lactam and Fluoroquinolone Antibiotics in *Escherichia coli* Results from Overexpression of *hipA*

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The basis of joint tolerance to β-lactam and fluoroquinolone antibiotics in *Escherichia coli* mediated by *hipA* was examined. An antibiotic tolerance phenotype was produced by overexpression of *hipA* under conditions that did not affect the growth rate of the organism. Overexpressing *hipA* probably decreases the period in which bacteria are susceptible to the antibiotics by temporarily affecting some aspect of chromosome replication or cell division.

β-Lactam and fluoroquinolone antibiotics exhibit a bactericidal action against growing cultures of most pathogenic bacteria (16). However, mutants that are as sensitive to growth inhibition by the antibiotics as the wild-type parent strains but which undergo only a slow loss of viability in the presence of the antibiotics have been described previously (9, 12, 14, 15, 17). The phenomenon has been called tolerance or high persistence (9, 12). Mutants tolerant to β-lactam antibiotics have been described in both laboratory mutant strains and clinical isolates (9). However, mutants tolerant to fluoroquinolones have so far only been described in laboratory mutants of *Escherichia coli* (12, 14, 17). These mutants also display tolerance to β-lactam antibiotics (3, 4, 12, 17). Tolerance to β-lactam antibiotics may have clinical significance (11), but it is not known whether joint tolerance to β-lactams and fluoroquinolones has clinical relevance.

In *E. coli*, joint tolerance to both peptidoglycan and DNA synthesis inhibitors is under the control of the *hip* (high persistence) locus (14, 17). Thus, strains containing chemically mutagenized *hip* exhibit a 1,000-fold reduction in the rate of killing by β-lactam and fluoroquinolone antibiotics (14, 17). The *hip* locus in *E. coli* consists of two genes, *hipA* (1,320 bp), which encodes a weakly expressed 50-kDa protein, and *hipB* (264 bp), which encodes a Cro-like protein which is a *hipA* repressor and responsible for low-level *hipA* expression (3). HipA is found exclusively in a tight complex with HipB, and the stop codon of *hipB* overlaps the start codon of *hipA* by one base (3, 4). This close relationship is essential since *hipB* mutant strains are nonviable, indicating that nonregulated expression of *hipA* might be lethal (4).

An understanding of antibiotic tolerance mediated by mutations in the *hipA* gene may provide the key to a link between β-lactam and quinolone mechanisms of action. In this paper, we report on the distribution of *hip* in bacteria and the role of *hipA* in tolerance of *E. coli* through studies on overexpression of *hipA*.

Distribution of *hipA*. A search for *hipA* and *hipB* homologues was performed with a range of gram-negative and -positive bacteria. By using standard techniques (13), PCR-amplified *E. coli hipA* and *hipB* were used to probe restriction digests of chromosomal DNA from *Shigella sonnei*, *Salmonella typhimurium*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Bacillus subtilis*, *Providencia vulgaris*, and *Serratia marcescens*. To determine the distribution of the *hip* locus in *E. coli*, the *hip* operon was amplified by PCR with two sets of primers designed to amplify the entire *hipA* gene and the entire *hipB* gene (3). Although both *hipA* and *hipB* were identified in *S. sonnei*, homologues could not be identified, even with low-stringency hybridization, in any of the other bacteria examined, including organisms such as *Salmonella typhimurium* and *Klebsiella pneumoniae*, which are closely related to *E. coli* and *Shigella sonnei*. However, three homologues were identified by amino acid database searches, one in *Haemophilus influenzae* and two in the *Rhizobium* symbiosis plasmid pNGR234 (7, 8). In *H. influenzae*, the gene (*HI0665*) is significantly disrupted and would be unlikely to express a protein with similar function to HipA. In *Rhizobium*, the genes (*y4mE* and *y4dM*) encode proteins with 28 and 27% identity to HipA, respectively. Significantly, as for HipB, these genes are under the control of strong transcriptional regulators (*y4mF* and *y4dL*).

Chromosomal DNA from 40 clinical isolates of *E. coli*, from different locations worldwide, was PCR amplified with two sets of primers designed for *hipA* and *hipB*. Approximately 20% of the strains tested were negative with both primer sets, and the remaining strains were amplification positive with both sets.

Chromosomal deletion of *hipA* in *E. coli*. The *E. coli* LN2666 (1) *hipA* gene was replaced with a copy which expressed only the first 25 amino acids of the protein, resulting in strain IC4. This was done by homologous recombination (6) with the plasmid pHp100, a pFC24 (6) derivative in which a 618-bp *Bss*HII fragment of *hipA* had been excised. To determine if *hipA* deletion or disruption exhibited a specific phenotype, strains IC4 and LN3559 (*hipA*::*tetA*), a gift from J.-M. Louarn, were compared to the parent strain, LN2666, with respect to growth rate, morphology, antimicrobial susceptibility, and total cellular protein profiles. For all these characteristics, there were no differences between the three strains (data not shown).

Overexpression of *hipA*. Growth of *E. coli* BL21 (DE3) carrying pLysS (Promega) and pHp200, a pET30b (Novagen) construct containing the entire *hipA* open reading frame, was induced with 0.05 to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Induction produced dose-dependent inhibition of cell division in *E. coli* BL21 (Fig. 1). The growth rates of cells

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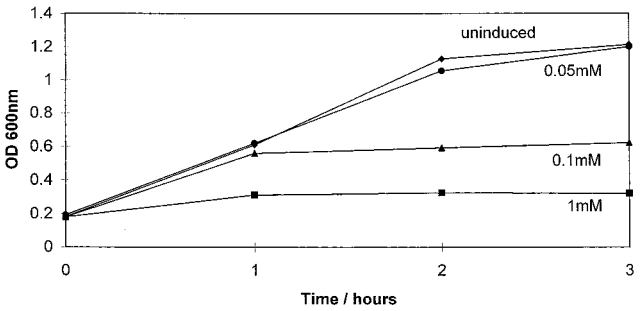

FIG. 1. Growth curve of *E. coli* BL21 (DE3) carrying pLysS and pHp200 with and without IPTG induction. OD_{600}, optical density at 600 nm.

exposed to IPTG concentrations of less than 0.03 mM were unaffected. However, when challenged with 100 μg of ampicillin per ml, *E. coli* containing pHp200, induced with 0.01 mM IPTG, exhibited significantly reduced killing in comparison to cells containing the vector alone (Fig. 2A). To determine if this phenomenon was joint tolerance as identified in the original mutants (14), survival studies were performed for cells containing pHp200 that had been exposed to the quinolone ciprofloxacin. In this case, there was a 10-fold difference in the killing of cells containing pHp200 compared to that of cells containing pET30b when exposed to 100 μg of the drug per ml (Fig. 2B). Expression of *hipA* was confirmed by S●Tag Western blot (Novagen), which showed HipA to be a protein of 49 kDa (data not shown), the size predicted by its sequence.

The ability to overexpress *hipA* has allowed us to demonstrate that joint tolerance to β-lactam and quinolone antibiotics in *E. coli* is due to expression of *hipA* in excess of *hipB* and that HipA is toxic to *E. coli*. Failure to isolate HipA unbound to HipB (4) and the observation made here that overexpression of *hipA* produced a tolerance phenotype similar to that observed in an earlier work (14) indicate that in the original mutants, reduced binding of HipA to HipB probably resulted in the observed tolerance. However, since neither cassette insertion inactivation (4) nor chromosomal deletion of *hipA* (this study) conferred tolerance, at least part if not all of HipA is required for the phenotype.

Since the *hip* locus is restricted to relatively few bacterial species, including not even all strains of *E. coli*, antibiotic tolerance resulting from *hip* mutations is unlikely to be clinically significant. In addition, persistence identified in species other than *E. coli*, e.g., the persistence of β-lactams in staphylococci (2), is unlikely to be related to *hip*. However, although the role of *hip* in *E. coli* tolerance to β-lactam and quinolone antibiotics has been established in this study, the mechanism of tolerance has not. Bigger (2) suggested that persisting bacteria are cells briefly existing in the nondividing phase of their life cycle and survive because penicillin kills only dividing cells. If bacteria are susceptible to β-lactam and quinolone antibiotics only during specific phases of their life cycle, then any phenomenon that reduced the window of opportunity for killing would enable more bacteria to survive the cidal effects of both antibiotics. It is tempting to speculate that HipA may decrease the period of time during which bacteria are susceptible to these antibiotics by affecting some aspect of chromosome replication or cell division. This may relate to the location of *hip* in the terminal domain of the chromosome within 100 bp of the *dif* locus and close to *terC*. These loci are involved in chromosome partitioning and termination of chromosome replication, respectively (5, 10).

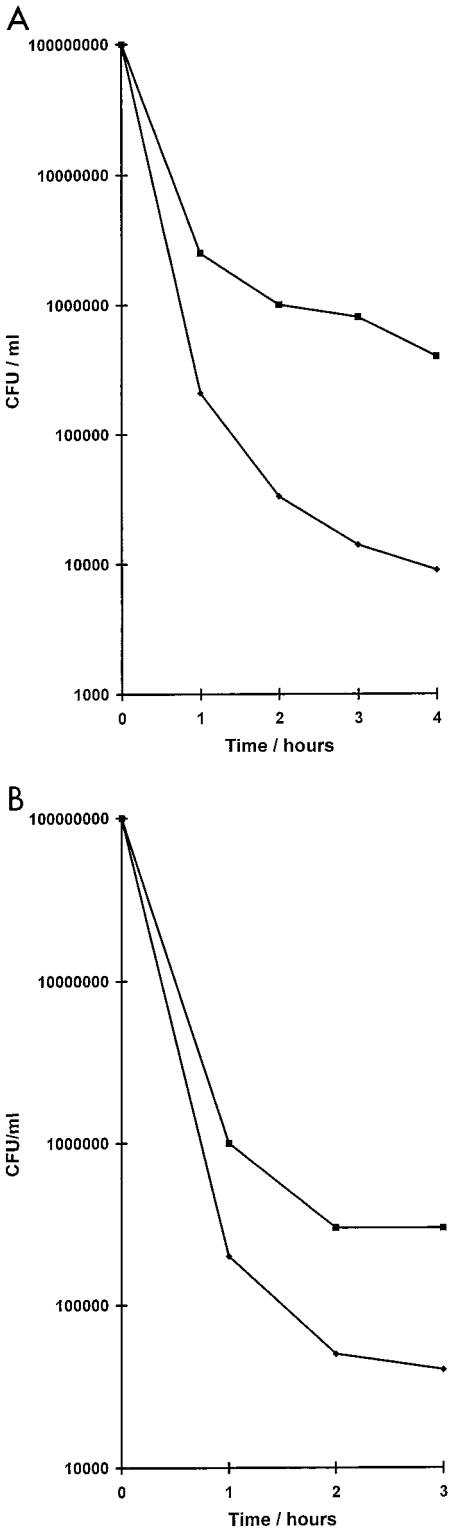

FIG. 2. Killing of *E. coli* BL21 (DE3) containing plasmid pHp200 (■) or pET30b (◆) with 100 μg of ampicillin per ml (A) or 100 μg of ciprofloxacin per ml (B). Cultures contained 0.01 mM IPTG.

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