

## Kinase Activity of Overexpressed HipA Is Required for Growth Arrest and Multidrug Tolerance in *Escherichia coli*<sup>∇</sup>

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**Overexpression of the HipA protein of the HipBA toxin/antitoxin module leads to multidrug tolerance in *Escherichia coli*. HipA is a “toxin” that causes reversible dormancy, whereas HipB is an antitoxin that binds HipA and acts as a transcriptional repressor of the *hipBA* operon. Comparative sequence analysis shows that HipA is a member of the phosphatidylinositol 3/4-kinase superfamily. The kinase activity of HipA was examined. HipA was autophosphorylated in the presence of ATP *in vitro*, and the purified protein appeared to carry a single phosphate group on serine 150. Thus, HipA is a serine kinase that is at least partially phosphorylated *in vivo*. Overexpression of HipA caused inhibition of cell growth and increase in persister formation. Replacing conserved aspartate 309 in the conserved kinase active site or aspartate 332 in the Mg<sup>2+</sup>-binding site with glutamine produced mutant proteins that lost the ability to stop cellular growth upon overexpression. Replacing serine 150 with alanine yielded a similarly inactive protein. The mutant proteins were then examined for their ability to increase antibiotic tolerance. Cells overexpressing wild-type HipA were highly tolerant to cefotaxime, a cell wall synthesis inhibitor, to ofloxacin, a fluoroquinolone inhibitor of DNA gyrase, and to topoisomerase IV and were almost completely resistant to killing by mitomycin C, which forms DNA adducts. The mutant proteins did not protect cells from cefotaxime or ofloxacin and had an impaired ability to protect from mitomycin C. Taken together, these results suggest that the protein kinase activity of HipA is essential for persister formation.**

Bacterial multidrug tolerance or persistence was initially observed by detecting a minor subpopulation of *Staphylococcus* spp. that survived treatment with penicillin (6). Tolerance is the ability of cells to resist killing by bactericidal antibiotics without the involvement of a resistance mechanism. Resistance allows cells to grow in the presence of an otherwise lethal factor, and the MIC of resistant cells is increased. Tolerant cells neither grow nor die in the presence of antibiotic.

Importantly, persisters are not mutants but phenotypic variants of the wild type (6, 17, 36), and we proposed previously that they emerge due to stochastic fluctuations in the level of persister proteins (18, 21, 22). Indeed, entry into the persistent state and reversal to normal growth occur randomly (3). There is also an apparent regulatory mechanism superimposed upon the stochastic persister formation. In several species examined, there is a sharp increase in persister production starting at mid-exponential growth phase and continuing through stationary phase (17). The mechanism of persister formation is unknown, but growing evidence points to dormancy as the basis of multidrug tolerance (18). Bactericidal antibiotics kill by corrupting their targets and are less active in a dormant cell. Consistent with this model, persisters are slow-growing or non-growing cells (3), with a decreased level of translation (31).

The first reports on the molecular nature of persistence came from Moyed and coworkers, who screened a mutagenized culture of *Escherichia coli* for increased survival to killing by ampicillin. This resulted in the discovery of a high-persistence *hipA7* allele, encoding a 50-kDa protein, which increased the frequency of persisters 1,000- to 10,000-fold (27, 28). The *hipA7* allele carries two mutations, G22S and D291A, the first of which is necessary for the initiation of high persistence (19). The wild-type gene, *hipA* (high persistence), was found to be cotranscribed with a smaller upstream gene, *hipB*, encoding a 10-kDa protein (8). The *hipA* and *hipB* genes overlap by 1 bp, suggesting that they are translationally coupled, and the two proteins were found to form a complex; furthermore, expression of HipA in the absence of HipB was toxic to the cell (8). HipB was shown to be a Cro/CI-like DNA-binding protein that negatively regulates the *hipBA* operon by binding at multiple upstream sites (7).

Subsequent studies have led to persister isolation by lysing a growing culture of *E. coli* with ampicillin or by sorting cells with a low level of green fluorescent protein expression. The gene expression profile of persisters points to overexpression of a number of toxin/antitoxin modules (18, 31). *E. coli* chromosomally encoded toxins, such as RelE and MazF, can cause reversible stasis by cleaving mRNA (30) and appear to be good candidates for persister genes (18). Overexpression of toxins, indeed, causes an increase in persister formation and drug tolerance (18, 35). *hipBA* seems to be a TA module as well. Overexpression of the HipA toxin causes stasis (12), and ectopic induction of HipB rescues cells overexpressing HipA

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TABLE 1. Strain list

Strain	HipA construct	Primers
KL319	Wild type	
KL320	D309Q mutant	GGTTGATTGGCGCAACGGACGGTTCATGCAAAAACTTC and GAAGTTTTTTCATGACCGTCCGTTGCGCCAATCAACC
KL321	D332Q mutant	CGACTCACGCCATTTTACGACATCATTTTCAGATTTCCGG and CCGGAAATGCTGAAATGATGTCGTAAATGCGCGTGA GTCG
KL322	Truncated after <i>hipA7</i> residue 167	
KL603	S150A mutant	GACTTTCGCATCGCGGTTGCTGGCGCACAG and CTGTGCGC CAGCAACCGCGATGCGAAAGTC

(20). Overexpression of HipA leads to multidrug tolerance (12, 20, 35). HipA causes inhibition of macromolecular synthesis (20), but its mechanism of action is unknown. In this study, we show that HipA belongs to a family of phosphatidylinositol and protein kinases and is capable of autophosphorylation. Mutants of HipA lacking either predicted active-site residues or the site of autophosphorylation are defective in producing multidrug-tolerant cells.

#### MATERIALS AND METHODS

**Plasmid construction.** Primers *hipA\_N\_His* and *hipAgerP2* were used to amplify *hipA* from HM21, a *hipA*-containing strain (27). The PCR products were digested with *NheI* and *SphI*, respectively, and ligated into the *XbaI* and *SphI* sites of pBAD33 (14). The cloning resulted in the insertion of 6 histidine residues plus serine and arginine immediately after the initiation methionine at the N terminus of HipA. The resulting plasmid, pHis21, was transformed into DH5 $\alpha$ , and the modified *hipA* insert was verified by sequencing.

**Construction of point mutants.** A QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make point mutations in pHis21 according to the manufacturer's recommendations. The primers and the resulting amino acid substitutions are listed in Table 1.

**Purification of HipA.** Cells were inoculated 1:1,000 from an overnight culture into 250 ml LB containing 50  $\mu$ g/ml chloramphenicol. Cultures were grown to an optical density at 600 nm ( $OD_{600}$ ) of approximately 0.3, at which point 20% L-arabinose was added to a final concentration of 0.2% in order to induce expression of HipA. Cells were collected by centrifugation after 1 h of induction and lysed in 1 $\times$  bind buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole, pH 8.0) containing lysozyme (50 mg/ml), 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. Benzonase (50 U/ml) was added to the cells once they lysed. The soluble, uncleared fraction was collected by centrifugation and applied directly to 1.0 ml Ni-nitrilotriacetic acid agarose (QIAGEN) that had been preequilibrated in 1 $\times$  bind buffer. The mixture was incubated at 4°C with shaking for 1 h and then applied to a column, where it was washed with 1 $\times$  wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0). HipA was eluted with 1 $\times$  elute buffer (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0) and collected in 0.5-ml fractions. Fractions containing HipA were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Antibiotic susceptibility measurements.** Cells were inoculated from an overnight culture (1:1,000) into 25 ml of LB containing chloramphenicol at 50  $\mu$ g/ml. When the  $OD_{600}$  of the culture reached approximately 0.3, a 1.0-ml control uninduced aliquot was removed and challenged with antibiotic for 3 h. Test samples (1.0 ml) were induced with L-arabinose (final concentration, 0.2%) for 60 min, and the following antibiotics were added at  $\geq$ 10-fold the MIC: cefotaxime (100  $\mu$ g/ml), mitomycin C (10  $\mu$ g/ml), ofloxacin (5  $\mu$ g/ml), and tobramycin (25  $\mu$ g/ml). Percent antibiotic challenge survival was calculated as the ratio of CFU/ml posttreatment divided by CFU/ml pretreatment.

**In vitro kinase labeling.** The following were added in a reaction volume of 20  $\mu$ l: buffer B (25 mM HEPES, pH 7.4, 25 mM  $\beta$ -glycerophosphate, 1 mM  $Na_3VO_4$ ), ATP (100  $\mu$ M),  $MgCl_2$  (100  $\mu$ M), and 33 nM [ $\gamma$ - $^{32}P$ ]ATP (5  $\mu$ Ci/ $\mu$ l). Purified HipA protein and its mutant variants in buffer B (100 ng each) were added to start the reaction, which lasted for 10 min at 30°C and was stopped by the addition of Li-sodium dodecyl sulfate loading solution (Invitrogen). Proteins were separated on NuPAGE 4 to 12% Bis-Tris gels by use of 1 $\times$  MES [2-(N-morpholino)ethanesulfonic acid] buffer (Invitrogen).

**Liquid chromatography-mass spectrometry (LC-MS) analysis.** Samples were analyzed using a nanoLC system (Ultimate; Dionex, Mountain View, CA) coupled to a hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT mass spectrometer; Thermo Electron, San Jose, CA). Approximately 2  $\mu$ l of the sample was loaded onto a 75- $\mu$ m, 15-cm column packed with 3- $\mu$ m Magic  $C_{18}$  particles (Michrom BioResources, Auburn, CA), followed by a 75-min linear gradient from 2% to 35% acetonitrile (vol/vol) in 0.1% formic acid using a 250-nl/min flow rate. In the data acquisition cycle, each high-resolution Fourier transform-MS scan (accumulation of  $2 \times 10^6$  ions) was followed by up to eight tandem MS (MS-MS) spectra in the linear ion trap (accumulation of  $3 \times 10^4$  ions), with dynamic exclusion set at 1 min. Each data acquisition cycle was completed in approximately 3 s. The resulting data were searched against a database consisting of two protein sequences, normal and mutant versions of HipA, by use of the Sequest algorithm (25). The precursor ion mass tolerance was set to 1.4 Da, and trypsin was assigned as the proteolytic enzyme with up to two missed cleavages. Phosphorylation specified as a variable modification on S, T, or Y. Peptide hits with cross-correlation values greater than 1.5 (1+), 2.0 (2+), and 2.5 (3+) were evaluated manually, using mass accuracy of 10 ppm for precursor ion and matching of high intensity MS-MS peaks to predicted b- and y-ion series as the most important criteria for a correct identification.

**Sequence analysis and phylogeny reconstruction.** The nonredundant protein sequence database (NCBI, NIH, Bethesda, MD) was iteratively searched using the PSI-BLAST program (2). The multiple sequence alignment was constructed using MUSCLE (11) and then manually corrected on the basis of high-scoring sequence pairs generated by PSI-BLAST. Phylogenetic analyses were performed using the MOLPHY program to build a maximum-likelihood, unrooted tree (1). The MOLPHY program was also used to compute the RELI bootstrap probabilities (1).

#### RESULTS

**HipA belongs to the PI 3/4-kinase superfamily.** A PSI-BLAST (2) search with the inclusion cutoff E value of 0.01, starting with the sequence of the HipA-like protein from *Geobacillus kaustophilus* (GK3292, gi 56381669), retrieved ~400 sequences from various prokaryotes and eukaryotes after seven search iterations. After the seventh iteration, the search picked up several sequences of the phosphatidylinositol (PI) 3/4-kinase superfamily with statistically significant E values (e.g., At1g64460, gi 15217655, from *Arabidopsis thaliana*, with an E value of  $2e-07$ ), and in subsequent iterations, numerous members of this protein superfamily were retrieved. Notably, all detected members of this superfamily were from bacteria or eukaryotes; no archaeal homologs could be identified. The multiple alignment of the HipA family and other, related bacterial protein families spans the entire catalytic core domain and contains several conserved motifs shared with proteins of the phosphatidylinositol 3-kinase superfamily; these motifs correspond to distinct structural elements in the available crystal structures of PI 3/4-kinase family proteins (Fig. 1A). Embedded within these motifs are the catalytic residue of phos-

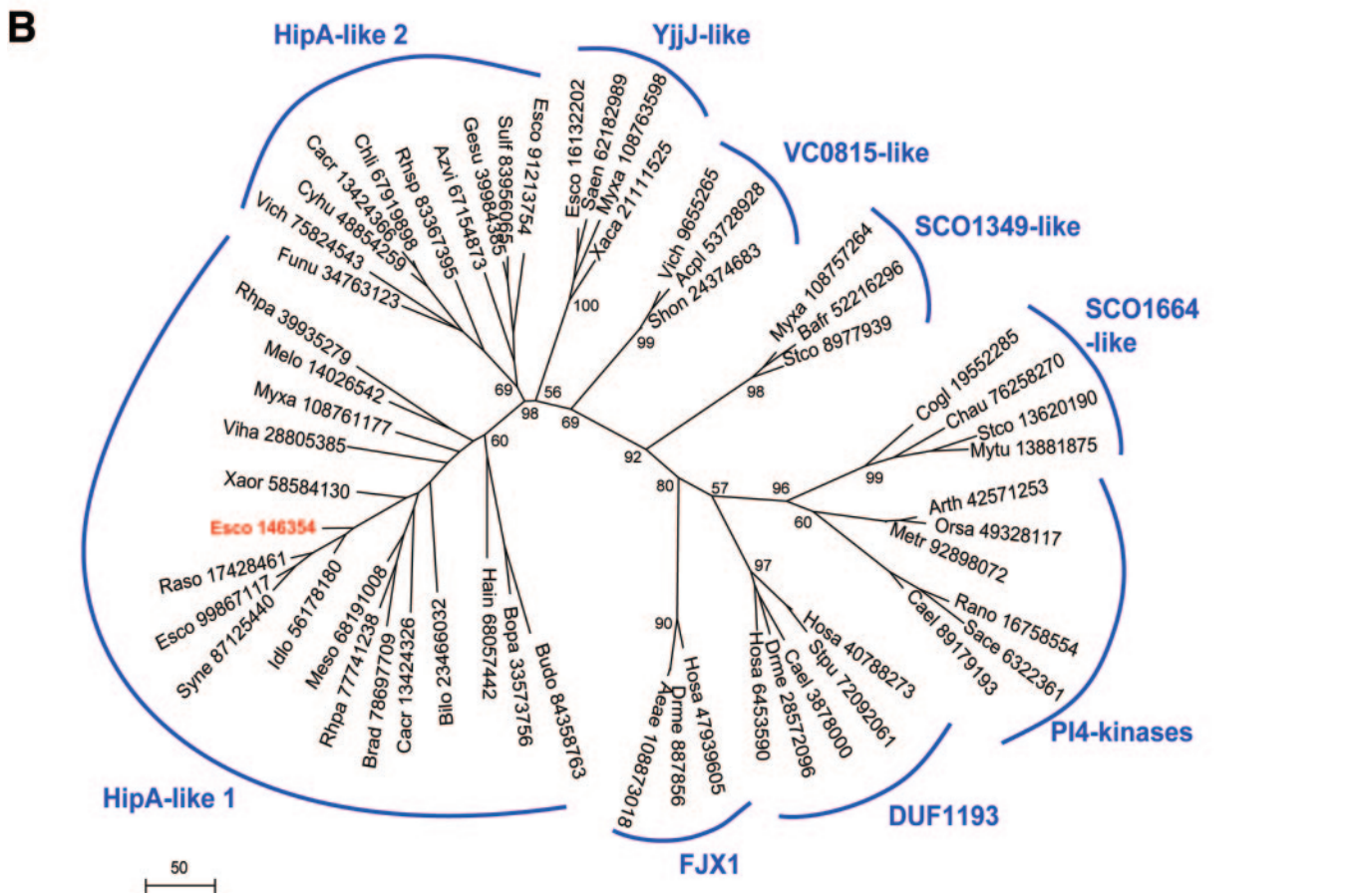
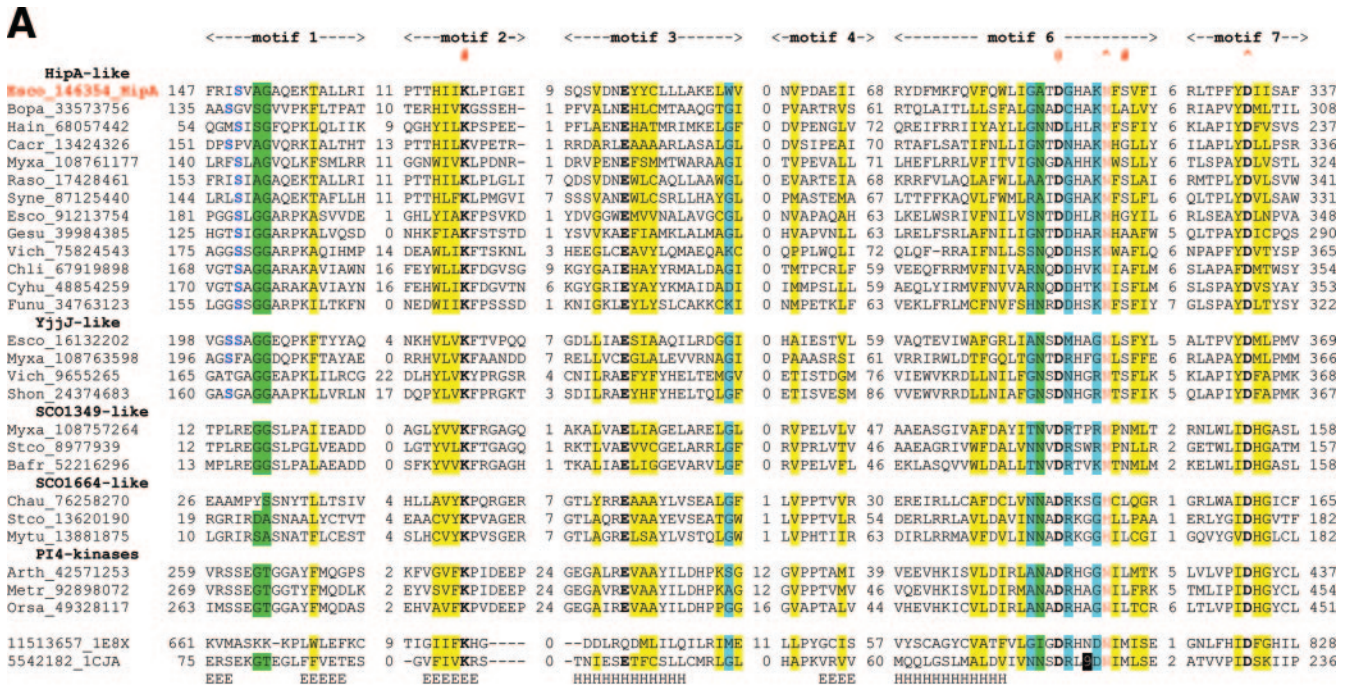


FIG. 1. HipA is a member of the PI 3/4-kinase superfamily. (A) Multiple alignment of the catalytic core of HipA, related families of bacterial proteins, and selected eukaryotic PI 3/4-kinase superfamily proteins. Selected sequences represent the HipA/COG3550, YjjJ-like, SCO1664, and SCO1349 families from bacteria and DUF1193 (uncharacterized family), FJX1 (four-jointed protein), and PI4 kinases from eukaryotes. Proteins with available crystal structures selected for comparison are 1cja (actin-fragmin kinase) and 1e8m (TOR1-like kinase). Sequences are denoted by abbreviated species names, which correspond to the following proteins: Esco\_146354, *E. coli* HipA; Bopa, *Bordetella parapertussis* BPP2765; Hain, *H. influenzae* NTHI0786; Cacr, *Caulobacter crescentus* CC2735; Myxa\_108761177, *Myxococcus xanthus* MXAN\_0097; Raso, *Ralstonia solanacearum*

phatidylinositol 3-kinases, aspartate 309 (motif 6), the  $Mg^{2+}$ -binding residues asparagines 314 (motif 6) and aspartate 332 (motif 7), and the ATP-binding lysine 181 (motif 2) (5).

The relationship between two families of uncharacterized bacterial proteins, typified by SCO1664- (SCO is a prototype name we assign to this group) and SCO1349-like proteins from *Streptomyces coelicolor*, with PI 3/4-kinases has been reported previously (5). We show here that two additional bacterial families, the HipA-like and the YjjJ-like proteins, possess a homologous catalytic domain. Within the PI 3/4-kinase superfamily, HipA shows the closest similarity to the relatively poorly characterized phosphatidylinositol 4-kinase II (PI4KII) family, a group of kinases whose only known substrate is phosphatidylinositol, which these enzymes phosphorylate at the D-4 position (4). Phylogenetic tree analysis reveals a close relationship between the SCO1664-like family of bacterial proteins and the PI4KII family but suggests that the HipA-like and YjjJ-like families form a distinct bacterial branch that is well separated from the eukaryotic homologs (Fig. 1B).

The detection of the phosphatidylinositol kinase-like catalytic domain and, in particular, the conservation of the catalytic and substrate-binding residues in HipA lead us to hypothesize that these proteins also possess kinase activity. The PI 3/4-kinase superfamily includes a variety of kinases that phosphorylate either lipids or proteins (e.g., TOR kinase or the DNA-dependent kinases). HipA and its bacterial homologs show the greatest similarity to a distinct family of phosphatidylinositol-specific kinases. However, given that phosphatidylinositides have not been reported for bacteria and that the sequence similarity between HipA and PI4KII kinases is distant, it does not seem possible to predict the substrate specificity of HipA with any confidence.

The catalytic core domain comprises the C-terminal part of HipA-like proteins, whereas ~100 amino acids at the N terminus correspond to a globular domain that does not have any detectable homologs outside the HipA family and the closely

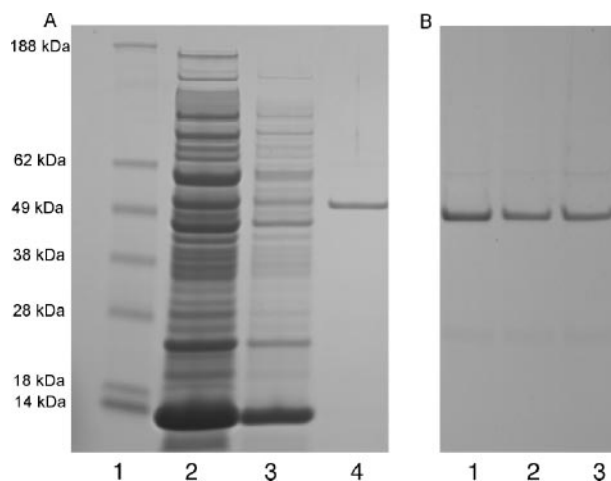


FIG. 2. Purification of HipA. (A) Coomassie-stained Ni-nitrilotriacetic acid affinity chromatography fractions. Lane 1, marker; lane 2, soluble protein load; lane 3, wash fraction; lane 4, eluted fraction. (B) Coomassie-stained eluted mutant proteins. Lane 1, D309Q mutant; lane 2, D332Q mutant; lane 3, S150A mutant. The band corresponds to the His-HipA molecular size of 50 kDa.

related families of bacterial proteins (YjjJ-like and VC0815-like proteins) (Fig. 1B). In several bacterial genomes (e.g., c5296 in *E. coli* CFT073, P700755\_01462 in *Psychroflexus torquis*, and HI0666 in *Haemophilus influenzae* Rd KW20), this domain is encoded by a separate gene that is often adjacent to and is predicted to form an operon with the gene encoding the HipA-like catalytic domain. This suggests an important role of the N-terminal domain in the function of HipA.

**Kinase activity of HipA.** To determine whether HipA has kinase activity, autophosphorylation of purified HipA was examined. For this purpose, *hipA* was first cloned in frame with an upstream histidine tag under control of the tightly regulated

RSc1446; Syne, *Synechococcus* sp. RS9917\_01666; Esco\_91213754, *E. coli* UTI89\_C4805; Gesu, *Geobacter sulfurreducens* GSU2399; Vich\_75824543, *Vibrio cholerae* RC385 VchoR\_01000367; Chli, *Chlorobium limicola* ClimDRAFT\_0642; Cyhu, *Cytophaga hutchinsonii* Chut02002929; Funu, *Fusobacterium nucleatum* subsp. *vincentii* FNV1338; Esco\_16132202, *E. coli* YjjJ; Myxa\_108763598, *M. xanthus* MXAN\_0015; Vich\_9655265, *V. cholerae* VC0815; Shon, *Shewanella oneidensis* SO3170; Myxa\_108757264, *M. xanthus* MXAN\_0652; Stco\_8977939, *S. coelicolor* A3(2) SCO1349; Bafr, *Bacteroides fragilis* BF2142; Chau, *Chloroflexus aurantiacus* CaurDRAFT\_1115; Stco\_13620190, *S. coelicolor* A3(2) SCO1664; Mytu, *M. tuberculosis* MT2191; Arth, *A. thaliana* AT2G46500; Metr, *Medicago truncatula* AC142526g2v1; Orsa, *Oryza sativa* OSJNBa0022J2.8; 11513657\_1E8X, *Sus scrofa* PI3Kc; 5542182\_1CJA, *Physarum polycephalum* actin-fragmin kinase. The positions of the first and last residues of the aligned region in the corresponding protein are indicated for each sequence. The numbers within the alignment refer to the lengths of inserts that are poorly conserved between all the families. Sequence motifs shared with PI 3/4-kinases correspond to previously established conserved regions and are shown at the top of the alignment (5). Conserved positions with predominantly hydrophobic residues are shown in yellow, positions with small residues in green, positions with turn-promoting residues in cyan, and positions with polar residues in red. Invariant, highly conserved, and functionally crucial residues in the PI 3/4-kinase superfamily are shown in boldface type. Serine residues that are potential targets for autophosphorylation are shown in blue. @, catalytic residue; ^,  $Mg^{2+}$ -binding residues; #, ATP-binding residues; H,  $\alpha$ -helices; E,  $\beta$ -strands (5). (B) Phylogenetic tree of HipA and a subset of the PI 3/4-kinase superfamily proteins. The maximum-likelihood unrooted tree was built using MOLPHY (1). Each terminal branch of the tree is labeled with the abbreviated name of the organism and the unique gene identifier or locus identifier. The REll bootstrap values are shown for the main branches. Abbreviations: Acpl, *Actinobacillus pleuropneumoniae*; Aae, *Aedes aegypti*; Arth, *A. thaliana*; Azvi, *Azotobacter vinelandii*; Bafr, *B. fragilis*; Bilo, *Bifidobacterium longum*; Bopa, *B. parapertussis*; Brad, *Bradyrhizobium* sp.; Budo, *Burkholderia dolosa*; Cacr, *C. crescentus*; Cael, *Caenorhabditis elegans*; Chau, *C. aurantiacus*; Chli, *C. limicola*; Cogl, *Corynebacterium glutamicum*; Cyhu, *C. hutchinsonii*; Drme, *Drosophila melanogaster*; Esco, *E. coli*; Funu, *F. nucleatum*; Gesu, *G. sulfurreducens*; Hain, *H. influenzae*; Hosa, *Homo sapiens*; Idlo, *Idiomarina loihiensis*; Melo, *Mesorhizobium loti*; Meso, *Mesorhizobium* sp.; Metr, *M. truncatula*; Mytu, *M. tuberculosis*; Myxa, *M. xanthus*; Orsa, *O. sativa*; Rano, *Rattus norvegicus*; Raso, *R. solanacearum*; Rhpa, *Rhodopseudomonas palustris*; Rhsp, *Rhodobacter sphaeroides*; Sace, *Saccharomyces cerevisiae*; Saen, *Salmonella enterica*; Shon, *S. oneidensis*; Stco, *S. coelicolor*; Stpu, *Strongylocentrotus purpuratus*; Sulf, *Sulfobacter* sp.; Syne, *Synechococcus* sp.; Vich, *V. cholerae*; Viha, *Vibrio parahaemolyticus*; Xaca, *Xanthomonas campestris*; Xaor, *Xanthomonas oryzae*. The HipA protein from *E. coli* is shown in red.

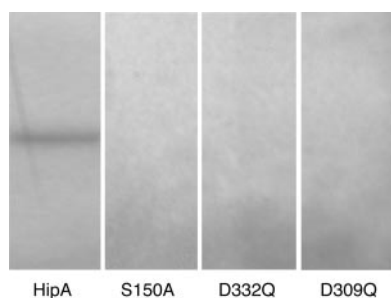


FIG. 3. In vitro phosphorylation of HipA. Purified HipA was incubated with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The figure depicts radiograms of the gels. A parallel gel was Coomassie stained to verify the presence of proteins at comparable levels, as shown in Fig. 2. The negative control included heat-denatured HipA (not shown).

arabinose  $P_{\text{BAD}}$  promoter. The resulting plasmid, pHis21, was transformed into strain BW25113 ( $\Delta\text{hipA}$ ) to produce KL319. Point mutations were introduced into pHis21 carrying *hipA*, replacing the putative active-site residue aspartate 309 or the predicted  $\text{Mg}^{2+}$ -binding site aspartate 332 with a glutamine, creating strains KL320 and KL321, respectively. The resulting HipA constructs were then expressed, and the modified proteins were affinity purified (Fig. 2A and B). Note that the wild-type (Fig. 2A, lane 4) and mutant (Fig. 2B, lanes 1 to 3) proteins were expressed at comparable levels.

Purified HipA and its mutant variants were then tested in an in vitro kinase autophosphorylation assay. Incubation of wild-type HipA in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  resulted in a single radioactively labeled band of approximately 50 kDa (Fig. 3, lane 1) (sizes were determined relative to molecular size markers which stained blue on the sodium dodecyl sulfate-polyacrylamide gel used for autoradiography), which corresponds well to the molecular size of His-tagged HipA. When either the D309Q mutant or the D332Q mutant was used in the assay, the

50-kDa band was not present (Fig. 3, lanes 3 and 4). Several-day exposure failed to show any phosphorylated bands for the mutant proteins. The 50-kDa radiolabeled band and purified His-HipA comigrate, suggesting that HipA has an autophosphorylating activity.

LC-MS analysis of a tryptic digest of HipA purified from cell lysate was conducted to identify the putative phosphorylation site. It was found that serine 150 was phosphorylated, and the MS-MS spectrum of the corresponding tryptic phosphopeptide ADIPLGMIREENDFRIS\*VAGAQEK, with annotation of individual fragments, is shown in Fig. 4. This peptide contained only a single potential phosphorylation site (serine 150), and the exact localization of the phosphorylation was confirmed by the presence of fragment ions  $b_{12}$  to  $b_{23}$ , as seen in Fig. 4. Besides the phosphorylated peptide, no other phosphorylation was observed (data not shown); however, peptide with no phosphorylation on serine 150 was found as well. Assuming similar ionization efficiencies for phosphorylated and nonphosphorylated peptides (34), it was estimated that more than 50% of HipA was phosphorylated on serine 150. Importantly, no phosphorylation was observed on peptides isolated from the D309Q substitution protein lacking the conserved D309 that is an essential residue in the ATP-binding consensus of other kinases belonging to the same phosphatidylinositol 3/4-kinase superfamily. This suggests that phosphorylated S150 is a product of HipA autophosphorylation rather than the result of phosphorylation by a different kinase.

To identify potential additional phosphorylation sites in HipA, a replacement S150A mutant was engineered. No labeling was apparent in the in vitro kinase assay with the S150A mutant protein (Fig. 3, lane 2). Thus, serine 150 appears to be the unique site of HipA autophosphorylation. To determine whether HipA is a protein kinase that is active in *trans*, we employed universal kinase substrates casein and histone 2A. Phosphorylation of either of these substrates was not detected

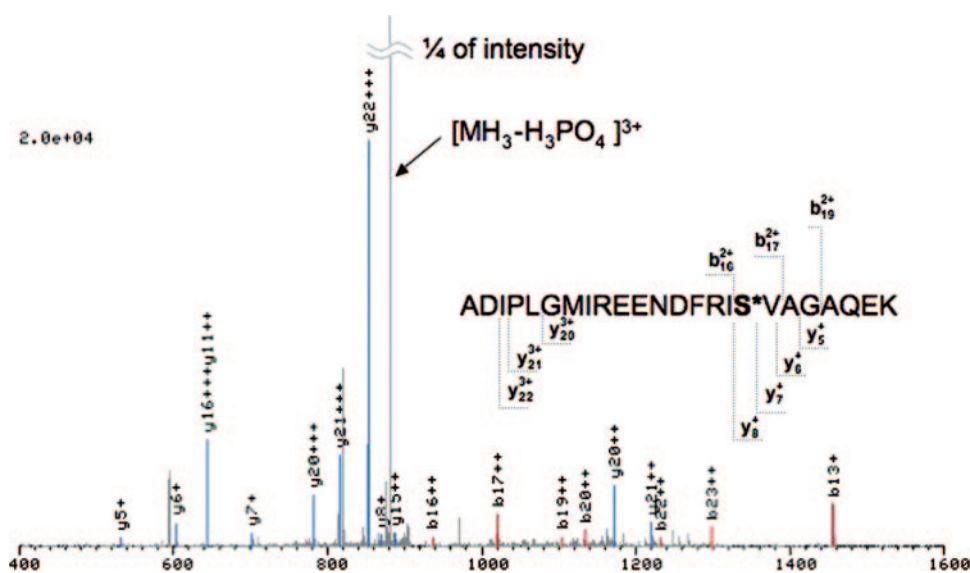


FIG. 4. MS-MS spectrum of phosphorylated peptide from wild-type HipA. The spectrum was obtained from HipA isolated from cells, and the result reflects in vivo-phosphorylated protein. The peak labeled  $[\text{MH}_3\text{-H}_3\text{PO}_4]^{3+}$  represents loss of phosphate from the precursor ion, and the intensity of this dominant peak was decreased fourfold for visualization.

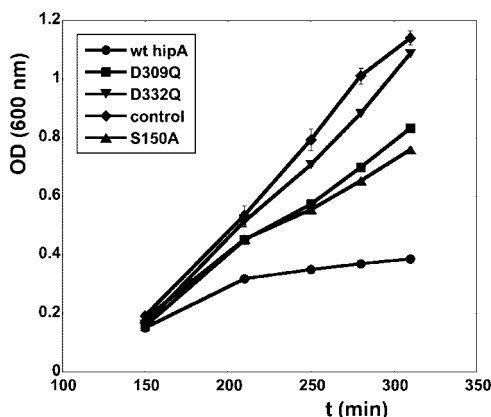


FIG. 5. Growth of strains expressing HipA and autophosphorylation-deficient mutants. Strains were grown in LB broth containing 50  $\mu$ g/ml chloramphenicol until they reached an OD<sub>600</sub> of approximately 0.250. HipA was then induced with L-arabinose (final concentration, 0.2%). The control strain carries the same vector with a truncated *hipA* gene. Means  $\pm$  standard deviations are shown. wt, wild type.

in the *in vitro* assay with purified HipA and [ $\gamma$ -<sup>32</sup>P]ATP. In the same experiment, HipA autophosphorylation was evident, and phosphorylation of casein by cyclic AMP-dependent casein kinase served as an additional positive control (not shown). Thus, HipA might be a kinase with fairly narrow substrate specificity or it might be able only to autophosphorylate.

**Effects of HipA expression on bacterial growth.** Expression of His-tagged HipA in KL319 (*hipA*) resulted in a near-complete cessation of growth within 45 min (Fig. 5). This agrees with previous reports on overexpression of HipA (12, 20, 35). This dramatic growth slowdown is reversible upon subsequent expression of HipB (20).

Expression of HipA mutants defective in the kinase active site or in the autophosphorylation site did not result in a substantial growth slowdown. Some of the strains expressing these mutant proteins grew only slightly more slowly than the control (Fig. 5). The essentially normal growth of cells expressing the mutant proteins strongly suggests that the autophosphorylating activity of HipA has a function in dormancy.

**Effect of HipA on antibiotic tolerance.** Expression of HipA causes an increase in tolerance to a variety of bactericidal antibiotics (12, 18, 35). We were interested to learn whether the kinase activity of HipA was essential for this function. HipA was induced in early, exponentially growing cultures with arabinose, and cells were then challenged with antibiotics at doses  $\geq 10$  times the MIC, which, according to our previous studies, are survived only by persister cells (17, 18, 32). Cells expressing HipA showed a high degree of tolerance to cefotaxime, a cell wall synthesis inhibitor (Fig. 6). The action of this antibiotic depends strongly on the growth rate of cells, and the high level of tolerance in this case is expected. Tolerance to cefotaxime was not affected in strains carrying the mutant forms of HipA that did not suppress growth. Next, cells were challenged with ofloxacin, a fluoroquinolone inhibitor of DNA gyrase and topoisomerase IV that has the ability to kill nongrowing cells (32). As expected, cells expressing HipA showed a high degree of protection from this antibiotic (Fig. 6). By contrast, the mutants defective in kinase activity were highly

susceptible to killing by ofloxacin. We then tested mitomycin C, another antibiotic that kills nongrowing cells by forming DNA adducts. Cells expressing HipA were almost completely protected from the action of this otherwise effective antibiotic (Fig. 6). The difference in persister level between a strain expressing HipA and the control was 10,000-fold, even greater than in the case of growth-dependent cefotaxim. Somewhat unexpectedly, mutants with abrogated kinase activity showed some degree of protection as well, suggesting that the function of HipA does not depend entirely on its kinase activity. Tobramycin, an aminoglycoside inhibitor of protein synthesis, had similar activity against the tested strains (Fig. 6). This result is rather unexpected, given that the action of tobramycin is growth rate dependent.

## DISCUSSION

Bacterial resistance to antibiotics has been studied extensively, and in most cases there is a reasonably good understanding of the underlying molecular mechanisms (23). In contrast, tolerance to antimicrobials, which was discovered at about the same time as resistance, is poorly understood, in spite of its considerable importance to human health. According to the CDC, 65% of all infections in developed countries are caused by biofilms, which exhibit multidrug tolerance largely due to the presence of persister cells (9, 15, 16, 22, 24, 32).

Persisters are phenotypic variants of the wild type, and their dormancy allows them to escape killing by bactericidal antibiotics. Several candidate genes have been implicated in persister formation. Isolation of persisters by lysing a culture with ampicillin or by physically sorting cells with decreased expression of green fluorescent protein enabled expression profiling, which pointed to overproduction of TA modules. Expression of several toxins, such as RelE (17), MazF (35), YgiU (31), and HipA (12, 18, 20, 35), has been shown to increase persister formation. TA modules are highly redundant, for example, there are at least 10 in *E. coli* (10) and >60 in *Mycobacterium tuberculosis* (13). Apart from TA modules, GlpD (glycerol-3-phosphate dehydrogenase) was recently identified as a potential persister component in *E. coli* (33).

The focus of the present study is the HipA toxin; we found it to belong to the phosphatidylinositol 3/4-kinase superfamily and examined its kinase properties.

Alignment of HipA with other members of the PI 3/4-kinase superfamily reveals conservation of all structural elements of the core catalytic domain and the key amino acids that contribute to the active-site formation and Mg<sup>2+</sup> binding. This strongly suggests that HipA is an active kinase, but beyond this general prediction, it is hard to predict the substrate specificity of HipA. The members of the PI 3/4-kinase superfamily that are most similar to HipA are the PI4KII kinases that appear to phosphorylate exclusively phosphatidylinositol, a lipid molecule that has not been found in bacteria. Given the diversity of substrates phosphorylated by members of the PI 3/4-kinase superfamily, it is conceivable that HipA family proteins are either lipid kinases with a distinct specificity or protein kinases.

HipA underwent autophosphorylation *in vitro* in the presence of ATP, and the purified HipA appeared to carry a single phosphate on Ser150. This showed that the protein is a serine

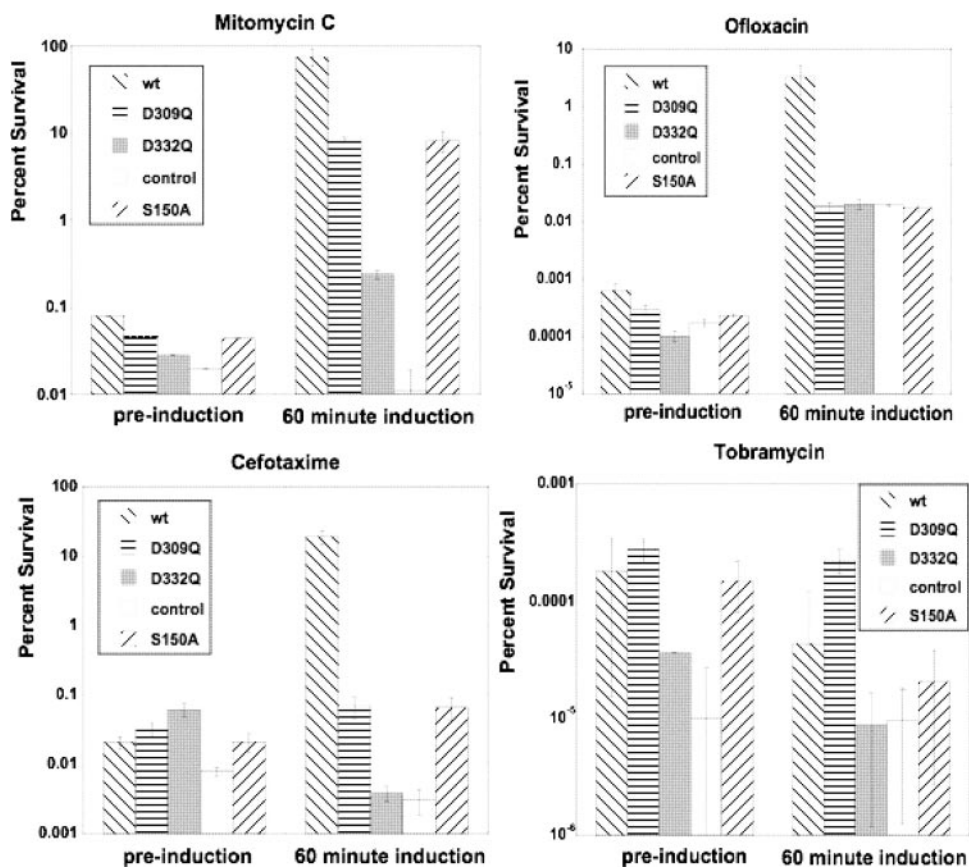


FIG. 6. Multidrug tolerance of cells expressing HipA. Strains KL319, KL320, KL321, KL322, and KL603 were cultured in LB to an  $OD_{600}$  of 0.3. Aliquots were removed and induced with L-arabinose for 60 min. Induced and control samples were challenged with antibiotic for 3 h. The final antibiotic concentrations were as follows: for cefotaxime, 100  $\mu\text{g/ml}$ ; for mitomycin C, 10  $\mu\text{g/ml}$ ; for ofloxacin, 5  $\mu\text{g/ml}$ ; and for tobramycin, 25  $\mu\text{g/ml}$ . Separate unchallenged samples were used to calculate total CFU/ml. Percent survival was calculated as the ratio of CFU/ml posttreatment divided by total CFU/ml. Means  $\pm$  standard deviations are shown. wt, wild type.

kinase and autophosphorylates *in vivo* as well. Attempts to find *trans*-kinase activity with universal, artificial kinase substrates casein and histone were unsuccessful, suggesting that HipA is a specific protein kinase or phosphorylates nonprotein substrates. Importantly, replacement of the active-site amino acids,  $\text{Mg}^{2+}$ -binding residues, and autophosphorylated Ser150 resulted in the loss of the ability of HipA to stop cell growth upon overexpression. This result shows that the kinase activity and phosphorylation of HipA are both required for its function in growth arrest.

HipA proteins with abrogated kinase activity were also found to be defective in their ability to produce persisters. Expression of wild-type HipA strongly protected cells from ofloxacin, a fluoroquinolone that has the ability to kill nongrowing cells, whereas the mutant proteins had no effect. Interestingly, HipA overexpression caused complete protection from mitomycin C, which forms DNA adducts. This was somewhat unexpected because dormancy alone probably would not prevent the chemical reaction of the drug with DNA. In this case, mutant HipA proteins also produced some degree of resistance to mitomycin, although the protection level was 10-fold lower than that with the wild type. Apparently, HipA might have some activity independent of its kinase activity and/or phosphorylation. Overexpression of HipA provided no

protection from tobramycin, an aminoglycoside antibiotic. This is surprising, given that activity of this aminoglycoside antibiotic depends on growth rate and cells expressing HipA are essentially nongrowing. Moreover, HipA has been reported to cause inhibition of DNA, RNA, and protein synthesis (20). Tobramycin, like other aminoglycosides, kills cells by interrupting translation, which yields truncated toxic peptides (other protein synthesis inhibitors, such as chloramphenicol, that simply stop translation are bacteriostatic). Conceivably, HipA-expressing cells have sufficient residual translation, and the inability of HipA to protect cells from tobramycin suggests that protein synthesis is not the target of this toxin.

TA modules are present on plasmids, where they constitute the maintenance mechanism, and on the chromosomes of most bacterial species. Toxins have been shown to inhibit translation by cleaving mRNA (RelE and MazF), inhibiting topoisomerase II (CcdB), and making holes in the membrane (29). To our knowledge, HipA is the first toxin that has been shown to possess protein kinase activity. Structural data indicated that the  $\zeta$  toxin of the  $\zeta\epsilon$  TA module encoded by plasmid pSM19035 is a P-loop-fold kinase, which is unrelated to the PI 3/4-kinase superfamily (26). Thus, the mechanism of HipA, which requires an active kinase domain and, apparently, autophosphor-

ylation, seems to represent a novel principle of bacterial toxin action.

Furthermore, HipA is the first bacterial member of the PI 3/4-kinase superfamily for which the phosphotransferase activity was experimentally demonstrated.

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