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A Requirement of TolC and MDR Efflux Pumps for Acid Adaptation and GadAB Induction in Escherichia coli

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Abstract

Background: The TolC outer membrane channel is a key component of several multidrug resistance (MDR) efflux pumps driven by H⁺ transport in Escherichia coli. While tolC expression is under the regulation of the EvgA-Gad acid resistance regulon, the role of TolC in growth at low pH and extreme-acid survival is unknown.

Methods and Principal Findings: TolC was required for extreme-acid survival (pH 2) of strain W3110 grown aerobically to stationary phase. A tolC deletion decreased extreme-acid survival (acidic resistance) of aerated pH 7.0-grown cells by 10³-fold and of pH 5.5-grown cells by 10-fold. The requirement was specific for acid resistance since a tolC defect had no effect on aerobic survival in extreme base (pH 10). TolC was required for expression of glutamate decarboxylase (GadA, GadB), a key component of glutamate-dependent acid resistance (Gad). TolC was also required for maximal exponential growth of E. coli K-12 W3110, in LBK medium buffered at pH 4.5–6.0, but not at pH 6.5–8.5. The TolC growth requirement in moderate acid was independent of Gad. TolC-associated pump components EmrB and MdtB contributed to survival in extreme acid (pH 2), but were not required for growth at pH 5. A mutant lacking the known TolC-associated efflux pumps (acrB, acrD, emrB, emrY, macB, mdtC, mdtF, acrEF) showed no growth defect at acidic pH and a relatively small decrease in extreme-acid survival when pre-grown at pH 5.5.

Conclusions: TolC and proton-driven MDR efflux pump components EmrB and MdtB contribute to E. coli survival in extreme acid and TolC is required for maximal growth rates below pH 6.5. The TolC enhancement of extreme-acid survival includes Gad induction, but TolC-dependent growth rates below pH 6.5 do not involve Gad. That MDR resistance can enhance growth and survival in acid is an important consideration for enteric organisms passing through the acidic stomach.

Introduction

Escherichia coli expresses a large number of multi-drug resistance (MDR) efflux pumps for the expulsion of antibiotics and metabolic wastes. An important group of inner membrane efflux pumps interacts with the outer membrane channel TolC proteins to form complexes that traverse the inner membrane, periplasm, and outer membrane. These complexes efficiently pump the materials outside of the cell [1–5]. The other components of these TolC-dependent tripartite efflux systems consist of an inner membrane bound transporter such as the “resistance nodulation division” (RND) family transporter AcrB or the major facilitator superfamily (MFS) transporter EmrB, both driven by H⁺ influx, or the ABC-superfamily transporter MacB driven by ATP hydrolysis [6]. Stabilizing the transporter-channel interaction is a cognate periplasmic membrane fusion protein (MFP) such as AcrA, EmrA and MacA. Homologs of the E. coli tolC are important in virulence for pathogens such as Salmonella typhimurium [7], Legionella pneumophila [8], Francisella tularensis [9], and Xylella fastidiosa [10]. The TolC-dependent efflux system is responsible not only for expulsion of toxic compounds but also for export of intracellular metabolites, such as enterobactin, porphyrin, and excess cysteine [4,11,12].

Several pieces of evidence link tolC expression to acid pH resistance. TolC shows acid-enhanced expression in the E. coli proteome [13]. In E. coli, tolC is a member of the EvgA acid resistance regulon [14,15] and, in F. tularensis, the tolC homolog is expressed in the same operon with gad (glutamate decarboxylase) [9], an important acid resistance factor (reviewed by [16,17]). The Gad acid resistance system (AR2) is active in stationary-phase cells grown at pH 7 or pH 5.5, in contrast to the glucose-repressed CRP system (AR1) which requires induction in acid, pH 5.5 [16]. Furthermore, assembly of TolC into efflux complexes requires low pH [18]. The acid-dependent expression and MDR assembly have been suggested to explain the increased sensitivity of bacteria to many antibiotics above pH 7 [18].
Nevertheless, the role of MDR pumps in *E. coli* acid growth and survival has not been tested. For comparison, at high pH, overexpression of the drug resistance pump MdfA has been shown to increase survival, and actually extends the *E. coli* growth range to pH 10 [19]. Since enteric pathogens must pass through the stomach, it is important to know whether MDR pumps have a role in growth or survival in acid. Here we report the contributions of *tolC, emrB,* and *mdtB* to extreme-acid survival (viability of cells following exposure to pH 2), the requirement of *TolC* for normal exponential growth at moderately low external pH (pH 4.5–6.0), and the requirement of *TolC* for Gad expression and induction at low pH.

**Results**

**Extreme-acid survival of *tolC, emrB,* and *mdtB***

*TolC* associates with at least nine different inner-membrane protein complexes (such as EmrAB or MdtABC) to form a connected efflux pump system [6]; several of these in the RND and MFS families, are driven by H\(^+\) influx. The growth and survival phenotypes of *tolC* deletions may result directly from the absence of *TolC* or from the combined inactivation of several inner-membrane efflux pumps. Therefore, we investigated whether these RND and MFS transporter pump components played a role in extreme-acid survival. Of the strains tested, only *tolC, emrB,* and *mdtB* deletions showed a significant effect on extreme-acid survival of aerobic cultures (Fig. 1). *TolC* deletion strains showed survival levels comparable to the wild-type (data not shown). Survivors was first determined for overnight cultures grown at external pH 7, where the Gad system is available but not the acid-inducible CRP system [16]. Extreme-acid survival (exposure at pH 2 for 2 hrs) was over 10\(^{-2}\)-fold lower for *tolC, 10\(^{-5}\)-fold lower for *mdtB,* and 100-fold lower for *emrB* compared to the wild-type strain W3110 (Fig. 1A). There was no increase or decrease in survival for a *mdtB* defect strain in which TolC expression is upregulated (data not shown) [20].

Acid survival was also tested for bacteria cultured overnight at pH 5.5, where RpoS- and CRP-dependent acid resistance systems are expressed [16]. Cultures grown at external pH 5.5 showed a 15-fold decrease in survival of *tolC* compared to W3110 (Fig. 1B). Thus, the *TolC* requirement was much greater for cells grown at pH 7 than for cells grown at pH 5.5. Complementation of *tolC* with plasmid pMX, which produces a functional TolC, grown at pH 5.5 and challenged at pH 2 restored the strain's acid survival comparable to that of the wild-type (data not shown). Strains defective for *mdtB* and *emrB* showed only a 6-fold and 2-fold decrease in survival under these conditions, respectively.

In extreme base (pH 10), the *tolC* strain (cultured aerobically to stationary phase at pH 8) showed comparable survival to the wild-type (data not shown). Thus, the pH sensitivity of *tolC* mutants was limited to acidic pH.

ToC is required for expression of the glutamate-dependent acid resistance system

A major contribution to acid resistance can result from the glutamate decarboxylase (Gad) system encoded by *gadA* and *gadBC*. The *gadA* and *gadBC* genes encode isoforms of glutamate decarboxylase and *gadC* encodes the glutamate transaminase. The glutamate-dependent acid antiporter [16]. Survival of strains MG1655 (wild-type) and MG1655T (*tolC::Tn10*) grown in LBK at pH 5.5 (100 mM MES) overnight and exposed to low external pH (pH 2.5) was tested in M9-glucose medium supplemented with 1.5 mM L-glutamic acid. After 30 min, survival of the *tolC* strain was decreased 10-fold relative to the wild-type; and after 60 min, survival of the *tolC* strain dropped to nearly 20-fold below the wild-type (data not shown). This is comparable to the acid-survival seen in complex LB medium (Fig. 1B) and suggests that the *tolC* strain is unable to utilize the glutamate-dependent acid resistance system, which is expressed during the overnight growth before exposure to pH 2.

Cultures of MG1655 and MG1655T (*tolC::Tn10*) were also assayed for activity and expression of the Gad system. Glutamate decarboxylase activity was assessed using the pH indicator dye bromocresol green; the dye changes from yellow to blue upon pH increase in the reaction mixture, following decarboxylation of L-glutamate (Fig. 2A). The wild-type strain behaved as expected with no decarboxylation at pH 5.5 and very clear evidence of decarboxylation at pH 7.5. The *tolC* strain, however, showed almost no Gad activity at pH 5.5. The *gadA* mRNA transcription was observed in wild-type but not in *tolC* cultures at pH 5.5, whereas the mRNA transcript of the lysine-dependent acid resistance system (*cadA*) was present in both wild-type and *tolC* strains (Fig. 2B). In the *tolC* strain at pH 5.5, the *cadA* mRNA transcript showed decreased expression compared to the wild-type, which may result from decreased regulation by GadE [21].
GadA and GadB proteins were absent in the tolC strain at pH 5.5 (Fig. 2C). Without TolC, no gadA or gadB expression could be detected.

Gad expression was restored by the pMX plasmid carrying the wild-type tolC gene (data not shown). Thus, TolC is required for expression of gadA mRNA and GadA and GadB proteins, as well as for activity of glutamate decarboxylases. Furthermore, in a tolC defective strain, plasmids expressing either GadB-C (pMF565) or the positive regulator GadE (pQEgadE) each restored extreme-acid survival at pH 2. This complementation confirms the role for Gad in the TolC requirement for extreme-acid survival.

Extreme-acid survival in a multiple-MDR efflux pump mutant

TolC acts as the outer-membrane conduit for export by several inner-membrane efflux pump complexes [5]. We investigated whether loss of several TolC-dependent pump complexes would affect acid resistance in a manner comparable to loss of TolC. Extreme-acid survival was tested for strain M6293, which is defective for the inner-membrane pumps of eight known TolC-dependent MDR efflux complexes (AcrAB, AcrAD, AcrEF, EmrAB, EmrKY, MacAB, MdtABC, MdtEF). Acid survival of the multi-pump defective strain was compared to its parent strain N7829, and to strains deleted for tolC grown to stationary phase at pH 5.5 (Fig. 3). The parent strain N7829 survived the acid challenge as well as other wild-type E. coli K-12 strains. M6293, the strain lacking the TolC-associated efflux pumps, including EmrB and MdtC, showed a 6-fold decrease in survival versus the parent strain N7829. This result is comparable to the survival percentages seen with strains lacking MdtB (6-fold) or EmrB (2-fold) in Fig. 1B. When tolC was also disrupted in these two strains, survival was decreased to below 1%. A strain with defects in both the EmrAB and MdtABC complexes (W3110 emrB::frt mdtB::kan) showed 4- to 10-fold decrease in survival (data not shown).

pH-dependent growth of tolC defective strains

While numerous genes are known to affect survival at pH 2, relatively few affect exponential growth at moderately low pH. The best studied case is the triple potassium transport deletion which results in K+-dependent growth at low pH [22]. To determine the role of TolC in acidic growth, we assessed the ability of a strain defective for tolC to grow in LBK with an external pH range of 4.5–9.0 (Fig. 4A). At pH 4.5, the growth rate of tolC::spc and tolC::frt emrB was near zero. Over the range of pH 4.5–6.0, the tolC strain grew at a slower rate than the parent. Over the range of external pH 6.5–9.0, there was no significant difference in growth; thus, the effect of the deletion of tolC on growth rate was limited to the range of acid stress. Wild-type growth at pH 4.5 was restored by complementation of the tolC strain with the tolC-carrying plasmid pMX (Fig. 4B). The tolC defect had no effect on cytoplasmic pH when cultures were suspended at pH 4.5 to pH 6.0 (data not shown), using GFPmut3b fluorimetry as described previously [22,23].

The pH-dependent growth of the tolC strain could be caused by a defect in the channel connection to one or more of its associated inner-membrane efflux complexes [24]. Growth at pH 5 was tested for a mutant deleted for the known TolC-dependent efflux porters (N7829 acrB::frt acrD::frt emrB::frt emrY::frt macAB::frt mdtC::frt mdtF::frt acrEF::spc). No difference was seen between the growth of mutant and parent (data not shown).

Given that a tolC strain does not appear to express the glutamate-dependent acid resistance system, we assessed the contribution of the Gad system to the pH-sensitive growth of a tolC mutant. The antiporter GadC imports glutamate in exchange for the decarboxylation product [16]. When a gadC tolC double
mutant (JLS1048) was grown in early log-phase at pH 5, growth rates similar to the toIC strain were observed, while the wild-type W3110 and a strain lacking gadC had comparable growth rates that were higher than the toIC strains (data not shown). No growth defect was seen in a gadC strain at pH 5.0; this finding is consistent with previous studies showing that the Gad regulon is needed only for extreme acid survival, not for growth in moderate acid [16]. Furthermore, growth rates in toIC cultures grown at pH 5 were unaffected by expression of GadE or GadB-C produced by plasmids pQEgadE and pMF565, respectively (data not shown), indicating that even over-expression of Gad system components on a low-copy plasmid could not restore wild-type growth rates. Thus, the poor growth at moderately low pH in toIC strains must involve a mechanism other than TolC-mediated induction of the glutamate-dependent acid resistance system.

**Discussion**

TolC is a component of several MDR efflux complexes that enable *E. coli* to expel both toxins and metabolic wastes across the periplasm and outer membrane, driven by H+ antiport [5]. Expression of TolC is regulated by MarA, SoxS and Rob [20] and by the EvgA acid resistance regulon [14], which suggests that TolC may function in acid adaptation. Consistent with acid adaptation, TolC-associated drug efflux of toxins is more active at low pH [25]. Our finding that TolC contributes to acid resistance is the first report of an MDR pump component that enhances acid adaptation. For comparison, at high pH over-expression of MDR pump MdtA confers alkali-tolerance and extends the upper pH range for growth [19]. Our results suggest a general possibility that when antibiotics select for gain of MDR pumps by enteric bacteria, the bacteria may show increased resistance to stomach acid.

Additionally, two TolC-associated inner-membrane MDR efflux pump components, EmrB and MdtB, contributed to extreme-acid survival. MdtABC comprises RND-family efflux pumps and a membrane fusion protein which, along with toIC, is under the regulation of the BacR regulon [26–28]. The BacSR two-component regulatory system is an envelope stress signaling pathway that responds to extracytoplasmic stress, which may include acidic pH [27,29]. EmrB is a major facilitator superfamily MDR efflux pump that is induced by permeant weak acids, such as salicylate [30]. Other TolC-associated inner membrane pumps that were tested for extreme-acid survival, such as AcrB, EmrY, and MdtF, showed comparable survival to that of the wild-type.

Of all MDR efflux pump components tested, TolC contributes the most to extreme-acid survival at pH 2, even in a background strain that lacks eight TolC-dependent inner membrane pumps (Fig. 3). While the multiple MDR efflux pump mutant pre-grown at pH 5.5 showed 6-fold decreased survival at pH 2 compared to its wild-type, a toIC deletion in either the parent strain or the MDR mutant showed a much larger decrease (45- and 12-fold, respectively; Fig. 3). Thus, either TolC itself plays a major role in extreme-acid survival independent of its associated inner-membrane pumps, or else an unidentified TolC-dependent pump is involved.

The mechanism of the TolC effect in acid survival was shown to include regulation of the Gad system. The decarboxylation of glutamate by GadA and GadB is one of the main pH homeostasis mechanisms active at low external pH and in stationary phase; cells lacking this system are unable to maintain cytoplasmic pH and perform poorly when challenged in acidic media [16]. In a strain lacking TolC grown at pH 5.5, we identified almost no glutamate decarboxylase activity (Fig. 2A), observed no gadA mRNA among total RNA isolated (Fig. 2B), and detected no GadA and GadB protein expression (Fig. 2C). Thus, TolC is required for induction of the glutamate-dependent acid-resistance system. The restoration of pH 2 survival by GadB-C or by GadE provided on a plasmid confirms that the TolC requirement involved Gad regulation. This may explain why the TolC requirement for acid survival was greatest for cells grown at pH 7 (Fig. 1A) where the acid-induced AR1 system that involves CRP is unavailable, and thus Gad offers the main mechanism of acid resistance [16].

On the other hand, the requirement for TolC for exponential growth in moderate acid (pH 4.5–6.0) was shown to be independent of Gad. Deletion of gadC significantly reduces extreme-acid survival [pH 2.5] [31,32], verifying that a gadC deletion inactivates Gad activity. Nevertheless, a gadC deletion strain did not exhibit a growth defect during exponential growth in...
ToLC Requirement for Acid Adaptation in E. coli

moderate acid and expression of the positive regulator GadE or GadBC from plasmids did not restore wild-type growth rates in a tolC mutant strain (LBK, pH 5.0; data not shown). Thus, while the role of ToLC in Gad regulation may be the reason ToLC is required for extreme-acid survival, the decreased growth rates of the tolC strain only in acidic conditions are not the result of a lack of Gad activity. ToLC was needed for maximal growth below pH 6.5, where cytoplasmic pH is less than optimal (pH 7.4–7.8) [Fig. 4].

The growth defect and the acid resistance defect of the tolC strain were both complemented with a complete tolC gene on a low-copy plasmid. The complementing plasmid pMX does not carry the ygiABC genes downstream from tolC that may be in the tolC operon [33]. Thus, the low pH growth effect is not due to YgiABC activity. Complementation confirms that tolC, and not adjacent genes, contributes to acid resistance.

Our findings suggest a novel physiological role for ToLC in pH homeostasis in acidic conditions. Previous reports demonstrate no growth defects in LB medium, but find impaired cell division and growth in minimal glucose medium [33]. The requirement of ToLC for growth at low pH is surprising because ToLC resides in the outer membrane, mediating exchange of the external medium with the periplasm; and the periplasmic pH generally equals the external pH [23]. Thus, it is hard to see why cytoplasmic pH homeostasis would require an outer-membrane channel. A possibility is that products excreted during metabolism at low external pH accumulate in the periplasm, if they cannot be removed without the ToLC channel.

The mechanism of TolC may or may not involve its interactions with the inner membrane efflux pumps [24]. The fact that deletion of eight major inner-membrane efflux pumps has no effect on growth and a relatively modest effect on extreme-acid survival of pH 5.5-grown cultures (Fig. 3) suggests that the significant reduction of extreme-acid survival in tolC deletion strains is independent of the channel’s association with these pumps. The proton motive force from the periplasm to the cytoplasm drives the functioning of many multidrug efflux transporters [34]. In addition to functioning as an outer membrane pore for many MDR pumps, ToLC may also play a physiological role in pH homeostasis through an interaction with the proton motive force that drives efflux. The original function of ToLC may have been to provide a pH homeostasis mechanism in acidic conditions that later was co-opted to function as a common outer membrane porin in multidrug resistance.

As we completed our manuscript, we became aware of an unpublished plate screen showing that E. coli colony growth at low pH requires several envelope and inner membrane components besides TolC, such as TolB and TolR; the report has since been published [35]. We have since confirmed with quantitative growth curves and survival assays the low-pH specific growth requirements for TolB and TolR (G. Garduque and J. Slonczewski, unpublished). It will be of interest to determine how all these envelope components relate to pH homeostasis.

Materials and Methods

Bacterial strains, media, and growth conditions

The E. coli K-12 strains used here are described in Table 1. W3110 [36] was used as the wild-type strain unless indicated otherwise. Deletion strain M6293 (N7829 aaeB:fta acdE:fts emrY::fts enter::fts mcbB:fts mcdC:fts mcd::fts acdE::sp) was compared to parental strain N7829 (GC4468). Deletion alleles containing a kanamycin resistance insertion (Km) were transduced from the Keio collection [37], obtained from the Coli Genetic Stock Center (Yale University), into the wild-type strain by P1 phage transduction. “fts” is the designation for the “scar” sequence remaining at the site of the cured Keio km insertion. Deletion strains were maintained with 50 µg/ml kanamycin. Plasmid pMX carrying the wild-type tolC gene on a low-copy-number vector pMW119 (derived from pSC101) was transformed into JLS1015 (W3110 tolC::kan) for complementation experiments [4]. Strains containing plasmid pMX were maintained with 50 µg/ml ampicillin in overnight cultures and 20 µg/ml ampicillin in growth cultures.

Bacteria were cultured in LBK medium (10 g/l tryptone, 5 g/l yeast extract, and 7.45 g/l KCl) supplemented with pH buffers as needed [38]. Overnight cultures of deletion strains were maintained with kanamycin (50 µg/ml). Media were buffered with 100 mM Homopiperazine-N, N’-bis-(2-ethanesulfonic acid) (HOMOPIPES; pKa = 4.55), 2-(N-morpholino) ethanesulfonic acid (MES; pKa = 5.96), 1,4-Piperazinethanesulfonic acid (PIPERES; pKa = 6.66), 3-(N-morpholino)propanesulfonic acid (MOPS; pKa = 7.01), N-Tris(hydroxymethyl)methyl-3-amino-2-hydroxypropansulfonic acid (TAPS; pKa = 8.11), 3-(1,1-Dimethyl-2-hydroxyethyl)amino-2-hydroxypropansulfonic acid (AMPSO; pKa = 9.10), or 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS; pKa = 10.08). At the end of the experiments, the pH of the cultures was checked to ensure that it was within 0.2 pH units of the original uninoculated medium.

Acid and base resistance assays

The conditions for testing acid resistance (survival in extreme acid) of aerated cultures were based on those previously described, with modifications [39,40]. Cells were cultured with rotary aeration overnight (16–18 hr at 37°C) to stationary phase in LBK pH 5.5 (100 mM MES) or LBK pH 7 (100 mM MOPS). Overnight cultures were diluted 200-fold into LBK pH 2 and incubated with rotation at 37°C. Following a 2 hr exposure, cultures were serially diluted and plated on LBK-agar. Overnight cultures were also diluted 200-fold into LBK 100 mM MOPS, pH 7 and immediately serially diluted and plated onto LBK-agar. Plates were incubated overnight at 30°C.

Percent survival was calculated as follows: since acid survival represents an exponential death curve, colony counts of surviving cells and control plates were log_{10}-transformed to provide a normal distribution of the data. The mean of the unexposed controls was then subtracted from the mean of exposed pH 2 colony counts, resulting in a log_{10} ratio that correlates to percent survival. All errors stated are the standard error of the mean (SEM). Each experimental condition consisted of six biological replicates from the same overnight culture. Each entire experiment was conducted at least twice.

For base resistance (survival in extreme base), bacteria were cultured with aeration in LBK pH 8.0 (100 mM TAPS) and diluted into LBK pH 10 (100 mM CAPS). Survival was measured and calculated as for acid resistance.

Glutamic acid decarboxylase assays

E. coli K-12 derivative strains MG1655 and MG1655T (tolC::Tn10), transduced by P1-phage from JA300T [41], were used in the assessment of glutamic acid decarboxylase activity. Glutamic acid decarboxylase activity was assessed using the GAD reagent for the GAD reagent. The conditions for testing glutamic acid decarboxylase activity were as follows: the reagent was added to the assay buffer, which comprised of LBK pH 7.5 and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pKa 6.1), 50 mM sodium phosphate (pKa 6.8), and 50 mM Tris (pKa 7.6). The reaction was incubated at 37°C for 2 hr. The reaction was stopped by the addition of 2% TCA, and 200 µl of the reaction mixture was added to a 96-well plate. Glutamic acid decarboxylase assays were performed in triplicate.

Plates were incubated overnight at 30°C.

Glutamic acid decarboxylase activity was assessed using the GAD reagent for the GAD reagent. The conditions for testing glutamic acid decarboxylase activity were as follows: the reagent was added to the assay buffer, which comprised of LBK pH 7.5 and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pKa 6.1), 50 mM sodium phosphate (pKa 6.8), and 50 mM Tris (pKa 7.6). The reaction was incubated at 37°C for 2 hr. The reaction was stopped by the addition of 2% TCA, and 200 µl of the reaction mixture was added to a 96-well plate. Glutamic acid decarboxylase assays were performed in triplicate.
The presence of gadA and cadA mRNA in both MG1655 and MG1655T at pH 7.5 and pH 5.5 was assessed using Northern analysis. Total cellular RNA was isolated using the RNeasy kit (Qiagen) and separated by formaldehyde-agarose gel electrophoresis (10% acrylamide) and stained with Coomassie Brilliant Blue. The 53-kDa protein band was cut out and analyzed by mass spectrometry (MALDI-TOF/TOF ultrafleXtreme, Bruker Daltonics).

Glutamate-dependent extreme-acid resistance was tested with overnight cultures grown in LB buffered with 100 mM MES and pH 7.5. Cells were harvested, suspended in a 50 mM sodium phosphate buffer (pH 7.0), and disrupted by sonication. After unbroken cells were removed, lysate proteins were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and stained with Coomassie Brilliant Blue. The 53-kDa protein band was cut out and analyzed by mass spectrometry (MALDI-TOF/TOF ultrafleXtreme, Bruker Daltonics).

Acid growth assays
To test acid growth, cells were cultured with aeration to stationary phase (16–18 hr, 37°C) in unbuffered LBK. Overnight cultures were diluted 100-fold into LBK pH 4.5–9.0 (in half unit increments) including 100 mM of the pH-appropriate buffer, and rotated at 37°C until cultures reached stationary phase. OD₆₀₀ was measured at regular intervals after the initial dilution. Growth rates were calculated as doublings per hour over the region of exponential growth (approximately OD₆₀₀ = 0.1 to 0.3). The wild-type strain W3110 and its tolC derivative (JLS1015) were also tested for loss of cytoplasmic pH homeostasis at low pH as described previously [22,23].

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Author Contributions
Conceived and designed the experiments: KNWD AH RDK RT JLR MW JLS. Performed the experiments: KNWD AH RDK RT. Analyzed the data: KNWD AH RDK. Contributed reagents/materials/analysis tools: JLS. Performed the experiments: KNWD AH RDK RT. Analyzed the data: KNWD AH RDK. Wrote the paper: JLS. Edited the manuscript: JLR MW JLS.

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