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Aquaporin Z of *Escherichia coli*: Reassessment of Its Regulation and Physiological Role

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Transcription of an *aqpZ-lac* fusion in a single copy on the *Escherichia coli* chromosome increased as cells entered the stationary growth phase. This was true in a variety of media, and increased transcription in enriched medium required the RpoS sigma factor. Expression of the *aqpZ-lac* fusion was not affected by up- or downshifts in osmolality. Disruption of *aqpZ* had no detectable adverse effects.

Aquaporins belong to a large family of proteins that increase the rate of diffusion of water and glycerol across cell membranes (18, 22, 24). They are prominent in multicellular eukaryotic organisms, whose large size and need for rapid water movement make such proteins essential (17, 25). Aquaporins (but not glyceroporins) occur only sporadically in bacteria and archaea. For example, *aqpZ* occurs in all four *Escherichia coli* strains for which whole genome sequences are available and in *Shigella flexneri*, but it does not occur in the closely related enteric bacterium *Salmonella enterica* serovar Typhimurium or in the γ-proteobacterium *Yersinia pestis* (*E. coli* Genome Project, University of Wisconsin—Madison, http://www.genome.wisc.edu/). Moreover, *aqpZ* appears to be missing from >80% of the 69 bacterial and archaeal genomes that have been completely sequenced (National Center for Biotechnology Information website, http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi). It is not clear whether small cells that lack internal organelles require aquaporins or whether unmediated diffusion of water across their cytoplasmic membranes is sufficient.

The *aqpZ* gene of *E. coli* was isolated by homology cloning (3). Calamita and colleagues (4) studied regulation of expression of this gene by using a plasmid carrying an *aqpZ-lacZ* fusion. They reported that *aqpZ* expression was induced very sharply in the middle of the exponential growth phase and declined thereafter. Calamita and colleagues reported that an *aqpZ*-null strain of *E. coli* (*aqpZ*::*lacZ-kan*) formed mostly small colonies on Luria-Bertani (LB) agar containing kanamycin and that it showed decreased viability upon prolonged incubation at osmolalities between 80 and 240 mosmolal.

Transcription of *aqpZ* is increased in stationary phase. To reexamine control of *E. coli aqpZ* expression, we constructed a strain carrying a single copy of an *aqpZ-lac* transcriptional fusion stably integrated on the chromosome (7). To create the fusion, *aqpZ*, which had been amplified by PCR from the genome of strain MG1655, was cleaved with *Pvu*II, resulting in destruction of the sixth codon. The upstream fragment, which carries 400 bp 5′ of the translational start site for *aqpZ*, was cloned into pRS551 (19) to yield pJES1320. The fusion was integrated at the *trp* locus as previously described (7) and then introduced into prototrophic strain NCM1458 (also known as RK4353 [21]) by P1-mediated transduction to yield strain NCM3342 [Kan^r-Φ(*aqpZ′-′lac*)]. As reported previously (4), expression of the fusion increased markedly during the late exponential growth phase (Fig. 1 shows both the plot used by Calamita and colleagues [4] [Fig. 1A] and a Monod or differential plot [11] [Fig. 1B]). However, the specific activity of β-galactosidase did not peak in mid- or late exponential growth (Fig. 1A), as previously reported (4), but rather increased into stationary phase (reflected in the constantly increasing slope in Fig. 1B). Similar patterns of expression of *aqpZ* were observed for cells grown in enriched medium (LB broth) (Fig. 1), nitrogen- and carbon-free (N^-C^-) minimal medium (5, 9) with glucose or glycerol as the carbon source, and N^-C^- minimal medium with glucose as the carbon source and glutamine, rather than ammonium, as the nitrogen source (data not shown). A similar pattern was also observed in N^-C^- medium diluted fivefold to decrease its osmolality (data not shown) and upon upward and downward shifts in osmolality (Fig. 2). The downshift was effected by diluting a culture in N^-C^- medium into 0.2× N^-C^- medium with glucose and ammonium as the carbon and nitrogen sources, respectively (Fig. 2A and B). The upshift was effected by adding NaCl to a N^-C^- medium culture to a final concentration of 0.3 M (Fig. 2C and D). Over osmolalities from 105 and ≥605 mosmolal (Fig. 2B and D), we were unable to confirm the previously reported large difference in *aqpZ* expression with increasing osmolality (4).

RpoS control. Transcription of the *aqpZ-lac* fusion was strongly controlled by σ^S when cells were grown in enriched medium. Introduction of an *rpoS*::Tn*10* allele (10) into strain NCM3342 eliminated the increase in β-galactosidase specific activity seen in the stationary phase in LB cultures (Fig. 1C and D). Note that β-galactosidase specific activity continued to rise in the *rpoS*$^+$ strain after growth had ceased. The *rpoS*::Tn*10* allele had little effect on *aqpZ-lac* expression in N^-C^- minimal medium with glucose (0.4%) as the carbon source (data not shown). Control experiments indicated that during growth on LB the *rpoS*::Tn*10* allele essentially eliminated expression of the *ddp* operon, which was previously determined to require

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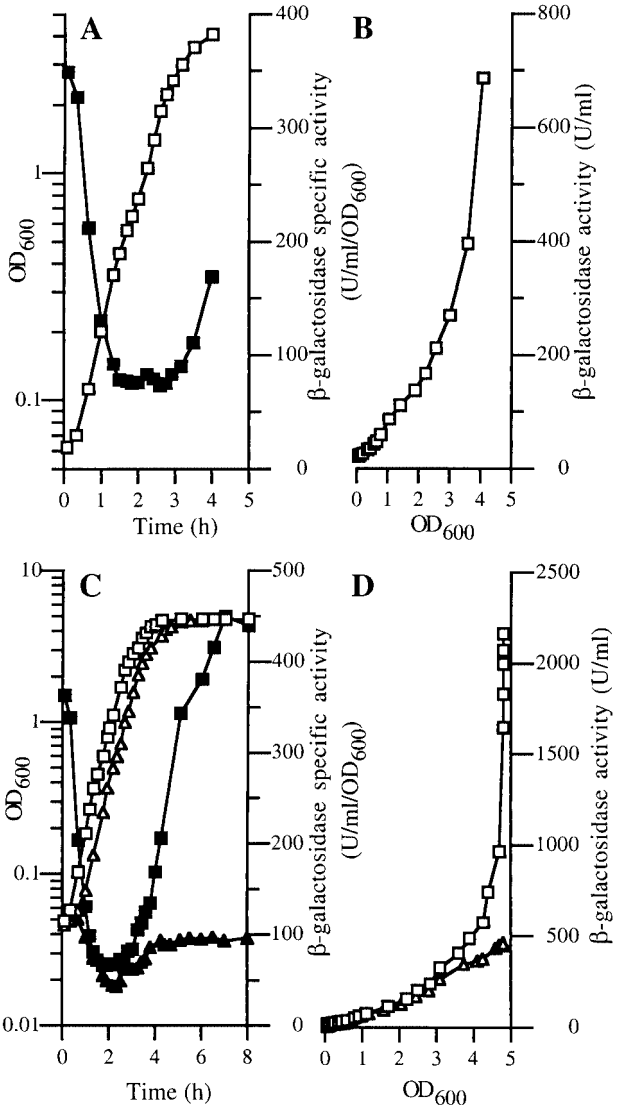

FIG. 1. Expression of a Φ(*aqpZ'-'lac*) fusion during growth of *E. coli* strain NCM3342 in LB with aeration at 37°C, and effects of *rpoS* disruption. (A) Growth (open squares) of strain NCM3342 (optical density at 600 nm [OD_{600}]) and the specific activity of β-galactosidase (in Miller units [16]) (closed squares) were plotted as a function of time. (B) β-Galactosidase activity (in units per milliliter of culture) was plotted as a function of growth (OD_{600}). The data are from panel A. (C) Growth (open symbols) and β-galactosidase specific activities (closed symbols) for strains NCM3342 (squares) and NCM3905 (*rpoS*::Tn*10*) (triangles) were plotted as a function of time. (D) β-Galactosidase activities for the two cultures were plotted as a function of growth. The starter cultures were grown to full yield in the same medium and were diluted 1/100 to initiate the experiment.

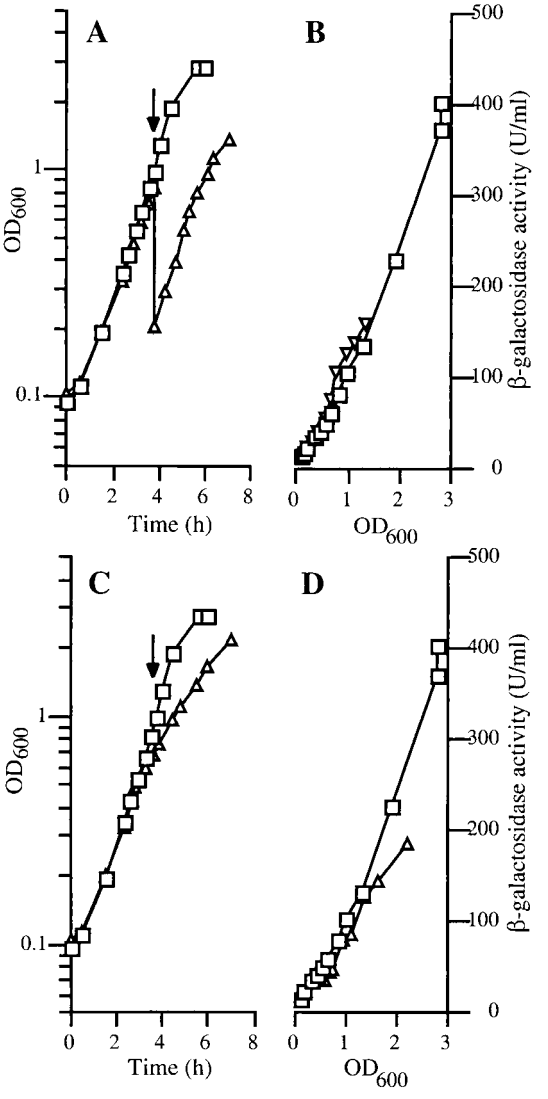

FIG. 2. Expression of a Φ(*aqpZ'-'lac*) fusion upon osmotic downshift (A and B) or upshift (C and D) of strain NCM3342. (A) At the time point indicated by the arrow, the experimental culture was diluted from full-strength N^-C^- medium with glucose (0.4%) and NH_4Cl (10 mM) as carbon and nitrogen sources, respectively, into 0.2× N^-C^- medium with glucose (0.4%) and NH_4Cl (10 mM). The dilute medium was supplemented with 0.3 mM $MgCl_2$ (5), and all media contained 0.1 mM tryptophan. Growth of the experimental culture (open triangles) and the control culture (open squares) was plotted as a function of time. (B) β-Galactosidase activity was plotted as a function of growth for the experiment shown in panel A; symbols are the same. (C) At the time point indicated by the arrow, NaCl was added to the experimental culture to a final concentration of 0.3 M (open triangles). The control culture (open squares) was maintained in full-strength N^-C^- medium with glucose (0.4%) and NH_4Cl (10 mM) as carbon and nitrogen sources, respectively. Growth was plotted as a function of time. (D) β-Galactosidase activity was plotted as a function of growth for the experiment shown in panel C; symbols are the same. OD_{600}, optical density at 600 nm.

RpoS (13), but had no effect on expression of *lacUV5* (data not shown).

Disruption of *aqpZ* did not affect the phenotype. We disrupted the *aqpZ* gene (carried on a 1.6-kb fragment in pJES1298) by digestion with *Nae*I, which cleaves at positions corresponding to the 37th and 158th codons (of 231 total), thereby deleting the internal *Nae*I fragment, and replacing it with a 1-kb fragment carrying a chloramphenicol resistance cassette (Fig. 3A) (20). The disrupted gene, carried on plasmid pJES1301, was integrated into the chromosome as previously described (20) and transferred by P1-mediated transduction to *E. coli* strains NCM1458 (see above) and MG1655 (obtained from the *E. coli* Genetic Stock Center [28]) to yield strains NCM3314 and NCM3306, respectively. That mutant strain

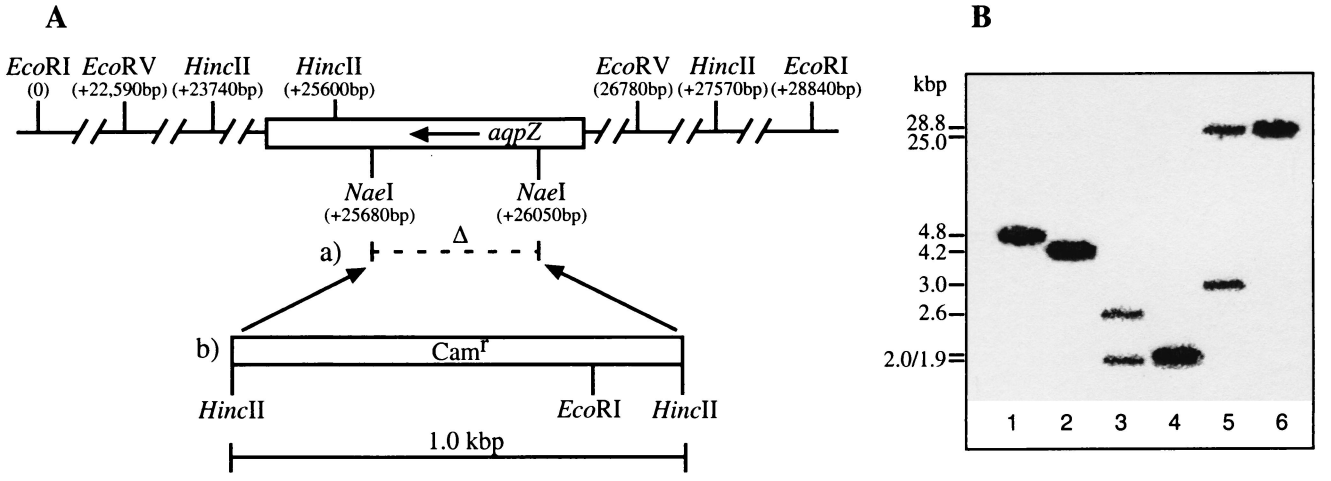

FIG. 3. Southern analysis of the *aqpZ* deletion/insertion mutant strain NCM3314 (Δ*aqpZ*::Cam^r). (A) Diagram indicating predicted cleavage sites and fragment sizes for the three restriction endonucleases in and around wild-type and mutant *aqpZ* genes. (B) Chromosomal DNA from parental strain NCM1458 (lanes 2, 4, and 6) and from mutant strain NCM3314 (lanes 1, 3, and 5) was digested with *Eco*RV (lanes 1 and 2), *Hin*cII (lanes 3 and 4), or *Eco*RI (lanes 5 and 6), and fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Hybridization was performed with a ^{32}P-labeled probe containing the entire coding region of *aqpZ*. The sizes of predicted fragments (panel A) are indicated to the left.

NCM3314 carried the disrupted *aqpZ* gene and not the intact gene was confirmed by finding a single PCR fragment that was approximately 0.6 kb longer than the fragment from its parental strain (data not shown) and by finding the expected patterns of fragments when chromosomal DNA digested with *Eco*RV, *Hin*cII, or *Eco*RI was analyzed by Southern blotting (Fig. 3B). The *aqpZ* strains formed normal-sized colonies on LB agar (data not shown). Strain NCM3314 grew normally when shifted from full-strength N^-C^- medium with glycerol as the carbon source to 0.2× N^-C^- medium (Fig. 4A) and maintained normal viability for up to 5 days after the start of incubation in either medium (Fig. 4A, inset). Hence, we obtained no evidence that AqpZ was required for normal growth at high or low osmolality or that it was required for viability in stationary phase under either set of conditions.

The *aqpZ* strains grew normally on N^-C^- minimal medium with different concentrations of glycerol or glucose as the carbon source, even with concentrations of glycerol as low as 1 mM (Fig. 4B [for glycerol] and data not shown [for glucose]). In the single experiment we performed, the *aqpZ* disruption strain NCM3306 had a normal cell yield in a glycerol-limited chemostat (300-ml culture vessel; N^-C^- medium with 5 mM glycerol and 10 mM NH_4Cl in the reservoir) (20) at dilution rates between 0.34 and 0.45 h^{-1} (data not shown). Like its parent strain, MG1655 (NCM3105), the *aqpZ* strain washed out of the chemostat at a dilution rate of ~0.5 h^{-1} because both strains became internally limited for uracil due to polar effects of the *rph1* mutation on expression of *pyrE* (12) (H. Lee and E. Soupene, unpublished data). Thus, we obtained no evidence that the AqpZ protein was involved in acquisition of glycerol.

Need for further study. Our conclusions regarding the regulation of *aqpZ* and its physiological importance to *E. coli* differ from those of Calamita and colleagues (4). Using a single-copy *aqpZ-lac* fusion inserted stably on the chromosome, we found that expression of *aqpZ* was increased as cell growth slowed and the cells entered stationary phase. This was confirmed by the finding that increased *aqpZ* transcription on enriched medium depended on σ^S, the stationary phase sigma factor (8, 14, 15), which is not known to mediate a peak of gene expression in the middle of exponential growth. Moreover, Wei et al. (26) reported that *aqpZ* was among the genes not expressed when cells were cultured in rich (LB) medium. Calamita and colleagues studied expression of an *aqpZ-lacZ* fusion on a multicopy plasmid (4), an inherently less reliable configuration.

Although purified *E. coli* AqpZ clearly increases the osmotic permeability of liposomes to water (1, 2), it remains to be determined whether water is the preferred substrate for this protein in vivo. In our hands, an *aqpZ*-null strain of *E. coli* had no defect in growth or viability at low or high osmolality or upon shifting between the two. Hence, we found no evidence that AqpZ mediates water permeativity under these conditions. We wonder whether the small-colony phenotype of the *aqpZ* strain described by Calamita et al. (4) and its viability problems and aberrant appearance in electron micrographs (6) might have been due to the insertion of a 3.9-kb *lacZ-kan* gene fusion cassette (23) into the middle of the *aqpZ* gene. Perhaps the first half of AqpZ or an AqpZ–β-galactosidase fusion protein is toxic when inserted into the membrane. It is difficult for us to understand how the growth problems of the disruption strain could have been complemented by a high-copy-number plasmid carrying the intact *aqpZ* gene because in our hands such a plasmid is toxic (E. Soupene and M. Lee, unpublished data).

Glycerol does not appear to be the natural substrate for AqpZ because the strain lacking that protein grew normally on glycerol even at low concentrations. Given that 13 of 25 residues lining the selectivity filter of bovine AQP1 are different in *E. coli* AqpZ (22), it may be worth considering whether the preferred substrate for AqpZ is something like ethanol, which is a major fermentation end product of *E. coli* (27). Whatever

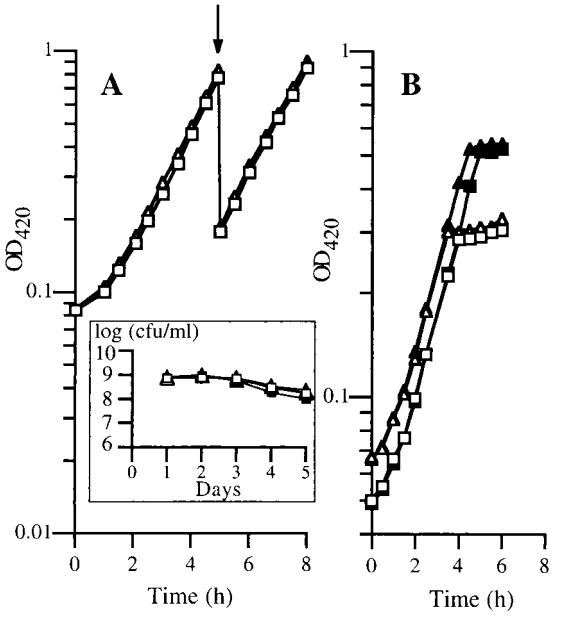

FIG. 4. Lack of effect of an *aqpZ* disruption on growth or survival under conditions of low osmolality (A) or low concentration of glycerol (B). (A) At the time point indicated by the arrow, cultures of strains NCM1458 (squares) and NCM3314 (Δ*aqpZ*) (triangles) were diluted from full-strength N^-C^- medium with glycerol (0.2%) and NH_4Cl (5 mM) as carbon and nitrogen sources, respectively, into 0.2× N^-C^- medium containing the same carbon and nitrogen sources and supplemented with 0.3 mM $MgCl_2$. Cultures were incubated with aeration at 37°C. The inset shows survival after incubation in both media for up to 5 days; symbols are the same as for the panel, with open and closed symbols for cultures in full-strength or dilute medium, respectively. Samples were diluted appropriately to obtain 150 to 300 colonies/plate and were spread on LB agar medium. The experiment was performed twice with two independent dilutions for each point in each experiment. Each point is an average of cell counts from eight plates with error bars indicated (but too small to be seen). (B) Cultures of strains NCM1458 (squares) and NCM3314 (Δ*aqpZ*) (triangles) were incubated with aeration at 37°C in full-strength N^-C^- medium containing NH_4Cl (5 mM) as the nitrogen source. The carbon source was glycerol at 2 mM (closed symbols) or 1 mM (open symbols). Cultures used for inoculation were grown in the same medium with 27 mM glycerol (0.2%). OD_{420}, optical density at 420 nm.

AqpZ's substrate, studies of the regulation of *aqpZ* indicate that its role is likely to be manifest during the transition to the resting state or in resting cells. Seeking phenotypes for *E. coli aqpZ*-null mutants under conditions in which the gene is highly expressed warrants further effort.

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