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Regulation of *kdp* Operon Expression in *Escherichia coli*: Evidence against Turgor as Signal for Transcriptional Control

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Kdp, an inducible high-affinity K^+ transporter in *Escherichia coli*, is encoded by genes of the *kdpABC* operon, and its expression is regulated by the products of *kdpD* and *kdpE*. Loss of cell turgor has been proposed to be the signal which induces *kdp* expression (L. A. Laimins, D. B. Rhoads, and W. Epstein, Proc. Natl. Acad. Sci. USA 78:464–468, 1981). We reexamined *kdp* expression during steady-state growth under a variety of conditions and were able to confirm earlier observations which had indicated that it is primarily affected by the concentration of K^+ in the medium and by mutations in genes encoding various K^+ transporters in *E. coli*. Changes in pH of the culture also altered *kdp* expression. In all of these cases, an increase in $[K^+]$ of the medium repressed the operon. Several ionic solutes induced steady-state *kdp* expression (but to differing extents), whereas nonionic solutes had no effect, indicating that *kdp* expression is not determined by osmolarity of the growth medium. *kdp* expression during steady-state growth was shown also to be unaffected by the accumulation of other intracellular compatible solutes such as trehalose or glycine betaine, which would be expected to restore cell turgor during growth in high-osmolarity media. Two mutants that are defective in perception of the signal regulating *kdp* were isolated, and the mutation in each of them was mapped to the *kdpDE* regulatory locus. Analysis of *kdp* expression in one of these mutants provided additional evidence against the turgor regulation model. On the basis of these data, we discuss alternative candidates that might serve as the signal for control of *kdp* operon transcription.

Potassium is the major cation present within all cells, including bacteria such as *Escherichia coli*. The intracellular K^+ concentration ($[K^+]_i$) in *E. coli* under normal growth conditions is around 150 mM, and this level is believed to be required for the optimal activity of many cellular enzymes, including those engaged in protein synthesis, for the regulation of intracellular pH, for DNA-protein interactions, and for controlled dissipation of the proton motive force (4, 12). In addition, $[K^+]_i$ has been shown by several workers to increase progressively with increase in osmolarity of the growth medium and is therefore thought to be an important determinant of cell turgor (6, 7, 12, 15, 44).

To cope with environments that vary widely in their concentrations of extracellular K^+ ($[K^+]_e$), *E. coli* uses many transport systems with differing affinities for K^+. The best characterized among them is Kdp, an inducible high-affinity transporter encoded by genes of the *kdpABC* operon (13, 38). The expression of *kdp* is induced by low $[K^+]_e$ and is repressed by excess $[K^+]_e$. *kdp* expression is subjected to positive regulation by the products of two genes, *kdpD* and *kdpE*, which are organized as an operon immediately downstream of, and transcribed in the same direction as, the *kdpABC* structural genes (35, 38). Analysis of the nucleotide sequences of *kdpD* and *kdpE* had suggested that they constitute the sensor and response elements, respectively, of a two-component control system (47), similar to that described for other operons in *E. coli* (41). More recently, biochemical evidence supporting the view that KdpD is a sensory protein kinase exhibiting autophosphorylation and KdpE phosphotransfer characteristics and that KdpE binds to DNA immediately upstream of the promoter for the *kdpABC* structural genes, in a region which is necessary in *cis* for normal regulation of the operon, has also been obtained (30, 31, 43). The deduced amino acid sequence of KdpD also suggests that it is a transmembrane protein, and cell fractionation studies indicate that it is resident in the cytoplasmic membrane (31, 47).

Under conditions in which $[K^+]_e$ is not limiting and the Kdp transporter genes are repressed, *E. coli* uses three major constitutively expressed, low-affinity K^+ uptake systems: TrkG, TrkH (both together formerly called TrkA, which is the designation used in this report), and TrkD (5, 11, 14, 39). Mutations in *trkD* abolish the function of the TrkD porter, whereas mutations in *trkA* render defective the function of TrkA (that is, both TrkG and TrkH). In mutants defective in one or more Trk systems, *kdp* is expressed at intermediate $[K^+]_e$ as well (17, 23, 39). The transport activity of each of the K^+ transporters, Kdp, TrkD, and TrkA, has been shown to be increased by an increase in osmolarity of the assay medium (10, 27, 37).

The environmental factors governing regulation of the *kdp* operon were first studied by Laimins et al. (23), using *kdp-lac* operon fusion strains. Their investigations revealed that during steady-state growth, *kdp* induction for each strain occurs at that $[K^+]_e$ at which the growth rate decreases because of K^+ limitation. They also showed that the $[K^+]_e$ at which *kdp* was induced during steady-state growth was dependent on osmolarity of the medium; *kdp-lac* expression occurred at a higher $[K^+]_e$ in cultures grown at high osmolarity than in those grown at low osmolarity. In another experiment, they showed that upon addition of ionic or nonionic impermeable solutes to cultures grown at a $[K^+]_e$ sufficient to repress *kdp*, an instantaneous transient increase in transcription of *kdp* occurred. Glycerol, which is freely permeable across the cell membrane, did not cause this transient induction. From these results, Laimins et al. (23) proposed that turgor pressure is the signal for regulation of *kdp* expression and that growth in K^+-limiting conditions imposes a turgor stress on the cells. That *kdp* transcription

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increases transiently upon instantaneous addition of impermeable solutes has also been confirmed recently by Sugiura et al. (43).

Other work, reported both from our laboratory (17) and by Sutherland et al. (44), showed that whereas ionic solutes were able to induce steady-state *kdp-lac* expression in a range of $[K^+]_e$ tested, nonionic solutes failed to do so. This result appeared to be inconsistent with the turgor regulation model of *kdp* expression (8, 9, 18), and we therefore decided to reexamine in detail the mechanism of *kdp* regulation. Toward this end, we have used *kdp-lac* operon fusion strains to analyze *kdp* expression under a variety of conditions and in different genetic backgrounds. The results of this study provide evidence against the hypothesis that turgor is the signal controlling *kdp* transcription.

MATERIALS AND METHODS

Chemicals and media. All antibiotics, medium constituents, and chemicals were obtained from commercial sources and were at least of reagent grade. Unless otherwise specified, the nutrient medium used was LB (29), and the defined growth medium was minimal A (29) with glucose as the C source. The routine temperature used for growth of all *kdp-lac* strains was 30°C. Any sugar as a C source was used at a final concentration of 10 mM. Stock solutions of sugars used as osmolytes were filter sterilized before use. Three other categories of media were used in this study.

(i) K medium (17) contained the following per liter: KH_2PO_4, 1 mM; $(NH_4)_2SO_4$, 1.5 mM; $MgCl_2$, 0.08 mM; $FeSO_4$, 0.5 mg; Casamino Acids (Difco), 5 g; and thiamine, 2 mg (pH adjusted to 7.0 with free Tris base). The contribution of Casamino Acids to $[K^+]_e$ was about 350 μM. In Na-substituted K medium, NaH_2PO_4 (1 mM) was substituted for KH_2PO_4. Lactose-K minimal medium was similar to K medium but had lactose instead of Casamino Acids as the C source.

(ii) Medium MBM (6, 7) contained morpholinopropanesulfonic acid (MOPS), 40 mM; tricine, 4 mM; NH_4Cl, 9.52 mM; KH_2PO_4, 1.32 mM; $MgCl_2$, 0.523 mM; Na_2SO_4, 0.276 mM; $FeSO_4$, 0.1 mM; and trace micronutrients (pH adjusted to 7.0 with NaOH). Glucose was used as the C source.

(iii) Phosphate-buffered media with reciprocally varying concentrations of Na^+ and K^+ were prepared as described earlier by Epstein's laboratory (14) by mixing together suitable proportions of 115 mM K^+-phosphate medium (K115Na0) and 115 mM Na^+-phosphate medium (K0Na115), each containing, as appropriate, $(K \text{ or } Na)_2 HPO_4$, 46 mM; $(K \text{ or } Na)H_2PO_4$, 23 mM; $(NH_4)_2 SO_4$, 8 mM; $MgSO_4$, 0.4 mM; $FeSO_4$, 6 μM; sodium citrate, 1 mM; thiamine hydrochloride, 1 mg/liter; and glucose or other carbon source. According to the nomenclature used in this report, K60Na55 medium, for example, refers to a mixture of the two media in a 60:55 ratio.

Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin, 50; chloramphenicol, 50; kanamycin, 15 in low-osmolarity medium and 50 in other media; and tetracycline, 15 and 5 in rich and minimal media, respectively. The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was used in plates at a final concentration of 25 μg/ml.

Bacterial strains, phages, and plasmids. All bacterial strains were derivatives of *E. coli* K-12 and are listed in Table 1. Phage P1*kc* was from our laboratory collection. Phages λpMu507 (26), λp*lac*Mu55 (26), and λ1105 (48) have been described earlier. λ1105 is a vector for achieving high-frequency transposition of an otherwise transposition-defective Tn*10*-derived insertion element encoding Kan^r (48), which is referred to below as Tn*10*dKan.

Plasmid pJG1, encoding Tet^r Amp^r, is a pBR322 derivative with a 6.3-kb *Eco*RI-*Hin*dIII fragment bearing the *trkD* gene as well as the *rbsA* gene (5) and was obtained from J. Beckwith. Plasmid pHYD94 (19), encoding Cm^r, was used in some conjugation experiments to provide a convenient contraselectable marker in the recipient strain.

λp*lac*Mu55(Kan) mutagenesis. One new *lac* operon fusion in each of the two operons, *kdp* and *proU*, was obtained during the course of this work as follows. The *recA lacZ* strain GJ602 was coinfected with the *lac* fusion phage λp*lac*Mu55 and the helper phage λpMu507, and clones with random transposition of and lysogenization by the former phage were selected as Kan^r colonies as described by May et al. (26).

From among 2,000 Kan^r colonies screened, one strain, GJ602.1, was identified as a *kdp-lac* fusion derivative on the basis of (i) inducibility of *lac* expression in the strain by NaCl, (ii) its repressibility by K^+, and (iii) its map position (data not shown). The mutation in this strain was designated *kdp-204*::λp*lac*Mu55(Kan).

Another strain, GJ602.2, was identified in the same experiment as a *proU*::*lac* derivative, because (i) it displayed an osmoresponsive Lac$^+$ phenotype identical to that of strains with other *proU*::*lac* alleles characterized earlier (17) and (ii) the *lac* fusion concerned was shown in P1 transduction experiments to map to the *proU* locus at 58 min (data not shown). This allele was designated *proU610*::λp*lac* Mu55(Kan).

The *kdp-204*::*lac* and *proU610*::*lac* mutations were subsequently transferred from GJ602.1 and GJ602.2, respectively, into other strain backgrounds by P1*kc* transduction, using Kan^r as a marker for selection.

Transpositions of Tn*10*dKan. Random transpositions of Tn*10*dKan into the chromosome were generated following infection of strains with λ1105 as described previously (48).

Isolation of mutants altered in *kdp* regulation. A strain with the *kdp-205* mutation and another with the *kdp-207* mutation were both isolated as explained below in a single experiment whose original aim had been to obtain transposon insertion-generated mutants altered in the expression of *kdp-lac*. Random insertions of Tn*10*dKan were generated in GJ18 by infection with λ1105, and a double selection was imposed on the population of infected cells by plating for colonies on kanamycin-supplemented lactose-K minimal medium. (GJ18 has sufficiently low β-galactosidase activity in K medium that it is unable to grow on lactose as the sole C source under these conditions [17]). Further studies with two of the mutants obtained by this procedure showed that in either instance, the mutation conferring increased *lac* expression was not linked to the Kan^r phenotype but was instead closely linked in P1*kc* transduction to the *kdp-lac*(Ap) allele of GJ18 itself. The mutations were designated *kdp-205* and *kdp-207*; for further characterization, isogenic derivatives of MC4100 carrying each of these two alleles along with *kdp-lac*(Ap) were constructed by P1*kc* transduction (with selection for Amp^r) and were named GJ618 and GJ619, respectively.

Transduction and conjugation. The methods for P1*kc* transduction and conjugation were as described earlier (17, 29).

β-Galactosidase assays. The specific activity of β-galactosidase in cultures was measured, after treatment with chloroform and sodium dodecyl sulfate, by the method of Miller

TABLE 1. Strains of *E. coli* K-12

Strain	Genotype[a]	Derivation or reference
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 araD 139 flbB5301 deoC1 ptsF25*	Laboratory stock
TL1105A	*lac thi nagA rha trkA405 trkD1 kdpA5*::*lac*	23
RJ70	MC4100 *glpF*::Tn*10*	45
UE60	MC4100 *otsA*::Tn*10*	3
GJ18	MC4100 *kdp-200*::λd*lac*(Ap)	17
GJ602	*lacI*^{q} *lacZ* Δ*M15 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25 recA srl*::Tn*10*	From MC4100 by P1*kc* transduction in two steps
GJ602.1	GJ602 *kdp-204*::λp*lac*Mu55(Kan)	This study
GJ602.2	GJ602 *proU610*::λp*lac*Mu55(Kan)	This study
GJ610	*thi rha gal lacZ*(Am) *kdp-204*::λp*lac*Mu55(Kan)	From FRAG1 (13) by P1*kc* transduction
GJ616	GJ18 *trkA203 trkD204 zhc-904*::Tn*10*	This study
GJ617	GJ18 *trkA203 zhc-904*::Tn*10*	From GJ39 (17) by P1*kc* transduction
GJ618	MC4100 *kdp-205 kdp-200*::λd*lac*(Ap)	This study
GJ619	MC4100 *kdp-207 kdp-200*::λd*lac*(Ap)	This study
GJ627	GJ18 *zhc-904*::Tn*10 zie-901*::Tn*10*dKan	This study
GJ628	GJ18 *trkA203 zhc-904*::Tn*10 zie-901*::Tn*10*dKan	This study
GJ629	GJ18 *trkD204 zhc-904*::Tn*10 zie-901*::Tn*10*dKan	This study
GJ630	GJ18 *trkA203 trkD204 zhc-904*::Tn*10 zie-901*::Tn*10*dKan	This study
GJ632	GJ18 *trkA203 trkD204 ilv-3164*::Tn*10*Kan Δ(*zhc-904*::Tn*10*)*461*	Derived from GJ616 in two steps
GJ636	GJ18 *zbb-3055*::Tn*10* [F(Ts)*lac*]	Derived from GJ18 in two steps
GJ642	*thi rha lacZ nagA trkA405 kdp-200*::λd*lac*(Ap) *zie-90*::Tn*10*dKan	Derived from TK2205 (38) in three steps
GJ644	*nagA thi rha lacZ trkA405 trkD1 kdp proU610*::λp*lac* Mu55(Kan)	From TK2205 (38) by P1*kc* transduction
GJ648	TL1105A *glpF*::Tn*10*	P1(RJ70) × TL1105A
GJ649	TL1105A *otsA*::Tn*10*	P1(UE60) × TL1105A
GJ650	GJ618 *trg-3120*::Tn*10*Kan (pHYD94 Cm^r)	Derived from GJ618 in two steps
GJ651	GJ619 *trg-3120*::Tn*10*Kan (pHYD94 Cm^r)	Derived from GJ619 in two steps
GJ652	GJ618 *trkA203 zhc-904*::Tn*10*	From GJ618 by P1*kc* transduction

[a] Genotype designations are those described by Bachmann (1). Allele numbers have been given where they are known. With the exception of GJ636, all strains listed are F$^-$. The allele *kdp-200*::λd*lac*(Ap) represents a *lac* fusion into the *kdpABC* operon and was derived as a stabilized version of a Mud1(*lac* Ap) insertion in *kdp* (17). The *trkD204* mutation has also been referred to as *sta-1* in the text.

(29), and the values are expressed in the units defined by Miller.

RESULTS

Effects of $[K^+]_e$ and pH on steady-state *kdp* expression. The expression of *kdp* during steady-state growth was measured as a function of $[K^+]_e$ in two *kdp-lac* operon fusion strains, GJ610 (*trkA*$^+$ *trkD*$^+$) and TL1105A (*trkA trkD*). Consistent with earlier reports (17, 23, 44), *kdp-lac* expression in the former increased with progressive K^+ limitation, and such induction was further enhanced in the *trkA trkD* mutant, TL1105A (Fig. 1).

We examined the effects of addition of various solutes on steady-state *kdp-lac* expression in cells growing in K medium. In the course of experiments that made use of fermentable sugars as solutes, we found that pH itself was a factor affecting *kdp* expression. For example, at $[K^+]_e$ <10 mM, *kdp* expression in TL1105A was induced approximately 20-fold in the presence even of 0.2 M glycerol (a permeable solute) compared with that in its absence (data not shown). The pHs of the culture media at the time of assay were 7.0 and 7.7, respectively, for cultures growing exponentially in the presence and absence of glycerol. *kdp* induction by glycerol did not occur in the *glpF*::Tn*10* strain GJ647, which cannot utilize glycerol (45) and in which the pH change associated with growth was not different between the glycerol-supplemented and -unsupplemented cultures (data not shown). Induction of *kdp* expression, with a concomitant decrease in pH of the medium, was also obtained in TL1105A when we used glucose, mannitol, or autoclaved sucrose as a solute added to the growth medium; some of the sucrose was presumably hydrolyzed to glucose and fructose during the autoclaving process (15).

These findings led us to systematically examine the effect of pH on *kdp* induction at a variety of $[K^+]_e$ and in strains

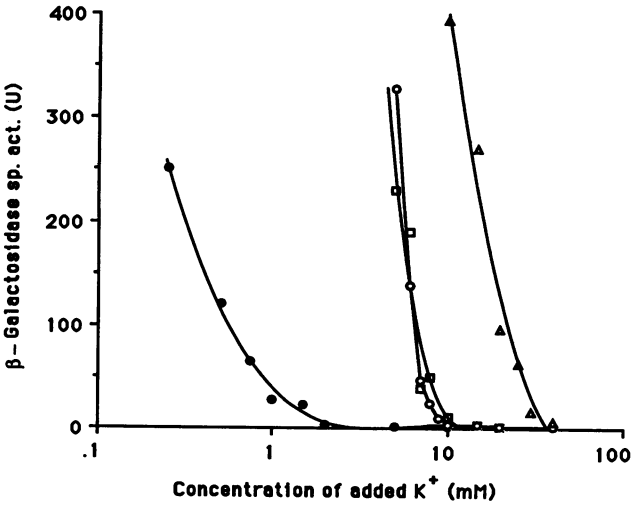

FIG. 1. β-Galactosidase specific activities in *kdp-lac* strains as a function of $[K^+]_e$ and of solutes added to the growth medium. GJ610 (*trkA*$^+$ *trkD*$^+$; closed symbols) and TL1105A (*trkA trkD*; open symbols) were grown to mid-log phase in media containing defined concentrations of KCl added to Na-substituted K medium and K medium, respectively, and that were unsupplemented (●, ○) or supplemented with 0.4 M sucrose (□) or 0.25 M NaCl (△).

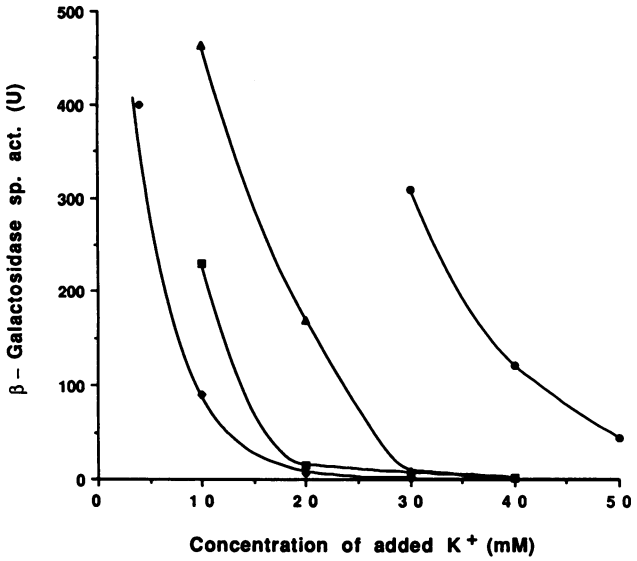

FIG. 2. Dependence of *kdp::lac* expression on pH of the growth medium. Cultures of TL1105A for assay were grown to mid-log phase in MBM-based media that differed in pH and in $[K^+]_e$. Symbols correspond to the following pH values at the time of inoculation: ●, pH 6.4; ▲, pH 6.9; ■, pH 7.4; and ◆, pH 7.85. The pH at the time of assay had decreased by 0.25, 0.1, 0.15, and 0.4 units, respectively.

defective in one or more K^+ porters. A decrease in pH of the medium at constant $[K^+]_e$ induced *kdp-lac* expression in all strains studied. The effect was most pronounced in TL1105A (Fig. 2) and moderate in its *trkD*$^+$ or *trkA*$^+$ derivatives (data not shown). In all of these instances, an increase in $[K^+]_e$ repressed *kdp* expression. The magnitude of *kdp* expression, for each of the various mutant combinations and pH values examined, exhibited an approximate inverse correlation with the $[K^+]_e$ required for half-maximal growth that had been determined earlier by Rhoads et al. (39). The triple mutant strain TL1105A failed to grow in a medium of pH 6.5 and $[K^+]_e$ of 20 mM, although it did grow if either of the two variables was increased, to 7 or to 30 mM, respectively. It is known that increased K^+ uptake is essential for pH homeostasis in cells growing in acidic environments (4).

The pH of the culture medium shows significant changes during growth, depending on various environmental factors such as C source, oxygen availability, choice of buffer, and so on. The respective pH values at time of inoculation and during the mid-logarithmic phase of growth in different media were as follows: K medium, 7.0 and 7.6; glucose-MBM medium, 7.0 and 6.5; and glucose-K115Na0 or glucose-K0Na115 medium, 7.0 and 6.95.

Since pH was not previously identified as a variable that affects *kdp* expression, the extent to which it might have contributed to changes in *kdp* expression in earlier studies (17, 23, 43, 44) is uncertain. In all of the experiments described below, comparisons of *kdp-lac* expression between different strains or between different conditions of growth have been made after controlling for pH.

Differential effects of ionic and nonionic osmolytes on steady-state *kdp* expression. The effect of osmolarity adjustments with ionic or nonionic solutes on *kdp-lac* expression was also studied. In the case of nonionic solutes, nonfermentable impermeable sugars such as D-arabinose or filter-sterilized sucrose were used as osmolytes, and as mentioned above, care was taken to ensure that interpretation of the results was not confounded by pH differences between solute-supplemented and -unsupplemented growth media. TL1105A was grown in media of varying $[K^+]_e$ in the presence and absence of isoosmotic concentrations of sucrose (0.4 M) or NaCl (0.25 M). NaCl supplementation was associated with a marked induction of *kdp* expression during steady-state growth, whereas sucrose did not have any significant effect (Fig. 1), confirming earlier observations made by one of us (17) and by Sutherland et al. (44).

We also tested whether the addition of different ionic solutes induced steady-state *kdp-lac* expression in GJ610. Many positively charged ionic species (added to a final concentration of 0.2 M in the K medium used for growth) induced *kdp* during steady-state growth but to different degrees; compared with the basal value of 20 U in K medium, the specific activity of β-galactosidase was highest with Na^+ or NH_4^+ supplementation (around 315 U), followed by Tris, arginine (at pH 7), and Mg^{2+}, which gave, respectively, 242, 200, and 110 U. In all of these instances, induction did not occur in media with high $[K^+]_e$. Lysine (at pH 7; 26 U) did not induce *kdp* expression in K medium, whereas Rb^+ (4 U) and, as expected, K^+ itself (3 U) repressed it. The different extents to which equiosmolar concentrations of ionic and nonionic solutes induced *kdp* expression during steady-state growth suggest that their effects on *kdp* induction may not depend on the osmotic properties of the solutes. The repressing effect of Rb^+ is also consistent with its reported ability to substitute for K^+, both for growth (24) and as a substrate for the various K^+ transporters in *E. coli* (5).

To test the effect of nonionic solutes on instantaneous induction of *kdp*, sucrose or D-arabinose (each dissolved in K60Na55 medium) was added, to a final concentration of 0.25 M, to a culture of TL1105A grown to log-phase in K60Na55 medium. The specific activity of β-galactosidase remained constant at 8 U for 10 min following solute addition, rose to a maximum of 35 U at 20 min, and gradually declined thereafter. Thus, instantaneous induction of *kdp* upon addition of nonionic solutes was confirmed, but the magnitude was less than that reported by Laimins et al. (23).

An osmosensitive mutation does not affect steady-state *kdp-lac* expression. Intracellular accumulation of solutes other than K^+ salts contributes to the restoration of turgor in cells growing in high-osmolar conditions. In *E. coli*, increased synthesis of trehalose occurs in response to growth at elevated osmolarity. The products encoded by the *otsA* and *otsB* genes are required for the osmotically regulated synthesis of trehalose, and mutations in either of the genes lead to an osmosensitive phenotype (3, 16, 42). We reasoned that if the hypothesis (12, 23) of inverse correlation between $[K^+]_e$ in the growth medium and turgor stress sensed by the cells were correct, an *ots* mutant may be expected to suffer turgor stress (and therefore to express *kdp-lac*) at a higher $[K^+]_e$ than would the corresponding *ots*$^+$ strain. We examined *kdp-lac* expression in the isogenic strains TL1105A (*ots*$^+$) and GJ649 (*otsA*::Tn*10*) after growing them in K medium supplemented with 0.4 M sucrose and with varying $[K^+]_e$. The range of $[K^+]_e$ chosen for study corresponded to the heel of the induction curve for TL1105A shown in Fig. 1, because it is in this range that expression of *kdp-lac* in the strain is expected to be most sensitive to perturbations in signal strength. The introduction of the *otsA* mutation did not have any significant effect on *kdp-lac* expression over this range of $[K^+]_e$ (Fig. 3A).

Effect of glycine betaine on *kdp-lac* expression. *E. coli* cells

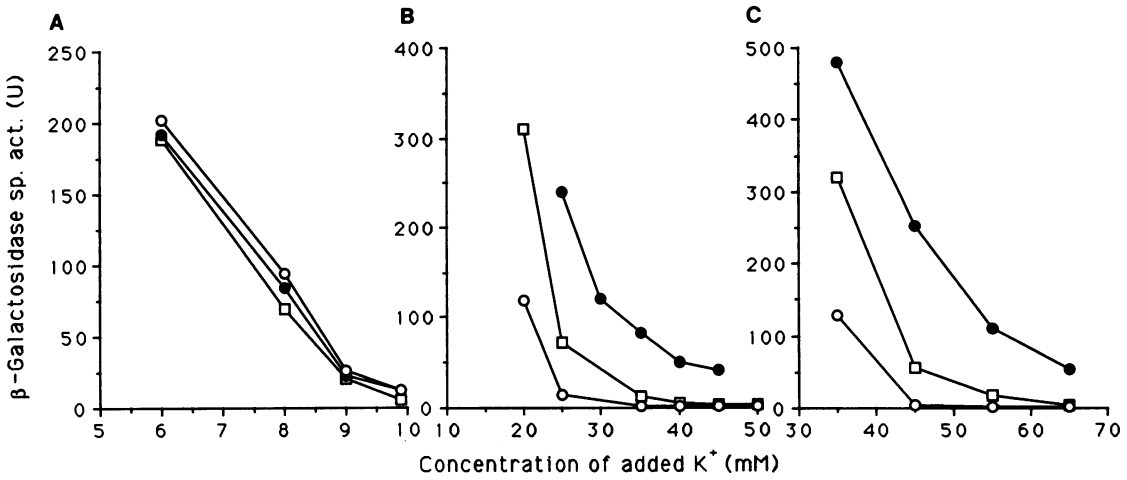

FIG. 3. Effects of glycine betaine and of an *otsA* mutation on *kdp-lac* expression in high-osmolarity media. Cultures of TL1105A (*otsA*$^+$) and GJ649 (*otsA*) for assay were grown without or with glycine betaine supplementation to mid-log phase in K medium plus 0.4 M sucrose (A), glucose-MBM plus 0.4 M sucrose (B), or glucose-MBM plus 0.25 M NaCl (C), each containing defined concentrations of added KCl as denoted on the abscissa. Symbols: ○, *otsA* without glycine betaine; □, *otsA*$^+$ without glycine betaine; ●, *otsA*$^+$ with 1 mM glycine betaine.

have two porters, ProU and ProP, for the active transport and intracellular accumulation of glycine betaine during growth in media of elevated osmolarity (8); each of these is believed to independently contribute to the alleviation of turgor stress under these conditions if, and only if, glycine betaine is provided in the growth medium. We exploited this fact to devise a test of the *kdp* turgor regulation model: the accumulation of glycine betaine in cells growing with 0.4 M sucrose over a range of $[K^+]_e$ should serve to partially relieve turgor stress and consequently reduce *kdp* expression. We found, however, that the *kdp-lac* induction profile of TL1105A growing in K medium supplemented with 0.4 M sucrose and a range of $[K^+]_e$ (as had been used above with the *otsA* derivative) was not significantly affected by the addition of 1 mM glycine betaine to the growth medium (Fig. 3A). The results of a control experiment demonstrated that the addition of 0.4 M sucrose induced *proU-lac* expression in the *kdp trkA trkD* strain GJ644 to 90% of normal (at the lowest $[K^+]_e$ used as the data point in Fig. 3A); the addition of 1 mM glycine betaine also had the expected feedback-repressive effect (8) on such induction (data not shown). These data ruled out the possibility (44) that *proU*$^+$ expression itself could be significantly impaired at these low values of $[K^+]_e$ in TL1105A.

Growth rate effect on *kdp* expression. One criticism of the experiments described above that looked at the effects of *otsA* or of glycine betaine on *kdp* expression is that they were done in K medium, which contains 0.5% Casamino Acids. It is possible that the presence in Casamino Acids of other osmoprotective compounds like L-proline (7, 8, 17) might have masked the true effects of trehalose and glycine betaine. We therefore repeated both sets of experiments by using defined glucose-MBM supplemented with 0.4 M sucrose as the basal growth medium. We observed once again that at no value of $[K^+]_e$ did either the *otsA* mutation or glycine betaine have the predicted effects of, respectively, increasing or decreasing *kdp-lac* expression (Fig. 3B). In fact, a consistent two- to fourfold alteration of expression in the opposite direction was observed in both instances (Fig. 3B). An essentially similar pattern of results (but with all curves shifted to the right) was also obtained when 0.25 M NaCl was substituted for 0.4 M sucrose in the experiments described above (Fig. 3C).

We considered the possibility that the observed reverse effect (from that expected) of *otsA* and of glycine betaine on *kdp-lac* expression in MBM-growth media was secondary to differences in growth rates of the different cultures, for the following reasons. First, it is known that the intracellular accumulation of glycine betaine or of trehalose is associated with an increase in growth rate in high-osmolarity media (7, 8, 16, 19, 34, 42). Second, Epstein and Schultz (15) had earlier shown that a direct relationship between $[K^+]_i$ and the richness of the C source used for growth possibly exists in *E. coli*. Finally, in cultures grown at different dilution rates in a chemostat, Tempest and Meers (46) also observed a correlation between growth rate and intracellular K^+ content per gram (dry weight) of cells.

We, therefore, examined whether *kdp* expression might also be influenced by changes in culture growth rate at constant $[K^+]_e$, pH, and osmolarity. TL1105A was grown to log-phase in K30Na85 medium supplemented with each of the following C sources (arranged in decreasing order of the corresponding growth rates): glucose plus Casamino Acids, glucose, maltose, and glycerol. The specific activities of β-galactosidase in these cultures were, respectively, 500, 250, 30, and 5 U, providing support for the notion that steady-state *kdp* expression is enhanced at elevated growth rates and that this is a sufficient explanation for the data depicted in Fig. 3B and C. We emphasize that the effect described above is obtained when growth rate is altered by means other than K^+ limitation; alterations in growth rate obtained by varying the $[K^+]_e$ have exactly the opposite kind of effect on *kdp-lac* expression (23).

Glycine betaine accumulation abolishes correlation between K^+-limited growth and *kdp* induction. In cultures grown in low-osmolarity medium, Laimins et al. (23) have shown that there is a close correlation between the $[K^+]_e$ at which growth rate is decreased and that at which steady-state *kdp-lac* induction occurs. In our experiments with glycine betaine addition to cultures grown at high osmolarity with varying $[K^+]_e$, we observed that this correlation breaks down (Fig. 4). Thus, the growth rates of TL1105A grown in

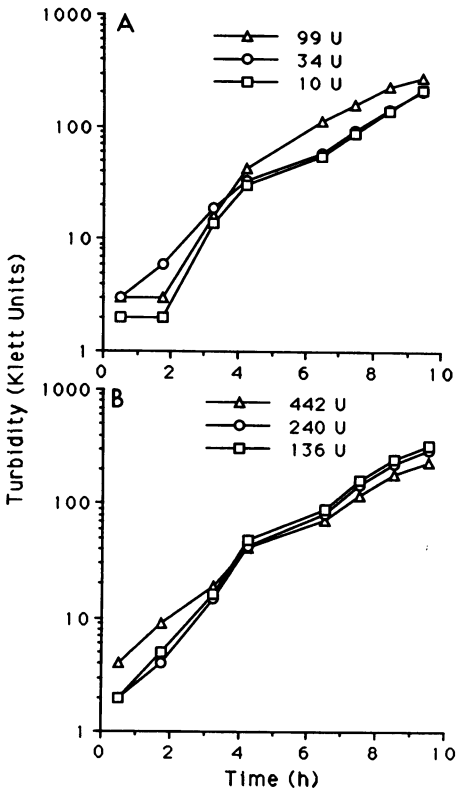

FIG. 4. Growth rates of TL1105A in glycine betaine-supplemented media of varying $[K^+]_e$. Cultures of TL1105A were grown overnight in glucose-MBM medium supplemented with 1 mM glycine betaine, either 0.4 M sucrose (A) or 0.25 M NaCl (B), and different concentrations of added KCl. Each was then subcultured 1:100 in the same medium, and optical density was monitored as a function of time in a Klett-type colorimeter. The symbols △, ○, and □ correspond respectively to added K^+ concentrations of 25, 30, and 40 mM in panel A and 35, 45, and 55 mM in panel B. Aliquots for enzyme assay were removed at approximately 5 h, and the measured specific activity (units) of β-galactosidase for each culture is marked.

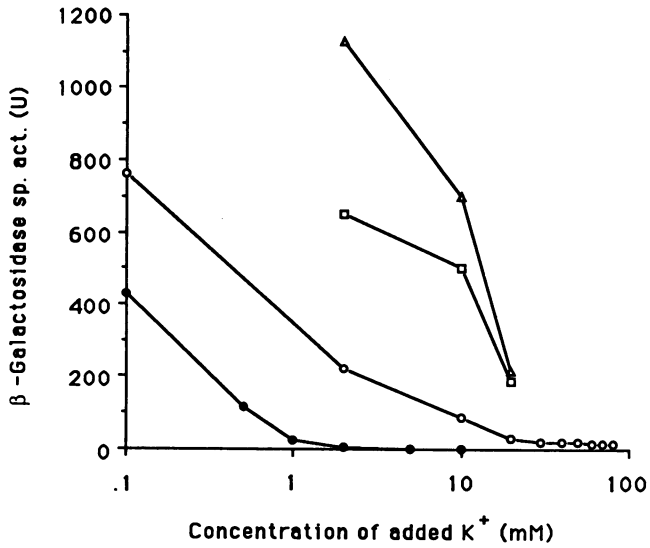

FIG. 5. Effect of *kdp-205* on *kdp-lac* expression. β-Galactosidase specific activities in cultures of GJ618 (*kdp-205*; ○) and GJ18 (*kdpD*$^+$ *E*$^+$; ●) are plotted as a function of [KCl] added to Na-substituted K medium. Also shown are curves corresponding to cultures of GJ652 (*kdp-205 trkA*) grown in the same medium (△) and of GJ618 grown in medium supplemented with 0.25 M NaCl (□).

sucrose-supplemented media at three values of $[K^+]_e$ were similar (Fig. 4), although the magnitude of *kdp-lac* expression was quite different among the three cultures. Similar results were obtained in NaCl-supplemented media (Fig. 4), as have also been reported earlier by Sutherland et al. (44). As discussed below, these data also support the conclusion that *kdp* transcription is not controlled by turgor.

Isolation and characterization of *kdp-lac* regulatory mutants. As described above, GJ618 and GJ619 were obtained as *kdp-lac*(Ap) strains carrying, respectively, the additional *kdp-205* and *kdp-207* mutations which conferred increased *lac* expression after growth in K medium. When P1*kc* lysates prepared on GJ618 and GJ619 were used to transduce MC4100 to Amp^r, more than 90% of the transductants (out of approximately 500 scored in each cross) exhibited a Lac phenotype similar to that of the respective mutants, whereas the remainder had the phenotype of GJ18, the wild-type *kdp-lac* strain; the mutations in both GJ618 and GJ619 were thus shown to be closely linked to, yet separable from, the *kdp-lac*(Ap) fusion itself. Transduction experiments with strains carrying Tn*10* insertions on either side of the *kdp* locus (from the collection of Singer et al. [40]) indicated that both *kdp-205* and *kdp-207* were situated to the counterclockwise (that is, promoter-distal) end of the *kdpABC* genes (data not shown), to the region where the *trans*-acting regulatory genes of the operon, *kdpD* and *kdpE*, have earlier been mapped (35, 38).

The map positions of the *kdp-205* and *kdp-207* mutations were each further confirmed as follows. The F-prime plasmid F(Ts)*lac114* was used to mobilize the chromosome of a *kdp-lac*(Ap) strain, GJ636, into the respective *kdp-205* and *kdp-207* mutant strains GJ650 and GJ651. It is known that chromosome transfer would be initiated within the region of homology shared between the *lac* region of the F-prime plasmid and that at the *kdp* locus (at 16 min) and that markers from the promoter-distal end (that is, counterclockwise) of *kdp-lac* would be transferred as early markers in conjugation (20). Recombinants were selected for a proximally located Tn*10* insertion allele at 11.5 min on the donor chromosome, and Cm^r (conferred by plasmid pHYD94 in the recipient) was used for contraselection. Approximately 60% of Tet^r exconjugants in both crosses (out of 400 scored on K medium plates supplemented with X-Gal) had also recombined at the *kdp-205* and *kdp-207* loci, respectively; none of 70 Tet^r exconjugants tested in either cross had recombined for a Tn*10*Kan allele located distally, at 31 min on the donor linkage map. These results clearly indicated that the *kdp-205* and *kdp-207* alleles were transferred as proximal markers in the conjugation experiments and thus established their map position to the *kdpDE* region.

The profile of steady-state *kdp-lac* expression in GJ618 as a function of varying $[K^+]_e$ in the growth medium is depicted in Fig. 5. A comparison of the results for GJ618 with those for the wild-type *kdp-lac* strain GJ18 indicates that the patterns of K^+ repression of *kdp* expression are similar, although the overall curve for GJ618 is shifted far to the right. Furthermore, the addition of NaCl to the culture medium or introduction of the *trkA* mutation into the strain evoked similar patterns of change in *kdp-lac* expression in

GJ618 and in the wild-type *kdp-lac* strain (Fig. 5). The addition of glycine betaine to a high-osmolar medium also did not alter *kdp-lac* expression in GJ618 (data not shown).

The *kdp-lac* induction profile of GJ618 is apparently similar to that of a *trkA trkD* mutant such as TL1105A. However, unlike the situation in the latter strain, in which enhanced expression of *kdp-lac* (compared with that in the *trkA*$^+$ derivative) at any particular $[K^+]_e$ was associated with a concomitant reduction in growth rate (23), that in GJ618 was not accompanied by a decrease in growth rate. The growth rates of GJ618 and of GJ18 at any of various $[K^+]_e$ were identical to one another (data not shown). The significance of these results with respect to the turgor regulation model for *kdp* is discussed below.

In contrast to that in GJ618, the expression of *lac* in GJ619 was constitutive (around 800 U) under all conditions and at all values of $[K^+]_e$ tested (data not shown). It may be noted that the maximal level of *kdp* expression observed in both GJ618 and GJ619 was approximately twofold higher than that in GJ18.

Isolation and characterization of a *trkD* gain-of-function mutant. Strains doubly mutant in *kdp* and *trkA* are extremely crippled for K^+ uptake and consequently grow slowly on most media. In the course of routine maintenance of a *trkA kdp-lac* strain (GJ617), a mutant, GJ616, was identified in which a presumed compensatory genetic alteration had resulted in the following changes in phenotype. (i) β-Galactosidase activity after growth in K medium was reduced to 6 U in GJ616, from 187 U in GJ617; consequently, GJ616 colonies were white on X-Gal-supplemented K medium, whereas those of GJ617 were blue on these plates. (ii) The presence of the *trkA* and *kdp* mutations in GJ617 renders it sensitive to growth at 42°C in K medium supplemented with 0.4 M NaCl (17), whereas GJ616 was able to grow under these conditions. The mutation in GJ616 conferring the new phenotype was not linked to either *kdp* or *trkA* (data not shown) and was tentatively designated *sta-1* (suppressor of *trkA*).

To map the *sta-1* locus, random transpositions of Tn*10*dKan were generated in MC4100, and a P1 lysate prepared on a population of these cells was used in transduction into GJ616, with selection for Kan^r on X-Gal-supplemented medium. Approximately 1% of the Kan^r transductant clones were blue on these plates, and upon further characterization, one of these latter clones was shown to have a Tn*10*dKan insertion which was 70% cotransducible with *sta*$^+$. Standard procedures (40) were then used to map *sta-1* to 84 min, 90% cotransducible with *ilv* in the gene order *bgl–sta-1–rbs–ilv* (data not shown).

The *trkD* locus is also located in the region between *bgl* and *rbs* and encodes one of the constitutive K^+ uptake systems in *E. coli*. We used an indirect test of marker rescue (25) to examine whether *sta-1* was allelic to *trkD*. Plasmid pJG1, carrying the cloned *trkD* gene and the adjacent *rbsA* gene (5), was introduced by transformation into the *sta-1* mutant GJ632 by selection for Tet^r. A P1*kc* lysate prepared on GJ632 (pJG1) was then used to transduce GJ642 (*kdp-lac trkA sta*$^+$) to Tet^r. The majority of transductants had acquired the original pJG1 plasmid and were dark blue on X-Gal-supplemented K medium indicator plates; however, approximately 1% of the colonies were pale blue on these plates, a phenotype characteristic of the *sta-1* mutant. Plasmid preparations from representative colonies of the two classes of transductants were used in subsequent transformation into GJ642 to demonstrate that the difference in *lac* expression in the latter class was indeed the result of an alteration in the plasmid molecules carried in them. The simplest explanation for these results would be to assume that *sta-1* is a dominant allele of *trkD* and that the pale blue colonies represent derivatives in which the mutation has been transferred by homologous recombination onto plasmid pJG1 (25). The *sta-1* mutation has accordingly been redesignated *trkD204*.

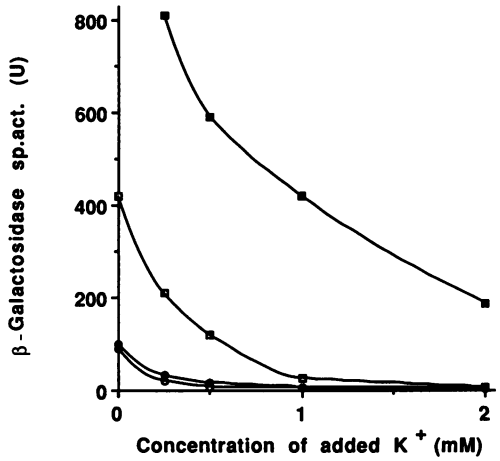

FIG. 6. β-Galactosidase specific activities in isogenic *kdp-lac* strains bearing different combinations of *trkA* and *trkD* alleles as a function of the concentration of added KCl in the growth medium (Na-substituted K medium). Symbols: □, GJ627 (*trkA*$^+$ *trkD*$^+$); ■, GJ628 (*trkA trkD*$^+$); ○, GJ629 (*trkA*$^+$ *trkD204*); ●, GJ630 (*trkA trkD204*).

The availability of the *zie-901*::Tn*10*dKan insertion near the *trkD204* locus and of *zhc-904*::Tn*10* near *trkA* enabled construction of a panel of isogenic *kdp-lac* strains that were *trkA*$^+$ *trkD*$^+$, *trkA trkD204*, *trkA*$^+$ *trkD204*, and *trkA trkD*$^+$. Measurement of β-galactosidase activity as a function of $[K^+]_e$ in each of these strains (Fig. 6) indicated that the *trkD204* mutation shifts the K^+ repression curve leftward in both *trkA*$^+$ and *trkA* backgrounds; that is, even though it was originally obtained in a *trkA* strain, its effect on *kdp-lac* occurs independently of the presence of the *trkA* mutation.

DISCUSSION

Mutations affecting *kdp* expression. Different mutations could affect *kdp-lac* expression either (i) by altering the strength of the environmental signal that is sensed by the cell in controlling *kdp* transcription, in which case the signal-transduction pathway, mediated by the products of *kdpDE* is itself normal; or (ii) by interfering with the latter pathway.

The data from this study and from earlier studies (17, 23) indicate that mutations in the K^+ transporter genes *kdpABC*, *trkA*, and *trkD* (including the gain-of-function *trkD204* allele) alter *kdp-lac* expression by the first mechanism. The hallmark of all these mutants is that there continues to be a close correlation between the value of $[K^+]_e$ at which growth limitation begins and that at which *kdp-lac* induction is observed (23). Various environmental factors that affect *kdp* expression, including pH, growth temperature (17), and the presence of other cationic solutes such as Na^+ or NH_4^+ in the culture medium, also do so by altering the strength of the signal. In all of these cases, *kdp* induction correlates with a decrease in growth rate, and both effects are reversible with K^+ supplementation.

On the other hand, mutants GJ618 and GJ619 appear to be

defective in the signal transduction pathway of *kdp* regulation. The mutation in either strain maps in *kdpDE*, and there is no correlation between the value of $[K^+]_e$ that becomes growth limiting and that at which sustained *kdp-lac* expression occurs. Of the two mutants, GJ619 remains fully constitutive at all values of $[K^+]_e$ tested and appears to be the first *trans*-acting *kdp* regulatory mutant of its kind that has been reported and characterized. In contrast, the *kdp-205* mutation in GJ618 confers a partially constitutive phenotype; a far weaker signal is sufficient to induce *kdp* expression in this mutant than in the isogenic $kdpD^+E^+$ strain.

An alternative explanation for the phenotype observed in GJ618 would be that the mutant regulatory protein (KdpD) requires a particular minimum $[K^+]_e$ to adopt a wild-type conformation and that this requirement has no relationship with the inducing signal per se. However, such a possibility is excluded by our observation that the curve describing *kdp-lac* expression as a function of $[K^+]_e$ in the *kdp-205* strain is shifted to the right by addition of Na^+ to the growth medium or by introduction of the *trkA* mutation. Thus, the $[K^+]_e$ at which repression is established in GJ618 is not invariant but can be perturbed to much the same extent as that in the $kdpD^+E^+$ parent by factors which are known to influence signal strength in the latter.

Is turgor the signal for *kdp* regulation? Several results obtained by us and by workers elsewhere are discordant with the proposal (23) that *kdp* expression is turgor regulated, and these are listed below. Some of the arguments presented here have also been discussed earlier by one of us (18).

(i) The expression of *kdp-lac* during steady-state growth was markedly induced by the presence of ionic solutes in the growth medium but was unaffected by nonionic solutes. The different ionic solutes tested also showed a gradation in the extent to which they induced *kdp* expression. If loss of cell turgor were the inducing signal, equiosmolar concentrations of various impermeable solutes would be expected to induce *kdp* expression to similar extents.

In this context, one line of evidence reported by Laimins et al. (23) in support of turgor regulation of *kdp* was that steady-state expression of *kdp-lac* for a given low $[K^+]_e$ was reduced when medium osmolarity was decreased; in their experiment, however, osmolarity was lowered by progressive dilution of the predominantly Na^+-containing growth medium, and we believe now that their results can be explained on the basis of Na^+-specific effects on *kdp* expression, described above and earlier (17, 44).

(ii) Accumulation of glycine betaine via ProU and ProP did not reduce *kdp* expression at any value of $[K^+]_e$. Glycine betaine is expected to contribute to restoration of cell turgor during adaptation of cells to high-osmolar conditions, and its instantaneous accumulation is known, for example, to be able to trigger K^+ loss from K^+-replete cells through turgor-activated efflux pathways (2, 28).

(iii) Furthermore, our results indicate that although *kdp-lac* is induced with decreasing $[K^+]_e$ during growth in glycine betaine-supplemented high-osmolarity media, the growth rates of the different cultures under these conditions were similar to each other (Fig. 4). The latter observation lends support to the notion that various properties of the intracellular steady-state (including ionic strength, concentrations of compatible solutes, and turgor) could not have been different between the various cultures.

(iv) The absence of trehalose accumulation at high osmolarity in an *ots* mutant also did not increase *kdp*::*lac* expression compared with that in an isogenic ots^+ strain. Since trehalose is another compatible solute whose intracellular accumulation is expected to contribute to restoration of turgor (42), one would have expected to observe such an increase in case of a turgor-regulated gene.

(v) As argued above, the strength of the environmental signal regulating *kdp-lac* in the partially constitutive *kdp-205* mutant GJ618, for any particular combination of growth conditions, is the same as that for the isogenic $kdpD^+E^+$ strain GJ18; the mutant is altered only in perception of the signal. The results with GJ618 (Fig. 5) indicate that the strength of the transcription-activating signal in a strain with defective Kdp and functional TrkA and TrkD porters does not remain constant for $[K^+]_e$ >2 mM (as may have been inferred from the GJ18 results) but indeed progressively decreases with increase in $[K^+]_e$ even in the range between 10 and 30 mM. It is unlikely that there would be a concomitant increase in cell turgor during steady-state growth over this range of $[K^+]_e$.

(vi) The finding that steady-state *kdp* expression at sufficiently high $[K^+]_e$ is not affected by the osmolarity of the medium had earlier been taken to imply that cell turgor is completely restored in cultures adapted to growth at elevated osmolarity (12, 23, 44). It has not been possible to perform direct measurements of turgor pressure in *E. coli*, but two lines of circumstantial evidence suggest that contrary to the assumption above, cell turgor during steady-state growth under K^+-replete conditions is indeed lower at elevated osmolarity than at low osmolarity (whereas *kdp* expression itself remains unchanged). First, in those bacteria in which turgor pressure has been directly measured from the value of external hydrostatic pressure required to collapse intracytoplasmic gas vesicles, an inverse correlation between cell turgor during steady-state growth and osmolarity of the medium has been demonstrated (22, 36). Second, several workers have observed that the steady-state values for intracellular and cytoplasmic water volumes of *E. coli* (expressed per gram [dry weight]) decrease with increasing medium osmolarity (6, 7, 10, 15, 34, 42, 44); this has been interpreted as evidence for a progressive decrease in cell turgor under these conditions, with a consequent reduction in stretch of the elastic cell envelope (9, 21, 42).

On the basis of the arguments above, we conclude that the signal involved in regulation of *kdp* expression is not cell turgor. One observation that then remains unexplained is induction of *kdp-lac* in response to a sudden osmotic upshock. We suggest that this occurs by a mechanism unrelated to the true physiological signal, reflecting perhaps a transient perturbation of KdpD activity consequent to plasmolysis.

Consideration of, and constraints on, alternative models. The facts that the strength of the environmental signal controlling *kdp* expression is influenced primarily by $[K^+]_e$ and that it is perturbed by mutations in each of three different K^+ transporter loci (*kdpABC*, *trkA*, and *trkD*) suggest that the signal must be related to $[K^+]_i$ or to the process of transmembrane K^+ transport. Three alternative possibilities may be considered, but each has its own limitations in providing a complete explanation.

The first is that $[K^+]_i$ itself serves as the signal. The argument against this has been that steady-state $[K^+]_i$ can be altered by changes in osmolarity of the growth medium (6, 7, 15, 44), under conditions in which *kdp-lac* expression remains constant. In its defense, however, several other reports suggest that $[K^+]_i$ increases only transiently when cultures are subjected to osmotic upshock and that it subsequently returns to a value close to that obtaining at low

osmolarity (10, 32, 33); furthermore, the growth rate of *E. coli* in high-osmolarity media has been shown not to be affected either by K^+ limitation (32) or under conditions in which K^+ is unable to accumulate intracellularly (33).

The second possibility is that intracellular K^+ is compartmentalized (18, 46) and that $[K^+]$ of one such compartment is the signal for *kdp* regulation. Recent reports by Wiggins (49) on the inhomogeneity of intracellular water and by Cayley et al. (6) postulating the polyelectrolyte model for distribution of cytoplasmic K^+ indicate the possible existence of distinct K^+ compartments within the cell, but the molecular details of such a model remain undefined.

The third candidate for the signal merits consideration because of the results that we have obtained with the regulatory mutant GJ618. The data from this strain impose a severe constraint on any model for *kdp* regulation, because they support the following conclusion: the strength of the signal which activates *kdp* expression (even in a *kdpD*$^+$*E*$^+$ strain) progressively decreases over a range of increasing $[K^+]_e$ across which homeostatically controlled parameters, such as turgor, $[K^+]_i$, or $[K^+]$ in any subcellular compartment, are expected to remain constant (12). Our observation that *kdp* expression in a wild-type strain grown in glycine betaine-supplemented media is altered at varying $[K^+]_e$ despite a constant growth rate is also formally analogous to the behavior of GJ618 and once again argues against a steady-state determinant serving as the signal for *kdp* regulation.

It is possible, therefore, that the signal is related to the specific rate of transmembrane K^+ influx (expressed per unit [dry weight] of cells), such that the operon is activated whenever the flux rate is decreased. Because the influx rate is expected to increase monotonically with increasing $[K^+]_e$ (with K^+ and turgor homeostasis under these conditions being achieved by concomitant increases in efflux rates [2, 28]), such a model would provide an explanation for the observations with both GJ618 and glycine betaine supplementation. The effects of solutes such as Na^+ or NH_4^+ and of pH (that is, H^+) on *kdp* induction may also be explained on the assumption that each of these cationic species decreases the rate of K^+ influx at a given $[K^+]_e$. However, some mechanism must exist by which the influx rates through each of the Kdp, TrkA, and TrkD transporters are integrated to provide a single measure of signal strength, and this remains as a major untested assumption of the model.

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