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Isolation and Characterization of Adenylate Kinase (*adk*) Mutations in *Salmonella typhimurium* Which Block the Ability of Glycine Betaine To Function as an Osmoprotectant

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Mutants of *Salmonella typhimurium* that were not protected by glycine betaine (GB) but could still use proline as an osmoprotectant in media of high osmolality were isolated. The mutations responsible for this phenotype proved to be alleles of the adenylate kinase (*adk*) gene, as shown by genetic mapping, sequencing of the cloned mutant alleles, complementation with the *Escherichia coli adk* gene, and assay of Adk enzyme activity in crude extracts. One of the mutations was in the untranslated leader of the *adk* mRNA, a second was in the putative Shine-Dalgarno sequence, and a third was in the coding region of the gene. The loss of osmoprotection by GB was shown to be due to the fact that the accumulation of this solute actually resulted in a severe inhibition of growth in the *adk* mutants. The addition of GB in the presence of 0.5 M NaCl resulted in a rapid decline in the ATP pool and a dramatic increase in the AMP pool in the mutants. Proline, which is not toxic to the *adk* mutants, did not have any significant effects on the cellular levels of ATP and AMP. The mutants exhibited two different phenotypes with respect to the utilization of other osmoprotectants: they were also inhibited by propiothiobetaine, L-carnitine, and γ-butyrobetaine, but they were stimulated normally in media of high osmolality by proline, choline-*O*-sulfate, and stachydrine.

Organisms adapt to conditions of high osmolality by increasing the intracellular concentrations of a few species of cytoplasmic solutes, known as compatible solutes. This response forestalls the efflux of water from the cells and therefore enables the cells to maintain their volume and pressure at levels that are necessary for growth in environments of high osmolality (11). A group of organic compounds, called osmoprotectants, can alleviate at low exogenous concentrations the inhibitory effects of high osmolality in bacteria. For *Salmonella typhimurium* and *Escherichia coli*, glycine betaine (GB) is the most potent osmoprotectant, but there are a number of other less potent ones: L-proline, stachydrine, choline-*O*-sulfate, propiothiobetaine, L-carnitine, ectoine, and γ-butyrobetaine.

The osmoprotectants are taken up by the ProP or ProU systems in *S. typhimurium* and *E. coli* (4, 5, 10, 11, 24, 33). The ProP system has a similar affinity for GB as for proline (44), but the ProU system recognizes GB at >100-fold higher specificity than proline (2, 5). The ProP system is coupled to the cotransport of cations (probably H^+ [44]), and therefore it is energized by the proton motive force. The ProU system, which is a member of the family of prokaryotic and eukaryotic transporters known as the ABC (ATP-binding cassette) proteins (30) or traffic ATPases (14), is energized by the hydrolysis of ATP (45).

To gain insights into the mechanism by which GB exerts its osmoprotective effects, we sought mutants in which GB could no longer counteract osmotic inhibition. Unexpectedly, the mutations in some of these strains proved to be in the *adk* gene, which specifies adenylate kinase (Adk), the enzyme catalyzing the reaction $Mg \cdot ATP + AMP \leftrightarrow Mg \cdot ADP + ADP$. In *E. coli* and *S. typhimurium*, the Adk reaction is the only route of de novo synthesis of ADP from AMP. Because its equilibrium constant is ~2.25 under standard conditions (16), the Adk reaction is readily reversible, and therefore it is thought to have a second function: to replenish ATP from ADP. It has been proposed that the levels of the three adenylates (AMP, ADP, and ATP) are maintained at ratios dictated by the equilibrium of the Adk reaction, and therefore Adk can buffer the ATP level during periods of rapid ATP consumption (1). The relative distribution of the adenylates has been expressed by a mathematical formalism known as the adenylate energy charge (EC_A), where $EC_A = ([ATP] + 0.5\,[ADP])/([ATP] + [ADP] + [AMP])$ (1). This ratio, which can vary from 1 (adenylates are completely in the form of ATP) to 0 (adenylates are completely in the form of AMP), has been suggested to coordinately regulate the activities of a number of key catabolic and biosynthetic enzymes (1). In exponentially growing cells, the EC_A is ≈0.7 to 0.9, while in carbon source-starved cells, it is <0.5 (7).

Most *adk* mutations in *E. coli* have been isolated as temperature-sensitive defects (9, 20, 29), suggesting that Adk is an essential enzyme. Shift of the *adk* mutants to the nonpermissive temperature results in rapid decline in the ATP pool, increase in AMP, and cessation of DNA, RNA, and protein synthesis (20, 21). However, there are a number of biochemical and genetic studies which indicate an even more complex role for Adk besides the synthesis of ADP or the maintenance of the equilibrium among the adenylates. Several *adk* mutants have emerged from selections designed to yield mutants with temperature-sensitive lesions in lipid synthesis (20, 32). Single mutations in the *adk* gene were found to be able to cause temperature sensitivity in *sn*-glycerol-3-phosphate acetyltransferase, which is a membrane-bound enzyme catalyzing one of the early steps of phospholipid synthesis. This observation was interpreted as evidence for a protein-protein interaction between Adk and *sn*-glycerol-3-phosphate acyltransferase (32). Lastly, in *E. coli*, it has been reported that 36% of the total Adk activity is located in the periplasmic space and 6% is membrane associated (55). The observations reported below sug-

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TABLE 1. Bacterial strains used

Strain	Genotype	Construction, reference, or source[a]
S. typhimurium		
JF1437	*aniB1054*::Mu dA	From SGSC (53)
MS1370	*zcc-5*::Tn*10*	From S. Maloy
PY13757	F′$_{ts114}$ *lac zzf-20*::Tn*10 zzf-3553*::TnMu dQ/*leuA424* r$^-$ m$^+$	57
PY13762	F′$_{ts114}$ *lac zzf-20*::Tn*10 zzf-*?::TnMu dP/*leuA 424* r$^-$ m$^+$	57
TL156	*galE496 metA22 metE55 nml rpsL120 xyl-404 Fels2*$^-$ *H1-b H2-enx ilv hsdL6 hsdSA29 zxx*::Mu *cts62 h7629*	12
TL179	*zcc-628*::Tn*5* Δ*putPA557 proP1654*	10
TL195	*zcc-628*::Tn*5* Δ*putPA557 proP1654 proU1655*::Tn*10*	10
TL235–TL242	*zcc-628*::Tn*5* Δ*putPA557 proP1654 bet-1 bet-2 bet-3 adk-4 adk-5 adk-6 adk-7 adk-8 adk-11*	24
TL1751	*zbb-900*::Mu dJ *proP1654 zcc-5*::Tn*10* Δ*putPA557*	24
TL1759, TL1765	*zbb-62 zbb-101*::Tn*10* dTc	24
TL1775, TL1777	*zbb-2 zbb-7*::Tn*10* dCm	24
TL1947	*zbb-2*::Tn*10* dCm *galE496 metA22 metE55 rpsL120 xyl-404 H1-b nml H2-enx ilv hsdL6 hsdSA29 Fels2 zxx*::Mu *cts62 h7629*/pEG5155	24
TL2159	*aniB1054*::Mu dA *galE496 metA22 metE55 rpsL120 xyl-404 H1-b nml H2-enx ilv hsdL6 hsdSA29 Fels2 zxx*::Mu *cts62 h7629*	24
TL2174, TL2176, TL2180	*zbb-101*::Tn*10* dTc *adk-4 adk-5 adk-8*	24
TL2204, TL2206, TL2210, TL2212	*zbb-7*::Tn*10* dCm *galE496 metA22 metE55 rpsL120 hsdSA29 nml xyl-404 H1-b H2-enx ilv hsdL6 Fels2*$^-$ *zxx*::Mu *cts62 h7629 adk-4 adk-5 adk-8 adk-11*	24
TL2228, TL2230, TL2234, TL2236	*aniB1054*::Mu dA *zbb-101*::Tn*10* dTc *adk-4 adk-5 adk-8 adk-11*	24
TN696	*apt-3 galE*	From SGSC (28, 53)
TN1731	*zbb-876*::Tn*10*	From SGSC (28, 53)
TR5657	*purE8 strA1*	From J. Roth (13)
E. coli		
BW5104	*zxx-5104*::Mu*1 lac-169 pho-510 hsdR514*	From B. Wanner
CV2	*adk-2 tonA22* Δ*phoA8 ompF627 fadL701 relA1 glpR2 glpD3 pit-10 spoT1* T_2R HfrPO2A	From A. Wittenhofer (8)
DH5α	F′ ϕ80 d*lac*Δ(*lacZ*) *M15*/*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*-169*	
KC543	BW5104/pJG2	Mu(TL1943)→BW5104 = Cm^r Km^r
KC755	*zxx*::Mu dA *adk-2 tonA22* Δ*phoA8 ompF627 fadL701 relA1 glpR2 glpD3 pit-10 spoT1* T_2R HfrPO2A	Mu(TL2159)→CV2 = Ap^r
KC756	KC755/pJG2380	Mu(TL2216)→KC755 = Ts$^+$ (Km^r)
KC757	KC755/pJG2391	Mu(TL2218)→KC755 = Ts$^+$ (Km^r)
KC759	KC755/pJG2410	Mu(TL2222)→KC755 = Km^r (Ts$^+$)
KC760	KC755/pJG2421	Mu(TL2224)→KC755 = Ts$^+$ (Km^r)

[a] Phage Mu/λ transduction is denoted as Mu(*A*)→B = *c* (*d*), where *A* and *B* are the donor and recipient strains, respectively, and *c* and *d* are the selected and screened phenotypes, respectively. SGSC, *Salmonella* Genetic Stock Centre (University of Calgary, Calgary, Alberta, Canada). A more detailed list of strains which includes all intermediates is available upon request.

gest that Adk may have an especially important role in maintaining a high ATP pool during the uptake of GB.

(The results of this work have been presented in preliminary form [25–27].)

MATERIALS AND METHODS

Chemicals, media, and growth conditions. All chemicals were reagent grade and obtained from commercial sources except for [*glycyl*-^{14}C]GB and [*methyl*-^{14}C]GB, which were the generous gifts of D. Rhodes and A. Hanson, respectively, and stachydrine, which was also from D. Rhodes. The rich medium was LB (13), and the minimal medium was M63 (8). The low-osmolar K medium was that of Kennedy (34), except that the yeast extract was replaced with 5 g of Casamino Acids (Difco) per liter. Solid media contained a 20 g of Difco agar per liter. Unless otherwise stated, the carbon sources in solid and liquid M63 were 10 mM glucose and 20 mM glucose, respectively. Osmotic stress was induced with 0.3 or 0.65 M NaCl in LB, 0.3 to 1.0 M NaCl in M63, and 0.5 M NaCl in K medium, with the pH adjusted to 7.2 with NaOH in each case. When used, osmoprotectants were at 1 mM. L-Azetidine-2-carboxylic acid and 3,4-dehydro-D,L-proline, used for scoring the presence of *putP*, *proP*, and *proU* mutations (10), were at 1 mM. When used, antibiotics were ampicillin at 25 mg/liter for Mu d1 and Mu dA lysogens and 100 mg/liter for strains harboring *bla*$^+$ plasmids, tetracycline at 15 mg/liter, kanamycin sulfate at 75 mg/liter for chromosomal determinants and at 250 mg/liter for selection of Tn*phoA* and Tn*phoA′-1* insertions into high-copy-number plasmids, chloramphenicol at 12.5 mg/liter, and spectinomycin at 50 and 1,000 mg/liter for *E. coli* and *S. typhimurium* strains, respectively. When used, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) and 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine (XP) were at 40 mg/liter. Lac phenotype was scored on half-strength MacConkey-lactose medium, which consisted of 25 g of MacConkey agar (Difco), 7.25 g of Bacto Agar (Difco), 5 g of lactose, and 15 mg of neutral red in 1 liter of H_2O. Growth in liquid media under aerobic conditions was in 10- to 20-ml cultures in 125-ml Erlenmeyer flasks, shaken at 250 rpm. Growth curves were obtained by periodic measurements of the optical density at 600 nm (OD_{600}) with a Gilford spectrophotometer, with appropriate dilutions of the cultures to account for the nonlinearity between cell density and OD_{600} at high cell densities.

Bacterial strains, bacteriophages, and genetic techniques. The relevant *S. typhimurium* (all LT2 derivatives) and *E. coli* strains used in this study are listed in Table 1. Bacteria were grown at 37°C, except for Mu d1 lysogens and the *E. coli adk-2* mutants, which were grown at 30°C and at room temperature, respectively. Generalized transduction was carried out with phage P22 HT1/105 *int-103* (referred to as P22 hereafter) as described by Davis et al. (13). The hybrid Mu d-P22, Mu dP, and Mu dQ phages, obtained from N. Benson and J. Roth, were used for the mapping of *adk* mutations as described in reference 57 except that P22 tail protein was not added to the phage lysates. Although this omission decreased the overall efficiency of transduction, it nonetheless gave satisfactory mapping data. Insertion mutagenesis of *adk*$^+$ plasmids was carried out with λ::Tn*phoA* (41) and λ::Tn*phoA′-1* (56), which were gifts of B. Wanner. λ::Tn*phoA′-1* element is a derivative of Tn*phoA* containing a promoterless *lacZ* gene for the construction of transcriptional fusions. P22 lysates of derivatives

TABLE 2. Plasmids used

Plasmid	Genotype or markers	Construction, reference, or source[a]
pBluescript II KS(+)	*lac*α *bla*$^+$	Cloning vector
pBR322	*bla*$^+$ *tet*$^+$	Cloning vector
pDO182	Promoterless *lacZYA spc*$^+$	48
pEak90	*bla*$^+$, *E. coli adk*$^+$	3; from A. Wittenhofer
pEG5005	Mini-Mu *kan*$^+$ *bla*$^+$	23; from B. Wanner
pEG5155	Mini-Mu *lac*$^+$ *kan*$^+$ *bla*$^+$	23; from B. Wanner
pJG2	*zbb-2*::Tn*10* dCm *adk*$^+$ *kan*$^+$	pEG5155 mini-Mu clone of TL1943; carries 14 kbp of *S. typhimurium* DNA
pJG201	*adk*$^+$ *kan*$^+$ *bla*$^+$ Δ(Tn*10* dCm)	*Bam*HI subclone of pJG2; lost 2.4 kbp including most of *zbb-2*::Tn*10* dCm and 0.1 kbp of mini-Mu DNA, cloned into the *Bam*HI site of pBR322; Tc^s Cm^s
pJG301	*adk*$^+$ *bla*$^+$	Partial *Cla*I digestion and religation of pJG201; contains a 1.8-kbp *Cla*I-*Cla*I fragment (0.2 kbp of pBR322 + 1.6 kbp of *adk*$^+$) in *Cla*I site of pBR322
pJG302	*adk-12*::Tn*phoA′-1 kan*$^+$ *bla*$^+$	Tn*phoA′-1* insertion into *adk* on pJG301; resistant to 0.25 mg of kanamycin per ml and Bet^s in *adk* mutant background; *lacZ* in opposite orientation with respect to *adk*
pJG303	*adk-12*::Tn*phoA′-1* Δ*MS* (Tnp$^-$) *kan*$^+$ *bla*$^+$	Internal *Sma*I-*Sac*II deletion of pJG302, which removed ~1.2 kbp of Tn*phoA′-1* DNA including part of the *tnp* gene
pJG310, pJG320	*adk*$^+$ *bla*$^+$	Subclone of 1.8-kbp *Cla*I-*Cla*I fragment of pJG301 ligated into *Cla*I-cut pBR322, oriA (pJG310) and oriB (pJG320)
pJG311, pJG321	*adk*$^+$ *bla*$^+$	1.6-kbp *Bam*HI-*Cla*I fragment from pJG310, ends converted to blunt, ligated into *Eco*RV site of pBR322; insert in oriA in pJG311 and in oriB in pJG321
pJG330	Δ*adk bla*$^+$	0.5-kbp *Bam*HI-*Eco*RI *adk′* fragment from pJG310, ends converted to blunt, ligated to *Bam*HI linkers, digested with *Bam*HI, and cloned into *Bam*HI site of pBR322, oriA
pJG341	*adk-13*::Tn*phoA kan*$^+$	Tn*phoA* inserted into *adk* of pJG321, resistant to 0.25 mg of kanamycin per ml and Bet^s in *adk* mutant background, *phoA* in same orientation and in frame with *adk*; PhoA$^-$
pJG350	*adk*$^+$ *bla*$^+$	1.5-kbp *Sca*I-*Cla*I fragment from pJG310, ligated between *Eco*RV and *Cla*I sites of pBR322
pJG401	*adk*$^+$ *bla*$^+$	Subclone of 1.8-kbp *Cla*I-*Cla*I fragment from pJG310 ligated into *Cla*I site of pBluescript II KS(+), oriB
pJG420	Δ*adk bla*$^+$	Internal *Eco*RV deletion of pJG401, religated; lost 0.35 kbp of *adk* insert and 0.15 kbp of vector DNA
pJG430	Δ*adk bla*$^+$	Internal *Eco*RI deletion of pJG401, religated; lost 0.5 kbp of *adk* insert and 0.3 kbp of vector DNA
pJG501	*adk*$^+$ *bla*$^+$	Same as pJG401 but insert in oriA
pJG610	Δ*adk bla*$^+$	Subclone of 0.5-kbp *Bam*HI-*Eco*RI *adk′* fragment from pJG310, *Eco*RI converted to blunt, ligated to *Bam*HI linker, digested with *Bam*HI and cloned into *Bam*HI site of pBluescript II KS(+), oriB
pJG620	Δ*adk bla*$^+$	Internal *Eco*RV deletion of pJG610, retained 0.3-kbp *Bam*HI-*Eco*RV region of *adk*, oriB
pJG1000	P(*adk*→)*lacZYA spc*$^+$	0.7-kbp *Bam*HI-*Bgl*II fragment containing the *adk* promoter cloned into the *Bam*HI site of the *lac* expression vector pDO182 (pSC101 replicon); Lac$^+$
pJG1001	P(*adk*←)*lacZYA spc*$^+$	Same as pJG1000 but insert in opposite orientation; Lac$^-$
pJG2380	*adk-4 kan*$^+$	pEG5005 mini-Mu clone derived from TL2216, confers Ts$^+$ in *E. coli* KC755 (*adk-2 ts*)
pJG2391	*adk-5 kan*$^+$	Same as pJG2380 but derived from TL2218
pJG2410	*adk-8 kan*$^+$	Same as pJG2380 but derived from TL2222
pJG2421	*adk-11 kan*$^+$	Same as pJG2380 but derived from TL2224
pJG2421-BR	*adk-11 bla*$^+$	1.5-kbp *Sca*I-*Cla*I fragment from pJG2421 cloned between the *Eco*RI and *Cla*I sites of pBR322

[a] oriA or oriB indicates that the transcriptional sense of insert is in the same or opposite orientation as that of the upstream promoter on the vector.

containing pools of transposon insertions (Mu dJ, Tn*10* dTc, and Tn*10* dCm) at random chromosomal sites were gifts of M. Mahan.

Mutagenesis and screening for mutants impaired in GB-mediated osmoprotection. Strain TL179 (Δ*putPA proP*) was mutagenized with ethyl methanesulfonate as described by Miller (43) and subjected to two rounds of ampicillin enrichment (43) in M63 plus 1 M NaCl plus GB. Survivors which were unable to grow on solid medium containing M63 plus 0.65 M NaCl plus GB but were able to grow on M63 plus 0.3 M NaCl or M63 plus 0.65 M NaCl plus proline were saved for further analysis. This procedure yielded eight not necessarily independent mutants, TL235 to TL242, which carry mutations initially designated *bet-1* through *bet-8* and *bet-11*, respectively. We demonstrated that *bet-4*, *-5*, *-6*, *-8*, and *-11* mutations are new alleles of *adk*, and consequently we will refer to them by the latter designation.

Cloning and DNA sequence determination of the wild-type and mutant *adk* genes. The plasmids used in this study are listed in Table 2. Plasmids were introduced into the host strains by $CaCl_2$-heat shock transformation (13), by electroporation (35), or by conjugation in triparental matings (53).

The wild-type and mutant *adk* alleles were cloned by the in vivo mini-Mu cloning procedure (23) except that phage Mu lysates were obtained by heat induction of Mu *cts* lysogens at 42° for 3 to 4 h. *E. coli* BW5104 (*hsdR* Mu d1) was infected with a Mu lysate of strain TL1947 (which carries the broad-host-range mutant phage Mu *cts62 h7629* (12), the *zbb-2*::Tn*10* dCm insertion (>99%

linked to *adk*$^+$), and the mini-Mu cloning vector pEG5515. Transductants that acquired both chloramphenicol resistance (Cm^r) (conferred by the *zbb-2*::Tn*10* dCm insertion) and kanamycin resistance (Km^r) (conferred by the mini-Mu plasmid) were selected at 30°C. One of these derivatives, KC543, carries plasmid pJG2, which contains a 14-kbp insert including the *adk*$^+$ gene and the *zbb-2*::Tn*10* dCm. Upon transfer into an *adk-11* Mu dA lysogen (TL1949), pJG2 restored the ability to use GB as an osmoprotectant to the host strain. Subcloning and insertion mutagenesis led to the identification of a 1.5-kbp *Sca*I-*Sal*I fragment that could complement the *adk-11* mutation (see Fig. 2). The location of the *adk* promoter was identified by subcloning a 0.7-kbp *Bam*HI-*Bgl*II fragment from the 5′ end of the gene (see Fig. 2) into the *Bam*HI site of pDO182, a low-copy-number promoterless *lac* expression vector (48); the presence of the promoter was deduced from the Lac$^+$ phenotype of transformants on half-strength MacConkey lactose plates. Two insertion mutations into the *adk*$^+$ gene on plasmids pJG302 and pJG341 were generated by infecting *E. coli* DH5α harboring pJG301 and pJG321 with λ::Tn*phoA* and Tn*phoA′-1*, respectively, and scoring for loss of the ability to complement the *adk-11* mutation. Restriction enzyme analysis and DNA sequence determination showed that the Tn*phoA* (on plasmid pJG341) was in the same transcriptional sense as the *adk* gene and resulted in an Adk-PhoA fusion protein (which did not have any detectable alkaline phosphatase activity on LB-XP plates) and that the Tn*phoA′-1 lac* insertion (on plasmid pJG303) was in the opposite orientation from the *adk* gene.

The mutant *adk* genes were cloned by complementation of the temperature-sensitive *adk-2* mutation in *E. coli* KC755, a Mu dA lysogenic derivative of strain CV2. First, the *adk-4*, *-5*, *-8*, and *-11* alleles were transduced together with the linked *zbb-7*::Tn*10* dCm transposon into a derivative of strain TL156, which is lysogenic for the broad-host-range mutant Mu *cts62 h7629*. The resulting strains (TL2204, TL2206, TL2210, and TL2212) were then electroporated with the mini-Mu cloning vector pEG5005. Phage Mu lysates obtained by heat induction of the resultant strains were used to infect *E. coli* KC755. Derivatives carrying the mutant *adk* genes on plasmids were obtained by selecting for Km^r (specified by the mini-Mu vector) and scoring temperature resistance (encoded by the mutant *adk* genes) or, conversely, by selecting growth at 42°C and scoring Km^r. Plasmid pJG2410 (*adk-8*) was obtained by the former approach, and pJG2380 (*adk-4*), pJG2391 (*adk-5*), and pJG2421 (*adk-11*) were obtained by the latter. We had also attempted to clone the *adk-6* allele by this procedure, but in repeated trials, we were unable to obtain a plasmid carrying it. Although strain KC755 (*adk-2*[Ts]) is lysogenic for phage Mu dA (which carries the temperature-sensitive repressor *cts62* mutation), we were nevertheless able to obtain stable plasmids that could complement the *adk-2* mutation at 42°C, probably because the temperature-sensitive repressor retained partial function at 42°C. Upon electroporation into the *adk-4*, *-5*, *-8*, and *-11* Mu dA-lysogenic tester strains TL2228 to TL2236, respectively, plasmids pJG2380, pJG2391, pJG2410, and pJG2421 bearing the mutant *adk* genes were able to complement the GB-sensitive (Bet^s) mutant phenotype of the host strains.

DNA sequencing. DNA sequencing of double-stranded templates pJG410 and pJG501 and their derivatives pJG420, pJG430, pJG510, and pJG620 was done by the method of Sanger et al. (54) with the U.S. Biochemical Sequenase II kit. Derivatives containing 5′ and 3′ deletions of pJG410 and pJG501 were generated with exonuclease III and S1 nuclease (Promega Corp., Madison, Wis.). The insertion sites of Tn*phoA* and Tn*phoA′-1* elements in plasmids pJG341 and pJG303 were determined by sequencing across the *adk*-Tn*phoA* junctions with *adk*-specific primers. These primers were also used to sequence the mutant genes as 1.5-kbp *Sca*I-*Sal*I fragments cloned into pBluescript II KS(+). Computer analysis of the sequence was carried out with the programs FASTA (search for homologies with the DNA sequences in the data bank) and PILEUP and BOX-SHADE (for alignments of the predicted amino acid sequence with those of other organisms) available from the AIDS Center Laboratory for Computational Biochemistry of Purdue University.

Enzyme assays. Adenylate kinase was assayed in membrane-free extracts by to the method of Huss and Glaser (32), with minor modifications. Cells were grown in glucose M63 to late exponential phase (~5×10^8 cells per ml), collected by centrifugation, and washed twice in 1/10 the original volume of 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$. The tubes containing the cells were immersed in an ice-salt bath, and the cells were lysed by sonication for 1 min with a Fischer sonic dismembrator (model 300). Particulate matter was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The supernatants were kept on ice until assayed. Routinely, they were used for adenylate kinase assays within 48 h, during which time the activities did not change significantly. Adenylate kinase was measured in an assay in which the reaction 2ADP → ATP + AMP was coupled to phosphorylation of glucose by hexokinase and oxidation of glucose-6-phosphate by NADP. The reaction mixtures contained 10 to 30 μl of cell extract for strains without *adk* plasmids and 1 to 10 μl of cell extracts for strains carrying *adk*$^+$ or *adk-11* plasmids, 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 21.3 mM β-mercaptoethanol, 2.5 to 5 U of hexokinase, 1.25 to 2.5 Units of glucose-6-phosphate dehydrogenase (Sigma), 2 mM ADP, 10 mM glucose, and 0.5 mM NADP in a 1-ml final volume. The rate of formation of NADPH at 25°C was monitored at 340 nm with a UV-160A Shimadzu UV-visible recording spectrophotometer. β-Galactosidase was assayed in mid-exponential-phase cells as described by Miller (43). Protein concentrations were determined by the Bradford assay with a Pierce kit.

Transport assays. Overnight cultures grown in M63 plus 0.3 M NaCl at 37°C were diluted 1:10 in the same medium and allowed to grow to a density of 4×10^8 cells per ml. For the transport assays, 100-μl samples of the cultures were mixed with 8 μM [*methyl*-^{14}C]GB (2×10^4 cpm); after various time points of incubation at room temperature, the mixtures were filtered through 0.45-μm-pore-size filters (Gelman GN-6 Metricel membranes), and the cells on the filters were washed immediately with 2 ml of M63 plus 0.3 M NaCl. The radioactivity in the cells was measured by scintillation counting. Transport rates are reported as nanomoles of [^{14}C]GB taken up per minute per milligram of protein. The protein concentration was calculated from the OD_{600} of cultures by the following conversion factors: OD_{600} = 1.0 corresponding to 0.5 mg of protein per ml for cultures grown in M63 plus 0.3 or 0.8 M NaCl, and OD_{600} = 1.0 corresponding to 0.11 mg of protein per ml for cultures grown in M63 without NaCl. These were established from the ratio of OD_{600} and the protein content of standard cultures, with the protein concentration determined by the Bradford assay.

Measurement of the intracellular adenylate pools. Overnight cultures grown in M63, M63 plus 0.3 or 0.5 M NaCl, and the indicated carbon and energy sources were subcultured in the same media to give cell densities of 0.4×10^8 to 0.8×10^8 cells per ml and grown with vigorous shaking for two doublings. At this time, the cultures were split into three portions, which received 1 mM GB, 1 mM proline, or an equal volume of water. Cells were extracted as described in reference 31: at various times before and after the addition of osmoprotectants, 10-μl samples were withdrawn from the cultures, transferred within 2 s into 500 μl of boiling 20 mM Tris-HCl (pH 7.7), and kept at 100°C for 2 min. ATP, ADP, and AMP were measured by the luciferin-luciferase method according to the procedure of Chapman et al. (7), with the modifications described by Holm-Hansen and Karl (31): the extracts were brought to 0.5 ml with water, and 10 μl of the samples thus diluted or of standard solutions containing known concentrations of adenylates was added to 190 μl of a mixture containing 0.1 M Tris acetate, 2 mM EDTA (pH 7.75), and 50 μl of reconstituted ATP Monitoring Reagent (luciferin-luciferase cocktail of the ATP Monitoring kit [BioOrbit, Turku, Finland]), and the increase in bioluminescence was recorded with a Monolight 2010 bioluminometer (Analytical Luminescence Laboratories). The lowest amount of ATP that was reproducibly detected was 0.01 pmol. The results are reported as nanomoles of adenylate per milligram of protein, with the latter being calculated from the OD_{600} of the cultures as described above.

Nucleotide accession number. The nucleotide sequence of the *S. typhimurium adk*$^+$ gene has been deposited in the Genome Sequence Data Base, Los Alamos National Laboratory, under accession number L26246.

RESULTS

Isolation and initial characterization of mutants unable to use GB as an osmoprotectant. In a search for *S. typhimurium* mutants that could not use GB as an osmoprotectant, we obtained eight derivatives of strain TL179 (*proP*) that were not stimulated by GB in M63 plus 0.65 M NaCl. These mutants fell into two classes. Class I included strains TL235 to TL237, which were very salt sensitive, not stimulated by GB, and stimulated only minimally by proline in media of high osmolality; the mutations in these strains probably caused general sensitivity to high osmolality. Class II consisted of strains TL238 to TL242, which were slightly salt sensitive and not stimulated by GB in media containing 0.5 to 1.0 M NaCl. Class II strains were stimulated nearly normally by proline in media containing 0.3 to 0.75 M NaCl, but at higher NaCl concentrations, they were not stimulated by proline as effectively as was the parental strain TL179. As shown below, class II strains have mutations in the *adk* gene; GB is toxic to these strains in media containing ≥0.15 M NaCl. The sensitivity of class II mutants to GB in media of high osmolality was slight in strains TL239 and TL240, intermediate in strain TL238, and very pronounced in strains TL241 and TL242. The GB-sensitive phenotype and the ability to use GB as an osmoprotectant will be indicated hereafter as Bet^s and Bet$^+$, respectively.

The Bet^s strains are not altered in accumulation of GB. To test whether the Bet^s mutants were impaired in GB accumulation, we assayed [^{14}C]GB transport in strains TL1765 (*adk*$^+$) and TL1774 (*adk-11*) in M63 plus 0.3 M NaCl. Although the initial rate of uptake of this solute was slightly lower in the mutant than in the wild type, the difference was not statistically significant (Fig. 1) and does not seem to be large enough to account for the inability of the mutant to use GB as an osmoprotectant. Similar results were obtained with all other class II

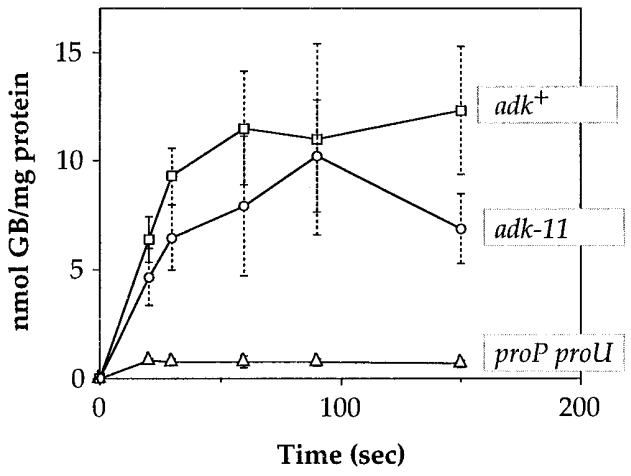

FIG. 1. Transport rates of [^{14}C]GB in the wild type and *adk-11* mutant. Assays were done in triplicate, as described in Materials and Methods, with strains TL1765 (*adk*$^+$ *proP*$^+$ *proU*$^+$) (squares), TL1774 (*adk-11 proP*$^+$ *proU*$^+$) (circles), and TL195 (*adk*$^+$ *proP1654 proU1655*::Tn*10*) (triangles) growing in M63 plus 0.3 M NaCl. Error bars are the standard deviations of three measurements. Initial transport rates for TL1765, TL1774, and TL195 were 19, 14, and 2 nmol/min/mg of protein, respectively.

mutants (data not shown). Assays of the steady-state pool sizes of GB in cells grown for several generations in media of high osmolality and [^{14}C]GB corroborated the conclusion that the lesions in the class II (*adk*) mutants did not affect the long-term accumulation of this osmoprotectant (data not shown).

To rule out the possibility that the *adk* mutations had activated a cryptic pathway of GB metabolism, we analyzed by thin-layer chromatography the metabolites extracted from cells grown in M63 plus 0.3 M NaCl plus [*glycyl*-^{14}C]GB or [*methyl*-^{14}C]GB; essentially 100% of the radioactivity in the cells was recovered as GB in both the *adk* mutants and the wild-type strain (data not shown).

Mapping of the class II mutations. We obtained a number of transposon insertions (*zbb-101* and *-62*::Tn*10* dTc, *zbb-2* and *-7*::Tn*10* dCm, and *zbb-900*::Mu dJ), which were linked in P22 transductions to the mutation causing Bet^s in strain TL242. The other class II mutations also proved to be linked to these insertions. We established with the locked-in Mu dP and Mu dQ mapping technique (57) that the approximate location of the *zbb-900*::Mu insertion is between *proBA* and *purE* (7 to 12 min). Fine-structure P22 mapping indicated the gene order *apt*, *zbb-900*::Mu dJ, *zbb-2*::Tn*10* dCm, *adk*, *zbb-101*::Tn*10* dTc, *zbb-876*::Tn*10*, *apeA* in the 11- to 12-min interval of the *S. typhimurium* chromosome (data not shown).

Cloning of the *adk*$^+$ gene of *S. typhimurium*. Cloning of the DNA proximal to the *zbb-2*::Tn*10* dCm tag is described in Materials and Methods. The first plasmid that we obtained, pJG2, had a 14-kbp insert (Fig. 2). Upon transfer into *S. typhimurium* TL1949 (*adk-11*), this plasmid could correct the Bet^s phenotype. By several successive subcloning steps, we obtained plasmid pJG350 (1.5-kbp *Sca*I-*Sal*I insert; Fig. 2), which could complement the Bet^s of strains TL238, TL239, TL241, and TL242. Further 5′ or 3′ deletions (pJG420, pJG430, pJG330, pJG610, and pJG620) or introduction of the *adk* insertions *adk-13*::Tn*phoA* (pJG341) and *adk-12*::Tn*phoA′-1* (pJG303) abolished complementing ability (Fig. 2).

The nucleotide sequence of a 1.6-kbp *Bam*HI-*Cla*I fragment capable of complementing the Bet^s phenotype was determined (Fig. 3). Computer analysis revealed the presence of a 214-codon open reading frame (ORF), whose predicted amino acid sequence exhibits a near identity (85% at the DNA level, 96% at the protein level) to the *E. coli adk* gene (3) and a high degree of similarity to two eukaryotic adenylate kinases: ADK1 of *Saccharomyces cerevisiae* (36, 37) and the human cytosolic Adk (42). There is a second, partial ORF downstream from the *adk* gene, with near identity to the 5′-region end of the *E. coli visA* gene, which specifies ferrochelase (18).

The suggestion that the mutations that cause Bet^s are in or near the *adk* gene was further corroborated by complementation experiments with *adk* mutants of *E. coli* and *S. typhimurium*. Plasmids pJG311 and pJG350 (Fig. 2) were able to complement the temperature-sensitive *adk-2* allele of *E. coli* CV2, and conversely, the *E. coli adk*$^+$ clone on pEak90 (3) could correct the sensitivity of the *Salmonella adk-11* mutant to GB in LB plus 0.3 M NaCl. Plasmids pJG303 and pJG341, which bear Tn*phoA* insertions in the *S. typhimurium adk* gene, did not complement the *E. coli adk-2* mutation.

To more precisely identify the location of the *adk* promoter and to study its regulation, a 0.7-kbp *Bam*HI-*Bgl*II fragment carrying sequences from the 5′ end of the gene was introduced into the pSC101-derived *lac* expression vector pDO182 (48), generating plasmids pJG1000 (*adk-lacZ* transcriptional fusion) and pJG1001 (*adk* in opposite orientation from *lacZ*). The β-galactosidase activities of cells grown in K and M63 media supplemented with 0.3 or 0.5 M NaCl, with and without GB, were very similar, suggesting that transcription of the *adk* gene is not subject to regulation by osmotic stress or by GB (Table 3).

Cloning and DNA sequence determination of the mutant *adk* genes. On the basis of the observation that the *adk* mutations in *S. typhimurium* did not result in a temperature sensitivity, we cloned the mutant genes by complementation of the temperature-sensitive *adk-2* mutation in *E. coli* and obtained plasmids pJG2380 (*adk-4*), pJG2391 (*adk-5*), pJG2410 (*adk-8*), and pJG2421 (*adk-11*) (see Materials and Methods). These multicopy plasmids were transferred into *S. typhimurium* Mu lysogens carrying *adk* alleles on the chromosome (TL2248, TL2250, TL2252, and TL2254); each plasmid could correct the Bet^s phenotype of the host strains, even when the identical *adk* mutant alleles were present both on the plasmid and on the chromosome. This result suggests that the mutant phenotype is the result of decreased Adk activity. Restriction mapping revealed in each of the plasmids a 1.6-kbp *Sca*I-*Cla*I and a 1.5-kbp *Sca*I-*Sal*I fragment, which were also present in the *adk*$^+$ plasmid pJG2. These fragments were subcloned into pBR322; the resultant plasmids could still correct both the temperature sensitivity of the *E. coli adk-2* mutant (CV2) and the Bet^s phenotype of the *S. typhimurium adk* mutants (data not shown).

Nucleotide sequence analysis revealed that the *adk-8* and *adk-11* mutations were the identical G→A change at nucleotide position 198 of the *adk* sequence (Fig. 3). This mutation lies within the putative Shine-Dalgarno sequence for *adk* and therefore might reduce the efficiency of translation initiation (22). The calculated free energy (ΔG°) of annealing of the wild-type *adk* Shine-Dalgarno sequence to 16S RRNA is −10.1 kcal (1 cal = 4.184 J)/mol, but that of the *adk-11* mutant is −3.8 kcal/mol (17). The *adk-4* allele is a C→T substitution at position 191, also in the untranslated leader of the *adk* mRNA (Fig. 3). It does not fall within any recognizable consensus element known to be important in translation initiation but might affect the formation of secondary structures in the leader RNA, recruitment of translation initiation factors, or the stability of the mRNA.

The *adk-5* allele, a change in the *adk* gene at nucleotide position 691, resulted in the replacement of Glu to Lys at

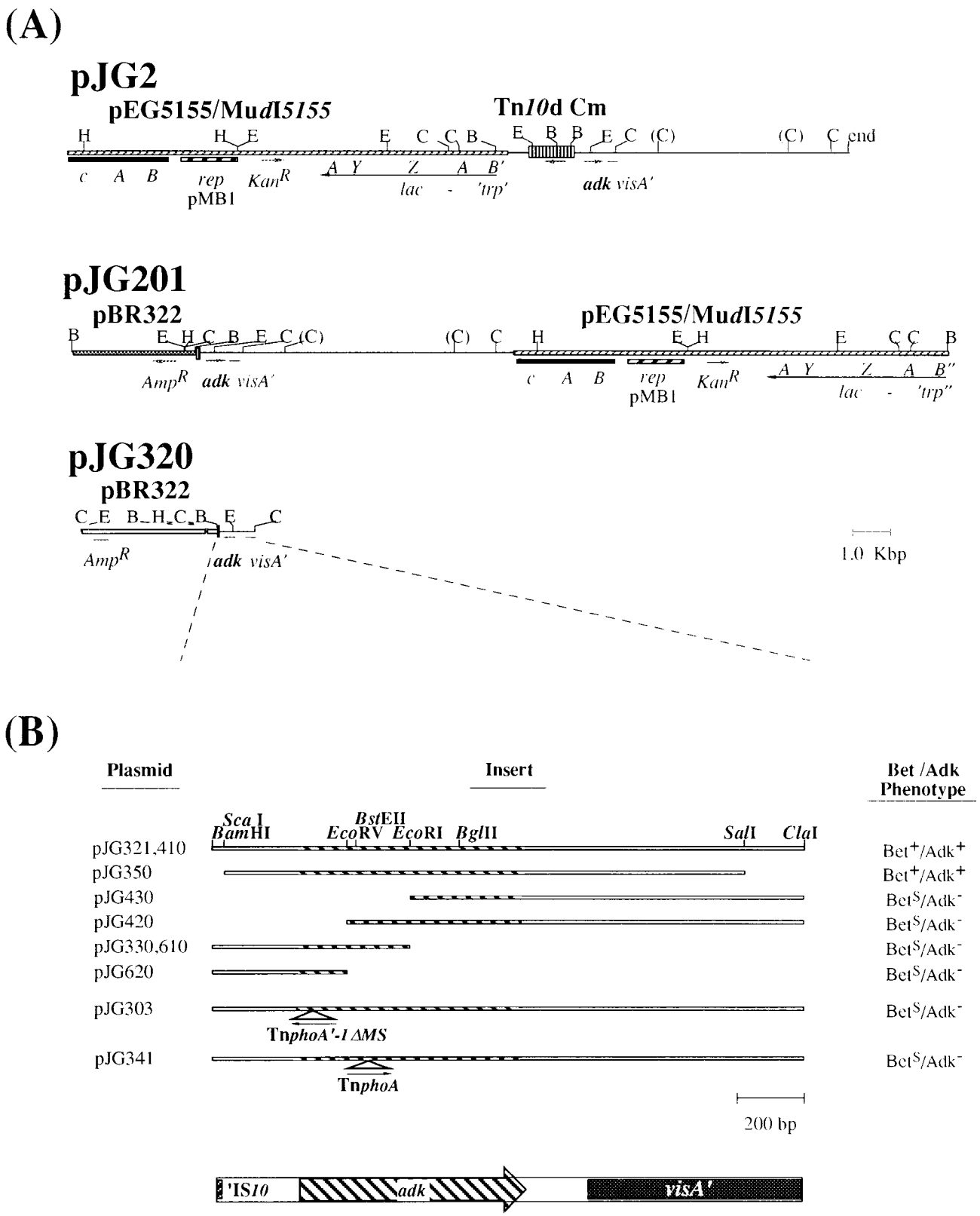

FIG. 2. Physical and genetic maps of the plasmids bearing the *adk*$^+$ gene. (A) Structures of the original mini Mu plasmid pJG2 and two *adk*$^+$ derivatives, pJG201 and pJG310. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII. (B) Deletion and insertion analysis of a 1.6-kb *Bam*HI-*Cla*I fragment that can complement the Bet^s phenotype of the *Salmonella adk* mutants and the temperature sensitivity of the *E. coli adk-2* mutant CV2. The deletion analysis was carried out with pJG410, and the insertion mutagenesis was carried out with pJG311.

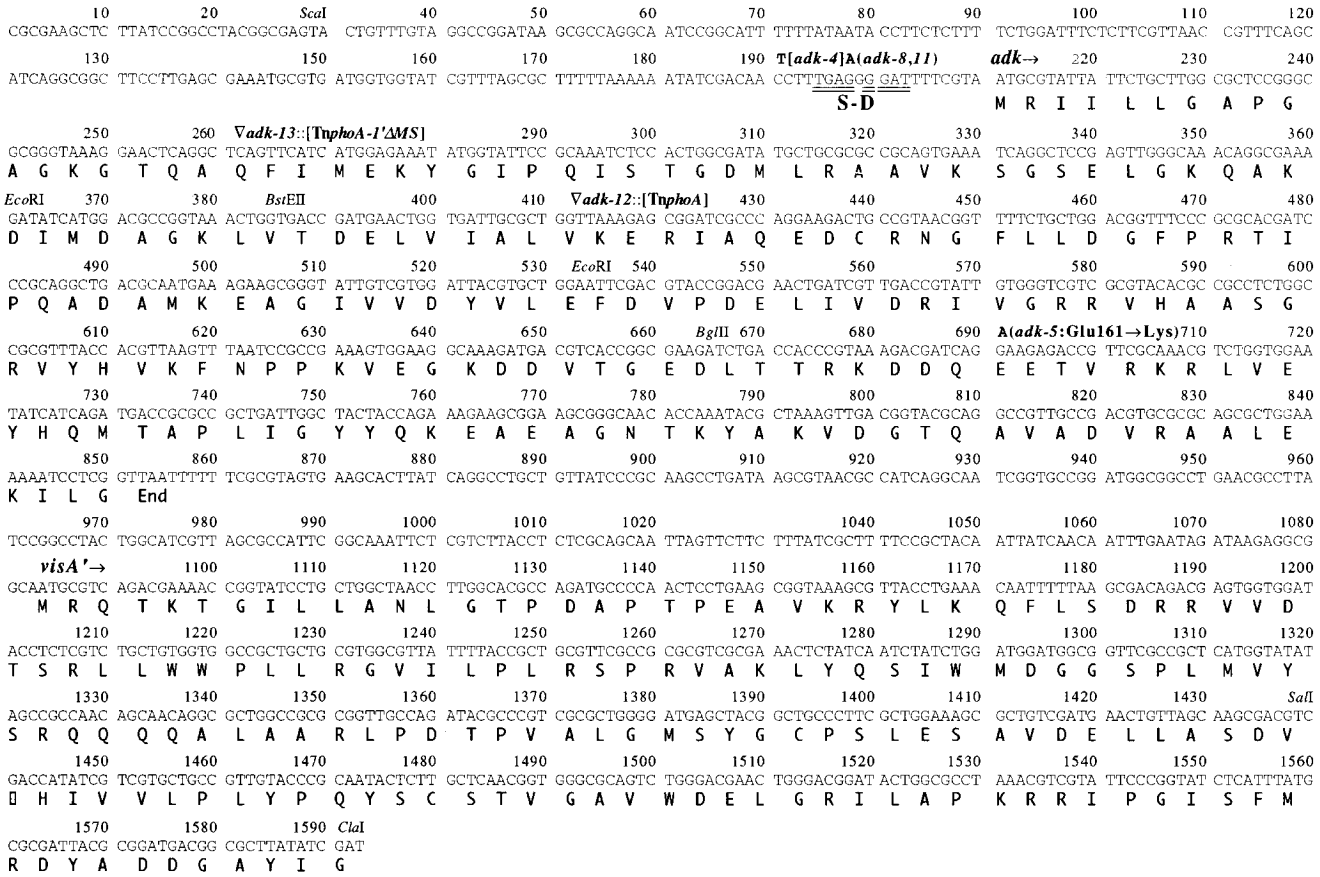

FIG. 3. Nucleotide sequence of a 1.6-kb fragment containing the *adk* gene of *S. typhimurium*. Also shown are the deduced amino acid sequences of an ORF with high degree of agreement to the *E. coli adk* gene product and of a partial ORF with high degree of agreement to the *E. coli visA* gene product. Putative signals for initiation of translation (Shine-Dalgarno [S-D] box) of the *adk* gene are indicated with double underlines. The locations of three point mutations and two insertions, *adk-4* (nucleotide 191), *adk-8*, *11* (nucleotide 198), *adk-5* (nucleotide 691), *adk-13*::Tn*phoA'-1ΔMS* (after nucleotide 261), and *adk-12*::Tn*phoA* (after nucleotide 412), are also displayed. Not shown are 67 nucleotides upstream of position 1 that are from the IS*10* element of *zbb-2*::Tn*10* dCm.

amino acid position 161 (Fig. 3). Glu-161 is contained in a 27-amino-acid loop of Adk, which is found in bacterial, yeast, and bovine mitochondrial Adks but absent from shorter cytosolic Adks from higher eukaryotes (19, 37).

The *adk* mutants have lower adenylate kinase activities. Assays of Adk activity in crude extracts of strains TL1765 (*adk*$^+$), TL2174 (*adk-4*), TL2176 (*adk-5*), TL2178 (*adk-8*), and TL1774 (*adk-11*) grown in M63 showed that the Bet^s mutants had lower Adk activities than the wild type (Table 4). The Adk activities of the mutants generally paralleled their degree of salt and GB sensitivities: the *adk-8* and *adk-11* mutants, which are the most sensitive, had the lowest Adk activity (65 to 71%

TABLE 3. Analysis of the control of the *adk* promoter[a]

Plasmid	Insert on plasmid	β-Galactosidase activity (nmol/min/mg of protein)					
		E. coli DH5α in K medium plus:			*S. typhimurium* TL1463 in M63 plus:		
		0 M NaCl	0.5 M NaCl	0.5 M NaCl + GB	0 M NaCl	0.3 M NaCl	0.3 M NaCl + GB
pDO182	*Bam*HI — *lacZYA*	1.7	0.9	0.4	5.1	3.1	2.7
pJG1001	*Bgl*II ← P_{adk} *Bam*HI — *lacZYA*	5.1	2.4	2.9			
pJG1000	*Bam*HI P_{adk}→ *Bgl*II — *lacZYA*	7.2×10^2	4.5×10^2	4.0×10^2	8.0×10^2	6.6×10^2	9.3×10^2

[a] A 0.7-kbp *Bam*HI-*Bgl*II fragment containing the *adk* promoter was placed in both orientations upstream of the *lacZ* gene on the expression vector pDO182 (48). The β-galactosidase activities synthesized from the resultant plasmids in *E. coli* DH5α and *S. typhimurium* TL1463 (*recA1 srl-2*::Tn*10*) were determined in exponentially growing cultures in the indicated media.

TABLE 4. Adenylate kinase activities in *adk* mutants

Strain	Relevant genotype	Adenylate kinase activity (μmol/min/mg of protein)[a]	
		M63	M63–0.3 M NaCl
TL1765	*adk*$^+$	1.44	1.10
TL2174	*adk-4*	0.53	0.47
TL2176	*adk-5*	1.09	0.82
TL2178	*adk-8*	0.42	0.33
TL1774	*adk-11*	0.50	0.32

[a] Measured in crude extracts by the reaction 2ADP → ATP + AMP as described in Materials and Methods. The strains were grown aerobically in M63 or M63 plus 0.3 M NaCl.

lower than wild-type activity), the *adk-4* mutant, which exhibited an intermediate sensitivity, had 63% decreased activity, and the *adk-5* mutant, which was the least sensitive, had the least reduction (24%) in Adk activity. When the cells were grown in M63–0.3 M NaCl, there was a slight (11 to 25%) decrease in the activities of both the wild-type and mutant strains relative to the values obtained with cultures grown in M63 without NaCl, but nevertheless, the Adk activities of the mutants were reduced by a similar factor as that of the wild type in the presence or absence of 0.3 M NaCl (Table 4).

Because most of the *adk* mutants of *E. coli* have thermolabile Adk activities, we compared the denaturation kinetics of the Adk activities of the *S. typhimurium* mutants with that of the wild type at 42°C. The Adk activity of the mutants in crude extracts did not exhibit increased heat sensitivity, which is consistent with the fact that strains TL238 to TL242 can grow at 42°C (data not shown).

When cloned into pBR322, the *adk-11* allele resulted in a 21-fold increase in the level of Adk over that of the host strain carrying the same allele in single copy on the chromosome (Table 5). Although the Adk activity of the strain carrying the cloned *adk-11* allele was sevenfold lower than that of a strain carrying the *adk*$^+$ allele on pBR322 (Table 5), the *adk-11* allele on pBR322 could correct the Bet^s phenotype of all of the *adk* mutants (data not shown). These results corroborate that the phenotypic defects caused by the *adk* mutations arise from a reduction in the Adk activity.

GB is toxic to the *adk* mutants. The *adk* mutants, which were isolated on the basis of their inability to use GB as an osmoprotectant in media containing ≥0.8 M NaCl, had an unanticipated phenotype: their growth was inhibited by the addition of GB in media of intermediate osmolality (0.3 to 0.5 M NaCl). The Bet^s phenotype was detectable in *adk* mutants within 10 min after the addition of GB and was evident in a variety of media (M63, LB, and K), provided that they contained solutes at sufficient concentration (≥0.5 osM) to induce uptake of GB (data not shown). The Bet^s phenotype of the *adk* mutants was not attenuated by single mutations in either *proP* or *proU*, and it was reduced, although not completely eliminated, by simultaneous *proP* and *proU* mutations (data not shown). Because in addition to the ProP and ProU systems, there is a third minor GB permease (15, 24, 39), these results suggest that the accumulation of GB is the cause for the growth inhibition of the *adk* mutants in media of high osmolality. The Bet^s phenotype was also manifested in media containing 0.3 M KCl or 0.6 M sucrose. GB at 0.1 μM was toxic to the *adk* mutants, and 10 mM proline could not relieve the toxicity of 0.1 μM GB. GB was bacteriostatic rather than bacteriocidal, because incubation of the *adk-11* mutant in M63–≥0.3 M NaCl–1 mM GB for 12 to 16 h did not decrease its subsequent viability in permissive media (data not shown).

TABLE 5. Adk activities of strains bearing wild-type or mutant *adk* alleles on plasmids[a]

Plasmid	*adk* allele on plasmid	Adk activity (μmol/mg protein/min)
pBR322	None	0.28
pJG301	*adk*$^+$	44.6
pJG303	*adk*::Tn*phoA-1′*	0.46
pJG2421BR	*adk-11*	6.14

[a] The host for each plasmid was TL1774 (*adk-11*). Adk activity was measured as described in Materials and Methods.

Two classes of compatible solutes in the *adk* mutants. In the wild-type strain, there is a hierarchy of the potency of osmoprotectants: GB is the most effective, followed by propiothiobetaine, stachydrine, choline-*O*-sulfate, proline, and L-carnitine, with intermediate potency, and γ-butyrobetaine, which is the weakest (Table 6). However, GB, propiothiobetaine, L-carnitine, and γ-butyrobetaine were inhibitory to the *adk* mutants, whereas stachydrine, choline-*O*-sulfate, and proline still acted normally as osmoprotectants. These results are illustrated with the *adk-11* mutant strain in Table 6. Thus, the potency of the osmoprotectants in the wild type does not appear to be related to their toxicity to the *adk-11* mutant.

Effects of GB and proline on adenylate levels in the *adk-11* mutant. The sensitivity of the *adk* mutants to GB might stem from their inability to buffer ATP levels sufficiently during uptake of GB. Therefore, we determined the effects of GB on the levels of the adenylates in strains TL1774 (*adk-11*) and TL1765 (*adk*$^+$) in M63 plus 0.5 M NaCl. The results of this experiment are in Fig. 4. The steady-state levels of ATP and ADP in the *adk-11* mutant were lower than those of the wild type by 38 and 55%, respectively, even before the addition of GB. After the addition of GB, the ATP level in the mutant decreased by 87% within 120 min, while in the wild type, it increased by 15%. During this time, the ADP level increased 138% in the mutant but only by 41% in the wild type. Lastly, there was over a 207% increase in the AMP pool in the mutant, whereas in the wild-type, the AMP pool increased by only 56%. After 120 min, the ATP pool of the *adk* mutant increased slowly but did not recover to the level found in the wild type. In the mutant, the AMP pool increased to levels that would necessitate additional de novo synthesis. This result, which is unexpected in view of the finding that AMP is an allosteric inhibitor of its own synthesis (47), raises the possibility that there is some uncharacterized regulation of the synthesis of adenylates which could override the allosteric inhibition of synthesis of AMP at low ATP concentrations. In contrast to GB, proline neither depleted the ATP pool nor resulted any significant long-term changes in the levels of ADP and AMP in the *adk-11* mutant (Fig. 4). In the other *adk* mutants, ATP pools declined by ~60% within 2 h after the addition of GB in M63 plus 0.5 M NaCl (data not shown). The drastic drop in the ATP pools in these mutants after the addition of GB could account for their sensitivity to this metabolite.

As calculated from the data in Fig. 4, the EC_A in the *adk-11* mutant was 0.6 before the addition of GB and fell to 0.2 within 60 min upon the addition of GB. In contrast, the EC_A was not affected by proline in the mutant. In the *adk*$^+$ strain, the EC_A was 0.75 and was not greatly altered upon addition of GB or proline.

TABLE 6. Effects of various osmoprotectants on the growth of an *adk-11* mutant aerobically[a]

Strain (genotype)	Growth rate (generation/h) in M63 + 0.5 M NaCl supplemented with:							
	Nothing	GB	Propiothiobetaine	Stachydrine	Choline-*O*-sulfate	Proline	L-Carnitine	γ-Butyrobetaine
TL1765 (*adk*$^+$)	0.37	0.77	0.71	0.67	0.58	0.56	0.56	0.53
TL1774 (*adk-11*)	0.35	0.16	0.18	0.53	0.56	0.53	0.27	0.28

[a] Exponential growth rates were determined aerobically in M63 plus 0.5 M NaCl plus 1 mM indicated osmoprotectants.

DISCUSSION

As part of our attempts to characterize the mechanism by which GB acts to alleviate osmotic inhibition, we isolated mutants that could no longer use this solute as an osmoprotectant. Unexpectedly, the mutations in these strains resulted in a 24 to 71% decrease in Adk activity and conferred sensitivity to GB in media of high osmolality. Thus, they represent a new class of conditional *adk* lesions. The addition of GB to cultures of the mutants resulted in a rapid depletion of their ATP pools in media of high osmolality but not when they were grown in media whose osmolality was not high enough to induce the uptake of GB. The GB-dependent growth stasis and depletion of the ATP pools were also attenuated if both the ProP and ProU systems were inactivated by mutations. These observations suggest that as a consequence of a partial decrease in the Adk activity, the mutants are unable to replenish their ATP pools properly during the energy-consuming process of GB uptake. It is surprising that relatively modest decreases in the Adk activity have any dramatic phenotypic consequences, such as inhibition of growth by GB. This result could be interpreted to indicate that, contrary to the generally held view (1), Adk may not be in great excess. The conclusion that Adk is not in excess is also supported by our observation that overexpression of Adk from mutant alleles in high copy suppressed the Bet^s phenotype of the *adk* mutants. The result obtained by Sakai et al. (52) that the overexpression of the *Saccharomyces cerevisiae* Adk1 gene product in *Candida* sp. led to increased ATP pool also suggests that Adk is not in excess in that organism.

Upon the addition of GB to cultures of the *adk-11* mutant growing in the presence of 0.3 M NaCl, its EC_A declined rapidly to ~0.2, which is well below the value characteristic of exponential growth (7). However, it has been questioned whether enzymes respond to the EC_A itself as an allosteric effector or rather respond to the concentrations of different specific nucleotides (46, 50). Thus, growth inhibition by GB might stem from a deficiency in ATP or an excess in AMP. The possibility that GB-dependent growth inhibition is due to ATP shortage is supported by the finding (24) that the Bet^s phenotype of the *adk* mutants could be partially corrected and their ATP pool could be boosted by growing them with phos-

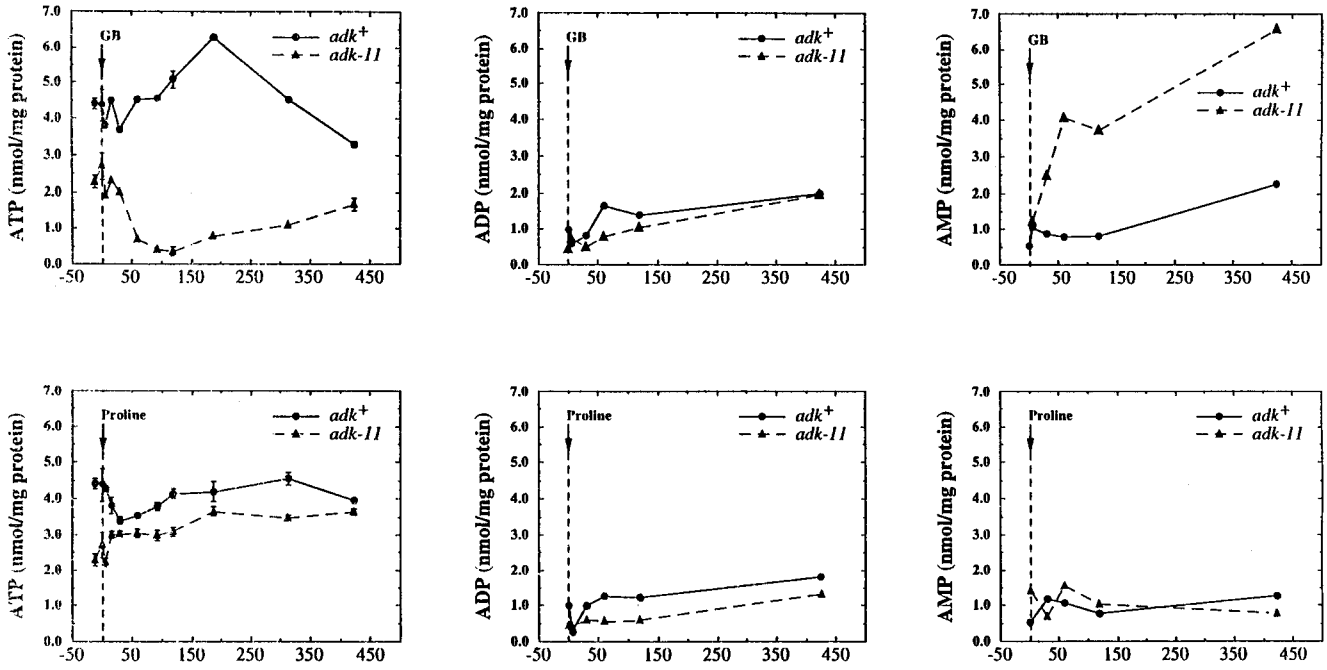

FIG. 4. Effects of GB and proline on the ATP, ADP, and AMP levels in the *adk-11* mutant. Strains TL1765 (*adk*$^+$) and TL1774 (*adk-11*) were grown in M63 plus 0.5 M NaCl plus 1 mM GB (top three panels) and proline (bottom three panels), added at time zero, indicated by the arrow. The levels of ATP (left panels), ADP (middle panels), and AMP (right panels) were determined at various time points as described in Materials and Methods. ATP values are the averages of triplicate measurements; ADP and AMP values are from single measurements for each time point.

phoenolpyruvate, 3-phosphoglycerate, or α-glycerophosphate (which are taken up by *S. typhimurium* without hydrolysis [51] and used to generate ATP by pyruvate kinase).

Solutes that are osmoprotectants for the wild-type strain fall into two classes with respect to their effects on *adk* mutants: GB, propiothiobetaine, L-carnitine, and γ-butyrobetaine are toxic to the mutants in media of moderate or high osmolality, whereas proline, stachydrine, and choline-*O*-sulfate are still able to function normally as osmoprotectants. Because these solutes are accumulated by the same active transport systems, it is difficult to explain, simply on the basis of the energetics of transport, why the accumulation of some osmoprotectants depletes the ATP pool of the *adk* mutants while the accumulation of others does not. The shift of a temperature-sensitive *adk* mutant of *E. coli* to the nonpermissive temperature was shown to result in a decrease in the ATP pool and an increase in the AMP pool (20, 21), which is similar to what we observed in the *adk-11* mutant of *S. typhimurium* upon the addition of GB. The inhibition of growth upon the uptake of osmoprotectants in the former class can be explained by postulating that these solutes may partially inhibit Adk activity while the osmoprotectants in the latter class do not, so that higher Adk activity would be required when cells accumulate osmoprotectants in the former class. However, preliminary results indicated that the Adk was not inhibited substantially by 1 M GB or 1 M proline in assays of crude extracts of the wild type or the *adk* mutants (24).

It has been proposed that Adk, which might have membrane-associated and periplasmic forms (55), can interact with another membrane-bound enzyme, *sn*-glycerol-3-phosphate acyltransferase (32). A second possible explanation for the differential effects that the two classes of osmoprotectants have on the *adk* mutants could be that there is an interaction between Adk and the transport systems for osmoprotectants, which regulates Adk such that its activity is partially inhibited as a result of the transport of GB and the other osmoprotectants that are toxic to the *adk* mutants. This explanation, however, is not attractive not only because it requires that Adk interact with both the ProP and the ProU systems, which have very different structures, but also because it involves the ad hoc assumption that the regulatory interaction between Adk and the two transport systems for osmoprotectants is sensitive to the nature of the substrate taken up.

The rapid depletion of the ATP pools that occurred in the *adk* mutants upon challenge with GB can account for the sensitivity of the mutants to this solute. However, this effect of GB cannot be explained satisfactorily solely by stoichiometric considerations of the energy cost of transport. In addition to GB, the cells take up a number of other nutrients by energy-linked transport systems, such as carbon sources, K^+, SO_4^{2-}, Mg^{2+}, and HPO_4^{2-}. Nevertheless, the *adk* mutants are sensitive to only GB and a few other osmoprotectants in media of high osmolality and exhibit nearly normal growth in most other media. In particular, they are not inhibited by proline, which can be accumulated to about 70% of the GB levels under conditions of high osmotic stress (6). In *E. coli*, the GB content in the presence of 0.5 M NaCl plus 1 mM GB was reported to be 1.2 μmol/mg (dry weight) of cells (6), whereas it was estimated that at least 43 μmol of ATP is required for the synthesis of 1 mg (dry weight) of cells (46). Mimmack et al. (45) concluded that in vivo, approximately two ATPs are hydrolyzed per one molecule of GB taken up by the ProU system. Therefore, on the basis of these values, the energetic burden for the transport of GB would be only 6% of the total ATP needed for biosynthesis. There are a number of factors which complicate the determination of the energy cost of GB transport. The accumulation of GB alters the intracellular levels of other osmolytes (K^+, glutamate, and trehalose) (6), and consequently, the energy cost for GB transport might be difficult to establish precisely because of possible changes in the energy input required for the accumulation of other compatible solutes. Furthermore, GB, which can be present in cells at concentrations of 0.5 to 1.0 M (49), has been shown to be excreted continuously in exponentially growing cells via at least one export system that is distinct from the ProP and ProU permeases (38–40). A futile cycle of simultaneous uptake and excretion of GB could greatly increase the energy required for the maintenance of this solute at high intracellular concentrations. If proline and the other solutes which can function as osmoprotectants for the *adk* mutants are not subject to a futile cycle of uptake and excretion, then it is possible that the toxicity of some of the osmoprotectants to the *adk* mutant is due to the excessive ATP burden imposed on them because of multiple cycles of uptake and excretion. This proposal might be tested by isolating mutations which can suppress the Bet^s phenotype of the *adk* mutants and determining whether any of the suppressor mutations can inactivate the excretion of GB.

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REFERENCES

1. **Atkinson, D. E.** 1971. Adenine nucleotides as stoichiometric coupling agents in metabolism and as regulatory modifiers: the adenylate energy charge, p. 1–21. *In* H. Vogel (ed.), Metabolic regulation. Academic Press Inc., New York.
2. **Barron, A., J. U. Jung, and M. Villarejo.** 1987. Purification and characterization of a glycine betaine binding protein from *Escherichia coli*. J. Biol. Chem. **262:**11841–11846.
3. **Brune, M., R. Schumann, and F. Wittinghofer.** 1985. Cloning and sequencing of the adenylate kinase gene (*adk*) of *Escherichia coli*. Nucleic Acids Res. **13:**7139–7151.
4. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. **164:**1224–1232.
5. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. **164:**1218–1223.
6. **Cayley, S., B. A. Lewis, and M. T. Record, Jr.** 1992. Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J. Bacteriol. **174:**1585–1595.
7. **Chapman, A. G., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108:**1072–1086.
8. **Cohen, G. N., and H. V. Rickenberg.** 1956. Concentration specifique reversible des amino acides chez *E. coli*. Ann. Inst. Pasteur (Paris) **91:**693–720.
9. **Cousin, D., and G. Buttin.** 1969. Mutants thermosensibles d'*Escherichia coli* K-12. III. Une mutation letale d'*E. coli* affectant l'activite de l'adenylate kinase. Ann. Inst. Pasteur (Paris) **117:**612–630.
10. **Csonka, L. N.** 1982. A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. J. Bacteriol. **151:** 1433–1443.
11. **Csonka, L. N., and A. D. Hanson.** 1991. Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. **45:**569–606.
12. **Csonka, L. N., M. M. Howe, J. L. Ingraham, L. S. Pearson III, and C. L. Turnbough, Jr.** 1981. Infection of *Salmonella typhimurium* with coliphage Mu *d*1 (Ap^r *lac*): construction of *pyr*::*lac* gene fusions. J. Bacteriol. **145:**299–305.
13. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Doige, C. A., and G. F.-L. Ames.** 1993. ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. Annu. Rev. Microbiol. **47:**291–319.
15. **Dunlap, J. V., and L. N. Csonka.** 1985. Regulation of the osmotically stimulated transport of proline and glycinebetaine in *Salmonella typhimurium*, p. 115–128. *In* J. L. Key and T. Kosuge (ed.), Cellular and molecular biology of plant stresses. Alan R. Liss, Inc., New York.
16. **Eggleston, L. V., and R. Herms.** 1952. Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. J. Biochem. **52:**156–160.

17. **Freier, S. M., R. Kierzek, J. A. Jaeger, N. Sugimoto, M. H. Caruthers, T. Neilson, and D. H. Turner.** 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83:**9373–9377.
18. **Frustaci, J. M., and M. R. O'Brian.** 1993. The *Escherichia coli visA* gene encodes ferrochelase, the final enzyme of the heme biosynthetic pathway. J. Bacteriol. **175:**2154–2156.
19. **Gerstein, M., G. Shulz, and C. Chothia.** 1992. Domain closure in adenylate kinase. Joints on either side of two helices close like neighboring fingers. J. Mol. Biol. **229:**494–501.
20. **Glaser, M., W. Nulty, and P. R. Vagelos.** 1975. Role of adenylate kinase of macromolecular biosynthesis in a putative mutant of *Escherichia coli* defective in membrane phospholipid biosynthesis. J. Bacteriol. **123:**128–136.
21. **Glembotski, C. C., A. G. Chapman, and D. E. Atkinson.** 1981. Adenylate energy charge in *Escherichia coli* CR341T28 and properties of heat-sensitive adenylate kinase. J. Bacteriol. **145:**1374–1385.
22. **Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. S. Singer, and G. Stormo.** 1981. Translational initiation in prokaryotes. Annu. Rev. Microbiol. **35:**365–403.
23. **Groisman, E. A., and M. J. Casadaban.** 1986. Mini-Mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J. Bacteriol. **168:** 357–364.
24. **Gutierrez, J. A.** 1993. Adenylate kinase of *Salmonella typhimurium* is involved in the maintenance of the energy charge during the uptake of the osmoprotectant glycinebetaine. Ph.D. thesis. Purdue University, West Lafayette, Ind.
25. **Gutierrez, J. A., and L. N. Csonka.** 1989. *Salmonella typhimurium* mutants that are not stimulated by glycinebetaine in media of elevated osmolarity, p. 89–98. *In* J. H. Cherry (ed.), Environmental stress in plants: biochemical and physiological mechanisms. Springer-Verlag, Berlin.
26. **Gutierrez, J. A., and L. N. Csonka.** 1992. Molecular and biochemical characterization of *Salmonella typhimurium* adenylate kinase mutants sensitive to the osmoprotectant glycinebetaine, abstr. K27, p. 261. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
27. **Gutierrez, J. A., and L. N. Csonka.** 1993. The involvement of adenylate kinase in the utilization of glycinebetaine as an osmoprotectant in *Salmonella typhimurium*, p. 21–29. *In* T. J. Close and E. A. Bray (ed.), Plant responses to cellular dehydration during environmental stress. The American Society of Plant Physiologists, Riverside, Calif.
28. **Heiman, C., and C. G. Miller.** 1978. Acylaminoacid esterase mutants of *Salmonella typhimurium*. Mol. Gen. Genet. **164:**57–62.
29. **Henson, J. M., A. Blinkowa, and J. R. Walker.** 1982. The *Escherichia coli dnaW* mutation is an allele of the *adk* gene. Mol. Gen. Genet. **186:**488–492.
30. **Higgins, C. F.** 1992. ABC-transporters from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
31. **Holm-Hansen, O., and D. M. Karl.** 1978. Biomass and adenylate energy charge determination in microbial cell extracts and environmental samples. Methods Enzymol. **57:**73–85.
32. **Huss, R. J., and M. Glaser.** 1983. Identification and purification of an adenylate kinase-associated protein that influences the thermolability of adenylate kinase from a temperature-sensitive *adk* mutants of *Escherichia coli*. J. Biol. Chem. **258:**13370–13376.
33. **Jebbar, M., R. Talibart, K. Gloux, T. Bernard, and C. Blanco.** 1992. Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. J. Bacteriol. **174:**5027–5035.
34. **Kennedy, E. P.** 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79:**1092–1095.
35. **Kilbane, J. J., II, and B. A. Bielaga.** 1991. Instantaneous gene transfer from donor to recipient microorganisms via electroporation. BioTechniques **10:** 354–365.
36. **Konrad, M.** 1988. Analysis and *in vivo* disruption of the gene coding for adenylate kinase (ADK1) in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **263:**19468–19474.
37. **Konrad, M.** 1993. Molecular analysis of the essential gene for adenylate kinase from the fission yeast *Schizosaccharomyces pombe*. J. Biol. Chem. **268:**11326–11334.
38. **Koo, S. P., and I. R. Booth.** 1993. Analysis of mutants affected in the efflux system for betaine in *Escherichia coli*, abstr. K173, p. 291. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
39. **Koo, S. P., C. F. Higgins, and I. R. Booth.** 1991. Regulation of compatible solute accumulation in *Salmonella typhimurium*: evidence for a glycinebetaine efflux system. J. Gen. Microbiol. **13:**2617–2625.
40. **Lamark, T., O. B. Styrvold, and A. R. Strøm.** 1992. Efflux of choline and glycine betaine from osmoregulating cell of *Escherichia coli*. FEMS Microbiol. Lett. **96:**149–154.
41. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
42. **Matsuura, S., M. Igarashi, Y. Tanizawa, M. Yamada, F. Kishi, T. Kajii, S. Miwa, M. Sakurai, and A. Nakazawa.** 1989. Human adenylate kinase deficiency associated with hemolytic anemia. J. Biol. Chem. **264:**10148–10155.
43. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Milner, J. L., S. Grothe, and J. M. Wood.** 1988. Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J. Biol. Chem. **263:**14900–14905.
45. **Mimmack, M. L., M. P. Gallagher, S. R. Pearce, J. R. Hyde, and C. F. Higgins.** 1989. Energy coupling to periplasmic binding protein-dependent transport systems: stoichiometry of ATP hydrolysis during transport *in vivo*. Proc. Natl. Acad. Sci. USA **86:**8257–8261.
46. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates Inc., Sunderland, Mass.
47. **Neuhard, J., and P. Nygaard.** 1987. Purines and pyrimidines, p. 445–473. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
48. **Overdier, D. G., and L. N. Csonka.** 1992. A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **89:**3140–3144.
49. **Perroud, B., and D. LeRudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **161:**393–401.
50. **Purich, D. L., and H. I. Fromm.** 1973. Additional factors influencing enzyme responses to the adenylate energy charge. J. Biol. Chem. **248:**461–466.
51. **Saier, M. H., Jr., and B. U. Feucht.** 1980. Regulation of carbohydrate transport activities in *Salmonella typhimurium*: use of the phosphoglycerate transport system to energize solute uptake. J. Bacteriol. **141:**611–617.
52. **Sakai, Y., T. Rogi, T. Yonehara, N. Kato, and Y. Tani.** 1994. High-level of ATP production by a genetically-engineered *Candida* yeast. Bio/Technology **12:**291–293.
53. **Sanderson, K. E., and J. R. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition VII. Microbiol. Rev. **52:**485–532.
54. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
55. **Watanabe, K., H. Fukumoto, and K. Isoi.** 1986. Intracellular localization of ATP:AMP phosphotransferase in *Escherichia coli*. Biochem. Biophys. Res. Commun. **134:**527–531.
56. **Wilmes-Riesenberg, M., and B. L. Wanner.** 1992. Tn*phoA* and Tn*phoA′* elements for making and switching fusions for study of transcription, translation, and cell surface localization. J. Bacteriol. **174:**4558–4575.
57. **Youderian, P., P. Sugiono, K. L. Brewer, N. P. Higgins, and T. Elliot.** 1988. Packaging specific segments of the *Salmonella* chromosome with locked-in Mu*d*-P22 prophages. Genetics **118:**581–592.