

A polyphosphate kinase 1 (*ppk1*) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects

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Contributed by Arthur Kornberg, November 6, 2006 (sent for review July 10, 2006)

Pseudomonas aeruginosa, of medical, environmental, and industrial importance, depends on inorganic polyphosphate (poly P) for a wide range of functions, especially survival. Mutants of PAO1 lacking poly P kinase 1, PPK1, the enzyme responsible for most poly P synthesis in *Escherichia coli* and other bacteria, are defective in motility, quorum sensing, biofilm formation, and virulence. We describe here multiple defects in the *ppk1* mutant PAOM5, including a striking compaction of the nucleoid, distortion of the cell envelope, lack of planktonic motility and exopolymer production, and susceptibility to the β -lactam antibiotic carbenicillin as well as desiccation. We propose that *P. aeruginosa* with reduced poly P levels undergoes ultrastructural changes that contribute to profound deficiencies in cellular functions.

carbenicillin | exopolymer | motility | desiccation | nucleoid

Polyphosphate (poly P), a linear chain of phosphate residues linked by phosphoanhydride bonds, is present in all cells and was likely present throughout evolution (1). Poly P is synthesized in prokaryotic cells from ATP by poly P kinases (PPKs) for which two families, PPK1 and PPK2, have been identified. PPK1 is responsible principally for the synthesis of poly P in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Vibrio cholerae*, *Helicobacter pylori*, *Bacillus cereus*, *Myxococcus xanthus*, and, as described here, *P. aeruginosa*. Homologous PPK1 amino acid sequences are in the databases of >40 organisms, including many bacterial pathogens (2). Knockout mutants of *ppk1* in several pathogens demonstrate phenotypes including responses to stresses, motility, and virulence (3–7) as well as developmental defects in other species (8, 9). The only apparent eukaryotic homolog is in the social slime mold *Dictyostelium discoideum* (2), the mutant of which exhibits growth and developmental phenotypes (10).

We describe here the construction and biochemical characterization of a *P. aeruginosa* *ppk1* knockout mutant, PAOM5, for which the motility, biofilm formation, burned-mouse, and ocular virulence phenotypes were reported in refs. 3, 6, and 11 and which, unlike the WT, is susceptible to predation by *D. discoideum* (10). Electron microscopy studies reveal significant differences in ultrastructure between the WT and mutant, including compaction of the nucleoid, withdrawal of the cytoplasm from the inner membrane, abnormal envelope structure, as well as a failure to produce exopolymer. Video microscopy studies show that the mutant is almost immotile in liquid medium, despite having about the same number of polar flagella. We also demonstrate that the *ppk1* mutant exhibits much reduced viability after exposure to a β -lactam antibiotic, carbenicillin, or the hyperosmolarity of desiccation.

Results

PPK1 Activity and poly P Levels Are Reduced but Not Abolished in the *ppk1* Mutant. *P. aeruginosa* PAO1 (WT) grown in rich (LB) medium exhibits high levels of PPK1 activity during mid-

exponential phase growth, almost wholly associated with the membrane fraction (Fig. 1). The levels decline as the culture enters stationary phase and continue to decline into stationary phase. ExopolyPase (PPX) activity, mostly solubilized after sonication, is inversely correlated with that of PPK1.

Three confirmed *ppk1* deletion/Tet insertion ($\Delta ppk1::Tet$) mutants were selected for enzymatic analysis and poly P determinations (Table 1). PPK1 activities in the membrane fractions of all three were reduced to <5% of the WT, whereas the soluble PPX activities (data not shown) were reduced by no more than 45%. Poly P levels in the WT and the three mutants were determined and checked for susceptibility to the yeast PPX, *Saccharomyces cerevisiae* PPX1 (12). Levels in the WT are \approx 2.8-fold lower than those determined in *E. coli* under the same conditions (13). All three mutants have considerably higher Poly P levels than the *E. coli* *ppk1* mutant, likely attributable to PPK2 and its homologs, apparently absent in the latter. One of these mutants, PAOM5 (M5), was selected for all further studies because it has intermediate levels of remaining PPK1 activity and poly P.

Addition of the amino acid analog serine hydroxamate to WT *E. coli* grown in minimal salts medium induces a stringent response, resulting in a >6-fold increase in poly P levels to \approx 13 nmol/mg (14). The same phenomenon was observed in PAO1 (WT) in the current studies, although the level was significantly higher, near 100 nmol/mg; the mutant had a 10-fold lower level of poly P compared with the WT (data not shown for either strain).

The mutant growth was comparable to that of the WT both in complex and minimal media. However, exponential phase mutant cells consistently were smaller than the WT under all conditions tested. The striking defects in motility, quorum sensing, and biofilm development became manifest only upon entry into stationary phase. When the mutant was complemented with plasmid pHEPAK11 expressing PAO1 *ppk1*, the PPK1 level increased 25-fold to 170,000 units/mg protein and these defects, as well as other phenotypes including virulence, disappeared (3, 6, 11).

Author contributions: C.D.F., M.H.R., S.S.K.L., R.G., J.H., P.J.W., M.R.W.B., and A.K. designed research; C.D.F., M.H.R., S.S.K.L., R.G., J.H., P.J.W., and M.R.W.B. performed research; C.D.F., M.H.R., S.S.K.L., R.G., J.H., P.J.W., M.R.W.B., and A.K. analyzed data; and C.D.F. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: poly P, polyphosphate; PPK, polyphosphate kinase; PPX, exopolyPase.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0609733104/DC1.

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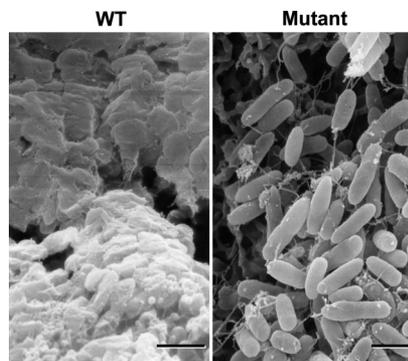


Fig. 3. Scanning electron micrographs show mutant biofilms fail to produce exopolymer. WT (Left) and mutant (Right) static biofilms were grown on nitrocellulose membranes in LB medium for 3 days (see *Materials and Methods*). (Scale bars: 1 μm .)

particularly β -lactams. The apparent defects in the mutant cell envelope suggested it might be deficient in responses to envelope-related stresses such as a β -lactam antibiotic. Late exponential phase WT and mutant cells were exposed to varying levels of the β -lactam antibiotic, carbenicillin (Cb). Four hours after exposure to 50 $\mu\text{g}/\text{ml}$, WT cells showed no difference in plate counts relative to untreated cells, whereas mutant cells exhibited a 4-fold drop (Table 2). At 100 $\mu\text{g}/\text{ml}$, mutant cells were \approx 200-fold more sensitive, a concentration only slightly inhibitory to the WT. This assay is significantly different from that for the minimum inhibitory concentration in that antibiotic is added to late exponential cells not exposed previously to the antibiotic.

Mutant Cells Fail to Survive Desiccation. The *E. coli ppk1* mutant is far more sensitive than the WT to stresses such as pH shock, oxidative agents, heat shock, and osmotic shock (16). Another environmental stress that all bacteria face in evolution is desiccation. Stationary-phase planktonic cultures of the WT and mutant were grown in LB medium; $\approx 10^6$ cells in 20 μl were placed on membranes and allowed to dry at room temperature. At time points up to 20 h, replicate samples were assayed for colony forming units (see *Materials and Methods*). Within 4 h, the mutant exhibits a 10-fold reduced survivability relative to the WT and 100-fold reduction after 11 h (Fig. 4 Left).

Inasmuch as bacteria in a hygroscopic biofilm likely show increased resistance to desiccation, failure of the mutant to produce exopolymer and a mature biofilm suggested it would be more sensitive to desiccation. After 24 h of growth in LB medium to produce static biofilms, cultures were assayed for desiccation survival over a period of weeks. After one week, survival of the

Table 2. Late-log phase mutant cells are sensitive to carbenicillin

Cb, $\mu\text{g}/\text{ml}$ *	Strain	Colony forming units, $\times 10^7$		Change, fold
		Time zero	4 hours	
50	WT [†]	73 \pm 25	220 \pm 26	+3
	Mutant	101 \pm 8	11 \pm 10	-9
100	WT	74	24	-3
	Mutant	58	0.1	-580

Drop in colony forming units 4 h after carbenicillin (Cb) addition to late-log phase cultures grown in LB medium. Data for each Cb concentration are obtained from independent experiments; those for 50 $\mu\text{g}/\text{ml}$ are the arithmetic mean \pm one standard deviation ($n = 3$).

*Final concentration.

[†]The growth of WT cells to 4 h after Cb addition was identical to that of untreated cells.

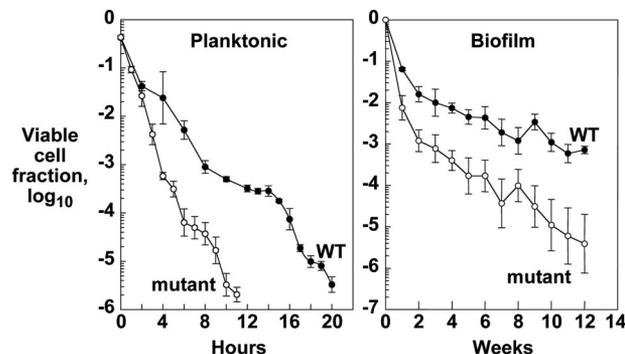


Fig. 4. Planktonic (Left) or biofilm (Right) mutant cells are sensitive to desiccation. Data are geometric means with error bars of 1 standard error of four to six replicate measurements (see *Materials and Methods*).

mutant decreased 10-fold relative to the WT and declined even more over the next 11 weeks (Fig. 4 Right).

Discussion

P. aeruginosa (PA01) is adapted to natural environments and considered an opportunistic pathogen in humans. The organism is unusual in the number and variety of substrates it can grow on, as well as its intrinsic and developed resistance to antibiotics. Poly P metabolism is crucial for PA01 as shown by null mutants of poly P kinase 1 (*ppk1*) with deficiencies in motility, quorum sensing, biofilm formation, and virulence (3, 6, 15). We report here that a *ppk1* mutant (PAOM5) also fails to produce exopolymer and survive exposure to the β -lactam antibiotic carbenicillin or desiccation. A remarkable compaction of the nucleoid and structural alterations in the cell envelope are further indications of the profound need for *ppk1* and/or poly P in PA01.

PPK1 of *P. aeruginosa* shares some genetic and biochemical features with its homolog in *E. coli* and also some distinct differences. In *E. coli*, the operon with the encoding gene and the exopolyPase encoded by *ppx* are organized in a colinear arrangement, whereas in PA01, the two adjacent genes are convergent and overlap and, thus, do not form an operon; the effects on transcriptional regulation of PA01 *ppk1* are not known. Most significantly, PA01, unlike *E. coli*, contains a family of three additional genes that encode enzymes for poly P synthesis and utilization that are nonhomologous to PPK1: PPK2, a cryptic PPK2, and PAP, a putative poly P-adenylate phosphotransferase (2), (K. Ishige and A.K., unpublished data). PPK1 of *E. coli* is a multifunctional enzyme with five demonstrated enzymatic activities, including the synthesis of poly P from ATP (the forward reaction) and the generation of ATP from poly P and ADP (the reverse reaction) (17), both of which have been demonstrated for PA01 PPK1, although the other three have not. In addition, PA01 PPK1 forms oligomers, likely octamers, in the presence of short-chain poly P (18). These, in turn, form filaments in the presence of ATP (K. Ishige, D. J. Griffith, and A.K., unpublished data), which is blocked by the addition of phalloidin (19), an inhibitor of actin filament formation.

PPK1 in PA01 is associated almost exclusively with the membrane fraction as in *E. coli*, and the WT activities in both organisms are similar. Poly P levels in minimal medium are similar in PA01 and *E. coli*, but under the stringent condition induced by the amino acid analog serine hydroxamate, PA01 levels are considerably higher (14). PPX activity in the growth cycle is inversely related to that of PPK1 in PA01 but directly related in *E. coli*. Thus, the two organisms, although closely related, have evolved significantly different patterns of poly P metabolism in response to their different habitats.

Three null mutants of PA01 ($\Delta ppk1::Tet$) showed marked reductions of PPK1 activity as in the *E. coli* $\Delta ppk1ppx::Kan$ mutant, <4% that of the respective WT (4). However, poly P levels in the PA01 mutants were reduced by 21–73% compared with the parental strain, due presumably to the additional enzymes of poly P synthesis; levels in the *E. coli* mutant are <5% of the WT. Of the three PA01 mutants isolated, one was selected for the studies presented here.

The PA01 mutant is deficient in all motilities on solid media: swimming, swarming, and twitching. Scanning electron microscopy showed both WT and mutant to have similar numbers of polar flagellae and normal type IV pili, as previously reported (11). The differences between mutant and WT motilities on agar of different densities indicated that the mutant defects might be surface-related and suggested that the flagellar-mediated swimming motility deficiency would be exacerbated under planktonic conditions for lack of a solid surface. Video microscopy showed the virtual lack of swimming motility in the mutant (<1% of WT), possibly due to structural defects in the flagellar motor or its function, although neither have been tested.

Transmission electron microscopy revealed major structural alterations in the nucleoid and envelope of the mutant as well as its smaller size, the first such exponential phase phenotypes observed in any *ppk1* mutant. Adaptation to stationary phase begins several generations before its onset (20), suggesting a role for PPK1 in the earliest stages of this process. By inspection, as in Fig. 2, nucleoids in the mutant are strikingly compacted and the cytoplasm is irregularly distributed within all cells. However, the digitally determined mean estimates for WT and mutant relative nucleoid areas in the micrograph cross-sections differ by <50% (41% and 26%, respectively, with considerable variations). This discrepancy is likely due to the difficulty in determining the precise location of the WT nucleoid because it is distributed within the cell with other components. It occupies most of the cell volume so the WT mean value reported is likely an underestimate.

This compaction resembles that observed in *E. coli* strains that overproduce the histone-like protein H-NS (21). That poly P can readily displace the bound DNA in isolated chromatin (K. Ishige and A.K., unpublished data) indicates the capacity of poly P to influence chromosomal structure and function. Further, DNA microarray data show large, global effects on gene expression in the mutant compared with the WT (K. Ishige and A.K., unpublished data). In the mutant, the arrangement of the inner and outer membranes and the interstitial periplasmic space appear distorted, perhaps as a consequence of the compacted nucleoid and its function. Although the defects in envelope ultrastructure and compacted nucleoid have not been demonstrated in any way to be related to the other observed mutant phenotypes, namely immotility and altered gene expression, respectively, such causal relationships are possible.

Mutant cells fail to produce a WT biofilm (3) for which two explanations are now possible. The lack of planktonic flagellar and twitching motilities, both required for *P. aeruginosa* biofilm formation (22), may be sufficient. In addition, the failure of the mutant to produce exopolymers, as observed in 3-day-old cultures by scanning electron microscopy, would result in a defective biofilm architecture. Poly P may regulate the expression of alginate, a principal component of *Pseudomonas* exopolysaccharides (23). A mucoid strain of PA01 has higher levels of poly P than a nonmucoid strain; these levels are greatly reduced in a mutant of *algR2*, which encodes a master regulator of alginate synthesis. Poly P may be a sensor for alginate production or the source of the GTP required for the synthesis of the GDP-mannose alginate precursor. The coordinated regulation of these two polymers may occur at the transcriptional level. Some *Pseudomonas* genes, whose products are critical for alginate biosynthesis, exhibit regulation by the alternate sigma factor σ^E

(24). Transcriptional analysis of *ppk1* in *P. aeruginosa* 8830, a stable mucoid strain isolated from cystic fibrosis patient sputum, suggested regulation by a σ^E -responsive promoter (25); this has not been tested in PA01.

The defects in the *ppk1* mutant described to this point indicate, as with the corresponding *E. coli* mutant, a failure to respond to a variety of stresses. Among them is the loss of the high level of resistance of PA01 to the β -lactam antibiotic, carbenicillin. This loss might be anticipated from the alterations in envelope structure and the involvement of the sigma factor RpoS, known from *E. coli* studies to depend on poly P (26), in responses to antibiotics. When assayed for sensitivity to carbenicillin, the mutant showed a several hundredfold decrease in survival at a drug concentration that has little effect on the WT. We are exploring the possibility of changes in the amount and/or reduction in cross-linking of peptidoglycan as cause(s) of the increased sensitivity. Other candidate mechanisms are changes in multi-drug efflux pumps or inducible lactamases.

Desiccation is a stress encountered by bacteria throughout evolution. After 10 h of drying, planktonic mutant cells lose viability >100-fold greater than the WT. The sensitivity of the PA01 mutant in static biofilms was 10-fold greater after one week and, by week 12, increased by >400-fold. Increasing hyperosmolarity may be the cause, because the *E. coli* *ppk1* mutant is 10- to 100-fold more sensitive than the WT when exposed to 2.5 M sodium chloride (16). Osmotic pressure induces the *E. coli* general stress response, reviewed in ref. 27, which is diminished in the *E. coli* *ppk1* mutant by the down-regulation of RpoS function (16, 26). Mutant cells exposed to desiccation thus may be unable to mount an adequate stress response for survival.

The reported studies show poly P (and/or PPK1) to be important for many functions and processes in *P. aeruginosa* PA01. The *ppk1* null mutant exhibits pleiotropic phenotypes including decreased virulence, defective motility and biofilm formation, and a compacted nucleoid. The mutant shares features with the *E. coli* *ppk1* mutant in responses to various stresses. Phenotypes in both organisms are manifest almost entirely upon entry into stationary phase and where tested are eliminated by complementation, indicating similar functions of PPK1 and/or poly P in these two organisms. However, differences between the two suggest that *P. aeruginosa* in its natural habitat and in infections, human or other, has evolved with special strategies to produce and use poly P. In the capacities that all cells must have to respond and adapt to environmental stresses, physical, chemical, nutritional, and biological, poly P plays a central role. The involvement of poly P in many cellular processes, including development and the fitness of both predator and prey, in all organisms tested demonstrates its widespread biologic importance.

Materials and Methods

Bacterial Strains, Media, and Growth. WT *P. aeruginosa* PA01 was a gift of Daniel W. Martin (East Carolina University, Greenville, NC). *P. aeruginosa* (WT PA01 and the derivative $\Delta ppk1::Tet$ mutant, PAOM5) cultures were grown at 37°C with aeration in LB medium or on *Pseudomonas* Isolation Agar (EM Science, Gibbstown, NJ). $\Delta ppk1::Tet$ derivative strains were selected with 250 $\mu\text{g/ml}$ tetracycline. Strains containing pHEPAK11 were grown in the presence of 300 $\mu\text{g/ml}$ carbenicillin. *E. coli* DH5 α was used for subcloning work.

Growth conditions for inducing poly P accumulations in Mops (3-(*N*-morpholino)propanesulfonic acid) minimal medium (Sigma, St. Louis, MO) due to phosphate (P_i) and amino acid downshifts were described in ref. 13. Briefly, cells were grown overnight with glucose (4 mg/ml) as a carbon source, 2 mM P_i (as K_2HPO_4), and 20 $\mu\text{g/ml}$ of all 20 aa followed by at least 1:100 dilution in fresh Mops with glucose (4 mg/ml), 0.1 mM P_i , and 2 $\mu\text{g/ml}$ amino acids. Induction of poly P accumulations by

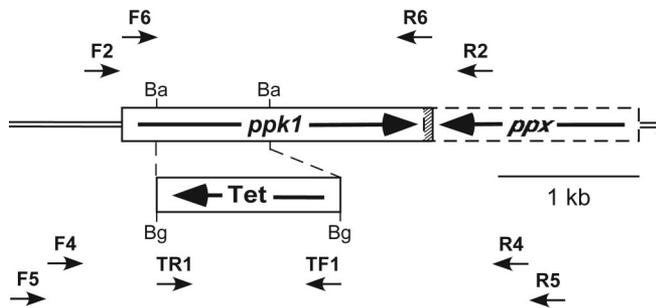


Fig. 5. Chromosomal organization of *ppk1* *ppx* and construction/verification of the $\Delta ppk1::Tet$ mutation. In PAO1, *ppk1* (PA5242; 2,210 bp) and *ppx* (PA5241; 1,520 bp) are convergently transcribed, and their ORFs overlap by 14 bp at the 3' ends, denoted by the hatched area. Construction of the mutation is described in *Materials and Methods*. Tet denotes the tetracycline resistance-encoding gene cassette from pDMB100 (28). Ba and Bg indicate BamHI and BglII endonuclease restriction sites, respectively. The small arrows denote primers (described in SI Table 3) used in cloning *ppk1* and in the genetic verification by PCR of the mutation (see *Materials and Methods*).

addition of the amino acid analog serine hydroxamate (SHX) to Mops medium to effect amino acid starvation was described in ref. 14. Briefly, cells in medium with 0.4 mM P_i and 40 $\mu\text{g/ml}$ of all 20 aa were grown to an OD_{540} of ≈ 0.2 , and SHX (0.5 $\mu\text{g/ml}$) was added.

Construction of $\Delta ppk1::Tet$ Mutant and Complemented Strains. A 2.45-kb DNA fragment containing the chromosomal *P. aeruginosa* PAO1 *ppk1* gene was amplified by PCR with primers F2 and R2 (Fig. 5; for primer information see SI Table 3) and cloned into the HindIII and XbaI sites in pBluescriptII SK(+) to generate pSKPAK1. The PCR fragment starts at 107 bp upstream of the A in the ATG translational start codon (bp 150 in GenBank locus AB007587) and ends 115 bp downstream of the A in the TGA stop codon, not as reported in ref. 11. A 0.8-kb BamHI fragment in the amino terminus half of the *ppk1* ORF in pSKPAK1 was replaced with a 1.3-kb BglII fragment containing the tetracycline resistance-encoding gene cassette from pDMB100 (ref. 28; kindly supplied by Daniel W. Martin) in the opposite transcriptional orientation to create pPAK1TC4. WT PAO1 was electroporated with pPAK1TC4, and transformants were selected on LB plates with tetracycline (250 $\mu\text{g/ml}$). Double cross-over recombination of the mutation was verified first by genomic PCR with combinations of primers F4, F5, R4, R5, TF1, and TR1 and, subsequently, by biochemical determinations of PPK1 activity and poly P levels. Mutant PAOM5 was complemented by transformation with a plasmid overexpressing *P. aeruginosa* *ppk1* under *tac* promoter control, pHEPAK11, constructed by PCR amplifying the *ppk1* gene in plasmid pPPK02F (ref. 29; kindly supplied by Toshikazu Shiba, Fujirebio, Inc., Tokyo, Japan) by using primers F6 and R6 and inserting the resulting product into the HindIII and EcoRV sites in the medium copy number *E. coli*-*P. aeruginosa* shuttle vector pMMB66H (30). Transformants were selected on LB agar with 300 $\mu\text{g/ml}$ carbenicillin.

Assays for PPK1 and PPK Activities and Poly P Levels. PPK1 activity was measured as the conversion of [^{32}P - γ]ATP to poly P by thin-layer chromatography analysis as described in ref. 31. PPK activity was measured as the conversion of poly P to P_i as described in ref. 32. Poly P was extracted by the glassmilk method and levels were determined by conversion to ATP by *E. coli* PPK1 followed by conversion to light in a luciferase reaction and quantification in a TopCount NXT microplate scintillation and luminescence counter (Packard Instruments (PerkinElmer), Boston, MA) as described in ref. 33.

Scanning Electron Microscopy. Strains were grown in LB medium to an OD_{600} of 0.6. Cells (10^9) were seeded onto individual nitrocellulose membranes in 24-well culture plates. The inoculated plates were incubated at 37°C and examined at 12, 24, 48, and 72 h. The membranes were removed from the plate and fixed in 2.5% glutaraldehyde and 0.15% ruthenium red for 3 h at 40°C. They were then rinsed in PBS solution, followed by postfixation in 1% osmium tetroxide/0.1 M sodium cacodylate (pH 6.5)/0.2% ruthenium red for 2 h at room temperature. The membranes were dehydrated in a series of graded ethanol solutions, dried with a critical-point dryer (Tousimis, Rockville, MD) with carbon dioxide, and mounted on aluminum stubs with silver paste. These were dried for 3 h, and then coated with gold-palladium with a Polaron cool-sputter coater (Quorum Technologies, Newhaven, U.K.). The membranes were examined in a scanning electron microscope (JSM-6300F; JEOL USA, Peabody, MA) at 15 kV.

Transmission Electron Microscopy and Negative Staining. A suspension of cells grown overnight in LB medium was inoculated into fresh LB medium and grown to an OD_{600} of 0.6. The log-phase bacteria were fixed in 2.5% glutaraldehyde in PBS, embedded in 1% agar, and postfixed in 1% osmium tetroxide. The fixed samples then were dehydrated through a series of graded ethanol solutions. After further dehydration in propylene oxide, the specimens were embedded in Spurr's low-viscosity embedding resin (Ted Pella, Inc., Redding, CA) and sectioned. For negative staining, uranyl acetate and lead citrate were added and reinforced with evaporated carbon. The specimens were examined with a transmission electron microscope (JEM-1200EXII; JEOL USA) at 80 kV. High-resolution digitized images were used to determine the cross-sectional areas of individual nucleoids relative to those of the corresponding whole cells, expressed as percentages, by using the associated size bar. Areas were calculated by the software program Volocity, version 3.7.0 (Improvision, Ltd., Coventry, U.K.) in the Cell Science Imaging Facility in the Beckman Center at Stanford University. Although Volocity used pixel contrast to define the apparent nucleoid boundaries in all cells, subjective determination was made in some cases, particularly in WT cells. Only whole, undividing cells were used, regardless of orientation, as long as some part of the nucleoid was observable. The values reported are arithmetic means ± 1 standard deviation.

Planktonic Motility Studies. A suspension of cells grown overnight in LB medium was inoculated into fresh LB medium and grown to an OD_{600} of 0.6. The cultures were spun down and resuspended in LB medium to a concentration of 1.6×10^8 cells per ml. The bacterial suspension (8 μl) was spotted onto a glass slide, covered with a no. 1.5 (22 \times 22 mm) glass coverslip and sealed with clear nail lacquer. The chambered bacteria were inverted and allowed to settle to minimize the influence of convection. Random microscopic fields were selected, and serial video images were captured for 10 sec. Bacteria were visualized with a $\times 100$ phase-contrast oil objective on a Diaphot (Nikon, Tokyo, Japan) inverted microscope. Serial video images were captured at 20 frames per second with a LG-3 frame-grabber (Scion, Frederick, MD), a Dage CCD-72 camera and a Digital Image Processor DSP-2000 (Dage-MTI, Michigan City, IN). The images were captured on a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) using Scion Image 1.62 software (Scion); a freeware version of NIH-Image to view the video can be found at <http://rsb.info.nih.gov/nih-image>. Estimates of bacterial motility by image analysis, as described in ref. 34, were obtained by using Metamorph software version 4.3 (Universal Imaging, Downingtown, PA). Briefly, serial images of the 200-frame animation sequences were subtracted by using automated Metamorph journals in the following order: the second from the

first, the third from the second, to the 200th frame. The subtractions were binarized, and the pixels were counted in every frame of the sequence. Motility in the sequence was expressed as the total number of pixels remaining after the subtraction of two serial video images and then was normalized for bacterial density. The WT is arbitrarily assigned a motility value of 100%.

Carbenicillin-Sensitivity Assays. Strains were grown overnight in LB and subcultured into replicate flasks of LB medium to a starting OD₅₅₀ of ≈ 0.04 . Approximately one generation before entry into stationary phase, at an OD₅₅₀ of ≈ 0.8 , the indicated concentrations of carbenicillin were added to the flasks. Survivability initially was assessed by OD, then by duplicate colony plate counts of serial dilutions in LB medium. The standard error of plating was $<20\%$.

Desiccation Survival Assays. Strains were grown in LB medium in baffled conical flasks. At 24 h, the ODs were measured and the cultures diluted to 5×10^7 cells per ml. A 45-mm diameter, 0.2- μm pore size, hydrophobic edge, cellulose nitrate membrane (Sartorius, Goettingen, Germany), prewashed in LB medium (10 ml) and dried, was placed in a 9-cm diameter, triple-vent Petri dish. Cells (10^6) ($20 \mu\text{l}$) were spotted onto the center of the

membrane, which was dried at room temperature (20–24°C). At sampling intervals of every hour for the mutant and every 2 h for the WT up to 12 h, then every hour, the membranes were rehydrated with 100 μl of saline solution and the bacteria were removed with a cell scraper. The scraper was rinsed in 5 ml of saline solution to which the scraped membrane was added and vortexed for 15 sec. Colony counting of the suspensions, either to inoculate the membranes or recovered from them, was performed on dilutions in saline solution plated onto LB agar by using a combination of 20- μl drops and spread plates (200 μl). The plates were incubated at 37°C for 24 h and colonies counted. The fraction of recovered cfu was calculated as the colony counts recovered from the membranes divided by the cfu of the inoculum.

We thank Drs. Daniel W. Martin and Toshikazu Shiba for plasmids, Kitty Lee at Stanford University's Cell Science Imaging Facility for expert assistance with the nucleoid area analyses, and Drs. A. Dale Kaiser and I. Robert Lehman for helpful comments on the manuscript. This work was supported by the National Institutes of Health (to C.D.F., M.H.R., and A.K.), the U.K. Department of Health (to P.J.W. and M.R.W.B.), and The Ellison Medical Foundation for funding (to A.K.) to support M.R.W.B. during his sabbatical stay in the A.K. laboratory.

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