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Osmoregulatory Systems of *Escherichia coli*: Identification of Betaine-Carnitine-Choline Transporter Family Member BetU and Distributions of *betU* and *trkG* among Pathogenic and Nonpathogenic Isolates

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Multiple transporters mediate osmoregulatory solute accumulation in *Escherichia coli* K-12. The larger genomes of naturally occurring strains such as pyelonephritis isolates CFT073 and HU734 may encode additional osmoregulatory systems. CFT073 is more osmotolerant than HU734 in the absence of organic osmoprotectants, yet both strains grew in high osmolality medium at low K^+ (micromolar concentrations) and retained locus *trkH*, which encodes an osmoregulatory K^+ transporter. Both lacked the *trkH* homologue *trkG*. Transporters ProP and ProU account for all glycine-betaine uptake activity in *E. coli* K-12 and CFT073, but not in HU734, yet elimination of ProP and ProU impairs the growth of HU734, but not CFT073, in high osmolality human urine. No known osmoprotectant stimulated the growth of CFT073 in high osmolality minimal medium, but putative transporters YhjE, YiaMNO, and YehWXYZ may mediate uptake of additional osmoprotectants. Gene *betU* was isolated from HU734 by functional complementation and shown to encode a betaine uptake system that belongs to the betaine-choline-carnitine transporter family. The incidence of *trkG* and *betU* within the ECOR collection, representatives of the *E. coli* pathotypes (PATH), and additional strains associated with urinary tract infection (UTI) were determined. Gene *trkG* was present in 66% of the ECOR collection but only in 16% of the PATH and UTI collections. Gene *betU* was more frequently detected in ECOR groups B2 and D (50% of isolates) than in groups A, B1, and E (20%), but it was similar in overall incidence in the ECOR collection and in the combined UTI and PATH collections (32 and 34%, respectively). Genes *trkG* and *betU* may have been acquired by lateral gene transfer, since *trkG* is part of the *rac* prophage and *betU* is flanked by putative insertion sequences. Thus, BetU and TrkG contribute, with other systems, to the osmoregulatory capacity of the species *E. coli*, but they are not characteristic of a particular phylogenetic group or pathotype.

Bacteria that cause food- and waterborne diseases face diverse and changing environments during processing and storage of feed, food, and water (1), within human or animal hosts (76), and outside those hosts on plants, in soil, or in water (39). Stresses faced by these bacteria may include nutrient deprivation, low pH, high organic acid levels, oxygen deprivation or exposure to reactive oxygen species, thermal fluctuations, osmotic stress (variations and extremes of salinity and/or osmolality), desiccation, or denaturant stress. Bacterial stress tolerance mechanisms are believed to increase the incidence and severity of food- and waterborne disease by increasing the frequency with which humans and animals are exposed to contaminated food or water and by enhancing bacterial virulence. Bacteria may also sense their own movement into and out of host tissues by detecting environmental changes.

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Cytoplasmic accumulation of particular organic solutes (often designated compatible solutes) is a widely recognized bacterial stress response (89). Growing evidence indicates that compatible solutes confer thermal, denaturant, and/or oxidative stress tolerance in addition to being key players in osmoregulation. Trehalose accumulates in *Escherichia coli* in stationary phase and in response to thermal and osmotic stress, protecting the bacteria from osmotic stress (41), freezing and desiccation (53), cold stress (46), lethal heat stress (42), and nonlethal high temperature (14). Trehalose (6) and dimethylsulphoniopropionate (79) also alleviate oxidative stress. Glycine-betaine, a widely used osmoprotectant (89), promotes chill tolerance in *Listeria monocytogenes* (77), yet it reduces the ability of other organisms to tolerate high temperatures (31, 32, 74). The membrane-permeant solute urea is present in the urine of humans and animals at levels that can inhibit bacterial growth (up to 0.5 M for human urine) (5). Glycine-betaine confers urea tolerance on *E. coli* (66), as well as on renal cells (13), by counteracting its effects as a cytoplasmic denaturant (83).

Multiplicity and redundancy of homeostatic mechanisms are hallmarks of bacterial stress response. They complicate efforts to elucidate relationships among stress tolerance mechanisms, bacterial virulence and the incidence of human or animal disease. The redundancy of bacterial osmoregulatory mechanisms

TABLE 1. Known and predicted bacterial osmoprotectant transporters[a]

Transporter family[b]	Energy supply[c]	Osmoprotectant transporter(s) of:		
		E. coli[d]	*C. glutamicum*	*B. subtilis*
MFS	$\Delta\mu_{H+}$	ProP, [YhjE]	ProP	Nil
TRAP	$\Delta\mu_{H+}$	[YiaMNO]	Nil	Nil
SSSS	$\Delta\mu_{Na+}$	(PutP)	(PutP)	OpuE
BCCT	$\Delta\mu_{Na+}$	BetT, BetU	EctP, BetP, LcoP	OpuD
ABC	ATP	ProU, [YehWXYZ]	Nil	OpuA, OpuB, OpuC

[a] The table lists known and predicted osmoprotectant transporters of *E. coli*, *B. subtilis*, and *C. glutamicum* (this article, (48, 57, 89)). Names specifying known osmoprotectant transporters are shown without parentheses or brackets. Predicted osmoprotectant transporters (*E. coli* only) are shown in square brackets, and transporters that are similar in sequence to known osmoprotectant transporters but are not osmoregulatory are shown in parentheses.

[b] The transporter families are MFS (major facilitator superfamily), TRAP, SSSS (sodium-solute symporter superfamily), BCCT, and ABC (ATP-binding cassette family) (27, 70).

[c] Each transporter class is associated with a particular energy supply (27), but the energy supplies utilized by the listed transporters have not been determined experimentally in every case.

[d] Listed transporters are encoded by all sequenced *E. coli* genomes (MG1655, EDL933, RIMD0509952, and CFT073) (Table 2), with the following exceptions. YiaMNO is encoded by the genomes of *E. coli* MG1655 and CFT073 but not by EDL933 or RIMD0509952. Gene *betU* is not present in any sequenced *E. coli* genome, but it is present in one-third of the *E. coli* isolates included in this study (Tables 4 and 5).

was first defined via studies of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium (17), and even greater redundancy has since been shown for the gram-positive bacteria *Bacillus subtilis* (48), *Corynebacterium glutamicum* (57), and *L. monocytogenes* (44). *E. coli* K-12 can achieve osmotolerance through the accumulation and release of K^+ or compatible solutes (88). Osmoregulatory K^+ uptake can be mediated by KdpFABC, a high-affinity K^+-transporting ATPase, or by Trk, a low-affinity system present in *E. coli* K-12 as two variants, TrkG and TrkH. Compatible solutes stimulate bacterial growth in high-osmolality media more effectively than does K^+, and compatible solute accumulation suppresses K^+ accumulation in response to osmotic stress (24). Unlike compatible solute accumulation, K^+ accumulation has not been reported to provide collateral thermo-, urea, or oxidative stress tolerance. Organic osmoprotectants are compounds that stimulate bacterial growth in high-osmolality media because osmoregulatory transporters, listed in Table 1, mediate their accumulation as compatible solutes. Osmoprotectants may also be converted to compatible solutes after uptake (e.g., choline uptake via BetT and conversion to glycine-betaine by BetBA) or be synthesized from central metabolic precursors (e.g., trehalose synthesis from cytoplasmic glucose mediated by OtsBA). To rigorously test the hypothesis that osmoregulatory mechanisms assist *E. coli* to cause human or animal disease, all systems that contribute to osmoprotection must be identified.

High urea levels and fluctuating osmolality distinguish the urinary tract from other mammalian tissues. The osmolality of urine from healthy humans with a normal diet and fluid intake varies in the range of 0.5 to 0.8 mol/kg, but urinary osmolality may vary from approximately 0.04 to 1.4 mol/kg (3, 50, 69). Urea, the primary contributor to the osmolality of human urine and of renal extracellular fluid, approaches a concentration of 0.5 M. Despite its high urea content, fluctuating osmolality, and low pH, urine supports rapid and extensive growth of *E. coli* (18, 34). Upon demonstrating that glycine-betaine and proline-betaine conferred osmoprotective activity on human urine, Chambers and Kunin (15) inferred that osmoregulatory betaine uptake may promote growth in urine and colonization of the human urinary tract by *E. coli* (16). We therefore chose to probe the relationship between osmoregulatory compatible solute accumulation and bacterial virulence by focusing our attention on uropathogenic *E. coli* strains. Our approach is to conduct detailed studies of two pyelonephritis isolates (HU734 and CFT073) and survey commensal and virulent *E. coli* strains to determine the prevalence and distribution of identified osmoregulatory mechanisms (19, 23, 51).

Earlier work showed that osmoprotectant transporters ProP and ProU are present and expressed in diverse *E. coli* strains, both commensal and pathogenic (19). *E. coli* strain CFT073 is more osmotolerant than strain HU734 in the absence of organic osmoprotectants, and a defect in stationary-phase sigma factor RpoS impairs the relative ability of HU734 to grow in media of very high (over 0.8 mol/kg) but not of moderate salinity by impairing trehalose accumulation (22). In addition, HU734 harbors an osmoregulatory betaine uptake activity (BetU) not evident in CFT073, but defects eliminating ubiquitous osmoprotectant transporters ProP and ProU impair the growth of HU734 and not CFT073 in high-osmolality human urine (18, 21). The latter data suggested that osmoregulatory betaine uptake is critical for osmoregulation (and growth in urine) by HU734 but not CFT073 and that CFT073 may harbor one or more additional glycine-betaine-independent osmoregulatory systems that contribute to bacterial growth in urine and are not present in HU734.

This paper reports that both pyelonephritis isolates retain osmoregulatory K^+ transporter TrkH but lack its homologue, TrkG. Both are able to grow on low-K^+, high-osmolality media in the absence of organic osmoprotectants. No known osmoprotectant stimulated the growth of CFT073 in high-osmolality medium, but analysis of the CFT073 genome revealed putative osmoregulatory transporters that may mediate accumulation of urinary osmoprotectants, which are as yet unidentified. We report the isolation of *betU* from HU734 and evidence that BetU is a member of the betaine-carnitine-choline transporter (BCCT) family. Phylogenetic and genomic sequence analyses are revealing striking genetic diversity among *E. coli* isolates and elucidating the evolution of virulence (8, 40, 62, 67, 86). Osmoregulatory loci *proP* and *proU* are ubiquitous (19, 22; this work) and likely part of the core *E. coli* genome. We have now examined the distributions of *trkG* and *betU* within the ECOR collection and collections of pathogenic *E. coli* isolates to fur-

TABLE 2. *E. coli* strains of clinical origin used during this study (the PATH collection)

Pathotype[a]	Name	Source[b]	Serotype[c]	Reference(s)
UPEC	HU734[d]	Urine, pyelonephritis	O75:K5	37, 80
UPEC	CFT073	Blood, pyelonephritis	O6:K2:H1	56
UPEC	J96	Urine, pyelonephritis	O4:K6:H5	45, 80
UPEC	536	Pyelonephritis	O6:K15:H31	25
EHEC	EDL933	Ground beef	O157:H7	49, 62, 68
EHEC	RIMD0509952	Bloody diarrhea	O157:H7	40, 84
EHEC	93-001	Bloody diarrhea	O157:H7	49
VTEC	FRIK920	Bovine feces	O157:H7	49
EPEC	E2348/69	Infantile diarrhea	O127:H6	52, 67
EPEC	B170	Infantile diarrhea	O111:NM	61, 67
EPEC	DEC12a	Infantile diarrhea	O111:H2	60, 67
ETEC	H10407	Liquid stool	O78:H11	30, 81
ETEC	339	Jejunal fluid	O15:H11	33
NEMEC	S3	Neonatal meningitis	NA	7, 10, 11
NEMEC	S4	Neonatal meningitis	NA	7, 10, 11
NEMEC	C5 (S25)	Cerebrospinal fluid	O18:K1:H7	7, 10, 11
NEMEC	S39	Neonatal meningitis	NA	7, 10, 11
NEMEC	S41	Neonatal meningitis	NA	7, 10, 11
NEMEC	S51	Neonatal meningitis	NA	7, 10, 11
NEMEC	S76	Neonatal meningitis	NA	7, 10, 11
NEMEC	S82	Neonatal meningitis	Ont:K1	7, 10, 11

[a] UPEC, uropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; VTEC, verotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; NEMEC, *E. coli* isolates from neonatal meningitis.
[b] Bacteria were isolated from humans unless otherwise noted, and all strains except FRIK920 were associated with human disease.
[c] NA, not available.
[d] HU734 is a *lac* mutant derivative of acute pyelonephritis isolate GR12 (22, 37).

ther assess their evolutionary origins and relationships to bacterial virulence.

MATERIALS AND METHODS

Bacterial strain. The *E. coli* K-12 derivatives used during this study included DH5α [F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA relA1*] (38), Frag-1 (F$^-$ *thi rha lacZ*$_{x82}$ *gal*) (29), MKH13 [F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF25 rbsR* Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*)*608*] (36), and TK2420 [Frag-1 *nagA trkD1* Δ(*trkA*) Δ(*kdpABC*)*5 kup*] (28). Pyelonephritis isolates HU734 and CFT073 and their derivatives deficient in transporters *putP*, *proP*, and/or *proU* were previously described (18, 22). HU734 is a *lacZ* derivative of acute pyelonephritis isolate GR12 with the following properties: streptomycin and spectinomycin resistance, cysteine auxotrophy, serotype O75:K5, possession of type 1 and P pili (the latter encoded by a single *pap* operon), carriage of a ColV plasmid, resistance to killing by human and mouse serum, and failure to produce hemolysin. CFT073 was isolated from the blood of a patient with acute pyelonephritis. It has no antibiotic resistance or auxotrophy, is O nontypeable and nonmotile, expresses type 1, S, and P pili (the latter encoded by two *pap* operons), produces hemolysin, and is cytotoxic for cultured human renal epithelial cells. The derivatives of these strains used for this study included WG695 [HU734 Δ(*putPA*)*566* Δ(*proP*)*218* Δ(*proV-proX*)*567*], WG696 [CFT073 Δ(*proP*)*218* Δ(*proV-proX*)*567*], WG745 [CFT073 Δ(*rpoS*)*2062*], and WG746 [CFT073 Δ(*proP*)*218* Δ(*proV-proX*)*567* Δ(*rpoS*)*2062*]. Two collections of *E. coli* strains representing diverse pathotypes were used. A collection of urinary tract and intestinal *E. coli* isolates was described previously (19, 23). The urinary tract infection (UTI) collection included the 30 urinary tract isolates from that collection (7 catheter-associated, 1 bacteriuria, 12 cystitis, 6 pyelonephritis [including strain HU734], and 4 unspecified UTI) plus strain CFT073. The *E. coli* pathotype (PATH) collection, including 21 strains with a broader array of pathotypes, is described in Table 2. Pyelonephritis isolates HU734 and CFT073 were common to both the UTI and the PATH Collections. Genomic DNA samples derived from the 72 *E. coli* reference (ECOR) collection strains (59) were a generous gift from C. Whitfield (University of Guelph), and R. Y. C. Lo (University of Guelph) provided plasmid pBR322 (9, 85).

Media and growth measurements. Culture media included Luria-Bertani (LB) (55), morpholinepropanesulfonic acid-based minimal medium (MOPS) (58), and K5 (24). MOPS minimal medium was supplemented with D-glucose (0.2% wt/vol) as the carbon source and NH_4Cl (9.5 mM) as the nitrogen source. Antibiotics were used at the following concentrations: ampicillin (AMP), 100 μg/ml; tetracycline, 25 μg/ml. The abilities of organic compounds to provide osmoprotection to *E. coli* were assessed, as described previously (54), by measuring bacterial plating efficiencies on MOPS medium supplemented with NaCl (0.6 M) and/or osmoprotectant (1 mM).

Isolation of gene *betU*. Molecular biological manipulations were performed as described by Sambrook et al. (71) unless otherwise stated. Plasmid DNA was prepared by using the QIAprep Spin Miniprep kit or Plasmid Midi kit (QIAGEN, Mississauga, Ontario, Canada). Electroporation was performed with the Micropulser Electroporater [Bio-Rad (Canada) Inc., Mississauga, Ontario, Canada] according to the manufacturer's instructions, and chemical transformation was performed as described by Hanahan (38).

To construct a DNA library, chromosomal DNA isolated from *E. coli* WG695 was partially digested with *Sau*3A to yield DNA fragments 3 to 10 kb in length. Vector pGEM-7z (Promega Corp., Madison, Wis.) was digested with *Bam*HI, dephosphorylated with shrimp alkaline phosphatase (USB Corp., Cleveland, Ohio), and treated with T4 DNA ligase [Boehringer Ingelheim (Canada) Ltd., Burlington, Ontario, Canada]. The resultant recombinant plasmids were introduced to *E. coli* DH5α via electroporation, and transformants were selected on LB plates containing AMP and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml). Plasmid DNA was isolated from the pooled transformants (approximately one-third of which were Lac$^-$) and stored.

To isolate gene *betU*, the DNA bank was introduced to *E. coli* MKH13 via electroporation and transformants were selected on MOPS supplemented with AMP, NaCl (0.6 M), and glycine-betaine (1 mM). Three clones that appeared after 48 h of incubation at 37°C were streak purified, and plasmid DNA was isolated and retransformed into MKH13 to confirm complementation. One of these clones, containing a plasmid with a 7.4-kb insert, was designated pAL1. The entire insert was sequenced by primer walking (Laboratory Services, Guelph, Ontario, Canada; MOBIX, Hamilton, Ontario, Canada). For sequences not represented in the genome of *E. coli* K-12, the reverse strand was also sequenced. DNA sequences were assembled and analyzed by using Sequencher (Gene Codes Corporation, Ann Arbor, Mich.).

To subclone *betU*, plasmid DNA (both pAL1 and vector pBR322) was digested with *Sca*I and *Pst*I restriction endonucleases, mixed, ligated with T4 DNA ligase, and transformed into DH5α. Colonies were selected on LB plates containing tetracycline. Plasmid DNA was isolated from 16 randomly selected clones and subjected to restriction analysis. Plasmids with the expected fragment sizes were further transformed into MKH13 to confirm complementation, and one clone, containing a plasmid designated pAL3, was retained as *E. coli* WG855.

TABLE 3. PCR primers

Primer	Sequence (5′ → 3′)	Amplicon	
		Origin	Size (bp)
putP1	GGT TGC GTG TGC ATA CCG A	bp 287–488 of *putP*	202
putP2	GCC GTT TCG TAG CTC ATG C		
proV1	GCA TCC ACA GCG AGC GTT CA	bp 48–301 of *proV*	254
proV2	GGA GTT CGG CGT CGG ATA T		
betU1	ACC GTC ATC CAC ATT AGC	bp 496–1118 of *betU*	623
betU2	GCG TTC TTC AGT TAC CG		
trkA1	CTG GTT GGC GAG AAC AAC	bp 55–263 of *trkA*	209
trkA2	GAG TAG GCT ACC TGG CAG		
trkG1	GCC CAC GCA CAC GAT AGT G	bp 992–1354 of *trkG*	362
trkG2	GCA CTC CGA ATG ACG ATG C		
trkH1	GCG ATC CGG AAT TTC GCA TG	bp 809–983 of *trkH*	175
trkH2	CGG GCA ATG CTG TCA GTT G		
sapD1	GAC ACG ACT GCG GTT CCT G	bp 53–304 of *sapD*	252
sapD2	GTG GGT TAA AGC CGT CGA C		
sapF1	GGC AAT CAG CCG TTT GGT G	bp 543–756 of *sapF*	214
sapF2	GGA TAT GTC GAT GCG TTC GC		

Analysis of the occurrence of genetic loci by PCR. Genomic template DNAs were prepared, duplex PCRs were performed, and amplicons were analyzed as described previously (23), except that the annealing temperature was 52°C and the extension time was 1 min for amplification of *putP* and *betU*. The primers are listed in Table 3. Amplification of *proV* was used as an internal positive control for the detection of *trkA*, *trkG*, *trkH*, and *sapF*, whereas *putP* was used as the positive control during detection of *sapD* and *betU*.

Analytical procedures. Initial rates of choline and glycine-betaine uptake were measured as described by Culham et al. (20) with [*methyl*-^{14}C]choline or [1-^{14}C]glycine-betaine (American Radiolabeled Chemicals, Inc., St. Louis, Mo.) at final concentrations and specific radioactivities of 8 μM and 10 Ci/mol for choline and 200 μM and 5 Ci/mol for glycine-betaine. The protein content of cell suspensions was determined with the bicinchoninic acid assay (78), with bovine serum albumin as the standard. Osmolalities of growth and assay media were measured with a vapor pressure osmometer (Wescor).

Nucleotide sequence accession number. The *betU* sequence was deposited in GenBank with accession number AF532988.

RESULTS

***E. coli* isolates HU734 and CFT073 lack TrkG but retain osmoregulatory K^+ uptake.** Pyelonephritis isolate CFT073 was similar in osmotolerance to *E. coli* K-12 when both were cultivated in NaCl-supplemented MOPS minimal medium (without osmoprotectants), but pyelonephritis isolate HU734 was less osmotolerant (18, 22). Failure to synthesize trehalose due to an RpoS defect accounted for the poor osmotolerance of HU734 at very high osmolality (more than 0.8 mol/kg) but not in the lower osmolality range characteristic of normal human urine (0.5 to 0.8 mol/kg) (22). In addition, elimination of ProP and ProU impaired the growth of HU734, but not CFT073, in high-osmolality urine (which contains osmoprotectants).

The difference in osmotolerance between *E. coli* HU734 and isolates K-12 and CFT073 could result from a difference in osmoregulatory K^+ uptake capacity. In *E. coli* K-12, transporters Kdp, Trk, and Kup contribute to K^+ uptake while Kdp and Trk also contribute to osmotolerance (26). Kdp is an optional, high-affinity K^+ uptake system that allows bacteria lacking Trk and Kup to grow on media that are very low in K^+ (micromolar concentrations) and low or high in osmolality (2). The abilities of strains HU734, CFT073, and WG745 (CFT073 Δ*rpoS*) to use K^+ were tested by comparing the growth of each strain on NaCl-supplemented K5 medium (24) with that of *E. coli* K-12 strain Frag-1 (wild type for K^+ uptake) and its K^+ uptake-null derivative TK2420 (28). As reported previously, strain TK2420 was unable to grow in K5 medium supplemented with less than 25 mM K^+. In contrast, strains HU734, CFT073, WG745, and Frag-1 grew well on K5 medium supplemented with up to 0.4 M NaCl even if no K^+ salt was added (typically, the level of K^+ contaminating such media is micromolar). Thus, no K^+ uptake deficiency was evident in *E. coli* HU734.

Since the low-affinity, high-capacity Trk systems are most likely to mediate osmoregulatory K^+ uptake in the relatively high-K^+ environments of the urinary tract or MOPS, we further tested the incidence of those systems in the pyelonephritis isolates. Trk refers to a pair of low-affinity, high-capacity K^+ transporters which are expressed constitutively in *E. coli* K-12 (17). Multiple components contribute to each Trk system, including TrkA and TrkG or TrkH (73). Mutations in *trkE* impair K^+ uptake via the TrkH system, and TrkE has been redefined as an ABC transporter (SapABCDF) that is probably not part of Trk itself (88). Primers targeting sequences internal to *trkA*, *trkG*, *trkH*, *sapD*, and *sapF* were used in PCR to compare the incidence of the *trk* loci in strains HU734, CFT073, Frag-1, and TK2420 with that of loci *putP* and *proV*, which are ubiquitous (23). PCR products representative of *putP* and *proV* were detected with template DNA from all four strains, whereas PCR products representative of *trkA* were obtained with template DNA from strains HU734, CFT073, and Frag-1 but not TK2420 (which is known to be Δ*trkA*). Products representative of *trkH*, *sapD*, and *sapF* were detected with template DNA from all four strains, whereas only Frag-1 and TK2420 DNAs served as templates for amplification of *trkG*. These observations are consistent with the fact that the *trkG* locus of *E. coli* K-12 is part of Rac (72), a lambdoid prophage that is absent from the sequenced genome of *E. coli* CFT073 (86). Since *trkG* is absent from both HU734 and CFT073, this *trk* defect does not account for the fact that HU734 is intrinsically less osmotolerant than CFT073 (22). Further studies will be required to determine the basis for that difference.

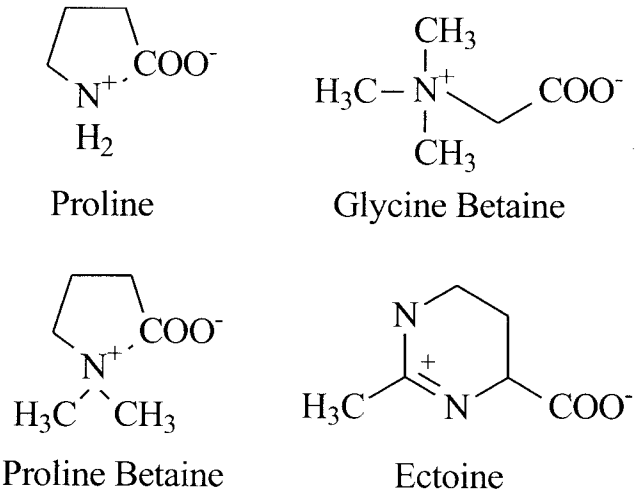


FIG. 1. Compatible solutes. The structures of compatible solutes commonly used by *E. coli* are illustrated. Glycine-betaine and proline-betaine (but not proline or ectoine) are substrates for BetU.

Osmoprotectant specificities of pyelonephritis isolates HU734 and CFT073. Transporters ProP and ProU accounted for all glycine-betaine uptake activity in strain CFT073 but not in strain HU734. The residual glycine-betaine uptake activity in HU734 was named BetU (22). Paradoxically, elimination of ProP and ProU impaired the growth of HU734, but not CFT073, in high-osmolality human urine (22). HU734 harbors an RpoS defect that blocked osmoregulatory trehalose accumulation, but deletion of *rpoS* did not impair growth of CFT073 in high-osmolality human urine (22). Analysis of extracts from CFT073 cells cultivated at high osmolality in the absence of osmoprotectants failed to reveal compatible solutes of biosynthetic origin other than trehalose (22). Choline is an osmoprotectant for *E. coli* K-12 because BetT mediates choline uptake while BetB and BetA mediate choline oxidation to glycine-betaine (88). Choline provided osmoprotection to HU734 and CFT073, both retain locus *betT* (data not shown), and the choline uptake activity of strain HU734 exceeded that of strain CFT073. For bacteria cultivated in NaCl-supplemented MOPS (0.94 mol/kg), the choline uptake activities of HU734 and CFT073 were 31 ± 0.4 and 7 ± 0.1 nmol/min/mg of cell protein, respectively, and they were not affected by deletion of loci *proP* and *proU*. Differences in BetT activity are thus unlikely to accelerate the growth of CFT073 over that of HU734 in high-osmolality human urine. HU734 is known to harbor a betaine uptake activity (BetU) that is not expressed by *E. coli* K-12 or CFT073. Perhaps CFT073 harbors an osmoprotectant uptake system that is not present in *E. coli* K-12 or HU734.

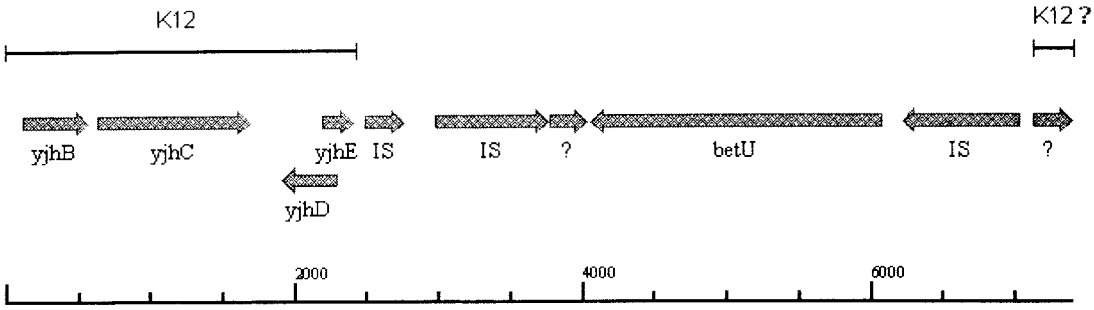
The transporters listed in Table 1 mediate accumulation of diverse osmoprotectants (some of which are illustrated in Fig. 1). We screened diverse compounds to identify osmoprotectant activity for derivatives of HU734 and CFT073 lacking transporters ProP and ProU (see Materials and Methods). Both glycine-betaine and proline-betaine increased the plating efficiency of strain WG695 (HU734 Δ*putPA* Δ*proP* Δ*proU*) on MOPS supplemented with 0.6 M NaCl. None of the protein amino acids provided osmoprotection to this strain, and none of them reduced the osmoprotective activity of glycine-betaine, indicating that BetU is not a broad-specificity amino acid transporter. Proline, ectoine, pipecolate, dimethyl glycine, sarcosine, and carboxymethyl pyridinium also failed to provide osmoprotection. BetU was thereby tentatively defined as a betaine-specific transporter. In contrast, neither the compounds listed above nor D-carnitine, L-carnitine, taurine, betonicin, butyrobetaine, thiaproline, or trigonelline conferred osmoprotection on *E. coli* WG696 (CFT073 Δ*proP* Δ*proU*). Further efforts will be required to identify urinary compounds, other than those listed, which are osmoprotective for *E. coli* CFT073. Additional, putative osmoprotectant transporters have been identified via analysis of the CFT073 genome (see Discussion).

Identification of the *betU* locus of HU734. *E. coli* MKH13, derived from *E. coli* K-12 derivative MC4100, lacks the BetT, ProP, and ProU transport systems and therefore cannot grow on high-osmolality minimal media containing glycine-betaine (36). A gene library prepared from *E. coli* WG695 (HU734 Δ*proP* Δ*proU*) was transformed into MKH13, and transformants were selected on MOPS containing NaCl and glycine-betaine as described in Materials and Methods. Plasmid pAL1, which contained a 7.4-kb insert, enabled *E. coli* MKH13 to grow on MOPS containing either glycine-betaine or proline-betaine but not choline or ectoine (1 mM). Therefore, the cloned insert encoded a system with the expected substrate specificity.

The entire 7.4-kb insert was sequenced, revealing a region identical with residues 4501566 to 4504677 of the *E. coli* K-12 genome, including genes *yjhB*, *yjhC*, *yjhD*, and *yjhE* (functions unknown) (Fig. 2, top). An open reading frame (ORF) flanked by putative insertion sequences was found upstream from the *yjh* genes and was not present in the *E. coli* K-12 genome, as expected given the absence of BetU activity from *E. coli* K-12 (22). The insertion sequences up- and downstream from *betU* (Fig. 2, top) (flanking *betU* to the left and right, respectively) encoded putative transposases identical to InsB and Hp1, present in IS*911* and IS*600* of *Shigella flexneri*, respectively. These insertion sequences and their close homologues (E values less than e^{-10}) are present in many copies in each of the sequenced *E. coli* and *S. flexneri* genomes, occurring least frequently in *E. coli* K-12 (4 copies each), at intermediate frequencies in the pathogenic *E. coli* isolates CFT073, EDL933, and RIMD0509952 (8 to 15 copies), and most frequently in *S. flexneri* (42 to 56 copies).

The ORF flanked by the insertion sequences was subcloned into vector pBR322, creating plasmid pAL3 that was transformed into *E. coli* MKH13. (To do this, the *Sca*I-*Pst*I fragment of pBR322 was replaced with the 3,061-bp *Sca*I-*Pst*I fragment from pAL1, which extended from 423 bp upstream to 635 bp downstream of the ORF.) Like pAL1, pAL3 restored growth of *E. coli* MKH13 on MOPS containing NaCl and glycine-betaine. These results indicated that the isolated ORF was *betU* and that plasmid pAL3 included the *betU* promoter. The *betU* sequence was deposited in GenBank with accession number AF532988. The putative insertion sequences flanking *betU* imply its arrival by lateral gene transfer. However, gene *betU* is not differentiated from the *E. coli* genome by its base composition (50.4% G+C).

E. coli MKH13 is devoid of glycine-betaine uptake activity



Organism	Transporter		Identity
	Name	Substrates	
Sinorhizobium meliloti	BetS	GB, PB	43%
Bacillus subtilis	OpuD	GB	40%
Listeria monocytogenes	BetL	GB	39%
Escherichia coli	BetT	GB, Ch	36%
Corynebacterium glutamicum	EctP	Ect, GB	35%
Corynebacterium glutamicum	BetP	GB	32%

FIG. 2. Isolation of *betU*. (Top) A 7,383-bp DNA fragment including *betU* was isolated from *E. coli* WG695 (HU734 Δ*putP* Δ*proP* Δ*proU*), inserted in vector pGEM-7z, and recovered by functional complementation of transporter defects in *E. coli* MKH13 (Δ*putP* Δ*betTIBA* Δ*proP* Δ*proU*) as described in Materials and Methods. Sequencing of this fragment revealed that *betU* is inserted adjacent to *yjhE* in the backbone sequence shared by *E. coli* K-12 and *E. coli* O157:H7 and that it is flanked by insertion sequences, as would be expected if it had appeared by lateral gene transfer. (Bottom) BetU is similar to known osmoregulatory transporters in diverse organisms (*Sinorhizobium meliloti* [12], *B. subtilis* [47], *L. monocytogenes* [75], *E. coli* [4], and *C. glutamicum* [63, 64]). These systems mediate accumulation of quaternary ammonium compounds including carnitine (Car), choline (Cho), ectoine (Ect), glycine-betaine (GB), and proline-betaine (PB).

(47). Initial rates of glycine-betaine uptake by *E. coli* strains WG695 [HU734 Δ(*putPA*)*566* Δ(*proP*)*218* Δ(*proV-proX*)*567*] and WG855 (MKH13 pAL3) were determined as a function of medium osmolality (Fig. 3). The glycine-betaine uptake activity of BetU in its native host (WG695) was half maximal at approximately 0.2 mol/kg (Fig. 3, inset) and reached a maximum of approximately 21 nmol/min/mg of cell protein. In contrast, the glycine-betaine uptake activity of *E. coli* WG855 was much higher (fivefold higher at an osmolality of 0.2 mol/kg) and it did not reach a limiting value within the osmolality range tested (Fig. 3, inset). These differences were unlikely to result solely from copy number effects.

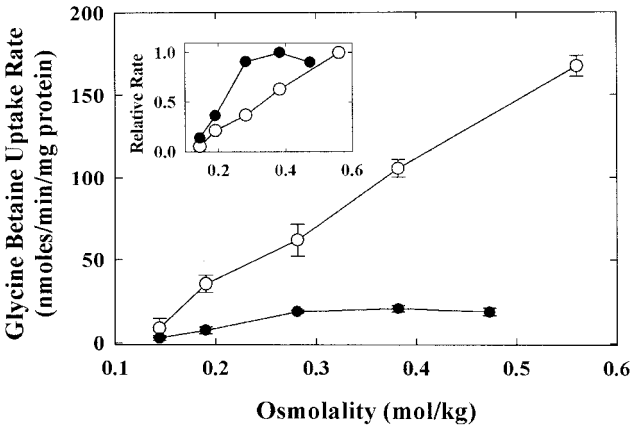


FIG. 3. *betU* encodes a glycine-betaine transporter. Initial rates of glycine-betaine uptake by *E. coli* strains WG695 [HU734 Δ(*putPA*)*566* Δ(*proP*)*218* Δ(*proV-proX*)*567*] (closed circles) and WG855 (MKH13 pAL3) (open circles) were determined as a function of assay medium osmolality as described in Materials and Methods. Relative rates of glycine-betaine uptake, calculated by setting the maximum rate to a value of 1 for each strain, are shown in the inset.

The *betU* gene encodes a 667-residue protein. A BLAST search showed that BetU is similar to members of the BCCT family (70) that are known to catalyze osmoregulatory accumulation of quaternary ammonium compounds such as glycine-betaine (Fig. 2, bottom, and Table 1). Transporters with strong sequence similarity to BetU are also predicted to occur in a number of other organisms, many of which are pathogens. They include the following (by organism and percent sequence identity): *Proteus vulgaris*, 66%; *Pseudomonas aeruginosa*, 58, 42, and 40%; *Xanthomonas campestris*, 41%; *Vibrio cholerae*, 41%; *Staphylococcus aureus*, 39%; *Mycobacterium tuberculosis*, 38%; *Yersinia pestis*, 37%; *Erwinia amylovora*, 37%; *Bacillus anthracis*, 36%; *Neisseria meningitidis*, 33%. No insertion sequences could be found flanking the genes encoding BetT, EctP, BetP, LcoP, and OpuD. Hydropathy analysis (e.g., TopPred) (82) predicts BetU to be a membrane protein with 12 membrane spanning α-helices and cytoplasmic termini.

TABLE 4. Incidence of *trkG* and *betU* in the ECOR collection strains[a]

EG[b]	Strain	Incidence of[c]:		EG[b]	Strain	Incidence of[c]	
		betU	*trkG*			*betU*	*trkG*
A	ECOR-01	−	+	B1	ECOR-69	+	−
A	ECOR-02	−	+	B1	ECOR-70	−	+
A	ECOR-03	−	+	B1	ECOR-71	−	+
A	ECOR-04	−	+	B1	ECOR-72	−	+
A	ECOR-05	−	−	B1	ECOR-42	−	+
A	ECOR-06	−	+				
A	ECOR-07	−	−	B2	ECOR-52	−	+
A	ECOR-08	−	−	B2	ECOR-53	+	−
A	ECOR-09	−	−	B2	ECOR-54	+	+
A	ECOR-10	−	−	B2	ECOR-55	+	+
A	ECOR-11	+	+	B2	ECOR-56	−	−
A	ECOR-12	+	−	B2	ECOR-57	−	+
A	ECOR-13	+	−	B2	ECOR-58	−	+
A	ECOR-14	+	+	B2	ECOR-60	−	+
A	ECOR-15	−	+	B2	ECOR-61	+	+
A	ECOR-16	+	+	B2	ECOR-62	+	+
A	ECOR-17	−	+	B2	ECOR-63	−	+
A	ECOR-18	−	+	B2	ECOR-64	+	+
A	ECOR-19	+	+	B2	ECOR-65	−	−
A	ECOR-20	−	+	B2	ECOR-66	−	−
A	ECOR-21	−	−				
A	ECOR-22	−	+	D	ECOR-35	+	−
A	ECOR-23	−	+	D	ECOR-36	+	−
A	ECOR-24	+	+	D	ECOR-38	+	+
A	ECOR-25	−	+	D	ECOR-39	+	+
				D	ECOR-40	+	+
B1	ECOR-26	−	+	D	ECOR-41	+	−
B1	ECOR-27	−	+	D	ECOR-45	−	+
B1	ECOR-28	−	−	D	ECOR-47	−	−
B1	ECOR-29	−	+	D	ECOR-48	−	+
B1	ECOR-30	−	+	D	ECOR-49	−	+
B1	ECOR-32	−	+	D	ECOR-50	+	+
B1	ECOR-33	−	−	D	ECOR-51	−	+
B1	ECOR-34	−	−				
B1	ECOR-46	−	+	E	ECOR-31	−	+
B1	ECOR-59	+	−	E	ECOR-37	−	−
B1	ECOR-67	−	−	E	ECOR-43	+	−
B1	ECOR-68	−	−	E	ECOR-44	−	+

[a] The incidence of each locus was determined by duplex PCR as described in Materials and Methods. To detect *betU*, PCR was performed with primers putP1, putP2, betU1, and betU2 (Table 3). A 0.2-kb *putP* amplicon was detected in every reaction mixture. To detect *trkG*, PCR was performed with primers proV1, proV2, trkG1, and trkG2 (Table 3). A 0.3-kb *proV* amplicon was detected in every reaction mixture. +, an additional amplicon of the expected size was observed (0.6 kb for *betU* and 0.4 kb for *trkG*); −, no additional amplicon was observed.

[b] EG, ECOR group. The ECOR groups represent distinct evolutionary clones of *E. coli* detected via multilocus enzyme electrophoresis and DNA sequence analysis of housekeeping loci (43, 87).

[c] Percent incidences of *betU* are as follows: group A, 28%; group B1, 6%; group B2, 50% group D, 58%; group E, 25%. Percent incidences of *trkG* are as follows: group A, 68%; group B1, 63%; group B2, 71%; group D, 67%; Group E, 50%.

Distributions of *trkG* and *betU* among *E. coli* isolates. The DNA sequences flanking *trkG* and *betU* suggest that both were or are components of mobile genetic elements. The distributions of these loci were determined to further assess their evolutionary origins and relationships to *E. coli* virulence. Locus *trkG* was present in 66% of the 72 ECOR collection isolates without strong bias among the ECOR groups (Tables 4 and 5). However, *trkG* was detected in only 16% of a group of isolates associated with UTI (the UTI collection) and 14% of a group of strains representing diverse *E. coli* pathotypes (the PATH collection) (Tables 2 and 5). The 22.9-kb *rac* prophage interrupts locus c1819 (function unknown) of *E. coli* K-12. A different, 1.7-kb DNA sequence occupies the corresponding position in *E. coli* CFT073 (86). That 1.7-kb insert encodes a homologue of *sitD*. Iron uptake locus *sitABCD* is present elsewhere in the genome of CFT073 and in the centisome 63 pathogenicity island of *S. enterica* serovar Typhimurium (91).

Locus *betU* was present in one-third of the ECOR collection strains, its incidence being highest (50% or more) in ECOR groups B2 and D (Tables 4 and 5). It has been suggested that genomic sequences common to group B2 organisms diverge deeply from those of commensal *E. coli* strains in ECOR groups A and B1 and have provided an essential context for the evolution of extraintestinal virulence (7, 65). Since *betU* is present in only one-half of the group B2 strains, either it is not part of that essential context or it has been selectively lost. *betU* was present in less than one-half of the isolates in the UTI and PATH collections (29 and 43%, respectively) (Table 5). Given that *betU* of HU734 is flanked by putative insertion sequences, it may be a nonessential gene that is present in a subset of

TABLE 5. Incidence of osmoregulatory loci in representative clinical isolates (the UTI and PATH collections)

Group or strain name[a]	Incidence of locus[b]	
	betU	*trkG*
UTI collection	29	16
HU734	+	−
CFT073	−	−
J96	+	+
536	−	−
EDL933	−	−
RIMD0509952	−	−
93-001	−	−
FRIK920	−	−
E2348/69	−	−
B170	+	−
DEC12a	+	−
H10407	+	+
339	+	+
S3	−	−
S4	−	−
C5 (S25)	−	−
S39	+	−
S41	−	−
S51	−	−
S76	+	−
S82	+	−
PATH collection	43	14

[a] The UTI collection includes 30 urinary tract isolates from a previously described collection (19, 23) plus *E. coli* CFT073. The PATH collection strains are listed in Table 2.

[b] The incidence of each genetic locus, determined by duplex PCR as described in Materials and Methods and footnote *a* to Table 4, is reported as percent occurrence for the collections and presence (+) or absence (−) for individual isolates. Loci *putP* and *proV* were detected in all strains.

pathogenic and nonpathogenic *E. coli* strains due to lateral gene transfer.

DISCUSSION

Our goal is to test the hypothesis that osmoregulatory betaine accumulation contributes to growth in urine and urinary tract colonization by uropathogenic *E. coli* (16). Before this hypothesis can be rigorously tested, all the osmoregulatory systems that contribute to osmoprotection must be identified. We are therefore conducting detailed studies of pyelonephritis isolates HU734 and CFT073 (18, 22) and surveying commensal and virulent *E. coli* strains to determine the prevalence and distribution of the identified osmoregulatory mechanisms (this study) (19, 23, 51). Earlier work (i) revealed BetU, an osmoregulatory system present in HU734 but not *E. coli* K-12 or CFT073, (ii) suggested that osmoregulatory betaine uptake is critical for osmoregulation (and growth in urine) by HU734 but not CFT073, and (iii) implied that CFT073 may harbor yet another glycine-betaine-independent osmoregulatory system that contributes to bacterial growth in urine and is not present in HU734.

In this paper, we report the isolation and characterization of the *betU* locus from *E. coli* HU734. Gene *betU* encodes a 667-residue protein that is a member of the BCCT family and predicted to have 12 transmembrane helices (Fig. 2). The BCCT family continues to grow (Fig. 2), and it appears to dominate osmoregulation in some organisms (e.g., *C. glutamicum*), whereas osmoregulatory ABC transporters appear to be dominant in others (e.g., *B. subtilis*) (Table 1). Gene *betU* could have coevolved with paralogue *betT* after a gene duplication event in *E. coli*. Alternatively, since *betU* is flanked by insertion sequences, it could have arrived by lateral transfer. In contrast, the genes encoding BCCTs BetT, BetP, EctP, and OpuD are not flanked by insertion sequences. Much higher glycine-betaine uptake activity could be attributed to BetU when *betU* was expressed from its own promoter in *E. coli* K-12 than when *betU* was expressed in its native genetic background (Fig. 3). This may indicate that elements required to regulate *betU* expression are absent from *E. coli* K-12.

One-third of the *E. coli* strains included in this study harbored locus *betU* (Tables 4 and 5). The incidence of *betU* among pathogenic *E. coli* strains included in this study (34% overall) was similar to that in the ECOR collection (32%) but lower than that in ECOR groups B2 and D (Tables 4 and 5). Clearly the presence of *betU* was not selected during the evolution of urovirulence. In contrast, locus *trkG*, which encodes an osmoregulatory K^+ transporter similar in structure and function to TrkH, occurred much less frequently among pathogenic *E. coli* isolates (overall incidence, 16%) than among the (predominantly) commensal isolates of the ECOR collection (overall incidence, 66%) (Tables 4 and 5). Indeed, the sequenced genomes of two *E. coli* O157:H7 isolates harbor lambdoid prophages at the Rac insertion site, but both lack *trkG*. Thus, TrkG is not essential for virulence and incorporation of *trkG* in the Rac prophage, loss of the genetic material that is replaced by Rac or the presence of genetic material within Rac may impair virulence. The presence of insertion sequences flanking *betU* (at least in the genome of *E. coli* HU734) as well as the fact that *trkG* is encoded by (and may be a latecomer to) the Rac prophage (at least in *E. coli* K-12) imply distribution of these genes by lateral transfer. The UTI and PATH collections are small (31 and 21 isolates, respectively). More extensive analyses conducted with larger numbers of isolates from each pathotype, each characterized by phylogenetic group, may reveal additional evolutionary relationships.

Differences in osmoregulatory trehalose synthesis, K^+, or choline uptake did not account for the greater osmotolerance of CFT073 relative to HU734, and no known osmoprotectant stimulated the growth of CFT073 derivatives lacking loci *proP*, *proU*, and/or *rpoS* in high-osmolality medium (see Results). The four sequenced *E. coli* genomes were analyzed to determine whether CFT073 might contain additional osmoprotectant transporters with new substrate specificities (Table 1). BLAST searches were conducted with known osmoregulatory transporters as query sequences. The significance of each identified relationship between a known osmoregulatory transporter and a protein of unknown function was assessed by comparing the percent identity and extent of sequence alignment with those parameters for pairs of known osmoregulatory transporters.

PutP, BetT, ProP, and ProU (the latter comprised of ProV, ProW, and ProX) are encoded by all four *E. coli* genomes, since according to BLAST analysis, these genomes share loci which show 99 to 100% sequence identity over alignments covering 100% of the query sequence length. Proline accumulation via PutP is not osmoregulatory for *E. coli* K-12 (88). PutP could be an osmoregulator in other *E. coli* strains, since

its homologues in *B. subtilis* and *S. aureus* have that activity (OpuE and PutP, respectively). However, this seems unlikely, since proline is not an osmoprotectant for a derivative of strain CFT073 which lacks ProP and ProU but not PutP (22). Each secondary transporter (PutP, BetT, or ProP) is comprised of a single integral membrane protein subunit. Homologues of those proteins were considered putative paralogues if there was more than 30% sequence identity over more than 80% of the query sequence length. On that basis, a putative paralogue was found for *E. coli* ProP but not for PutP or BetT. YhjE, encoded by all four *E. coli* genomes, shares 33% sequence identity with ProP over an alignment that covers 84% of the ProP sequence. (By comparison, *C. glutamicum* ProP, a known osmoregulatory transporter, is 39% identical and *E. coli* ShiA, a shikimate transporter that is not an osmoregulator, is 31% identical to *E. coli* ProP.)

ABC transporters (e.g., ProU) are comprised of an ATP binding cassette (ABC subunit, e.g., ProV), an integral membrane protein subunit (e.g., ProW), and a periplasmic substrate binding protein (PBP subunit, e.g., ProX). The components of *E. coli* YehXYWZ were similar to the corresponding components of *E. coli* ProU but even more closely related to those of OpuC from *B. subtilis*. Taking into account the presence of *yehXYWZ* in all four sequenced *E. coli* genomes, the encoded proteins showed more than 40% sequence identity over more than 70% of the query sequence length (ABC subunit OpuCA), more than 40% sequence identity over more than 80% of the query sequence length (integral membrane protein subunits OpuCB and OpuCD) or more than 25% sequence identity over 97% of the alignment length (PBP subunit OpuCC). Similar relationships were seen to OpuA and OpuB of *B. subtilis*, with the exception of the PBP subunits. Thus, YehXYWZ is likely paralogous to ProU and may be orthologous to OpuC, a glycine-betaine transporter, but differ in substrate specificity.

No osmoregulatory tripartite ATP-independent periplasmic (TRAP) transporter has been identified in an organism with a sequenced genome, but Gramman et al. have shown that TRAP transporter TeaABC is an osmoregulatory ectoine transporter in *Halomonas elongata* (35). YiaOMN of *E. coli* may be orthologous with TeaABC, since the subunits show 24, 24, and 33% identity over alignments that cover 95, 62, and 87% of the query (Tea) sequence length, respectively. However, the YiaOMN subunits are also similar in sequence to those of transporters not implicated in osmoregulation, and *yiaOMN* is part of a gene cluster implicated in carbon metabolism by *E. coli* (reference 90 and references cited therein). Interestingly, YiaOMN is encoded by the genomes of *E. coli* MG1655 and CFT073 but not by the sequenced genomes of *E. coli* O157:H7 isolates.

Further work will be required to determine whether YhjE, YehXYWZ, and YiaOMN are transporters, whether they transport osmoprotectants, what their substrate specificities are, and how they are distributed among *E. coli* isolates. Past failure to detect contributions of these systems to osmoregulation in *E. coli* K-12 or CFT073 could result from failure to offer the appropriate osmoprotectant or failure of these systems to be expressed under past experimental conditions. For example, the latter problem has to date prevented study of *C. glutamicum* ProP in its native context (64). These data suggest that *E. coli* and other organisms share a pool of genes encoding osmoregulatory transporters, some of which can be readily transferred among organisms. No particular complement of osmoregulatory systems is common to all *E. coli* strains.

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