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Regulation of the *Escherichia coli* water channel gene *aqpZ*

(bacteria/water transport/osmoregulation/aquaporins)

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ABSTRACT **Osmotic movement of water across bacterial cell membranes is postulated to be a homeostatic mechanism for maintaining cell turgor. The molecular water transporter remained elusive until discovery of the *Escherichia coli* water channel, AqpZ, however the regulation of the *aqpZ* gene expression and physiological function of the AqpZ protein are unknown. Northern analysis revealed a transcript of 0.7 kb, confirming the monocistronic nature of *aqpZ*. Regulatory studies performed with an *aqpZ*::*lacZ* low copy plasmid demonstrate enhanced expression during mid-logarithmic growth, and expression of the gene is dependent upon the extracellular osmolality, which increased in hypoosmotic environments but strongly reduced in hyperosmolar NaCl or KCl. While disruption of the chromosomal *aqpZ* is not lethal for *E. coli*, the colonies of the *aqpZ* knockout mutant are smaller than those of the parental wild-type strain. When cocultured with parental wild-type *E. coli,* the *aqpZ* knockout mutant exhibits markedly reduced colony formation when grown at 39°C. Similarly, the *aqpZ* knockout mutant also exhibits greatly reduced colony formation when grown at low osmolality, but this phenotype is reversed by overexpression of AqpZ protein. These results implicate AqpZ as a participant in the adaptive response of *E. coli* to hypoosmotic environments and indicate a requirement for AqpZ by rapidly growing cells.**

Although transport of water across cell membranes is fundamental to life, discovery of the aquaporin family of water channel proteins provided the first molecular explanation for this process (1). Presently eight aquaporins have been reported in mammals where they contribute to multiple physiological functions and clinical disorders (see review, ref. 2). Aquaporins have also been described in amphibia (3) and insects (4). Several dozen aquaporin DNAs have been found in plants, where they are believed to encode proteins involved in diverse functions, such as mediation of transpiration, maintenance of cell turgor, and inhibition of self pollination (5).

The first known prokaryotic aquaporin water channel gene, *aqpZ,* was identified in wild-type *Escherichia coli*. The functional characterization of AqpZ demonstrated water-selectivity with negligible glycerol transport (6), whereas the sequence-related protein GlpF exhibited the opposite specificity (7). The genomic organization of *aqpZ* has been defined in *E. coli* (8–10), and Southern blot analysis demonstrated cross-hybridizing bands in multiple Gram-negative and Gram-positive bacteria (6). Genomic sequencing has uncovered *aqpZ*-like genes in *Haemophilus influenzae* (11); *Mycoplasma genitalium,* a prokaryote lacking a cell wall (12); and *Synechocystis* sp. PCC6803, a cyanobacterium permanently living in fresh water (13). The genetic mechanisms for regulating *aqpZ* and the physiological functions of the AqpZ protein remain undefined.

Movement of water across the cytoplasmic membrane is believed to be a part of the osmoregulatory response by which prokaryotes adjust cell turgor within the range needed for growth or survival (14, 15). Because of similarities shared with the plant and animal kingdoms, bacteria provide a model system for biochemical and genetic aspects of osmoregulation. *E. coli* is the most extensively studied prokaryotic osmoregulatory model, and multiple genes are controlled at the transcriptional level (14, 15). *E. coli* responds to hyperosmolar environments by accumulating organic osmolytes (compatible solutes) in the cytoplasm and by extruding inorganic ions. Hypoosmotic downshock results in a sudden influx of water into the bacterial cells, leading to an increase of intracellular pressure (14). In these conditions, bacteria remain viable and dissipate excessive turgor by jettisoning small osmolytes (16, 17).

Although various transporters have been postulated to respond to hyperosmotic stress, little is known about the molecular identities of genes activated by hypoosmotic stress. Neither the normal roles of bacterial water channels nor the consequences of the AqpZ null phenotype have been explored in the adaptive response to osmotic stress. Here we describe regulation of *aqpZ* gene expression and report a potential physiological function for the AqpZ protein.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* K-12 strains DH5α (18) and MC4100 (19) were used for cloning and expression studies. The *E. coli* K-12 *rpoS* mutant strain ZK1000 and its *rpoS*$^+$ parent ZK126 (20) were kindly provided by Roberto Kolter, Harvard Medical School. The *E. coli* K-12 strain GM37 carries the osmoregulated Φ(*proV-lacZ*)(Hyb2)(λplacMu15) gene fusion in its chromosome (21). The *E. coli* MM294 (22) and SY327λ*pir* (23) strains were used as wild-type and host for the mutant construction. The *E. coli* K-12 strains MM294-strept^R (parental wild-type) and MM1211 (knockout, *aqpZ*::*lacZ-kan*) were prepared in this study. The M9 minimal medium (24) was supplemented with 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2% casamino acids. Media included ampicillin, kanamycin, and streptomycin at final concentrations of 50, 15 and 25 μg/ml.

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Abbreviations: cfu, colony-forming units, β-gal, β-galactosidase; LB, Luria–Bertani.

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Osmolalities of the media were checked with a vapor pressure osmometer (Wescor 5100).

Plasmid Constructions. An *aqpZ*::*lacZ* gene fusion was prepared by inserting a promoterless *lacZ-kan* cassette (25) in frame with the fourth codon of an *aqpZ* coding region carrying 532 bp of its 5′-flanking region. The gene fusion construct was inserted into the *Eco*RV site of pcDNA I/amp (Invitrogen) yielding pANG532; pANG-PL was derived by deleting the 532 bp of the *aqpZ* 5′-flanking region. The suicide vector pZSV was derived from pCVD442 (23) by subcloning the *aqpZ* coding region surrounded by 1.774 kb of 5′-flanking and 0.7 kb of 3′-flanking DNA and mutated by the insertion of a *lacZ-kan* cassette into the *Bsm*I site. The pBSK′ plasmid employed as control in the phenotypical studies was derived from pGC94q by releasing the *Pst*I/*Kpn*I fragment containing the whole *aqpZ* gene.

Northern Blot Analysis. Total RNA (25 μg) extracted from MC4100 harvested at the mid-logarithmic phase of growth (OD_{578} = 0.7) was electrophoresed, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *aqpZ* coding region.

***β*-Galactosidase Assays.** For the growth-phase control studies, DH5α or MC4100 cells carrying pANG532 were grown overnight and used to inoculate fresh M9 medium. Samples were harvested at intervals during exponential and stationary phases of growth, immediately diluted with M9, and plated to determine the number of colony forming units (cfu). At the same time, samples were pelleted, washed once with ice-cold Z-buffer (21), resuspended to OD_{578} = 0.5, and assayed for β-galactosidase (β-gal) activity, expressed as μmol of 2-nitrophenyl-β-D-galactopyranoside cleaved per min per mg of protein (21). For studies of osmoregulation, fresh M9 media of various osmolalities were inoculated with overnight cultures of pANG532 or pANG-PL transformed into MC4100 or DH5α cells. When cultures reached OD_{578} = 0.7, aliquots were removed for cfu and β-gal assays.

Construction of the aqpZ Null Mutants. The *E. coli* MM294-strept^R strain was selected by plating a high-density suspension of parental strain MM294 on Luria–Bertani (LB)-streptomycin plates. Insertional disruption of *aqpZ* in MM294-strept^R was performed by allelic exchange (23). *E. coli* SY327λ *pir* was electrotransformed with the plasmids pZSV, and transformants were selected with ampicillin. The plasmid was introduced into MM294-strept^R from SY327λ *pir* by filter mating with selection for streptomycin and ampicillin resistance. Resulting exconjugates were also confirmed by kanamycin resistance. A resulting *aqpZ* knockout colony, MM1211, was confirmed by genomic Southern hybridization to contain the *lacZ-kan* cartridge in the *aqpZ* ORF. Southern analyses were performed at high stringency by hybridizing genomic DNAs with a DIG-labeled probe containing the entire *aqpZ* coding region.

Phenotypic Characterizations. The cellular morphology of the MM1211 (knockout) was investigated by Gram staining of exponential and stationary phase liquid cultures. The thermic phenotype was determined by coculturing MM294-strept^R (wild-type) and MM1211 at 25°C or at 39°C. Each series of experiments was repeated three to five times, and all cultures were tested in duplicate. Osmotic phenotypes were characterized as a time course of the ratio between the cell viabilities (cfu/ml of culture) of MM294-strept^R and MM1211 cocultured in M9 minimal medium containing streptomycin at different osmolarities. A starting culture was prepared by inoculating 1.0 ml of M9 medium with single overnight colonies of MM294-strept^R and MM1211 and then incubating for 3 hr at 37°C. The starting culture was used to inoculate M9 media at 80, 240, and 975 mosM that were simultaneously incubated at 37°C. Samples from each culture were collected, diluted, and plated on LB-streptomycin and LB-kanamycin to determine the cfu. The same protocol was followed in control experiments by coculturing pBSK′- and pGC94- transformed MM294-strept^R and MM1211 in M9-ampicillin media (see above).

RESULTS

Growth Regulation of *aqpZ* Expression. Northern blot analysis was undertaken to evaluate expression of *aqpZ* during the mid-logarithmic phase of growth. RNA from *E. coli* MC4100 cells was hybridized with a probe corresponding to the entire *aqpZ* ORF, and a distinct 0.7-kb transcript was identified (Fig. 1*A*).

Regulatory studies were performed with pANG532, carrying the *aqpZ*::*lacZ* gene fusion inserted 12 bases downstream from the *aqpZ* ATG start codon. Because the 532 bp 5′-flanking region including the *aqpZ* promoter and *cis*-regulatory region were expected to drive *lacZ*, the plasmid pANG-PL that lacks the *aqpZ* 5′-flanking region was used as a control. When *E. coli* strains DH5α or MC4100 were transformed, pANG532 led to expression of an active hybrid β-gal with four AqpZ residues at the NH_2 terminus. In each of four separate experiments, β-gal activity peaked during the mid-logarithmic phase of growth ($OD_{578} \approx 0.75$), and declined to a lower level during late stationary phase (Fig. 1*B*). In contrast, negligible β-gal activity was detected in cells carrying pANG-PL.

Osmotic Regulation of *aqpZ* Expression. *E. coli* were grown at mid-logarithmic phase in a series of steady-state osmotic conditions. Addition of NaCl to the M9 medium significantly reduced the level of β-gal activity measured with cultures of DH5α cells carrying pANG532. Up to a 30-fold decrease in β-gal activity was noted when cultures grown at 80 and 1,530 mosmol/kg H_2O were compared. Cell survival is reduced at high osmolalities, but the reduction in β-gal activity was still striking after normalizing for cell viability at all osmolalities (Fig. 1*C*). Similar results were obtained with KCl and with a different *E. coli* strain, MC4100 (not shown). Downregulation of *aqpZ* by high external osmolality was not an artifact due to reduced copy numbers of pANG532, because no significant changes in the amount of this plasmid were found (not shown). In contrast, GM37, an *E. coli* strain carrying the *proU*::*lacZ* fusion gene in its chromosome (26), was studied in parallel and demonstrated the anticipated positive induction by hyperosmolar exposure (Fig. 1*C*).

Genetic Analysis of the *aqpZ* Null Mutant. Disruption of the *aqpZ* gene in the *E. coli* chromosome was undertaken by inserting a *lacZ-kan* cassette into the coding region, resulting in the *aqpZ* knockout strain, MM1211 (Fig. 2*A*). The construct was verified by genomic Southern analysis of the mutant and three wild-type *E. coli* strains: MC4100, MM294, and MM294-strept^R. When chromosomal DNAs digested with *Bgl*I and *Ava*II were probed with the *aqpZ* ORF, an intense band of about 560 bp was detected with the wild-type DNAs, whereas a band of 420 bp was observed in the MM1211 lane (Fig. 2*B*). The weak band detected at 1.2 kb represents the fragment of DNA between the two *Bgl*I sites located 1,108 bp upstream and 109 bp downstream of the *aqpZ* ATG start codon. The 109-bp segment of the *aqpZ* ORF was not physically modified by the insertion. The construct was further verified by PCRs using primers overlapping regions of the *aqpZ* ORF located 225 bp upstream and and 135 bp downstream of the mutational insertion (not shown).

Phenotypic Analysis of the *aqpZ* Null Mutant. In basal conditions, the MM1211 knockout strain grew in rich media (LB) and in minimal media (M9), demonstrating that disruption of the *aqpZ* gene is not lethal. Nevertheless, most MM1211 colonies were smaller than MM294-strept^R, the parental wild-type (Fig. 3*A*). Few small colonies (1.38 ± 0.7%; n = 4) were present on the MM294-strept^R plates, and only a few large colonies (1.42 ± 0.6%; n = 4) were present on the MM1211

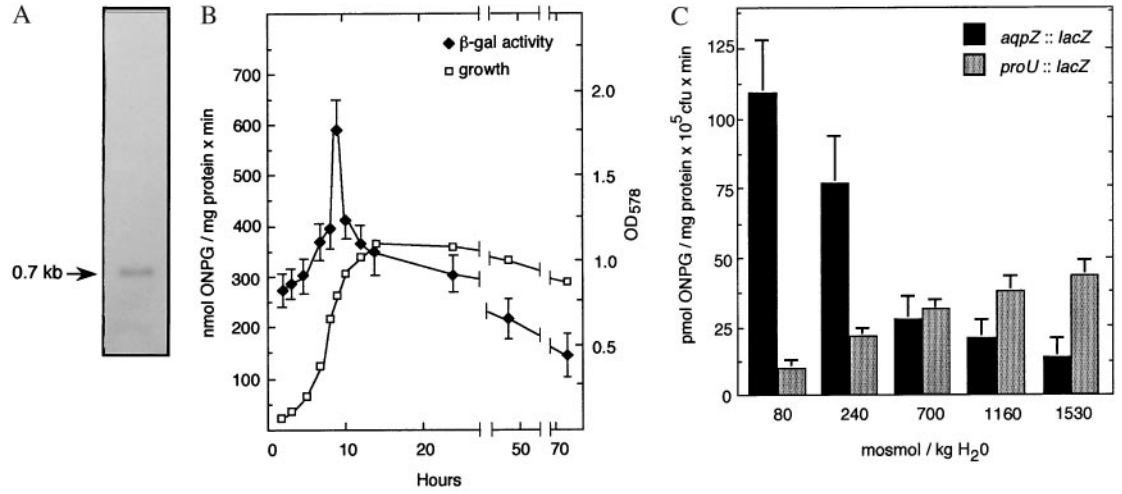

FIG. 1. Expression of *aqpZ* in *E. coli.* (*A*) Northern analysis of total RNA (25 μg) extracted from *E. coli* MC4100 cells in the logarithmic phase of growth and hybridized with a ^{32}P-labeled *aqpZ* coding sequence (see *Materials and Methods*). A single 0.7-kb signal was observed (arrow). (*B*) Expression of *aqpZ* during growth. *E. coli* DH5α cells transformed with pANG532 bearing the *aqpZ*::*lacZ* fusion gene, were evaluated for β-gal activity during growth in M9 medium (240 mosM) and assessed by OD_{578} (see *Materials and Methods*). Shown are the mean and standard error for four separate experiments. (*C*) Effect of the steady-state extracellular osmolality on the transcription rate of *aqpZ*. *E. coli* DH5α cells transformed with pANG532 when grown in M9 media made hypotonic (80 mosmol/kg H_2O) by dilution with water or made hypertonic (700, 1160, and 1530 mosmol/kg H_2O) by adding NaCl. β-Gal activities were measured at the mid-logarithmic growth (OD_{578} = 0.7) and normalized to the number of cfu. Solid columns represent *aqpZ*::*lacZ* expression. Stippled columns represent GM37, an *E. coli* strain carrying a single *proU*::*lacZ* hybrid gene inserted into its chromosome, studied in parallel as a positive control for hyperosmolar induction. Shown are the mean and standard error for three to five separate experiments.

plates. This phenotype was observed on selective as well as nonselective plates (not shown). Likewise, no apparent differences in cell morphology were observed with Gram stain in exponential or stationary phase liquid cultures grown in LB or M9 media (not shown).

To search for a relative growth phenotype, colony formation of mixed cultures of MM1211 (knockout) and MM294-strept^R (wild-type) strains were compared at a temperature supporting a reduced growth rate (25°C) and at a temperature where the growth rate is maximum (39°C). While at 25°C the MM1211 strain exhibited 80% of colony formation of MM294-strept^R strain, the MM1211 strain exhibited <15% of the colony formation at 39°C, indicating that expression of the *aqpZ* gene is linked to the rate of growth.

To further demonstrate the phenotypes, the viabilities of MM294-strept^R (wild-type) and MM1211 (knockout) strains were compared in competition by coculturing in M9 media at different osmolarities. Striking reductions in the viability of the MM1211 strain relative to MM294-strept^R were observed at 80 and 240 mosM, whereas no significant decrease in the cell viability was found at 975 mosM (Fig. 3*C*). The reduced growth in hypotonic media was ameliorated when the MM1211 strain was rescued by transformation with a high copy number plasmid (pGC94) containing the *aqpZ* gene (Fig. 3*C*).

DISCUSSION

Discovery of the aquaporin family of membrane water channels (1) has yielded a molecular understanding of membrane water transport and has led to a new set of challenges: (*i*) determination of the aquaporin protein structure at high

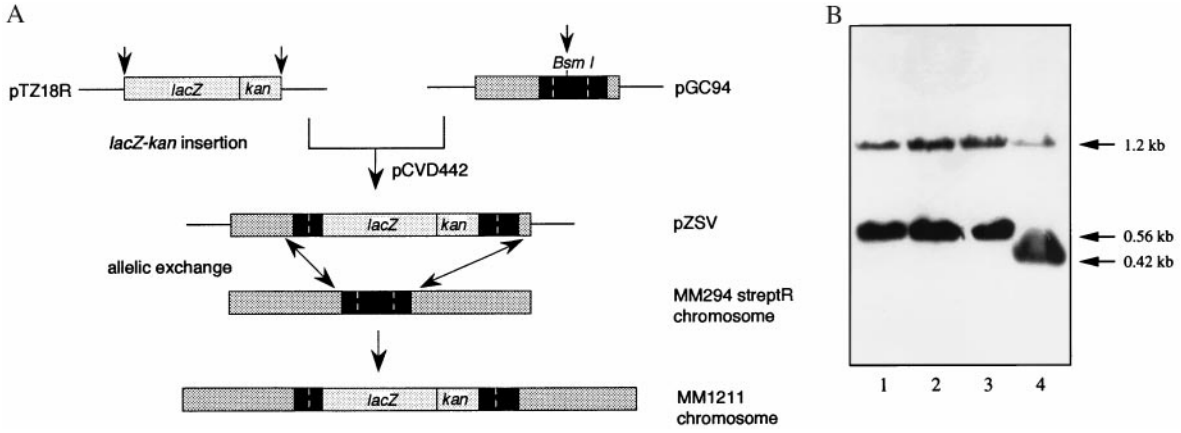

FIG. 2. Genetic analysis of *aqpZ* knockout in *E. coli. (A)* Construction of an *aqpZ*::*lacZ* transcriptional fusion gene on the *E. coli* MM294-strept^R chromosome. The *aqpZ* gene is represented by the solid rectangle with the ORF bracketed by vertical dashed lines. A promoterless 3.9-kb *lacZ-kan* cartridge was inserted into the *Bsm*I site of the *aqpZ* gene carried by the pZSV suicide plasmid. This yielded a *aqpZ*::*lacZ-kan* fusion gene that was used to replace the *aqpZ* gene in the wild-type chromosome (MM294 strept^R) by a double allelic recombination. The resulting *aqpZ* knockout strain, MM1211, contains the intact *aqpZ* promoter linked to *lacZ-kan.* (*B*) Southern analysis of disrupted *aqpZ* gene. Genomic DNAs from wild-type *E. coli* strains MC4100 (lane 1), MM294 (lane 2), MM294 strept^R (lane 3), and the *aqpZ* gene disruption construct MM1211 (lane 4) were cut with *Bgl*I and *Ava*II and electrophoresed into agarose gel. The DNA fragments were transferred to a membrane and hybridized at high stringency with a probe containing the entire coding region of *aqpZ* labeled with Digoxigenin.

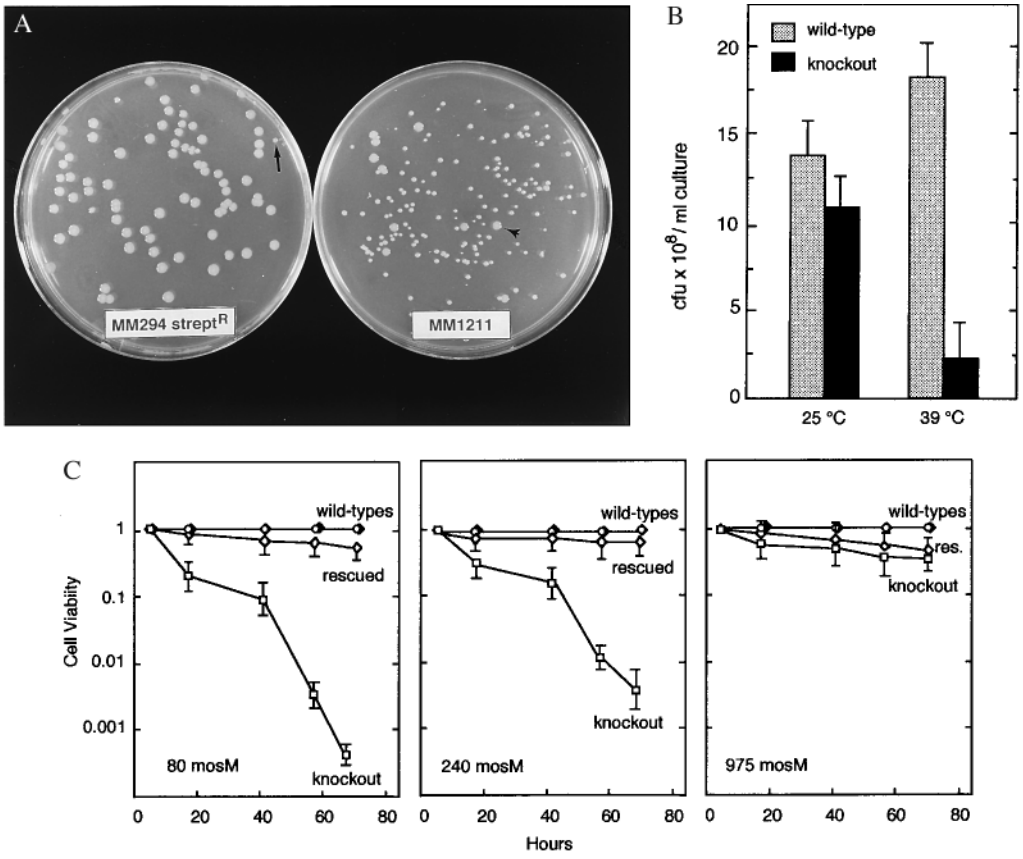

FIG. 3. Phenotypic analysis of *aqpZ* knockout in *E. coli. (A)* Overnight M9 cultures of MM294-strept^R (wild-type) or MM1211 (knockout) were diluted and plated on LB-agar containing streptomycin or kanamycin and incubated at 37°C for 20 hr. Most of the colonies observed in the MM1211 plate are significantly smaller than those seen in the MM294-strept^R plate. A few large colonies are indicated on MM1211 plate (arrowhead), and few small colonies are indicated on the MM294-strept^R plate (arrow). (*B*) Effect of culture temperature on the viability of the *aqpZ* knockout in *E. coli*. Starting cocultures containing equivalent numbers of cfu from MM294-strept^R and MM1211 were used to inoculate M9 media (240 mosM) and incubated at 25 or 39°C. Cell viabilities (cfu $\times$ 10^8/ml culture) were calculated after a 24 hour incubation. Shown are the mean and SEM for three or four separate experiments. (*C*) Effect of culture medium osmolality on the relative viability of *aqpZ* knockout in *E. coli*. A coculture of MM294-strept^R and MM1211 or a coculture of MM294-strept^R-pBKS′ and MM1211-pGC94 (*aqpZ* rescued) were grown in different osmolalities (80 mosM, 240 mosM, or 975 mosM) at 37°C. At the designated intervals, three or four aliquots were removed from each culture for growth and cell viability measurements. Values for MM1211 were normalized to the MM294-strept^R wild-type viabilities arbitrarily set at 1.0 (note log scale). Shown are the mean and SEM for quadruplicate determinations made in each of three to five separate experiments.

resolution; (*ii*) identification of molecular controls for aquaporin gene regulation; (*iii*) elucidation of physiological functions by analysis of mutant aquaporin phenotypes. Mammalian and plant systems are highly complex, so recognition of an *E. coli* member of the aquaporin water channel family (6) has provided a prokaryotic model system for biophysical investigations, molecular studies of gene regulation, and evaluation of the physiological need for aquaporin-mediated water transport.

Cryoelectron microscopic studies of AQP1 reconstituted into two dimensional membrane crystals have revealed the structure of the protein at a resolution of 3–6 Å (see review, ref. 27). Nevertheless, it is not clear if significantly higher resolution studies can be obtained with glycosylated, partially degraded AQP1 purified from human red cells. In addition, the lack of a convenient heterologous expression system has prevented structural studies of other wild-type or mutagenized aquaporins by cryoelectron microscopy or x-ray diffraction of three-dimensional aquaporin crystals. Expression of AqpZ in *E. coli* may remedy these problems.

Analysis of the molecular genetic controls for expression of mammalian and plant aquaporins is proceeding, however, the inability to reproducibly induce expression of most known aquaporins in culture systems has obscured the significance of published studies. Here we document the expression of *aqpZ* in *E. coli* by Northern analysis (Fig. 1*A*). The recent determination of the organization of the *aqpZ* gene in *E. coli* predicted that it is a monocistronic message (8, 10). As shown here, the size of the 0.7-kb transcript (Fig. 1*A*) coincides closely to the predicted size coding sequence of *aqpZ* plus transcribed flanking DNA (693 + 30 + 8 bp). This contrasts with the sequence-related gene *glpF* that encodes a glycerol transport protein in an operon also containing genes that encode glycerol metabolizing enzymes (7, 28). Moreover, the lack of differences in *aqpZ* expression in *rpoS*$^+$ and *rpoS*$^-$ *E. coli* (not shown) and the decline during stationary phase growth (Fig. 1*B*) indicate that RpoS is not involved.

The issue of whether aquaporins are essential for life is debated. As reviewed (2), mammalian aquaporin mutants have been identified with phenotypes including lens cataracts (AQP0), loss of the red cell Co blood group antigens (AQP1), nephrogenic diabetes insipidus (AQP2), and incomplete renal concentration (AQP4). Nonmammalian aquaporin-related phenotypes have been identified in other species including Drosophila (big brain) (29), and plants (increased root mass and inhibition of self pollination) (30). The mutant phenotypes of microbial genes encoding sequence-related glycerol transporters are not as severe. Mutations in the bacterial gene *glpF* result in only a subtle reduction of membrane permeability (31), while mutations in the yeast homolog *FPS1* exhibit somewhat reduced glycerol efflux (32).

Before dismissing an aquaporin as physiologically nonessential, careful testing of possible conditional phenotypes is

needed. Our first evaluations of the *aqpZ* knockout failed to reveal a distinct phenotype. Subsequently, clues generated with the *aqpZ*::*lacZ* plasmid led us to evaluate the *aqpZ* knockout for reduced colony formation in conditions of maximum growth (Fig. 3*B*) and in hypoosmotic media (Fig. 3*C*). While peak *aqpZ* expression coincides with the mid-logarithmic growth phase, this is not likely to simply reflect a need for increased bulk water uptake for volume expansion during rapid growth. Assuming a 20–30 min generation time, cell volume of $\approx 1\ \mu m^3$, and surface area of $\approx 5\ \mu m^2$, the doubling of bacterial volume could easily be provided by the diffusional water permeability of simple lipid bilayers, P_d from 2–50 μm/sec, (33), especially because the surface-to-volume ratio is much higher for bacteria than mammalian cells. Diffusional water permeability is known to have a high Arrhenius activation energy (≈10 kcal/mol), so water permeability should be maximum at the highest temperatures, suggesting that aquaporins would be less important. This contrasts with the observed greater need for AqpZ in *E. coli* grown at 39°C rather than 25°C. Moreover, if flaccidity is deleterious to bacteria, the presence of aquaporins would not be desired in hyperosmolar environments. Consistent with this, our studies of hyperosmolar environments demonstrated that the expression of the *aqpZ* gene is strongly reduced (Fig. 1*C*), and the *aqpZ* knockout suffers no growth deficit (Fig. 3*C*).

Together these observations all suggest that bacterial aquaporins are needed for rapid movements of water rather than slower movements of large volumes of water. Interestingly, a gene with a striking sequence-identity to the *E. coli aqpZ* has been recently identified in the chromosome of *Synechocystis* sp. PCC6803, a cyanobacterium permanently living in fresh water (9, 13). While additional roles for the AqpZ protein are probably still unknown, our studies lead us to propose that the physiological significance of AqpZ for the bacterium is that it permits the maintenance of cell turgor while facilitating volume expansion. Thus while sufficient water for cell division may be absorbed by simple membrane diffusion over the course of several minutes, maintenance of high intracellular turgor will require the fast entry of water into the cytoplasm as the cavity formed by the bacterial cell wall increases during rapid growth.

This work is dedicated to the memory of Jorge Chevesich, Ph.D. (1955–1997), exemplary friend and scientist. We thank Drs. Angela Ludovico, Susanne Kneip, Giovanna Valenti, Antonietta Monte, Maria Svelto, Kenn Rudd, and Paul Blount for important discussions, and Drs. Mary Berlyn, Roberto Kolter, and Michael Donnenberg for bacterial strains. This work was supported by National Institutes of Health grants (P.A., W.R.B.), Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie (E.B.), and Foundation pour la Recherche Médicale and Bourse de formation à l'étranger Institut National de la Santé et de la Recherche Médicale (M.B.).

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