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Accumulation of Trehalose by *Escherichia coli* K-12 at High Osmotic Pressure Depends on the Presence of Amber Suppressors

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When grown at high osmotic pressure, some strains of *Escherichia coli* K-12 synthesized substantial levels of free sugar and accumulated proline if it was present in the growth medium. The sugar was identified as trehalose by chemical reactivity, gas-liquid chromatography, and nuclear magnetic resonance spectroscopy. Strains of *E. coli* K-12 could be divided into two major classes with respect to osmoregulation. Those of class A showed a large increase in trehalose levels with increasing medium osmolarity and also accumulated proline from the medium, whereas those in class B showed no accumulation of trehalose or proline. Most class A strains carried suppressor mutations which arose during their derivation from the wild type, whereas the osmodefective strains of class B were suppressor free. When amber suppressor mutations at the *supD*, *supE*, or *supF* loci were introduced into such *sup*^{0} osmodefective strains, they became osmotolerant and gained the ability to accumulate trehalose in response to elevated medium osmolarity. It appears that the original K-12 strain of *E. coli* carries an amber mutation in a gene affecting osmoregulation. Mutants lacking ADP-glucose synthetase (*glgC*) accumulated trehalose normally, whereas mutants lacking UDP-glucose synthetase (*galU*) did not make trehalose and grew poorly in medium of high osmolarity. Trehalose synthesis was repressed by exogenous glycine betaine but not by proline.

When the osmolarity of the external medium increases it becomes necessary for bacteria to compensate by increasing their internal osmotic pressure (OP). This is normally achieved partly by increased accumulation of potassium ions from the surrounding medium (12, 13) and partly by increased synthesis or uptake of amino acids such as proline and glutamic acid (7, 8, 10, 18). Although many algae and yeasts are known to accumulate intracellular carbohydrate in response to increased external OP (3), this has rarely been reported in bacteria. In *Escherichia coli* B, Roller and Anagnostopoulos reported increased sugar accumulation with increasing osmolarity (21). These authors identified the sugar as glucose. However, we and Strom et al. (23) have found that the osmoinduced sugar is in fact a dimer of glucose, namely trehalose. The glucose originally observed (21) is apparently due to hydrolysis of trehalose during the extraction procedures. The trehalose is probably synthesized from UDP-glucose since *galU* mutants which lack UDP-glucose synthetase do not make trehalose. Trehalose accumulation is repressed by the addition of exogenous glycine betaine but is not affected by proline. Further investigation of a variety of strains revealed that many derivatives of *E. coli* K-12, including the original K-12 isolate, were defective in accumulating the osmoprotective solutes trehalose and proline in response to elevated osmolarity. Other derivatives of *E. coli* K-12 together with *E. coli* B and *Salmonella typhimurium* showed a normal osmotic response; i.e., they grew well at moderately high osmolarity and accumulated both trehalose and proline. We show here that the original K-12 strain of *E. coli* and many early derivatives apparently carry an amber mutation in a gene involved in the osmotic response. *E. coli* K-12 derivatives carrying amber suppressors gave a normal osmotic response. *E. coli* B and *S. typhimurium* are presumably wild type for this osmoresponsive gene.

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MATERIAL AND METHODS

Bacterial strains and media. Bacterial strains (Table 1) were *E. coli* K-12, except for JK116, which is *E. coli* B, and one strain of *S. typhimurium* LT2. The derivations of many strains are given in the pedigree charts of Bachmann (2), who kindly provided us with cultures of the original K-12 strain and many key descendants. Most experiments were done in half-strength M9 medium (19) supplemented with casein hydrolysate (0.1% [wt/vol]) and 0.4% potassium lactate, as previously described (4). In addition, the effects of a variety of modifications were tested as detailed in the Results section. Modular basal medium contained Na_2SO_4 (2.5 mM), KCl (5 mM), NH_4Cl (5 mM), Na_2HPO_4 (2.5 mM), $MgSO_4$ (1 mM), $FeSO_4$ (50 μM), $MnSO_4$ (5 μM), and $ZnSO_4$ (5 μm). Buffers (20 mM, pH 7.4) were added as required, as were carbon sources. Sodium chloride (2% [wt/vol] final concentration) was added to these media to induce the osmotic response. Although higher salt concentrations induced higher osmolyte levels in normal strains, the osmodefective derivatives grew poorly at NaCl levels above 2%.

Extraction and assay of sugars. Soluble carbohydrates were extracted by 50% ethanol as previously described (4) from cells in the late exponential phase. This procedure gives a mixture of trehalose with glucose derived from it by enzymatic hydrolysis (23). The total hexoses were quantitated by the anthrone assay (20). Selected cultures were extracted with 5% aqueous trichloroacetic acid (TCA), which inactivates the degradative enzyme(s) and gives extracts containing equivalent amounts of total hexose consisting of trehalose alone (23). Since both extraction procedures gave identical amounts of hexose, the results of this work are expressed as the amount of hexose per 10^9 cells throughout.

TABLE 1. Strains of *E. coli* used[a]

Strain(s)	Relevant characteristics	Source
K-12	F$^+$ wild type	CGSC[b]
58	F$^+$ *bio-1 spoT1*	CGSC
58-161	F$^+$ *bio-1 spoT1 metB1*	CGSC
679	F$^+$ *thr-1*	CGSC
679-680	F$^-$ *thr-1 leuB6*	CGSC
CA10	HfrH *galU*	CGSC
CA274	HfrH *trp-49*(Am) *lacZ125*(Am) *relA1 spoT1*	S. Brenner
CA374	HfrH *supD67* (=*serU67*) of CA274	S. Brenner
CR63	F$^+$ *supD60 lamB63*	CGSC
CS101	HfrC *relA spoT1 metB1 ompF627 garB10 tonA22*	CGSC
DC271	*mel-1 fadR supF58* (=*tyrT*)	Laboratory collection
DC300	*mel-1 fadR supF58* (=*tyrT*) *adhC zch*::Tn*10*	Laboratory collection
DC719	*malT*::Tn*10* of LCB498	P1 (TST3) × LCB498
DC720	*malT*::Tn*10* of LCB618	P1 (TST3) × LCB618
DC729	*glgA malT*::Tn*10* of UB1005	P1 (DC719) × UB1005
DC732	*glgC malT*::Tn*10* of UB1005	P1 (DC720) × UB1005
DC825	*zch*::Tn*10* of UB1005	P1 (WL20) × UB1005
DC826, DC827	*zch*::Tn*10 galU* of UB1005	P1 (WL20) × UB1005
DC829, DC830	*glgA malT*::Tn*10* of K10	P1 (DC729) × K10
DC832, DC833	*glgA malT*::Tn*10* of 679	P1 (DC729) × 679
DC835, DC836	*glgA malT*::Tn*10* of W208S^R	P1 (DC729) × W208S^R
DC843	*aroB ompR331*::Tn*10* of UB1005	Laboratory collection
DC844	Δ(*asd-bioH*) of UB1005	P1 (MD2) × DC843
DC846	*aroB ompR331*::Tn*10* of MG1655	Laboratory collection
DC847	Δ(*asd-bioH*) of MG1655	P1 (MD2) × DC846
DC885	*gal*$^+$ of DC826	P1 (W1485) × DC826
DC903	*aroL*::Tn*10 proC29 relA1 spoT1 metB1*	P1 (JP3123) × X342
DC906	*fuc*::Tn*10 cysI*(Am) of W6	P1 (PRC144) × W6
DC907	*fuc*::Tn*10 cysI*(Am) of W1485	P1 (PRC144) × W1485
DC926	*cysI*(Am) of W6	P1 (W1485) × DC906
DC927	*cysI*(Am) of W1485	P1 (W1485) × DC907
DC928	*aroL*::Tn*10 proC* of DC926	P1 (DC903) × DC926
DC929	*aroL*::Tn*10 proC* of DC927	P1 (DC903) × DC927
DC930	*cysI*(Am) *lac*(Am) of W6	P1 (CA274) × DC930
DC931	*cysI*(Am) *lac*(Am) of W1485	P1 (CA274) × DC931
DC932, DC933	*cys*$^+$ of DC906	Spontaneous Cys$^+$ (Tre$^-$)
DC934, DC935	*sup*$^+$ of DC906	Spontaneous Cys$^+$ (Tre$^+$)
DC936	*supD67* of DC930	P1 (CA374) × DC930
DC937	*supD60* of DC930	P1 (CR63) × DC930
DC938	*supE42* of DC930	P1 (W1485E) × DC930
DC939	*supE44* of DC930	P1 (Y10) × DC930
DC940	*supF58* of DC930	P1 (Ymel) × DC930
DC941	*supD67* of DC931	P1 (CA374) × DC931
DC942	*supD60* of DC931	P1 (CR63) × DC931
DC943	*supE42* of DC931	P1 (W1485E) × DC931
DC944	*supE44* of DC931	P1 (Y10) × DC931
DC945	*supF58* of DC931	P1 (Ymel) × DC931
DC946	Sup$^+$ of DC930	Spontaneous Lac$^+$ Cys$^+$
DC947	Sup$^+$ of DC930	Spontaneous Lac$^+$ Cys$^+$
DC948	Sup$^+$ of DC930	Spontaneous Lac$^+$ Cys$^+$
DC949	Sup$^+$ of DC931	Spontaneous Lac$^+$ Cys$^+$
DC950	Sup$^+$ of DC931	Spontaneous Lac$^+$ Cys$^+$
DC951	Sup$^+$ of DC931	Spontaneous Lac$^+$ Cys$^+$
HfrC	HfrC *relA1 spoT1 metB1*	CGSC
Hfr3000	HfrH *relA1 spoT1 thi-1 supQ80*	CGSC
JK1015	*fuc*::Tn*10*	Jack Parker
JM246	*cysI53*(Am) IN(*rrnD-rrnE*)	M. Jones-Mortimer
JP3123	*aroL478*::Tn*10 purE355 tyrR366 lac-352 rpsL741 tsx?*	J. Pittard
K10	HfrC *relA1 spoT1 tonA22 ompF627 garB10*	CGSC
LCB498	*thr-1 leuB6 pro-33 purE43 hisG1 argH1 thi-1 lacY1 gal-6 malA1 xyl-7 mtl-2 rpsL9 tonA2 glgA1 supE44*	CGSC
LCB618	*thr-1 leuB6 pro-33 hisG1 argH1 thi-1 lacY1 gal-6 malA1 xyl-7 mtl-2 rpsL9 tonA2 glgC3 supE44*	CGSC
MC4100	F$^-$ Δ(*argF-lac*)U169 *araD139 rpsL flbB relA thiA*	M. Casadaban
MG2	HfrG6 Δ29(*asd-bioH*) *hisA*	J. Cronan
MG1655	F$^-$ of W1485	CGSC
P4X	HfrP4X *metB1 relA1 spoT1*	CGSC
PRC105	*zch*::Tn*10 adhC supF58* (=*tyrT*) *cysI*(Am)	P1 (DC300) × JM246
PRC106	*zch*::Tn*10 adhC sup*^{0} *cysI*(Am)	P1 (DC300) × JM246

Continued on following page

TABLE 1—*Continued*

Strain(s)	Relevant characteristics	Source
PRC114	*cysI*(Am) *adhC81 galU* sup^0 *zch*::Tn*10*	Laboratory collection
PRC122	*cysI*(Am) sup^0	P1 (DC271) × PRC114
PRC123	*cysI*(Am) *supF58* (=*tyrT*)	P1 (DC271) × PRC114
PRC144	*fuc*::Tn*10 cysI*(Am)	P1 (JK1015) × JM246
TST3	*malT54*::Tn*10*	CGSC
UB1005	F^- *relA1 spoT1 metB1 nalA*	Laboratory collection
W6	F^+ *relA1 spoT1 metB1*	CGSC
W208	F^- *thr-1 leuB6 thi-1 lacZ4 supE44 rfbD1*	CGSC
W1485	F^+ wild type	CGSC
W1485E	*supE42* of W1485	CGSC
W1655F$^+$	F^+ *metB1 relA1*	CGSC
W1655F$^-$	F^- *metB1 relA1*	CGSC
W1872	F^+ wild type	CGSC
W3110	F^- IN(*rrnD-rrnE*)	CGSC
WL20	*galU zch*::Tn*10*	P1 (JW380) × CA10
X342	*proC29 metB1 relA1 spoT1*	R. Curtiss
Ymel	F^+ *mel-1 supF58*	CGSC
Y10	F^- *thr-1 leuB6 thi-1 rfbD1 supE44*	CGSC
Y53	F^- *thr-1 leuB6 thi-1 lacY1 supE44 rfbD1*	CGSC
JK116	Wild-type strain B	J. Parker

[a] *S. typhimurium* LT2 wild type (from J. Parker) was also used.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University; curator, B. Bachmann.

Some extracts were also analyzed by Somogyi, orcinol, and cysteine-sulfuric acid assays (20).

Sugars were analyzed by gas-liquid chromatography (GLC) after formation of the trimethylsilyl derivatives. A Varian instrument with flame ionization detectors was fitted with a column of OV17 resin and temperature programmed from 140 to 250°C. The carrier gas was oxygen-free nitrogen. Retention times of sample peaks were compared with those for a series of authentic sugars and derivatives including sucrose, maltose, trehalose, glucose, fructose, sorbose, mannitol, sorbitol, arabinose, xylose, ribose, arabitol, and erythritol.

NMR. Cultures were grown in 200 ml of minimal lactate medium containing various NaCl concentrations. The cell pellets were extracted as described above, using 5 ml of 10% TCA per 200 ml of original culture. After removal of TCA by ether counterextraction, the extracts were evaporated to dryness under vacuum and redissolved in deuterium oxide. Proton nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz on a Varian VXR-300 spectrometer operating in the pulsed Fourier transform mode. The parameters used were as follows: spectral band width, 4 kHz; pulse width, 90°; repetition time, 10 s. From 25 to 250 scans were accumulated depending on sample concentration. The field was locked on the solvent (D_2O), and internal H_2O was used as a reference peak (4.65 ppm). Commercially available sugars were run as standards under the same conditions.

RESULTS

Previous work suggested that sugars accumulate in *E. coli* grown at elevated osmolarity (21). Therefore, we investigated a variety of strains grown with and without 2% NaCl in the growth media. This rise in OP was selected because it gives a three- to fourfold increase in carbohydrate accumulation in strain UB1005 and fully induces the high-OP-specific proteins described previously (5). Furthermore, the addition of more than about 2% salt results in severe growth retardation in many derivatives of *E. coli* K-12 (data not shown).

Nature of carbohydrates at high osmolarity. Preliminary work suggested that the sugar accumulated at high OP differed from the membrane-derived oligosaccharides found at low osmolarity in being uncharged and lacking organically bound phosphate (data not shown). We therefore analyzed this material by GLC. Figure 1 shows GLC traces of 50% ethanol extracts of *E. coli* grown at low and high osmolarity. Extracts from strains grown at low OP showed no significant peaks irrespective of the strain used (Fig. 1A, UB1005; Fig. 1C, W1485). When a type A strain such as UB1005 was grown at high osmolarity, three well-defined peaks were observed (Fig. 1B). In contrast, when W1485 (type B) was grown at high osmolarity, these three peaks were barely significant (Fig. 1D). The three peaks were identified as trehalose, glucose, and proline by comparison with appropriate standards. This was confirmed by running a mixture of trehalose, glucose, and proline (Fig. 2A) and comparing it

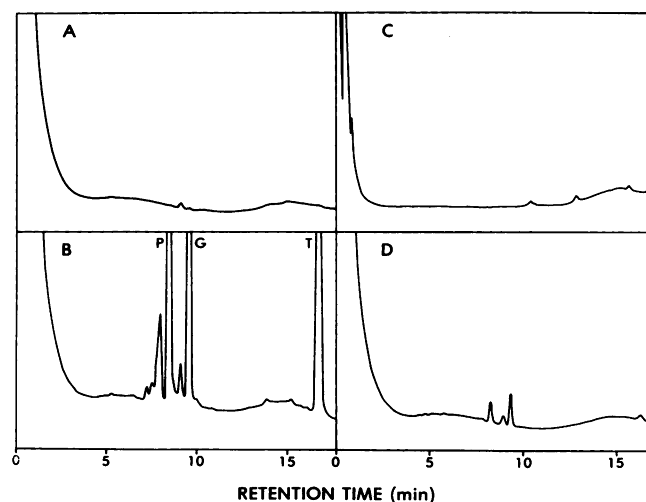

FIG. 1. Gas chromatography of cell extracts. (A) UB1005 grown at low osmolarity. (B) UB1005 plus 2.5% NaCl. (C) W1485 at low osmolarity. (D) W1485 plus 2.5% NaCl. All cultures were grown in HEPES (pH 7.4)-buffered modular basal medium with glycerol and Casamino Acids. P, Proline; G, glucose; T, trehalose.

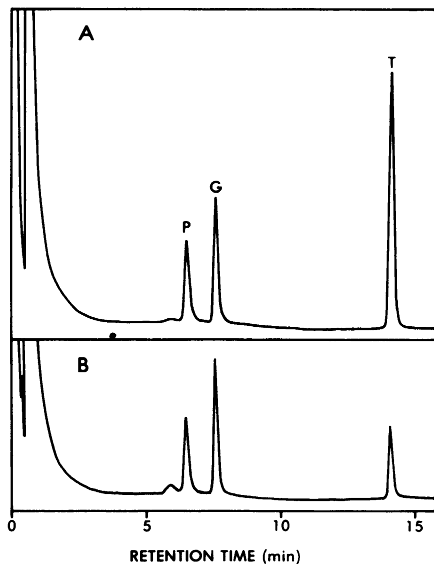

FIG. 2. Identification of cell extract constituents. (A) Mixture of authentic proline (P), glucose (G), and trehalose (T). (B) Extract from UB1005 grown at high osmolarity. Growth conditions were as described in the legend to Fig. 1.

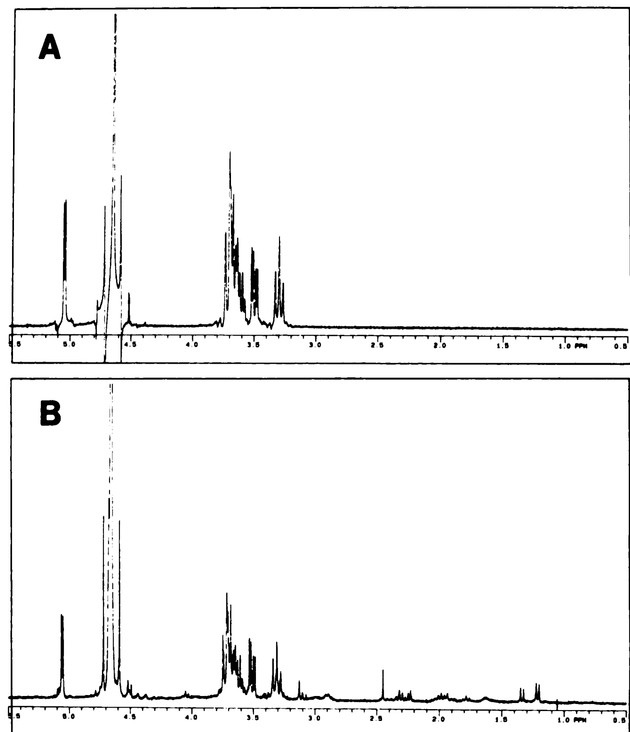

FIG. 3. NMR spectrometry of cell extracts. (A) Sample of authentic trehalose compared with (B) a cell extract of MG1655 grown with 2.5% NaCl.

with a high-osmolarity extract of UB1005 run under identical conditions (Fig. 2B). The two GLC traces are clearly superimposable. In these experiments proline was accumulated only when present in the growth medium. We also tested selected extracts with the orcinol assay for pentoses and with the cysteine-sulfuric acid reagent for keto sugars (20). Negligible amounts of pentoses and keto sugars were found irrespective of medium osmolarity.

When *E. coli* cells grown at high osmolarity were extracted in 5% TCA instead of 50% ethanol, only two peaks, corresponding to trehalose and proline, were observed (data not shown). The absence of glucose in TCA extracts was also demonstrated by using the Somogyi assay for reducing sugars. For extracts made by using 50% ethanol, somewhat less than half of the sugars were reducing sugar (e.g., glucose), whereas TCA extracts contained no detectable reducing sugar (data not shown). Since trehalose has no reducing end, it does not react with the Somogyi reagent. The glucose is presumably a breakdown product of trehalose, and its formation is prevented by use of the enzyme denaturant TCA. In contrast to *E. coli*, extracts of *S. typhimurium* grown at high OP yielded only proline and trehalose irrespective of the extraction procedure. This was demonstrated by GLC of extracts and confirmed by assay with the Somogyi reagent. Thus, *S. typhimurium* LT2 appears to lack the degradative enzyme(s) which converts trehalose to glucose in *E. coli*.

Representative strains were tested for growth on minimal medium M9 plus 0.2% trehalose. *S. typhimurium* LT2, *E. coli* B, and the *E. coli* K-12 strains 58, 679-680, Ymel, W1485, MG1655, W1872, Y10, W6, K10, HfrC, and UB1005 all used trehalose as the sole carbon and energy source. Thus, the strain differences in trehalose accumulation and its conversion to glucose in cell extracts are independent of the trehalose degradation system.

Confirmation of trehalose by NMR. To confirm that the osmoinduced sugar was trehalose, we ran NMR spectra of TCA extracts from *E. coli* K-12 strain MG1655 grown in minimal lactate medium with 2.5% NaCl. Figure 3 compares the extract from MG1655 with commercial α-α-trehalose. The spectra are essentially identical except for a few very small peaks that are probably due to traces of minor unidentified components in the cell extract. Extracts from *S. typhimurium* gave the same spectra as those from *E. coli* (data not shown). Several sugar standards other than trehalose were run; however, those spectra were quite distinct. In particular, no peaks due to glucose or maltose could be observed in the cell extracts (data not shown).

Strain variation in osmoregulation. During this work we noted that many common strains of *E. coli* K-12 failed to accumulate trehalose at high osmolarity (type B strains). We therefore undertook a systematic survey of the *E. coli* K-12 family tree (2). We found that the original wild-type *E. coli* K-12 failed to produce trehalose at elevated osmolarity, as did many of its immediate derivatives (Table 2). The relationships of these strains are shown in abbreviated form in Fig. 4. Other derivatives (type A strains) showed trehalose accumulation in response to high salt concentrations, as did wild-type *E. coli* B and *S. typhimurium* LT2 (Table 2). We assayed strains carrying mutations in several genes affecting envelope composition (*ompF*, *ompR*, and *envZ*) and lipid and membrane-derived oligosaccharide synthesis (*mdoA*, *mdoR*, *plsB*, and *plsX*). However, no significant effects upon trehalose accumulation were found (data not shown).

Correlation of osmoresponse and suppressor mutations. The pedigree charts of *E. coli* K-12 (2) suggested two possible hypotheses to account for the strain variation. The first theory tested was that loss of the F factor, present in the original K-12 strain but not in *S. typhimurium* or *E. coli* B, correlated with the ability to produce trehalose at high OP. This was suggested by comparison of MG1655 F^- with its

TABLE 2. Strain variation and carbohydrate content

Strain	Lineage[a]	Carbohydrate class	Amt of carbohydrate (μg of glucose/10^9 cells) accumulated in medium:[b] Without NaCl	With NaCl
E. coli				
K-12	Wild type	B	12.7	16.5
W1485	Wild type	B	14.1	10.3
MG1655	W1485	A	15.2	86.7
W1485E	W1485	A	40.9	95.4
W3110	W1485	C	65.0	80.0
58	Wild type	B	13.8	13.0
58-161	58	B	3.1	2.5
W6	58-161	B	10.9	7.2
HfrC	W6	B	9.3	9.5
CS101	HfrC	B	7.4	6.1
W1655F$^+$	W6	B	4.9	3.9
W1655F$^-$	W1655F$^+$	A	10.4	37.5
UB1005	W1655F$^-$	A	12.1	45.4
679	Wild type	C	43.8	70.7
679-680	679	B	18.0	7.9
Y10	679-680	A	36.4	60.5
Y53	Y10	A	14.7	44.1
W208	Y10	A	27.9	55.4
W1872	Wild type	A	11.1	23.2
Ymel	Wild type	A	22.5	90.7
MC4100	Mixed	A	4.5	27.9
B (JK116)		A	3.4	28.2
S. typhimurium		A	8.1	29.7

[a] Lineage is as given by Bachmann (2).
[b] The standard deviations of assays for replicate cultures of the above strains and many others ranged from 5 to 10%.

F$^+$ ancestor W1485 and of the F$^-$ and F$^+$ versions of W1655. We cured several strains of their F factor by using acridine orange and reintroduced F' factors into several F$^-$ strains. However, these manipulations did not affect trehalose accumulation (data not shown).

The second theory tested was that the appearance of amber suppressors in several ancestral lines of *E. coli* K-12 correlated with the ability to produce trehalose. This was suggested by a comparison of Ymel (*supF58*), W1485E (*supE42*), and Y10 (*supE44*) with their parents: K-12 wild type, W1485, and 679-680, respectively. Starting with a trehalose-negative, suppressor-free strain carrying a *cysI* (Am) mutation, we cotransduced in *supF58* using the nearby *zch*::Tn*10* insertion. We found that transductants receiving the *supF58* mutation gained the ability to synthesize trehalose at high osmolarity. Transductants receiving the *zch*::Tn*10* insertion but which still carried *sup*^{0} showed no osmoinduced trehalose production (Table 3).

Effects of different suppressor mutations. To test the effects of various suppressor alleles, we constructed strains carrying two amber mutations in a trehalose-negative, suppressor-free background (Fig. 5). Strains W6 and W1485 were selected as the parental strains, as these are both suppressor free and trehalose negative and are the ancestors to many widely used strains. The *cysI*(Am) mutation of JM246 was transduced into W6 and W1485 by using the nearby *fuc*::Tn*10* insertion. Tetracycline-resistant transductants which required cysteine were kept. Next, Tn*10* was removed by transduction to *fuc*$^+$ by using P1 grown on a wild-type strain, and transductants which retained *cysI*(Am) were kept. The third step involved moving in *proC* by using the neighboring *aroL*::Tn*10*. The final step consisted of transducing these strains to *pro*$^+$ with P1 grown on CA274 *lac*(Am). Transductants which were tetracycline sensitive and carried a *lac*

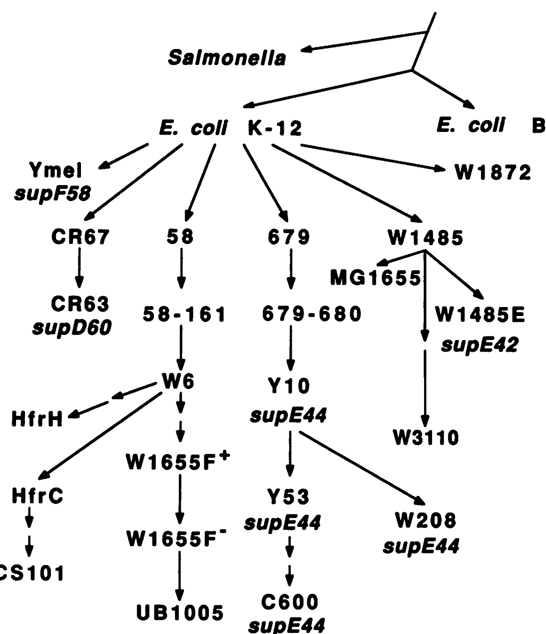

FIG. 4. Ancestry of strains used. The derivations of most of the strains used in this work are shown. Most of the pedigrees are from Bachmann (2). The first appearances of the suppressors *supE42*, *supE44*, *supF58*, and *supD60* are shown. The HfrH strains used in this work are derivatives of Hfr3000, a recombinant of the original HfrH with W677.

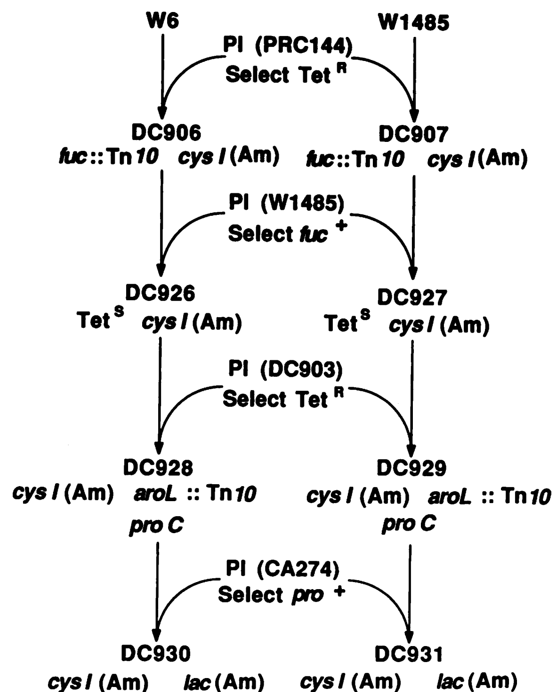

FIG. 5. Construction of strains. The construction of the *cysI* (Am) *lacZ*(Am) strains DC930 (from W6) and DC931 (from W1485) is shown. Details of the construction are given in the text.

TABLE 3. Suppressors and trehalose accumulation

Strain	Parent	Suppressor	Amt of hexose at high OP (μg of glucose/ 10^9 cells)
PRC105	JM246	*supF58*	88.8
PRC106	JM246	Wild type	13.9
PRC122	PRC114	*supF58*	94.1
PRC123	PRC114	Wild type	33.5
DC930		Wild type	17.3
DC936	DC930	*supD67*	81.0
DC937	DC930	*supD60*	54.0
DC938	DC930	*supE42*	75.6
DC939	DC930	*supE44*	53.2
DC940	DC930	*supF58*	53.4
DC931		Wild type	14.6
DC941	DC931	*supD67*	81.2
DC942	DC931	*supD60*	60.4
DC943	DC931	*supE42*	86.7
DC944	DC931	*supE44*	60.2
DC945	DC931	*supF58*	48.2

TABLE 4. Trehalose accumulation in spontaneous mutants

Strain	Parent	Growth response[a]	Amt of hexose at high OP (μg of glucose/ 10^9 cells)
DC906		S	8.5
DC932	DC906	S	12.1
DC933	DC906	S	13.6
DC934	DC906	R	86.0
DC935	DC906	R	82.8
DC930		S	17.3
DC946	DC930	R	55.9
DC947	DC930	R	61.8
DC948	DC930	R	76.1
DC931		S	14.6
DC949	DC931	R	50.0
DC950	DC931	R	54.7
DC951	DC931	R	56.2

[a] Grows well (R) or poorly (S) on minimal agar in the presence of 3% NaCl.

mutation were kept. These constructions (Fig. 5) produced DC930 (from W6) and DC931 (from W1485). Both strains were Cys$^-$ Lac$^-$, grew poorly at high osmolarity, and were trehalose negative.

A variety of suppressor mutations were transduced into DC930 and DC931. P1 was grown on strains carrying *supD67* (CA374), *supD60* (CR63), *supE44* (Y10), *supE42* (W1485E), and *supF58* (Ymel) and crossed with both DC930 and DC931. Transductants were selected on minimal medium lacking cysteine with lactose as the carbon source. Such a double selection with two amber mutations provides a direct unambiguous selection for the introduction of suppressor mutations since the likelihood of obtaining revertants at two loci simultaneously is insignificant. Selected Sup$^+$ transductants from each cross were assayed. All were found to produce trehalose when grown at elevated osmolarity (Table 3). Thus, all of the *supD*, *supE* (two alleles each), and *supF* mutations tested were effective in suppressing the osmotic defect in both DC930 and DC931.

Isolation of spontaneous suppressor mutations. Strains DC906 and DC907, which carry *cysI*(Am) and are osmosensitive and trehalose negative, were plated onto medium lacking cysteine. Spontaneous Cys$^+$ revertants were picked, purified, and tested for growth on minimal glucose agar containing 3% (wt/vol) NaCl. Approximately 50% of the Cys$^+$ revertants had also gained the ability to grow at high salt concentrations, whereas the others resembled their parents in being unable to grow at 3% NaCl. Selected revertants of both classes were grown in liquid with and without 2% NaCl and assayed for trehalose production. Those which were salt resistant produced trehalose in the presence of 2% NaCl, whereas the parental strains and the Cys$^+$ salt-sensitive revertants did not (Table 4). The Cys$^+$ Tre$^+$ revertants are presumably spontaneous suppressor mutants, whereas the Cys$^+$ salt-sensitive isolates are true revertants of the *cysI*(Am) mutation.

Another series of spontaneous suppressor mutants were selected by plating DC930 and DC931 onto lactose minimal medium without cysteine. Spontaneous Lac$^+$ Cys$^+$ mutants were isolated at low frequency (approximately 1 in 10^9). It should be noted that the frequency of isolation of spontaneous Sup$^+$ (Cys$^+$ Lac$^+$) mutants was 100- to 1,000-fold less than the frequency of transduction of known suppressors into the same strains. All of these presumably Sup$^+$ derivatives proved to accumulate trehalose at elevated osmolarity, unlike their parents (Table 4). As expected, these derivatives all grew well at elevated osmolarity.

Sugars not excreted. One conceivable explanation of the behavior of those strains which do not accumulate sugars at high OP is that sugars are indeed synthesized but are lost into the culture medium. We therefore grew high-osmolarity cultures of two pairs of closely related strains, one of each pair being a sugar accumulator and the other a nonaccumulator. The cell extracts and culture media were assayed for sugars. The nonaccumulators (W1485 and W1655F$^+$) had 4 to 5 μg of hexose per ml of culture medium after growth to approximately 10^9 cells per ml. When grown under the same conditions, comparable accumulator strains (MG1655 and W1655F$^-$) produced 35 to 70 μg of sugar per ml of culture, which was essentially all found in the cell extract. Moreover, measurement of sugar in culture medium is an overestimate, since relatively large samples have to be used and many organic substances react nonspecifically in the anthrone assay to a small extent. Also, certain amino acids, e.g., tryptophan, give substantial color formation (20). In contrast, measurement of sugar in cell extracts is a slight underestimate, since 100% extraction of sugars in one round of extraction is assumed. Thus, excretion of sugars into the growth medium does not account for the difference between the two types of *E. coli*.

TABLE 5. Effect of *galU* and *glgC* on hexose accumulation

Strain[a]	Genotype	Amt of hexose (μg/10^9 cells) accumulated with:	
		0% NaCl	2% NaCl
DC825	Wild type	9.4	93.0
DC826	*galU*	11.7	5.1
DC827	*galU*	13.1	6.3
DC885	Wild type	9.3	81.6
UB1005	Wild type	7.0	88.2
DC844	Δ*glg*	15.3	84.3
DC729	*glgA*	8.4	86.0
DC732	*glgC*(Up)	91.4	102.7
MG1655	Wild type	15.2	86.7
DC847	Δ*glg*	13.3	93.3

[a] All strains are derivatives of UB1005 except for DC847, which is derived from MG1655. Derivations of MG1655 and UB1005 are given in Table 1.

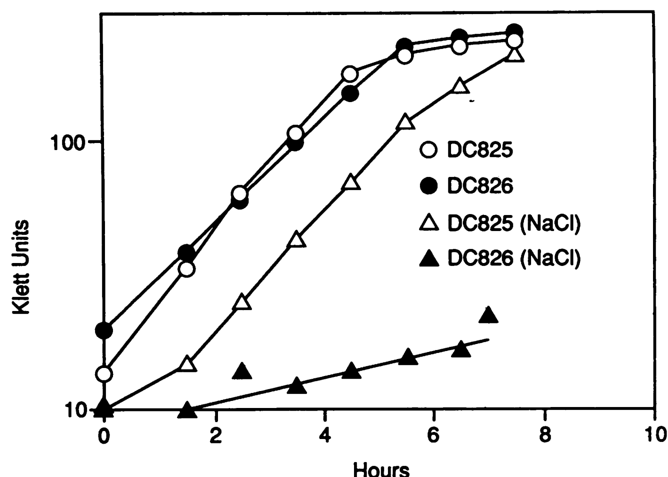

FIG. 6. Osmosensitivity of *galU*. DC826 carries *galU*, and DC825 carries gal^+. Minimal medium contained 0 or 2% NaCl.

Mutations in *pgi*, *galU*, and *glgC*. The *galU* gene encodes UDP-glucose synthetase, and *glgC* encodes ADP-glucose synthetase. We introduced mutations in *galU* and *glgC* into two strains which both accumulated trehalose at high OP: MG1655 and UB1005. A *galU* defect abolished the accumulation of trehalose in response to increasing osmolarity (Table 5). When the wild type (DC825) and a corresponding *galU* strain (DC826) were compared, it was clear that the growth of the *galU* mutant was highly osmosensitive (Fig. 6). The wild type showed little inhibition by 2% NaCl, whereas the *galU* mutant was severely inhibited. The effect of the *pgi* mutation, which blocks the synthesis of glucose 6-phosphate when cells are grown on gluconeogenic substrates, was also examined. There was no difference in osmosensitivity between a *pgi* mutant and the corresponding wild type when they were grown on glucose minimal medium. However, when grown on lactate medium, the *pgi* mutant showed pronounced osmosensitivity and failed to accumulate trehalose (data not shown).

Mutations in *glgC* and *glgA* and deletions of the entire *glg* region had no effect. However, mutations in *glgC* with increased ADP-glucose synthetase activity resulted in substantial amounts of sugar being accumulated at low OP. GLC analyses of this material showed no glucose, trehalose, or other mono- or disaccharide (data not shown). Whether the *glgC*-associated materials are membrane-derived oligosaccharides, maltodextrin precursors to glycogen, or some other material is unknown. During our survey of *E. coli* K-12 strains, we found several strains which produced excessively high amounts of sugar at low OP (Table 2). Furthermore, these strains stained very dark with I_2 in KI, as do glycogen-overproducing strains. We therefore hypothesized that these anomalous type C strains were spontaneous *glgC*(Up) mutants. Three of these strains (K10, 679, and W208S^R) were transduced with P1 grown on DC729 (*glgA malT*::Tn*10*), and glycogen-negative (i.e., *glgA* $glgC^+$) colonies were retained. All *glgA* $glgC^+$ derivatives of all three strains had lost the ability to overproduce sugar at low osmolarity (Table 6), supporting the contention that these anomalous strains (K10, 679, and W208S^R) are spontaneous *glgC*(Up) variants.

Growth at elevated osmolarity. The strains which were assayed for trehalose accumulation were also tested for growth at high OP on minimal medium with glucose. In strains derived from W6 and W1485, the production of trehalose correlated well with the ability to grow at elevated osmolarity (Table 4). This correlation extends to most other strains listed in Table 2. However, strains derived from MC4100 and HfrH produced trehalose in medium with 2% NaCl yet failed to grow at high salt levels (i.e., 3% NaCl). Figure 7 shows representative data for the growth of DC931,

TABLE 6. Effect of *glgC* on hexose accumulation in anomalous strains

Strain	Genotype	Amt of hexose (μg/10^9 cells) accumulated with:	
		0% NaCl	2% NaCl
K10	*glgC*	44.1	66.9
DC829	*glgA* of K10	7.1	86.4
DC830	*glgA* of K10	9.5	87.9
679	*glgC*	43.8	70.7
DC832	*glgA* of 679	9.2	69.9
DC833	*glgA* of 679	14.3	78.7
W208S^R	*glgC*	182.8	76.7
DC835	*glgA* of W208S^R	13.2	82.3
DC836	*glgA* of W208S^R	17.7	79.6

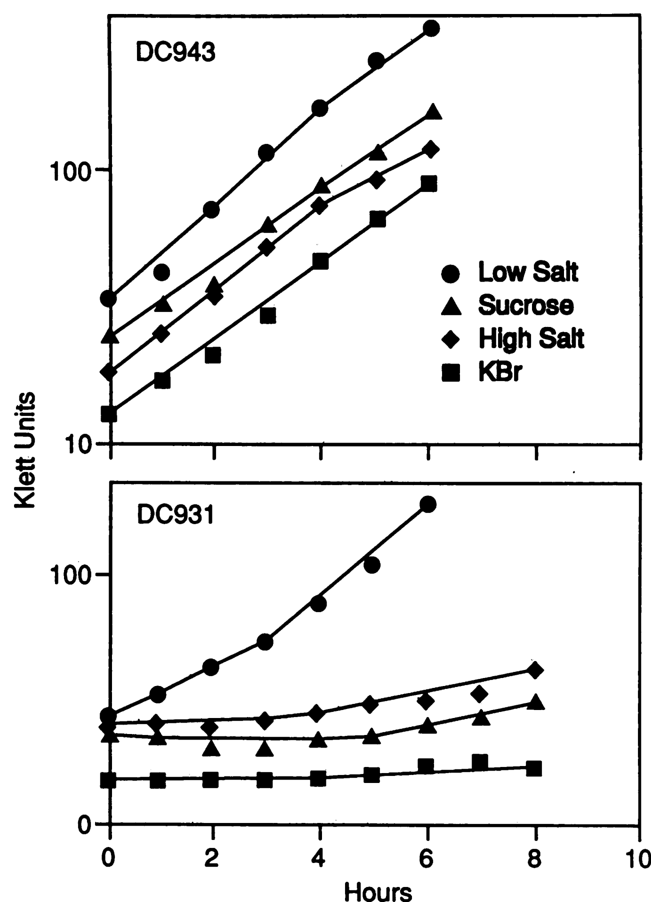

FIG. 7. Effect of trehalose accumulation on osmosensitivity. Strains DC931 *sup*^{0} and DC943 *supE42* were grown in the presence and absence of 2% NaCl or equiosmolar concentrations of KBr and sucrose.

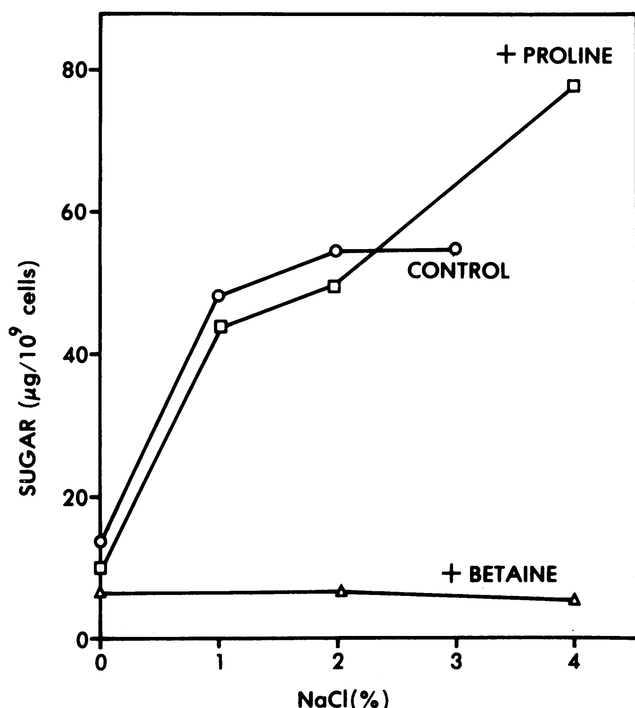

FIG. 8. Repression of trehalose synthesis by betaine. UB1005 was grown with the indicated concentrations of NaCl added to minimal medium. Hexose accumulation was measured in the presence of 1 mM proline or 1 mM glycine betaine or without either.

(sup^0; trehalose negative) and DC943 (a *supE42* transductant of DC931; trehalose positive). The growth of DC943 was little affected by 2% NaCl or equiosmolar amounts of KBr or sucrose. In contrast, all three osmotic agents severely depressed the growth of DC931. These results demonstrate also that growth inhibition is not specific to one ion (e.g., Na^+) but is a general osmotic effect.

Effect of culture medium. The effect of the growth medium on trehalose accumulation was investigated by independently varying both the nutrients and the buffer. The carbon source was varied for strain UB1005 grown in M9 medium by replacing the potassium lactate with 0.4% (wt/vol) glycerol, acetate, succinate, gluconate, or sorbitol. The response to increased OP was little affected by altering the carbon source. Rich broth gave results similar to M9 medium. The effect of varying the buffer was studied in modular basal medium, which contains minimal levels of necessary inorganic ions but lacks nutrients and buffer. Both W1485 and UB1005 were grown in this medium supplemented with glycerol (0.4%), casein hydrolysate (0.1%), and 20 mM Tris, phosphate, or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (all pH 7.4). The results indicated that the nature of the buffer makes little difference (data not shown).

Repression of trehalose synthesis by betaine. Betaine is a potent osmoprotectant (16), and we therefore tested the effect of exogenous glycine betaine upon the osmoinduced accumulation of trehalose by UB1005. Glycine betaine at 0.125 mM completely repressed the accumulation of hexose at high OP, whereas proline had no effect (Fig. 8). Although betaine had a major effect at high OP, it exerted negligible effect on the accumulation of hexose (i.e., membrane-derived oligosaccharides) at low osmolarity (Table 7). In addition, the effect of betaine was independent of the carbon source, and growth in lactate, sorbitol, or glycerol gave similar results (Table 7).

TABLE 7. Effect of betaine on sugar accumulation[a]

Carbon source	NaCl (%)	Betaine (1 mM)	Amt of hexose (μg/10^9 cells) accumulated
Lactate	0	−	15.4
	0	+	9.5
	2	−	57.9
	2	+	12.0
Sorbitol	0	−	11.9
	0	+	12.4
	2	−	44.9
	2	+	4.8
Glycerol	0	−	16.4
	0	+	16.0
	2	−	37.7
	2	+	11.3

[a] UB1005 was grown in minimal medium with 0.4% of the indicated carbon source plus 0.1% Casamino Acids.

DISCUSSION

We have found that many derivatives of *E. coli* K-12, together with *E. coli* B and *S. typhimurium*, respond to elevated osmolarity by synthesizing trehalose. Roller and Anagnostopoulos (21) observed the accumulation of sugars by *E. coli* at elevated osmolarity. These authors stated that glucose or arabinose was the sugar accumulated and that these sugars were taken up unchanged from the culture medium. In our experiments the growth substrate was lactate or glycerol, and any sugars produced must thus have been synthesized by the gluconeogenic pathway. In addition to glucose, which was present in substantial amounts in ethanol extracts, trehalose also was found in approximately equal quantities. When cells were extracted with the enzyme denaturant TCA, we found trehalose but no glucose, suggesting that the glucose is a product of trehalose hydrolysis. We found no pentose accumulation at elevated osmolarity; however, we have not examined whether pentoses are accumulated in *E. coli* K-12 if they are provided in the growth medium. While this work was in progress, we heard that trehalose had also been identified in *E. coli* by Arne Strom et al. using NMR (23). Our initial identification of trehalose was with GLC; however, we also confirmed our results using NMR.

Trehalose is the blood sugar of insects and has been found in other invertebrates and fungi (11). It has recently been reported in *Rhizobium* bacteroids, although its osmotic dependence was not investigated (22). Trehalose has been shown to accumulate in certain cyanobacteria under osmotic stress (17) and was demonstrated to protect biological membranes against damage from dessication (6). In other organisms trehalose is synthesized via trehalose phosphate, which is made from the condensation of UDP-glucose with glucose 6-phosphate (11, 23). We found that *galU* mutants of *E. coli*, which lack UDP-glucose synthetase (1, 23), were unable to accumulate trehalose, whereas a *glgC* mutant lacking ADP-glucose synthetase could still synthesize trehalose. Furthermore, *galU* mutants were very sensitive to growth inhibition by NaCl, whereas *glgC* mutants showed no significant growth defects at high osmolarity.

Many *E. coli* K-12 strains failed to synthesize sugars or to accumulate proline from the medium in response to elevated

osmolarity. We have found that normal osmotic responses are given by wild-type strains of *E. coli* B and *S. typhimurium* LT2 and by derivatives of *E. coli* K-12 which carry amber suppressors (type A strains). In contrast, the parental *E. coli* K-12 and suppressor-free derivatives were osmodefective, both in growth and trehalose accumulation (type B strains). Although we have focused on trehalose accumulation, our original observations by GLC indicated that proline accumulation from the medium was also defective in at least some of these nonresponding type B strains. Thus the defect is not merely in trehalose synthesis. This suggests that a presently unlocated gene involved in response to increased osmolarity carries an amber mutation in strains derived from the ancestral *E. coli* K-12. The three prominent *hop* proteins which increase in response to high OP as seen on two-dimensional polyacrylamide gels (5) are still produced in type B strains (P. E. Goodlove and J. M. Parker, unpublished data). Thus, the osmotic response is only partly compromised in these strains.

We confirmed the effects of suppressors by introducing known amber suppressor mutations into osmodefective strains. In all cases observed, the osmotic defect was suppressed, indicating that it was an amber mutation. Several known suppressors including *supD67*, *supD30*, *supE42*, *supE44*, and *supF58* (1, 2) were effective at suppression of the osmotic defect. We also isolated spontaneous suppressor mutations in nonosmoresponsive strains by using a selection procedure which involved no exposure to increased osmolarity and relied on suppressing previously characterized *cys* and *lac* amber mutations. These also showed suppression of the osmotic defect and produced trehalose in response to elevated osmolarity. We found that two strains, MG1655 and UB1005, were suppressor free as judged by their inability to suppress the *cysI*(Am) mutation from JM246 yet produced trehalose in response to high osmolarity. Whether these strains are spontaneous revertants of the amber defect or whether they contain suppressor mutations effective on the osmotic defect but not against *cysI*(Am) is unknown.

The present finding resembles the situation with *ilvG*. *E. coli* K-12 is sensitive to growth inhibition by valine due to a defect in the isoleucine-valine regulatory system (9, 14). By definition, the original K-12 strain is the wild type, and so the defective *ilvG* gene is the wild-type allele. Revertants to normal function are therefore mutants. Although wild type implies an organism isolated from the wild, the original strain of *E. coli* K-12 was maintained by continuous subculture from its isolation in 1922 until the 1940s, when standard isolates were first lyophilized (15; B. Bachmann, personal communication). Whether the defects in the standard K-12 strain were actually present at the time of its isolation or whether they arose during the following two decades of subculture can thus never be known.

One intriguing point concerns the frequency of appearance of spontaneous mutations in laboratory stocks of *E. coli*. Of some 10 major lines of descent depicted in the genealogical charts of *E. coli* done by Barbara Bachmann (2), four have acquired suppressors within three generations from the original K-12 ancestor (Y10, CR63, W1485E, and Ymel). In contrast, direct selection of amber suppressors in Lac(Am) Cys(Am) double mutants gave a frequency of about 1 in 10^9. However, it must be remembered that many early K-12 strains were subjected to substantial doses of UV light or X rays and probably contain many resulting mutations, most still unidentified. The frequency of such amber suppressors suggests that laboratory bacteria have been subjected to strong selection pressures. In the case of osmolarity, perhaps the tendency of slovenly bacteriologists to let their plates dry out is responsible for the high frequency of reversion to salt tolerance by means of amber suppression.

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