CONSTRUCTION AND CHARACTERIZATION OF *yciGFE* MUTANTS IN *Escherichia coli*

A Thesis

by

GAYATHRI KALYANARAMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Biology

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ABSTRACT

Construction and Characterization of *yciGFE* Mutants

in *Escherichia coli.* (December 2003)

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Chair of Advisory Committee: Dr. Deborah A. Siegele

Escherichia coli has served as a model organism for studies in molecular genetics and physiology since the 1960s. Yet the function of 20% of the 4288 known and predicted proteins in *E. coli* is still not known. Almost two-thirds of these proteins have homologs in other microorganisms, but their function(s) is not known in any organism. One such protein is YciG. YciG was chosen as the focus of this study because, intriguingly, an ortholog of YciG is found in the genome of the fungus *Neurospora crassa.* The gene encoding YciG is predicted to be in an operon with two other genes, *yciF* and *yciE*. Genes in the same operon often encode proteins with related functions, so the study was extended to include YciF and YciE. To determine the function of these proteins, in-frame deletion alleles were constructed and strains lacking one or more of the three proteins were tested for mutant phenotypes. Expression of the *yciGFE* operon is induced by several stresses and is regulated by RpoS, which controls the general stress response in *E. coli*. Therefore, we tested the ability of the mutant strains to survive environmental stresses. Our results revealed that YciG was important for stationary-phase resistance to thermal stress, oxidative stress and, in particular, acid

stress. Both RpoS-dependent and RpoS-independent acid resistance mechanisms are found in *E. coli*. YciG was shown to be required for RpoS-independent acid resistance, but further experiments are needed to determine whether YciG also is required for RpoS-dependent acid resistance. YciG was not required for normal exponential growth of *E. coli*, as mutants lacking YciG had the same growth rate as the wild-type parent. No mutant phenotypes have been found yet for mutants lacking YciF or YciE. *yciE* deletion mutants showed the same growth rate and the same level of acid resistance as wild-type cells. The acid resistance of *yciF* mutants has not yet been tested, and strains lacking YciE and/or YciF need to be assayed for their ability to survive stresses other than acid stress.

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CHAPTER I

INTRODUCTION

The advent of the genomic era has resulted in the availability of the complete genomic sequences of many organisms. This has facilitated the identification and prediction of genes and their corresponding functions in an organism, which is prerequisite for complete understanding of the physiology of an organism.

Escherichia coli, an enteric, gram-negative bacterium, has served as a model organism for studies in microbial genetics and physiology for many decades. In 1997, it became the third free-living organism to have its genome completely sequenced (7). The *E. coli* K-12 genome is composed of 4,639,221 bp of circular, duplex DNA. Annotation of the genome identified 4288 actual and predicted protein coding genes. 62% of these proteins were assigned to functional classes based on their known functions or predicted functions based on homology. However, 38% (1632) were classified as “hypothetical, unclassified, unknown” (7).

More recent bioinformatics analysis has assigned all but 851 proteins to functional classes (2). However, it is important to recognize that the specific role(s) of many of these proteins still remains to be determined. Of these 851 proteins, 61 have a functional annotation derived from tentative homologs, 511 have homologs in other organisms but their function(s) are not known in any of those organisms, and the remaining 279 do not have any probable homologs in any genome available for

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This thesis follows the style of the Journal of Bacteriology.

comparison. Complete understanding of the physiology of *E. coli* requires identifying the role(s) and function(s) of these proteins in *E. coli.* This involves a systematic and thorough characterization of each of these protein-coding sequences.

YciG is one of the *E. coli* proteins of unknown function. We chose to study YciG because, intriguingly, an ortholog of YciG, CON-10, is present in the genome of the fungus *Neurospora crassa* (D. Ebbole, personal communication, G. O'Toole, personal communication). Expression of CON-10 is induced during conidiation and by conditions of stress (23). In *E. coli*, the gene encoding YciG is predicted to be in an operon with two other genes, *yciF,* and *yciE* (32). Since genes in the same operon often, but not always, code for products with related functions, we extended our study to include the YciF and YciE proteins.

yciGFE **ORFs in** ***E. coli***

yciG, yciF, and *yciE* are three adjacent genes located in the *trpA-tonB* intergenic region (at 28.3 minutes in the genetic linkage map) (32). The *yciG, yciF*, and *yciE* ORFs are 236, 500, and 506 bp in length respectively (7). The predicted YciG, YciF and YciE proteins are composed of 78, 166, and 168 amino acids, respectively. The structure of the *yciGFE* chromosomal region is shown in Fig 1.

A BLASTP search (1) with each of the three predicted proteins against the nonredundant GenBank database reveals that they are conserved in several other gram-negative bacteria. Orthologs of all 3 proteins were identified in other members of the γ-

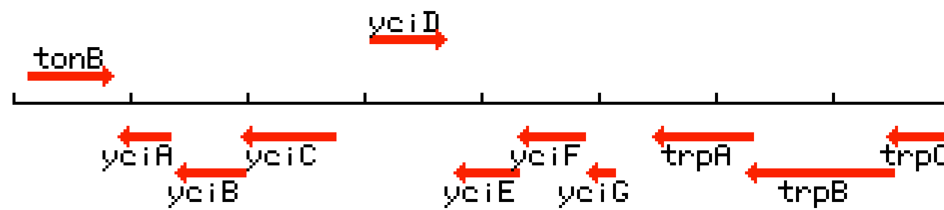


FIG. 1 . Structure of the *yciGFE* chromosomal region. Modified from genomic maps on Colibri, (http://genolist.pasteur.fr/Colibri/). An 8000 bp region of the *E. coli* chromosome is shown. Tick marks are present every 800 bp. The arrows indicate gene orientations.

TABLE 1. Orthologs of YciG, YciF, and YciE

Organism	E value for YciG ortholog	E value for YciF ortholog	E value for YciE ortholog
E. coli O157:H7 [a]	$7e^{-10}$	$4e^{-52}$	$5e^{-84}$
S. enterica serovar Typhi [a]	$1e^{-06}$	$2e^{-47}$	$8e^{-74}$
S. serovar Typhimurium [a]	$2e^{-06}$	$2e^{-47}$	$8e^{-74}$
CP-933X (prophage of *E. coli* O157:H7)	$2e^{-06}$	$6e^{-45}$	$9e^{-77}$
Mesorhizobium loti [b]	-	$9e^{-16}$	$5e^{-04}$
Sinorhizobium meliloti [b]	-	$2e^{-15}$	$1e^{-36}$
Agrobacterium tumefaciens [b]	-	$9e^{-18}$	0.032
Nostoc sp. PCC7120 [c]	-	$6e^{-05}$	$5e^{-05}$
Pseudomonas aeruginosa PA01[a]	$3e^{-04}$	-	$1e^{-38}$
Neurospora crassa [d]	$2e^{-06}$	-	-
Xanthomonas axonopodis [a]	-	$9e^{-33}$	-
Xanthomonas campestris [a]	-	$1e^{-32}$	-

[a] These bacteria belong to the class γ - proteobacteria.

[b] These bacteria belong to the class α- proteobacteria.

[c] This is a cyanobacterium.

[d] This is a fungus that belongs to the class Hymenoascomycetes.

proteobacteria. The *yciG*, *yciF*, and *yciE* ORFs are present contiguously in these organisms. In addition, the BLAST search also reveals the presence of homologs of one or two of the three predicted proteins in other bacteria and a fungus (see Table 1).

Regulation of *yciGFE* expression

Although these proteins are conserved in many organisms, their function(s) is not known in any of these organisms. Some clues to the possible role(s) of these proteins in the cell can be obtained from the spatial and temporal pattern of their expression and regulation.

yciG, *yciF,* and *yciE* have been shown to be cotranscribed in *Salmonella enterica* serovar Typhimurium (27) and most likely constitute an operon in *E. coli* also (32). In both these bacteria, a number of stresses are known to induce the expression of some or all of these three genes.

In *S*. serovar Typhimurium, the expression of *yciG*, *yciF*, and *yciE* is induced in stationary phase (19, 27). In *E. coli*, the *yciG* promoter is induced by treatment with sulfometuron methyl (35), which inhibits acetolactate synthase, the first common enzyme in the biosynthesis of isoleucine and valine. Transcription from the *yciG* promoter is also induced by treatment with the weak acid salicylate (35) and by increased salinity (29). In *S.* serovar Typimurium, YciE is an acid inducible protein (ASP) (5).

In *S.* Typimurium, expression of all three genes has been shown to be regulated by the stress-induced sigma factor RpoS (19, 27). In *E. coli*, the expression of *yciG* has

also been found to be regulated by RpoS (35). The expression of the YciF and YciE proteins is enhanced in an *hns* deletion mutant (39). H-NS is a major component of the *E. coli* nucleoid and lowers the expression of many RpoS-dependent genes (4). Another global transcription regulator Lrp (leucine response protein) also regulates the expression of *yciG* and *yciF* during stationary phase in *E. coli* (33).

Role of RpoS in the stress response of *E. coli*

The natural environments of bacteria are subject to constant fluctuations and bacteria have a remarkable capacity to sense and adapt to changing environments. They can survive prolonged periods of starvation and many other stress conditions such as osmotic stress, oxidative stress, thermal stress and pH stress.

When starved for nutrients, *E. coli* enters a non-differentiated state called the stationary phase. Entry into stationary phase is characterized by numerous changes in physiology and the pattern of gene expression (reviewed in 18). The growth rate slows down and eventually stops, the cells become smaller and more spherical and the starving cells accumulate reserve polymers like glycogen and polyphosphate, protective substances like trehalose, stimulate turnover of stable RNA and protein, and dimerize 70S ribosomes. An important change that occurs in response to starvation is the increased resistance of starving *E. coli* cells to various environmental stresses. The bacterial membranes become more resistant to freeze-thawing and to autolysis induced by penicillin and other agents. Starvation induces cross protection to various

environmental stresses such as heat shock, oxidative stress, osmotic challenge, alkylating agents, ethanol, acetone, toluene, and acidic or basic pH (18, 20, 21).

The alternate sigma factor, σ^s, is the gene product of *rpoS* and plays a key role in the survival of *E. coli* under stress conditions. RpoS regulates the expression of 40 to 50 genes in *E. coli* and is induced during the onset of stationary phase and when cells are exposed to certain stress conditions during exponential growth (24). These include *katE* and *katG*, which encode catalses that protect the cells against H_2O_2; *dps*, which encodes a non-specific DNA-binding protein that protects DNA from damage; *otsBA*, which encode proteins involved in the synthesis of trehalose; and *xthA*, which encodes the DNA repair enzyme Exonuclease III. During starvation, *rpoS* mutants are larger than wild-type cells, retain a more distinct rod-shape and fail to develop thermotolerance, osmoresistance, or resistance to oxidative stress. The intracellular concentration of σ^s is controlled at the levels of translation and protein stability (22). Different stress conditions affect different control mechanisms to generate a complex regulatory profile. A number of environmental signals regulate RpoS levels, including growth rate (22), cell density (22), CAP-cAMP (22), ppGpp (16), and UDP-glucose (9).

Determining the function of the YciG, YciF, and YciE proteins will lead to a better understanding of the mechanisms that allow *E. coli* to survive stress conditions.

CHAPTER II

MATERIALS AND METHODS

Strains and plasmids

The bacterial strains used in this study are listed in Table 2. The plasmids used in this study are listed in Table 3.

Growth media

Difco LB broth, Miller (Becton, Dickinson, & Co. Sparks, MD) was used for rich media. For solid support medium, 2% Difco Granulated Agar (Becton, Dickinson, & Co. Sparks, MD) was included. MacConkey agar base (Becton, Dickinson, & Co. Sparks, MD) with 1% L-rhamnose (Sigma Chemical Co. St.Louis, MO) was used to check for the use of L-rhamnose as a carbon source. Minimal medium was M63 salts (25) supplemented with 0.1% glucose, 0.02% L-rhamnose (when present), 1 mM $MgSO_4$, 0.1% vitamin-free casamino acids (Difco), 10 μg/ml uridine and 2 μg/ml thiamine. This medium will be referred to as MGC medium. For plates, 1.5% Difco Granulated Agar and 10 mM KNO_3 were included. Tetracycline-sensitive cells were selected on tetracycline-sensitive-selective (TSS) agar (8). When present, antibiotics were added at the following concentrations: ampicillin, 150 μg/ml; kanamycin, 50 μg/ml; tetracycline 12.5 μg/ml. Antibiotics were purchased from Sigma Chemical Co.

Molecular biology methods

All PCR amplifications were carried out with the high fidelity thermostable

TABLE 2. *E. coli* K-12 strains used in this study

Strain	Genotype	Source/Reference
DS631	MG1655 λ^- F^- *rfb-50 rph-1*	Carol Gross
DS649	DS631 pCP20	This study
DS 724	W3110 λ^- F^- Δ(*tonB-trpB*) *his*$^-$ IN(*rrnD-rrnE*)1 *rph-1*	CGSC[a] #7691 (38)
DS727	*galK*2(Oc) λ^- Δ(*tonB-cysB*)*206 his-68 xylA*7 *mtlA*2 *gyrA*223 (Nal^r) *tyrA*2 *rpsL125* (Str^r) *malT1* (λ^r) *thi-1*	CGSC[a] #6368 (14)
DY329	W3110 Δ*lacU169 nadA*::Tn*10 gal490* λ*cI857* Δ(*cro-bioA*)	(40)
GK20	DS631 Δ*yciG*::FRT	This study
GK119	DS631 Δ*rpoS*::Kan	This study
GK238	DS631 Δ*rpoS*::Kan Δ*yciG*::FRT	This study
GK263	DY329 *nadA*$^+$ Tet^s	This study
GK287	GK263 P_{rhaB}–*yciGFE*	This study
GK292	DS631 P_{rhaB}-*yciGFE*	This study
GK299	DS631 P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan	This study
GK303	DS631 P_{rhaB}-*yciGFE* Δ*yciE*::Kan	This study
GK311	DS631 P_{rhaB}-*yciGFE* Δ*yciF*::Kan	This study

[a]Coli Genetics Stock Center

TABLE 3. Plasmids used in this study

Plasmid	Description	Construction/ Source
pCP20	*FLP*$^{+}$ λ*cI857* Rep(Ts) Ap^{r} Cm^{r}	Barry Wanner (11)
pDZ1	A 1.9kb fragment containing the *yciGFE* operon was cloned into pRLG770	This study
pGK1	P_{rhaB}- *yciGFE rhaD' tetAR bla* $oriR_{R6k_{\gamma}}$	Subcloned the 1.3kb *Bam*H1- -*Sph*I *yciGFE* ORF from pDZ1 into pLD78
pLD78	P_{rhaB}-*'rhaD tetAR bla oriR*$_{R6k_{\gamma}}$	Barry Wanner (17)
pRATT04	$oriR_{R6k_{\gamma}}$ *npt-2* flanked by FRT sites	Robert Pratt, TAMU
pRLG770	*bla*, pBR322 origin of replication	(28)

DNA polymerase ThermalAce (Invitrogen, Carlsbad, CA). Oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). The Wizard® PCR purification system (Promega, Madison, WI) was used for the purification of PCR products. Restriction enzymes were from New England Biolabs (Beverly, MA). QIAGEN (Hilden, Germany) columns were used for the isolation of plasmid DNA. All sequencing reactions were performed with Sequenase (Applied Biosystems, Inc.) The reactions were analyzed on an automated sequencer (Gene Technologies Lab, Texas A&M University).

Construction of pDZ1

The *yciGFE* operon was amplified from DS631 genomic DNA using primers *yciG*-RI (5'-CGGAATTCCGGTCGAACGTCAACTTACGTCATTTTTCC-3') and *yciE*-H3 (5'-GTGCGAAGCTTTGATAATGGATTTAACGATCATGGCAAAGAGGCA-3'). The amplified *yciGFE* fragment acquired *Eco*RI and *Hin*dIII restriction sites (underlined) at its 5' and 3' ends, respectively. This PCR amplified fragment was digested with *Eco*RI and *Hin*dIII and ligated into the *Eco*RI and *Hin*dIII sites of pRLG770 to give pDZ1. The nucleotide sequence of the PCR amplified fragment in pDZ1 was checked by DNA sequencing.

Making the single copy P_{rhaB}-*yciGFE* construct

A DNA fragment carrying the promoterless *yciGFE* genes was cloned into the allele replacement vector pLD78 (17). This plasmid confers resistance to tetracycline

and has the γ-origin of replication from plasmid R6K, which requires the Pir protein for replication. The *yciGFE* fragment was derived by PCR amplification from plasmid pDZ1 with the oligonucleotide primers 5'-TTTGACTAATTCTAGGATCCACTGATTTTAATGAGTGTCG-3' and 5'-GGGCATGCTATAAAGGGTATTATTTCTTCGCTTCTACGCCATCAGTTTCAG–3'. The underlined bases indicate a *Bam*HI or *Sph*I recognition site. The fragment was cloned as an *Sph*I-*Bam*HI fragment downstream of the P_{rhaB} promoter in pLD78 to give pGK1. The presence of the insert was confirmed by PCR analysis with pairs of primers where one primer was complementary to sequences in the plasmid and the other was complementary to sequences in the *yciGFE* fragment.

Plasmid pGK1 was recombined into the chromosome of *E. coli* strain MG1655 (pir^- Rha^+). Transformants with the integrated plasmid were selected as tetracycline-resistant colonies, which were colony purified on selective medium and screened for a Rha^- phenotype on MacConkey rhamnose plates. The locus of plasmid insertion was confirmed by PCR analysis with primers complementary to either P_{rhaB} or *rhaD* in combination with primers complementary to *yciG, yciF,and yciE*. The location of the insert at the *rhaBAD* locus was also confirmed by determining the co-transduction frequency with linked markers. Two markers, *trpB83*::Tn*10,* which is linked to the *yciGFE* locus, and *zih-3166*::Tn*10*Kan, which is linked to the *rhaBAD* locus, were used for the linkage analysis.

To select for the loss of the plasmid sequences, colonies were purified once non-selectively to allow accumulation of tetracycline sensitive (Tet^S) segregants within the colonies and were then plated on TSS agar. The resulting colonies were purified non-

selectively and then tested for tetracycline sensitivity. The presence of the allele replacement was indicated by a Rha⁻ phenotype on MacConkey rhamnose plates and confirmed by PCR analysis.

Construction of FRT alleles of *yciG*, *yciF*, and *yciE*

The in-frame deletions of *yciG*, *yciF*, and *yciE* were constructed in two steps. The deletion/replacement alleles were made using the λ *red* recombination system (40) to replace all but the first six and last seven bases of each ORF with the selectable marker *npt2* (neomycin phosphotransferase), which confers resistance to the antibiotic kanamycin. The *npt2* gene was flanked by FRT sites, which are recognition sites for the Flp site-specific recombinase. In the second step, the *npt2* gene was excised by Flp to leave behind an in-frame "FRT scar".

The linear PCR product for generation of the deletion/replacement alleles was obtained by amplifying the *npt2* gene flanked by FRT sites from plasmid pRATT04. The oligonucleotide primers used contained sequences complementary to the region of the plasmid to be amplified. The 5' termini of these primers also included 50 nt extensions, complementary to sequences flanking the ORF to be deleted. The primers used are shown in Table 4.

The PCR product was introduced into strain DY329, which carries a defective prophage containing the λ *exo*, *bet*, and *gam* genes under the control of the temperature-sensitive *cI857* allele of λ repressor. The expression of these lambda recombination proteins was induced at 42 C and the strain was electro-transformed with the PCR

TABLE 4. Primers for construction of the deletion/replacement alleles

Name	Description	Sequence[a]
YciG Pr1	*yciG* 5' primer	5'cttaatttgactaattctcattagcgactaattttaatgagtgtcgacagctggagc tgcttcgaag 3'
YciG Pr2	*yciG* 3' primer	5'caattatctttatcagctgatattaataattaacggtgaatcaggatttgatatcctc cttagttcct 3'
YciF Pr1	*yciF* 5' primer	5'gtataagtataaaaagaggtcactttagtgacgctcggtatgccgggcgtctgc tggagctgcttcgaag 3'
YciF Pr2	*yciF* 3' primer	5'aatgtcctctttataaaatgactaaaagttaaaattcatatttcaggctttaatatcct ccttagttcct 3'
YciE Pr1	*yciE* 5' primer	5'tatgaattttaacttttagtcattttataaagaggacattttcatgaatcgtgctgga gctgcttcgaag 3'
YciE Pr2	*yciE* 3' primer	5'cccgtaatatacggggtcaataaggacatggtataaagggtattatttcttcatat cctccttagttcct 3'

[a]Underlined bases are complementary to sequences of the kanamycin resistance cassette in pRATT04. The rest of the primer is complementary to sequences in the *yciGFE* locus.

product. Homologous recombination between the PCR product and the desired locus in the chromosome results in the replacement of the ORF in the chromosome with the *npt2* gene with flanking FRT sites. Recombinants were selected as kanamycin resistant transformants. Transduction by P1vir was used to introduce the deletion/replacement mutations into strain DS649, which contains a plasmid with the gene for Flp under the control of the λ*cI857* repressor. Induction of Flp expression at 42 C results in the loss of the kanamycin resistance gene and leaves an in-frame FRT scar of 30 codons in place of the ORF. The pCP20 plasmid is also cured by growth at 42 C and the cells become Amp^s.

At every step, the presence or absence of the kanamycin resistance cassette was confirmed by the ability /inability of cells to grow on medium containing kanamycin. The presence of the kanamycin resistance gene was confirmed by the presence of a 1.6 kb fragment obtained by PCR amplification of genomic DNA with primers complementary to the *npt2* gene. The insertion of the *npt2* gene at the *yciGFE* locus was confirmed by PCR analysis with primers complementary to sequences flanking the replaced ORF and primers complementary to the *npt2* gene (see Fig 2).

Measurement of growth rates

For measurement of growth rates, cells from a fresh overnight culture or a freshly streaked colony resuspended in 1ml of M63 salts were diluted to OD_{600} = 0.01 into 10 ml of the same medium in a 125ml Erlenmeyer flask and grown at 37 C in a New Brunswick shaking water bath with vigorous aeration (250 rpm). At various times

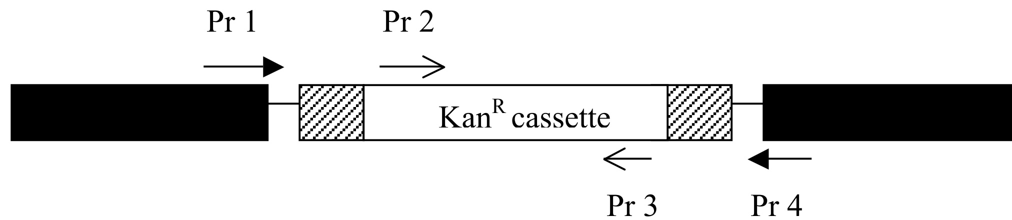


FIG. 2. Positions of primers used for the PCR analysis of the deletion/ replacement mutants. The figure (not drawn to scale) shows the relative positions of the primers used to confirm the presence and position of the deletion/replacement insert. The hatched boxes indicate the sequences remaining from the deleted gene. The filled boxes indicate sequences flanking the deleted ORF. Pr2 and Pr3 are primers complementary to sequences in the *npt2* gene. Pr1 and Pr4 are complementary to sequences upstream and downstream of the replaced ORF. PCR analyses with Pr1 X Pr3 and with Pr2 X Pr4 were used to confirm the presence of the kanamycin resistance gene at the correct locus.

after inoculation, samples were withdrawn from the culture and optical densities at 600 nm were measured with a Beckman DU-65 spectrophotometer. In some experiments, growth rate was monitored by plating appropriate dilutions of the withdrawn culture sample and calculating the number of colony forming units (CFU) per ml of the culture. Measurement of growth rates of the promoter fusion strains carrying the various deletion/replacement mutations were performed by inoculating cells grown on MGC plates containing 0.02% L-rhamnose into 10ml MGC with or without the inducer. 50 μl samples were withdrawn from the cultures at various time points and appropriate dilutions were plated on MGC containing L-rhamnose.

Stress response experiments

Survival during long-term starvation was assayed by growing cells in LB or MGC medium at 37 C with aeration for 10 days. After overnight growth and then each day for 10 days, samples were withdrawn for determination of viable cell number on Luria-Bertani (LB) plates after appropriate dilution in M63 salts. All experiments were performed at least thrice on independent cultures, except when noted otherwise.

Survival at pH 2.5 was determined as described previously (3). Cells grown overnight in LB or in MGC medium with 0.02% L-rhamnose (when present) at 37 C with aeration were diluted 1:40 into 4 ml of M63 salts or 4 ml of 10 mM citric acid at pH 2.5 and incubated for 1 h at 37 C without aeration. Each sample was then diluted in M63 salts and aliquots were plated on LB plates or MGC plates containing 0.02% L-

rhamnose to determine the number of viable cells. Percent survival was defined as the CFU per ml after acid shock divided by the CFU per ml in the M63 control.

Thermotolerance was determined at 55 C as described previously (3). Cells grown overnight in LB or in MGC medium with 0.02% L-rhamnose (when present) at 37 C with aeration were washed and diluted into 0.9% NaCl to about 5000 cells per ml. The samples were then put in prewarmed glass tubes either at 55 C or at room temperature and 0.1 ml samples were withdrawn at 2-min intervals and plated directly onto LB or MGC plates containing 0.02% L-rhamnose to determine the viable cell numbers. Percent survival was defined as the CFU per ml after incubation at 55 C divided by the CFU per ml in the room temperature control.

Oxidative stress was determined as described previously (3). Cells grown overnight in LB or in MGC medium with 0.02% L-rhamnose (when present) at 37 C with aeration were washed and resuspended in the original volume of 0.9% NaCl. H_2O_2 was added to a final concentration of 15mM and cells were incubated for 1h at 37°C without aeration. Samples were removed before and at 15-min intervals after the addition of H_2O_2, serially diluted in M63 salts and plated onto LB or MGC plates containing 0.02% L-rhamnose to determine the number of viable cells. Percent survival was defined as the CFU per ml after the addition of H_2O_2, divided by the CFU per ml present before the addition of H_2O_2.

Response to increased salinity was determined as described previously (21). Cells grown overnight in LB or in MGC medium with 0.02% L-rhamnose (when present) at 37°C with aeration were washed and resuspended in the same volume of

0.9% NaCl. The cells were then diluted 10-fold into 4 ml of 2.5 M NaCl or M63 salts and incubated for 4 h at 37°C without aeration. Samples were removed at 30 min and at 1 h intervals after the transfer into 2.5M NaCl for 3 h. The samples were serially diluted in M63 salts and plated onto LB or MGC plates containing 0.02% L-rhamnose to determine the number of viable cells. Percent survival was defined as CFU per ml after transfer to 2.5 M NaCl divided by the CFU per ml in M63 control.

CHAPTER III

RESULTS

Construction of the Δ*yciG*::FRT allele

As a first step towards determining the function of YciG in *E. coli*, I constructed an in-frame deletion of *yciG* to screen for any detectable phenotypes displayed by the mutant. The in-frame deletion was obtained in two steps. In the first step, all but the first six and the last seven bases of the *yciG* ORF was replaced with a kanamycin resistance gene (*npt2*) flanked by FRT sites as described in Materials and Methods. In the second step, the FRT sites were recombined by Flp recombinase and the kanamycin resistance gene was excised in the process, leaving a 90 nt in-frame FRT scar in place of the *yciG* ORF. The Δ*yciG*::FRT allele was confirmed by PCR analysis and by DNA sequencing.

The Δ*yciG*::Kan deletion/replacement mutant displayed a slow growth phenotype. The Δ*yciG*::Kan colonies were much smaller (≤ 1mm in diameter after 24 hrs incubation at 37 C in LB) than wild-type colonies (≥ 3mm under similar conditions). The final yield of the mutant in liquid LB culture after 18 hours of growth with aeration at 37 C was 2.6×10^8 CFU/ml, only 10% that of the wild type parent, which was 2.3×10^9 CFU/ml (the numbers are the mean of two independent cultures).

The Δ*yciG*::FRT mutant did not have any detectable growth defect. The colony morphology and growth in liquid culture of the mutant was indistinguishable from that of the wild-type parent.

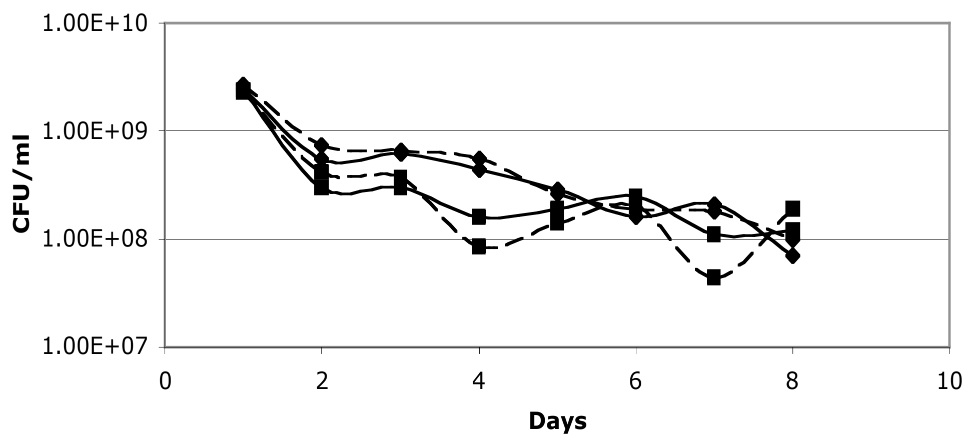
Stress responses of the Δ*yciG*::FRT mutant

In *E. coli*, stresses like cytoplasmic acidification and increased osmolarity are known to induce RpoS dependent expression from the *yciG* promoter (29, 35). To determine the role, if any, of YciG in the stress response of *E. coli*, stationary phase cultures of the Δ*yciG*::FRT mutant and the wild-type parent were subjected to heat shock, acid shock, oxidative stress, osmotic stress, and long term starvation survival as described in Materials and Methods.

Long-term starvation survival of the mutant was assayed in both rich LB and MGC medium as described in Materials and Methods. The results are shown in Fig 3. There was no difference in the long-term viability of the wild-type and the Δ*yciG*::FRT mutant. In LB medium, 3.2% of the wild type and 6.6% of the Δ*yciG*::FRT mutant cells remained viable after 8 days in stationary phase. In MGC medium, greater than 90% of the wild-type and the mutant cells remained viable after 8 days in stationary phase.

The ability of the Δ*yciG*::FRT mutant to survive several other stress conditions was measured and the results are shown in Fig 4. The Δ*yciG*::FRT mutant was significantly defective in surviving at pH 2.5 ($p = 0.01$). There was a 60-fold difference in survival between the mutant and the wild-type after exposure to low pH. 0.25±0.1% of the Δ*yciG*::FRT mutant cells survived the acid shock, while 15±2% of the wild-type cells survived. The Δ*yciG*::FRT mutant was also defective in surviving oxidative stress and thermal stress. A 4-fold difference in the ability to survive oxidative stress was observed.

A



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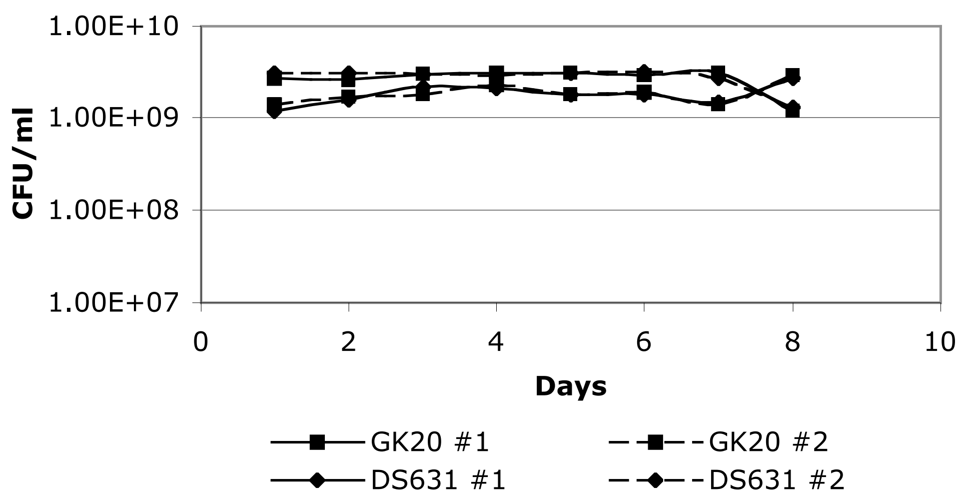

FIG. 3. Long-term starvation survival of the Δ*yciG*::FRT (GK20) mutant. Two independent cultures (#1 and #2) of both the wild-type (DS631) and the Δ*yciG*::FRT mutant (GK20) strains were maintained in LB medium (A) and MGC medium (B) for 8 days at 37 C with aeration and samples were withdrawn every day to determine the CFU per ml present.

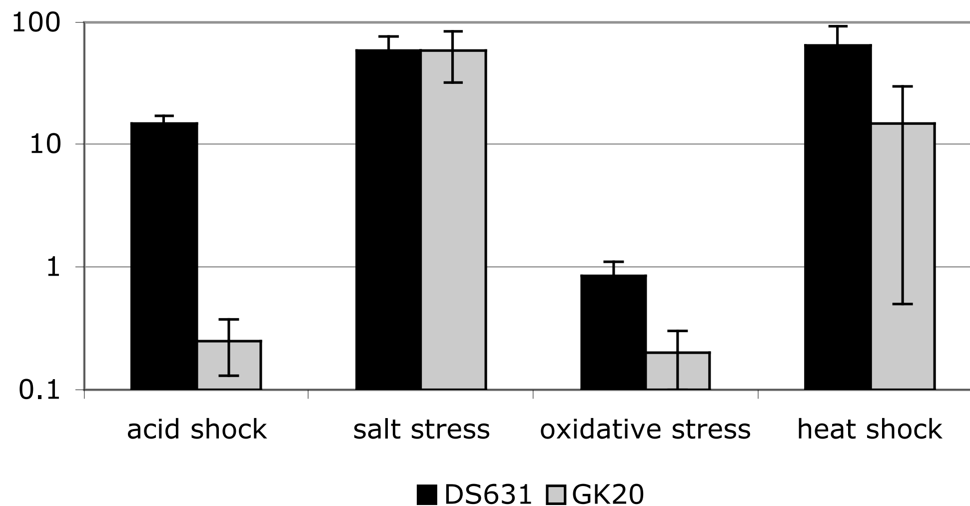

FIG. 4. Survival of the Δ*yciG*::FRT mutant after exposure to different stress conditions. The dark bars represent the wild-type (DS631) and the light boxes represent the Δ*yciG*::FRT mutant (GK20). The cultures were grown overnight in LB at 37°C and percent survival was calculated as described in Materials and Methods. Except for oxidative stress, the values shown are the means of three independent experiments and the error bars indicate the standard deviation. The oxidative stress response experiment was repeated twice and the error bars for this condition indicate the range.

0.2±0.05% of the mutants survived after 60 min in 15 mM H_2O_2, while 0.85±0.25% of wild-type cells survived. A 4-fold difference was also observed in the ability of the mutant and the wild-type cells to survive thermal stress. After 10 min at 55 C, 15±14% of the mutant cells survived, while 64±29% of the wild-type cells survived ($p = 0.08$).

There was no significant difference in the ability of mutant and wild-type cells to survive increased salinity. After 2 h in 2.5 M NaCl, 51±18% of the mutant and 66±20% of the wild-type cells survived ($p = 1.00$).

These results indicate that the *yciG* gene product is important for several of the resistance phenotypes characteristic of stationary phase *E. coli*, in particular acid resistance.

Role of RpoS in the stress responses of the Δ*yciG*::FRT mutant

Transcription from the *yciG* promoter is regulated by RpoS (29, 35). To determine the role of RpoS in the YciG-mediated stress responses, we compared the ability of Δ*yciG*::FRT and Δ*rpoS*::Kan single mutants and an Δ*rpoS*::Kan Δ*yciG*::FRT double mutant to survive different stress conditions. The results are shown in Fig 5.

As expected, the Δ*rpoS*::Kan mutant was defective in surviving all the stresses tested compared to the wild-type strain. The behavior of the Δ*rpoS*::Kan mutant relative either to the Δ*yciG*::FRT mutant or the Δ*rpoS*::Kan Δ*yciG*::FRT double mutant was different under different stress conditions.

At pH 2.5, 4±2%($p = 0.001$) of the Δ*rpoS*::Kan mutant cells survived, while only 0.25±0.1% ($p = 0.01$) of the Δ*yciG*::FRT mutant cells survived. The difference in

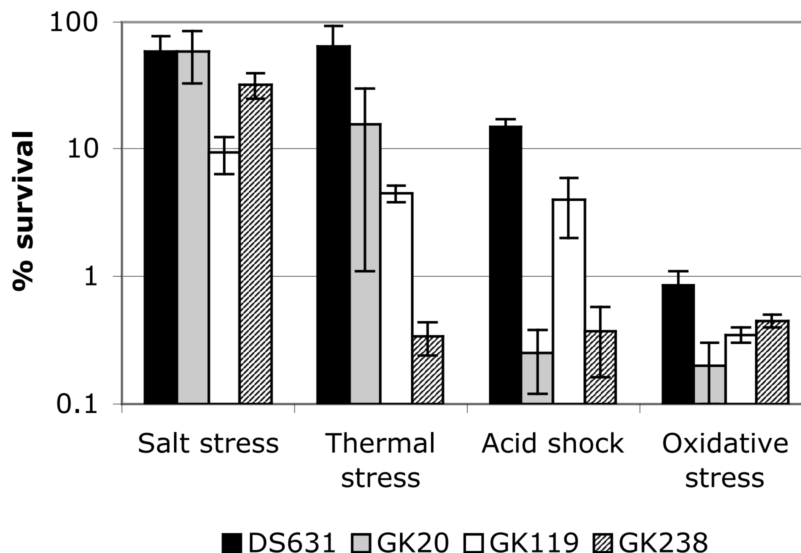

FIG. 5. Ability of the parent strain and mutants Δ*yciG*::FRT, Δ*rpoS*::Kan, and Δ*yciG*::FRT Δ*rpoS*::Kan to survive different stresses. DS631 is the parent, GK20 is the Δ*yciG*::FRT mutant, GK119 is the Δ*rpoS*::Kan mutant and GK238 is the Δ*yciG*::FRT Δ*rpoS*::Kan double mutant. Assays were performed as described in Materials and Methods. Except for oxidative stress, the values shown are the means of three independent experiments and the error bars indicate the standard deviation. The oxidative stress response experiment was repeated twice and the error bars for this condition indicate the range.

the mean % survival of these two strains is significant at $p = 0.08$. For the double mutant, 0.4±0.2% ($p = 0.01$) of the cells survived. This is comparable to the mean % survival of the Δ*yciG*::FRT single mutant ($p = 0.5$), but is significantly less than that of the Δ*rpoS*::Kan single mutant ($p = 0.08$). Because addition of the Δ*yciG*::FRT mutation to a strain already lacking RpoS lowers acid resistance, YciG must be important for RpoS-independent acid resistance. Because the Δ*yciG*::FRT single mutant and the double mutant have the same acid sensitive phenotype, we cannot discern if YciG has a role in an RpoS-dependent acid resistance pathway.

No marked difference was seen in the survival of the single mutants and the double mutant after exposure to oxidative stress. After exposure to H_2O_2 for 1 h, 0.35±0.01% of Δ*rpoS*::Kan cells, 0.2±0.05% of the Δ*yciG*::FRT cells, and 0.45±0.01% of the double mutant cells survived. These results indicate that RpoS and YciG act in the same oxidative resistance pathway. It appears that RpoS mediates the resistance to oxidative stress through YciG.

The Δ*rpoS*::Kan Δ*yciG*::FRT double mutant survived heat shock less well than either of the single mutants. After 10 min at 55 C, 15±14% ($p = 0.08$) of the Δ*yciG*::FRT cells, 4.5±0.7% of the Δ*rpoS*::Kan ($p = 0.06$) cells, and 0.34±0.1% ($p = 0.07$) of the double mutant cells survived. The difference between the mean % survival of the Δ*rpoS*::Kan mutant and the double mutant is significant at $p = 0.01$ and the difference between the mean % survival of the Δ*yciG*::FRT mutant and the double mutant is significant at $p = 0.2$. These data indicate that YciG and RpoS probably act independently to mediate survival during thermal stress.

The Δ*yciG*::FRT mutation had no effect on the ability to survive salt stress. In contrast, only 9.5±3% of the Δ*rpoS*::Kan mutant cells survived after 2 h at 2.5M NaCl ($p = 0.04$). Surprisingly, the Δ*rpoS*::Kan Δ*yciG*::FRT double mutant survived 3-fold better than the Δ*rpoS*::Kan mutant. The difference between the mean % survival of these two strains is significant at $p = 0.02$.

Construction of a single-copy P_{rhaB} promoter fusion to study the role of YciF and YciE in *E. coli*

The growth defect observed for the Δ*yciG*::Kan mutant but not for the Δ*yciG*::FRT mutant indicated that that mutants lacking YciF and/or YciE would grow poorly and would be difficult to study. Therefore, to study the role of YciF and YciE, the expression of the *yciGFE* operon was placed under the control of the *rhaBAD* operon promoter, P_{rhaB}. Expression from the P_{rhaB} promoter is tightly controlled as it is subject to a regulatory cascade (13). L-rhamnose induction of the *rhaBAD* operon, which encodes the enzymes required for L-rhamnose catabolism, is a multistep process that requires two transcription factors, RhaR and RhaS (13). In the absence of the inducer L-rhamnose, RhaR and RhaS are present at a low basal level. In the presence of L-rhamnose, the RhaR protein binds the sugar and activates transcription from the *rhaSR* operon promoter leading to the accumulation of RhaR and RhaS. RhaS activates transcription from P_{rhaB}, which leads to the accumulation of RhaB, RhaA, and RhaD. RhaS also activates the expression of *rhaT*, which encodes the L-rhamnose transport protein (6). Full induction of the *rhaBAD* operon also requires binding of the CRP-

cAMP complex (13). Because transcription from P_{rhaB} is subject to catabolite repression, the level of expression from this promoter can be modulated by varying the carbon source. In the presence of the inducer L-rhamnose, expression from P_{rhaB} increases ca. 400 fold with glucose as the carbon source. With glycerol as the carbon source, there is ca. 5800-fold increase in expression (17).

The P_{rhaB}-*yciGFE* fusion was constructed by cloning the *yciGFE* structural genes downstream of P_{rhaB} in plasmid pLD78, which requires the Pir protein for replication as a plasmid. The resulting plasmid, pGK1, was introduced into a Rha^+ pir^- strain of *E. coli* and the integrants were selected as Tet^r colonies. Colonies carrying the insert at the *rhaBAD* locus were recognizable by their Rha^- phenotype. The presence of the insert in these Tet^r Rha^- colonies was also confirmed by PCR analysis.

To obtain recombinants that have lost the plasmid backbone, cells were plated on TSS (tetracycline sensitive selection) medium following growth in the absence of tetracycline. Recombination could result either in the restoration of the wild type chromosomal structure or an allele replacement. The recombinants that have the allele replacement will be Rha^- and have the P_{rhaB}-*yciGFE* construct in single copy at the *rhaBAD* locus (details are described in Materials and Methods). In order to be able to move this construct to different strain backgrounds, a Tn*10* linked to the *rhaBAD* locus (*zih35*::Tn*10*) was introduced by P1 transduction.

The P_{rhaB}-*yciGFE* construct was introduced by transduction into the λ *red* recombination strain GK263 and the deletion/replacement mutations of *yciF*, *yciE*, and

the entire *yciGFE* operon were made in the presence of L-rhamnose. The structure of the deletion/replacement alleles was confirmed by PCR as described in Materials and Methods.

Phenotypes of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant

Growth of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan (GK 299) strain without the inducer L-rhamnose will result in the repression of transcription from P_{rhaB} and turn off expression of the *yciGFE* operon. The intracellular levels of the YciG, YciF, and YciE proteins will decrease with each generation.

To assay the effect of depleting the YciG, YciF, and YciE proteins from the cell, the growth rates of GK299 (P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan) and GK292 (DS631 P_{rhaB}-*yciGFE*) were measured in the presence and absence of the inducer L-rhamnose. Since the cells were initially grown on medium containing L-rhamnose, they were grown in the absence of L-rhamnose for almost 20 generations to ensure that the pre-existing YciG, YciF, and YciE proteins were diluted out. Cultures were inoculated with 10^5 CFU per ml and after ~6.6 generations were diluted 100-fold into fresh medium, allowed to grow for another 6.6 generations, and then diluted again 100-fold into fresh medium. The results are shown in Fig 6.

No significant difference in the growth rate or the final yield of the cultures was observed for the Δ*yciGFE::*Kan mutant whether grown in the presence or absence of inducer. There was also no observable difference in growth rate between the mutant and the *yciG*$^+$*F*$^+$*E*$^+$ parent. These data are inconsistent with the growth defect exhibited by

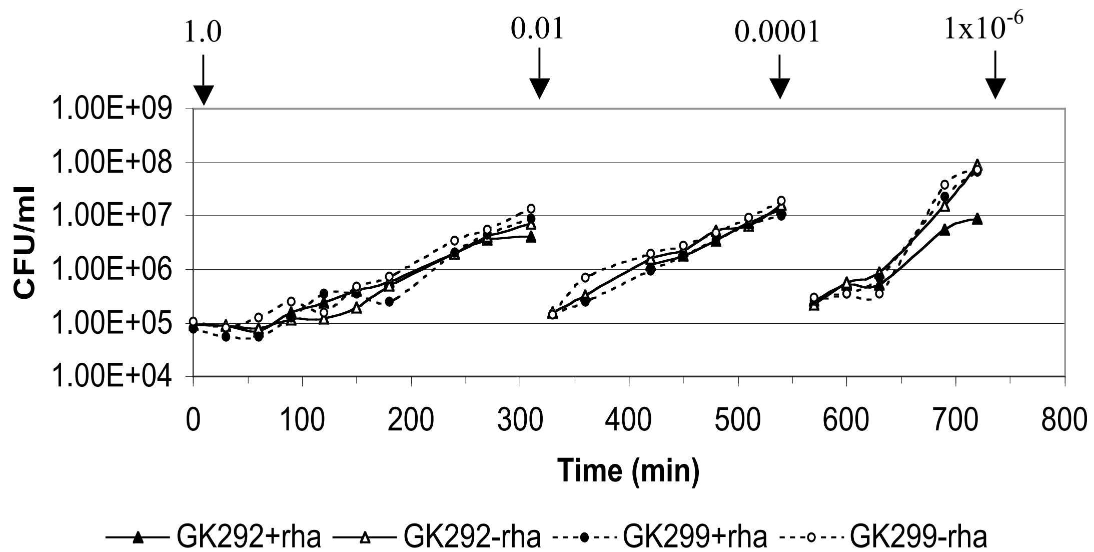

FIG. 6. Growth of GK292 and GK299 in MGC medium with (+) and without (-) the inducer L-rhamnose. GK292 is P_{rhaB}-*yciGFE* and GK299 is P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan. The numbers above the arrows indicate the predicted relative levels of the YciG, YciF, and YciE proteins in the cells after removal of the inducer.

the Δ*yciG::*Kan mutant, which was described above. It is possible that YciF and YciE are not required for the normal growth of *E. coli* and the growth defect observed for the Δ*yciG::*Kan mutant is not due to the lack of YciF and/or YciE. However, it is also possible that these proteins are indeed required for normal growth and are being synthesized in the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan strain, even in the absence of the inducer. Basal level of transcription of *yciF* and/or *yciE* from the P_{rhaB} promoter may be sufficient for normal growth. Alternatively, there may be an internal promoter upstream of *yciF* or *yciE*, which would allow for L-rhamnose independent transcription. An internal promoter upstream of *yciE* has been predicted (32).

P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan (GK299) is acid sensitive after growth in the absence of L-rhamnose

To determine if the expression of YciG, YciF, and YciE was being turned off in the absence of inducer, cultures of GK299 were grown overnight in MGC medium in the presence and absence of L-rhamnose and tested for acid resistance as described in the Materials and Methods. The P_{rhaB}-*yciGFE* Δ*yciE::*Kan mutant was also tested. The wild-type parent (DS631) and GK292 (DS631 P_{rhaB}-*yciGFE*) were used as positive controls. The results are shown in Fig 7.

Wild type (DS631) cells survived equally well at pH 2.5 whether grown in the presence or absence of inducer. 0.25±0.2% of wild-type cells grown in the presence of L-rhamnose survived, and 0.2±0.2% wild-type cells grown in the absence of L-rhamnose survived (p = 0.73).

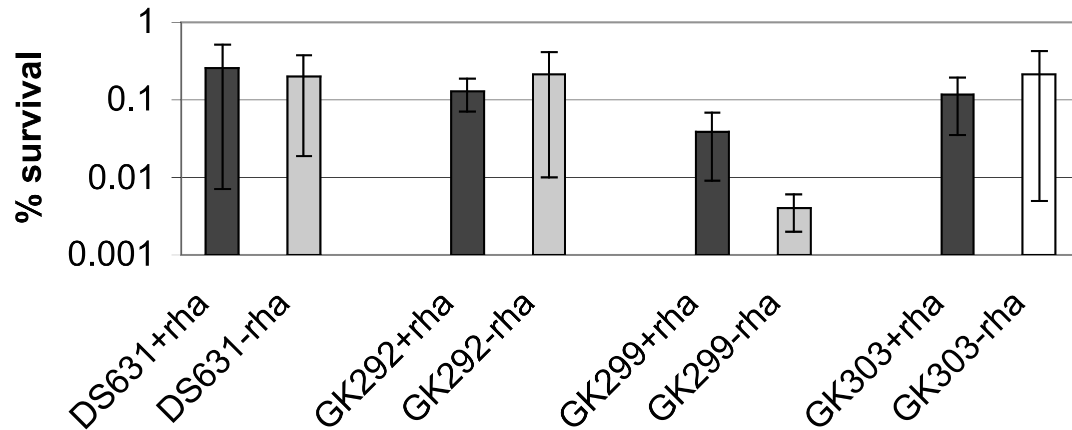

FIG. 7. Survival of stationary phase cultures of DS631, GK292, GK299, and GK303 at pH 2.5. Acid resistance was assayed as described in Materials and Methods. DS631 is the wild type parent, GK292 carries the P_{rhaB}-*yciGFE* construct, GK299 is P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan, and GK303 is P_{rhaB}-*yciGFE* Δ*yciE*::Kan. The values indicated in the figure are the mean of four independent experiments and the error bars indicate the standard deviation.

The presence or absence of L-rhamnose also did not affect the acid resistance of GK292, which carries the P_{rhaB}-*yciGFE* construct. After 1 h at pH 2.5, 0.1±0.06% of cells grown in the presence of inducer survived, while 0.21±0.2% of cells grown in the absence of inducer survived (p = 0.44) This indicates that constitutive expression of the *yciGFE* operon from P_{rhaB} did not have any significant effect on acid resistance.

Cultures of GK299 (P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan) grown in the presence of L-rhamnose survived low pH 10-fold better than when grown in the absence of L-rhamnose. After an hour at pH 2.5, 0.04±0.03% of cells grown in the presence of L-rhamnose were still viable, while only 0.004±0.002% of cells grown in the absence of inducer survived (p = 0.13). This result indicates that growth in the absence of L-rhamnose led to sufficient depletion of the YciG protein to cause acid sensitivity, and that enough YciG is produced during growth in the presence of L-rhamnose to increase the level of acid resistance. However, even when grown in the presence of L-rhamnose the Δ*yciGFE*::Kan mutant (GK299) survived less well at pH 2.5 than either DS631 or GK292 (DS631Δ*PrhaB-yciGFE*), p = 0.18 and 0.17, respectively. This suggests that the *PrhaB-yciGFE* construct does not express enough YciG at the appropriate time to confer a fully wild-type phenotype.

The DS631 P_{rhaB}-*yciGFE* Δ*yciE*::Kan mutant (GK303) did not show any significant difference in acid resistance whether grown in the presence or the absence of inducer. 0.114±0.08% of cells grown with inducer survived and 0.215±0.21% of cells grown without inducer survived (p value = 0.43). This result indicates that YciE is not

involved in acid resistance. Alternatively, YciE levels may not have been depleted enough to cause an acid sensitive phenotype.

YciG is required for acid resistance in both rich and minimal media

Interestingly, the parent strain DS631 showed a 60-fold higher level of acid resistance when grown in LB medium than when grown in MGC minimal medium, either with or without L-rhamnose (compare the results shown in Fig 5 and Fig 8). To test whether the requirement for YciG differs in these two growth conditions, the Δ*yciG::FRT* mutant (GK20) was assayed for acid resistance after growth in MGC medium. The results are shown in Fig 8.

The data show that the requirement for YciG for acid resistance is similar in the two media. The difference in mean % survival for the wild-type (DS631) and the mutant Δ*yciG*::FRT (GK20) after 1 h at pH 2.5 was 68±26-fold when cells were grown in LB medium. The difference was 44±42-fold when cells were grown in MGC medium. (Because the presence or absence of L-rhamnose did not significantly affect the acid resistance of either strain, the fold-difference in MGC medium combines the results from the cultures grown with and without L-rhamnose.)

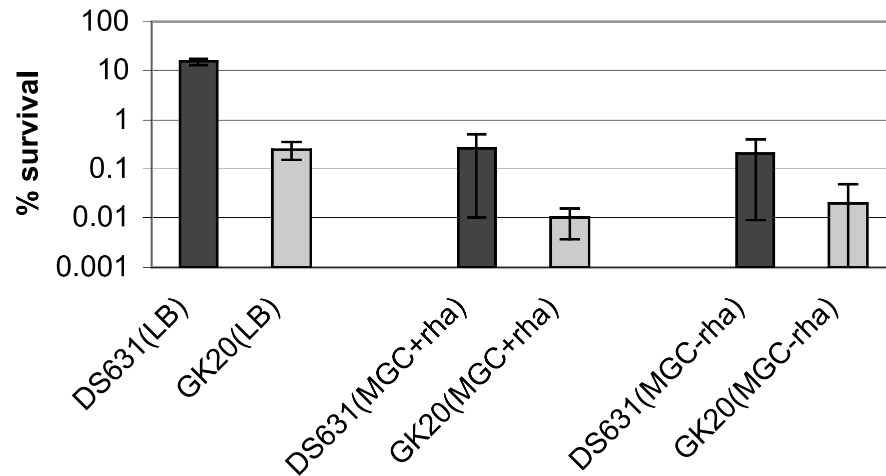

FIG. 8. Acid resistance of GK20 (Δ *yciG*::FRT) after growth in MGC medium with and without inducer. DS631 is the wild type parent, GK20 is DS631 Δ*yciG*::FRT. The values shown for DS631 are the mean of four independent experiments. The values shown for GK20 are the mean of three independent experiments. In both cases, the error bars indicate the standard deviation.

Transduction of the deletion/replacement mutations into strains with and without the P_{rhaB}-*yciGFE* construct

Two sets of results indicated that *yciF* and/or *yciE* are important for normal growth of *E. coli*. First, as described above, we had observed that derivatives of strains DY329 and DS649 that contained the Δ*yciG*::Kan mutation grew poorly. Second, it had been reported that transposon insertions in *yciF* are lethal (P. Sharpe, personal communication). However, the results in Fig 6 showed that the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant had the same growth rate as the $yciG^{+}F^{+}E^{+}$ parent strain even after ca. 20 generations of growth in the absence of inducer.

It is possible that the basal level of expression of *yciF* and/or *yciE* from P_{rhaB} was sufficient for normal growth. Alternatively, there may be an internal promoter upstream of *yciE* (32), which would allow L-rhamnose-independent transcription. To eliminate these possibilities we introduced the Kan^{r} deletion/replacement mutations into strains with and without the P_{rhaB}-*yciGFE* construct by transduction, selecting for a linked marker (trp^{+}) and then screening for the presence of the deletion/replacement mutation by checking for Kan^{r}. The recipient strains were DS631 *trpB83*::Tn*10* (DS709) and DS631 P_{rhaB}-*yciGFE trpB83*::Tn*10* (GK306). Trp^{+} transductants of GK306 were selected on minimal medium lacking tryptophan and containing L-rhamnose.

The transduction results are shown in Table 5. The cotransduction frequency between the selected marker and each of the unselected markers was identical

TABLE 5. Transduction of the deletion/replacement alleles into DS709 and GK306

Transduction[a]	Total transductants tested	No. of Kan^r Trp$^+$ colonies	Cotransduction Frequency
DS709[b] + P1/Δ*yciGFE*::Kan	79	65	82%
GK306[c] + P1/Δ*yciGFE*::Kan	130	107	82%
DS709 + P1/Δ*yciG*::Kan	24	21	88%
GK306 + P1/Δ*yciG*::Kan	24	20	83%
DS709 + P1/Δ*yciF*::Kan	25	20	80%
GK306 + P1/Δ*yciF*::Kan	25	21	84%
DS709 + P1/Δ*yciE*::Kan	30	26	86%
GK306 + P1/Δ*yciE*::Kan	30	25	83%

[a] Transductions were performed as described (25)

[b] DS709 is DS631 *trpB83*::Tn*10*

[c] GK306 is DS631 P_{rhaB}-*yciGFE trpB83*::Tn*10*

regardless of whether or not the recipient strain carried the P_{rhaB}-*yciGFE* construct, confirming that these genes are not required for normal growth.

Analysis of strains with deletions in the *trp-tonB* region

Deletion mutations that are thought to remove the *yciGFE* operon have been isolated and grow normally. We obtained two such strains from the Coli Genetics Stock Center and used PCR analysis with oligonucleotide primers flanking each of the three genes in the operon to determine whether any of the genes were still present in these mutants. The strains analyzed were DS724 Δ(*tonB-trpB)873*, which had acquired a requirement for tryptophan simultaneously with resistance to phage T1 (38), and DS727 Δ(*tonB-cysB)206*, which was isolated as a cysteine auxotroph that also required tryptophan (14). The regions deleted in each strain are shown in Fig 9.

The approximate positions of the oligonucleotide primers used to analyze these strains are shown in Fig 10. Each of the three genes was amplified with their respective flanking primers. In addition, combinations of flanking primers were used to test if the genes are present contiguously in these strains. The combinations of primers used and the sizes of the expected products are shown in Table 6.

The results from the PCR analysis are shown in Fig 11. Bands corresponding to the predicted size were detected for each of the three genes in both strains, indicating the presence of *yciG*, *yciF*, and *yciE*. PCR with combinations of flanking primers showed that the genes are present contiguously in these strains (Fig 11B).

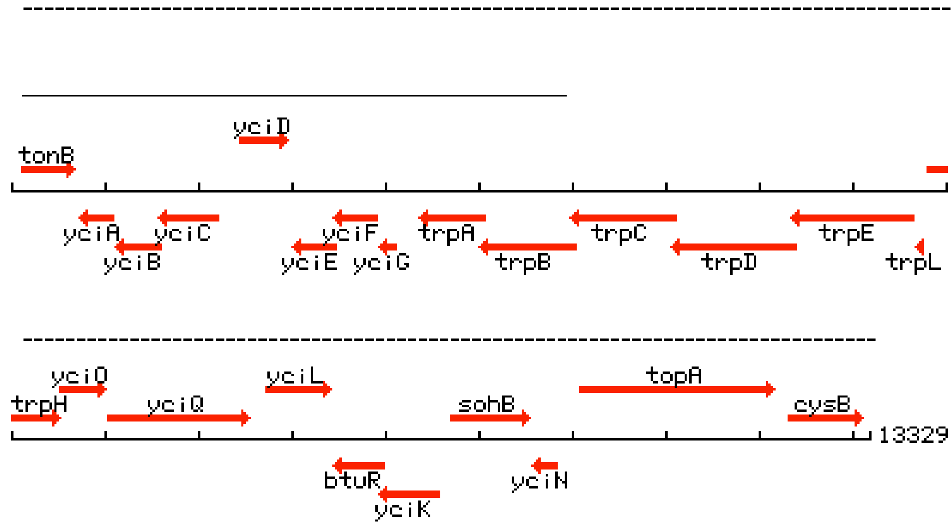

FIG. 9. Structure of the regions deleted in DS724 (Δ*tonB-trpB*)*873* and DS727 (Δ*tonB-cysB*)*206*. A 24000 bp region of the *E. coli* chromosome is shown. Tick marks are present every 1200 bp. The arrows indicate gene orientations. Modified from genomic maps in Colibri (http://genolist.pasteur.fr/Colibri). The solid line indicates the region deleted in DS724 and the dashed line indicates the region deleted in DS727.

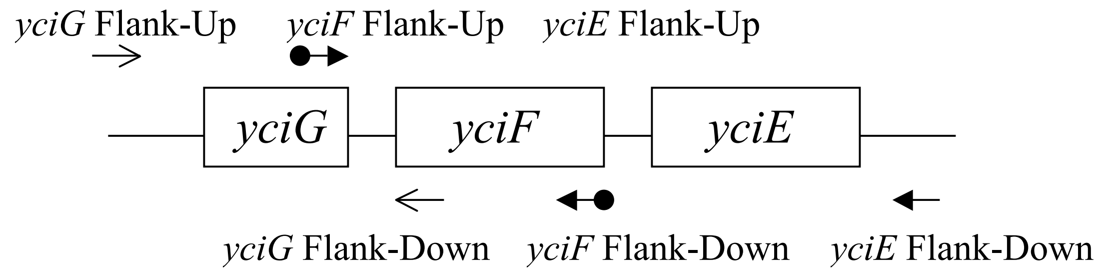

FIG. 10. Positions of the primers flanking the *yciG*, *yciF*, and *yciE* genes. The arrows indicate the orientation of the primers. The primer pair used to amplify each of the three genes is shown in the same style.

TABLE 6. PCR analysis of DS724 and DS727

Primer pairs used	Expected sizes	Results with DS724 DNA[a]	Results with DS727 DNA[a]
yciG-Flank Up X *yciG*-Flank Down	770 bp	+	+0
yciF-Flank Up X *yciF*-Flank Down	820 bp	+	+
yciE-Flank Up X *yciE*-Flank Down	1.03 kb	+	+
yciG-Flank Up X *yciE*-Flank Down	1.9 kb	+	+
yciG-Flank Up X *yciF*-Flank Down	1.23 kb	+	+
yciF-Flank Up X *yciE*-Flank Down	1.46 kb	-	-
yciF-Flank Up X *yciG*-Flank Down	330 bp	+	+
yciE-Flank Up X *yciF*-Flank Down	390 bp	+	+

[a] (+) indicates the presence of a product of the expected size and (-) indicates the absence of any product.

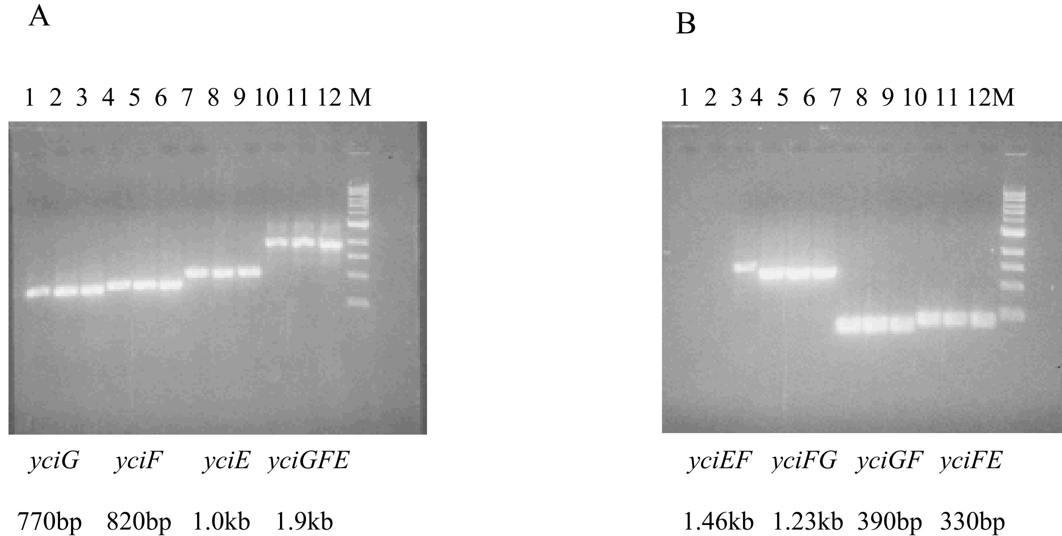

FIG. 11. PCR analysis of DS724, DS727, and DS631. (A) PCR analysis of the three strains with primers flanking *yciG* (lanes 1-3), *yciF* (lanes 4-6), *yciE* (lanes 5-8) and the entire operon (lanes 9-12). (B) PCR analysis with combinations of flanking primers. Lanes 1-3 are products amplified by *yciF*-Flank Up and *yciE*-Flank Down; lanes 4-6 are products amplified by *yciG*-Flank Up and *yciF*-Flank Down; lanes 7-9 are products amplified by *yciF*-Flank Up and *yciG*-Flank Down and lanes 10-12 are products amplified by *yciE*-Flank Up and *yciF*-Flank Down. For lanes 1, 4, 7 and 10 the template DNA was from DS724 (Δ*tonB-trpB*); for lanes 2, 5, 8 and 11 the template DNA was from DS727 (Δ*tonB-cysB*), and for lanes 3, 6, 9 and 12 the template DNA was from DS631 (wild-type). The lanes marked M contain a 1 kb ladder DNA molecular weight marker (New England Biolabs). From the bottom up, the sizes of the marker fragments are 0.5, 1.0, 1.5, 2, 3, 4, 6, 8, 9, and 10 kb.

CHAPTER IV

CONCLUSIONS

We have started to characterize three *E. coli* proteins, YciG, YciF, and YciE, whose function is unknown. The genes encoding these three proteins are thought to be in an operon (27, 32). Expression of these genes is induced by several different environmental stresses and is regulated by RpoS, the sigma factor that controls the general stress response in *E. coli* and other enteric bacteria (19, 27, 29, 35). We constructed in-frame deletions of each gene and of the entire operon and began to look for mutant phenotypes. Because transcription of these genes is known to be induced by stress, we started by looking for a role for these proteins in stress survival.

Our results revealed that YciG is important for stationary-phase resistance to thermal stress, oxidative stress, and, in particular, acid stress (Fig 4). A 41 to 60-fold difference was observed in the ability of stationary phase wild-type and Δ*yciG*::FRT mutant cells to survive at pH 2.5. Acid resistance is an important property of *E. coli*, because it enables the organism to survive gastric acidity. Acid resistance is also important for colonization of the lower intestine where the presence of short chain fatty acids can cause cytoplasmic acidification even at neutral external pH (10).

E. coli has both exponential phase and stationary phase acid resistance pathways (15). At least three stationary phase acid resistance pathways are known. One pathway requires RpoS and the CAP-cAMP transcription factor and is most important during growth in minimal media. A second pathway is also controlled by RpoS and requires the presence of glutamate. This system uses two glutamate decarboxylases and a

glutamate/γ-amino butyric acid antiporter to help maintain the internal pH near neutrality. The third stationary-phase acid resistance pathway requires arginine and an inducible arginine decarboxylase. RpoS does not appear to be involved in this third pathway.

The role of RpoS in YciG-mediated acid resistance was investigated by comparing the ability of stationary phase cells of Δ*rpoS*::Kan and Δ*yciG::FR*T single mutants and an Δ*rpoS*::Kan Δ*yciG*::FRT double mutant to survive low pH (Fig 5). Both of the single mutants survived less well than the wild-type strain. However, the absence of YciG was more deleterious than the absence of RpoS. Only 0.2±0.1% of Δ*yciG*::FRT cells survived the acid shock, while 4±2% of the Δ*rpoS*::Kan cells survived (p = 0.08). The Δy*ciG::FRT* Δ*rpoS*::Kan double mutant also was more acid sensitive than the Δr*poS::Kan* mutant. The level of acid resistance of the double mutant was comparable to what was observed for the Δ*yciG*::FRT single mutant (0.4±0.2% and 0.2±0.1% survival, respectively, p = 0.5). These results show that YciG functions in an RpoS-independent stationary-phase acid resistance pathway.

Whether or not YciG also has a role in one of the RpoS-dependent acid resistance pathways cannot be determined from these data. To answer this question, the Δ*yciG*::FRT mutation must be combined with mutations that inactivate the RpoS-independent pathway(s). We predict that YciG will function in RpoS-dependent acid resistance, because transcription of *yciG* is strongly induced by cytoplasmic acidification and this response is regulated by RpoS (35).

YciG is assigned to a cluster of orthologous proteins (COG3279), which includes the general stress protein GsiB from *Bacillus subtilis* (E = 0.007) (34). GsiB is involved in an adaptive non-sporulation response to nutrient deprivations (26), and its expression is induced by starvation for glucose or phosphate and by heat shock, salt stress, and oxidative stress (36). Another ortholog of YciG is the Con-10 protein in *Neurospora crassa,* which is a stress responsive protein induced during conidiation and by stresses such as heat shock (23).

The precise function of YciG in stress survival needs to be determined. The severe acid sensitive phenotype displayed by the Δ*yciG*::FRT mutant indicates that it has a key role in stationary-phase acid resistance. Multiple proteins are known to be important for survival at low pH and help to maintain cytoplasmic pH in different ways (15). This suggests the possibility that YciG could be involved in mediating expression of these proteins. Comparison of global changes in gene expression during entry to stationary phase or after acid shock in cells with and without YciG will help address this possibility.

Analysis of the amino acid sequence of YciG reveals that it contains a Walker A motif, commonly found in proteins involved in nucleotide binding (37).

Consensus for the Walker A motif: GXXXXGK(S,T)

YciG from *E. coli:*……….. QSGGNKSGKS

Mutations in the conserved GK residues of this motif are known to inactivate nucleotide binding (31). These residues in the YciG protein will be replaced by alanines to determine if nucleotide binding is necessary for the role of YciG in acid resistance.

As discussed earlier, there was evidence that YciF and/or YciE are required for normal growth of *E. coli*. Therefore, to investigate the role(s) of YciE and YciF, the expression of the *yciGFE* operon was placed under the control of the L-rhamnose inducible promoter P_{rhaB}. The acid resistance of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant was assayed after growth in the absence of the inducer, which should result in the depletion of the YciG, YciF, and YciE proteins from the cell. The ability of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant to survive at pH 2.5 was 10-fold higher when the mutant cells were grown in the presence of inducer than when grown without inducer (Fig 7). This shows that transcription of *yciG* from a heterologous promoter complements the Δ*yciGFE*::Kan mutation.

Interestingly, when grown in the absence of L-rhamnose, the strain carrying the P*rhaB-yciGFE* construct (GK292) had the same level of acid resistance as its wild-type parent (DS631) ($p = 0.9$) (Fig 7). In contrast, when acid resistance was assayed after the strains were grown in the presence of L-rhamnose, DS631 survived almost 3-fold better than GK292. This indicates that constitutive expression of the *yciGFE* operon interferes with normal stress responses. No difference was seen in the ability of the P*rhaB-yciGFE* Δ*yciE*::Kan mutant to survive low pH, whether cells were grown in the presence or absence of inducer. This indicates that YciE does not play a significant role in stationary phase acid resistance or, alternatively, that the intracellular level of YciE is not low enough even after growth for ~7 generations in the absence of L-rhamnose to allow for a detectable phenotype. The stress response of this mutant needs to be tested

after a longer period of growth in the absence of L-rhamnose to allow for complete depletion of the YciE protein.

The Δ*yciF*::Kan mutant has not yet been tested for acid resistance. To determine if YciE has and/or YciF have a role in other stress resistance pathways, the ability of strains lacking these proteins to survive other stresses, such as nutrient limitation or oxidative stress, needs to be investigated. YciF and YciE are paralogs and are predicted by the FUGUE threading program (30) to have the same fold as bacterioferritin, which is involved in iron storage and transport (12). The possibility that YciE and YciF are involved in metal binding needs to be explored.

We had gone to the effort of placing the *yciGFE* operon under the control of a regulatable promoter, because there was evidence that YciF and/or YciE are required for normal growth. The Δ*yciG*::Kan mutant we first constructed had a severe growth defect. Normal growth was restored when the Kan cassette was excised, generating an in-frame deletion. Since *yciG* is the first gene in the putative operon, it seemed plausible that the growth defect was due to a polar effect of the Kan cassette on the expression of the downstream genes, *yciF* and *yciE*. Also, we had learned from another laboratory that transposon insertions in *yciF* are lethal (P. Sharpe, pers. comm.). However, the growth rates of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant (GK299) were the same as the growth rates of the P_{rhaB}-*yciGFE* parent (GK292) during growth in media with and without L-rhamnose.

The acid sensitivity of the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant when grown in the absence of L-rhamnose showed that there was sufficient depletion of YciG to cause

an acid sensitive phenotype. There is a predicted internal promoter upstream of *yciE* in the coding region of *yciF* (Fig 12). It is possible that expression of YciE from this promoter explains why no growth defect was observed when the P_{rhaB}-*yciGFE* Δ*yciGFE*::Kan mutant was grown for almost 20 generations in the absence of L-rhamnose. It is also possible that the growth defect we observed in the Δ*yciG*::Kan mutant was not due to decreased expression of YciF and/or YciE.

Experiments should be performed to determine if there is any transcription from the predicted internal promoter and when transcripts are produced. The presence of an internal promoter suggests that there are growth conditions where YciE is expressed, but not YciG or YciF. Identification of such conditions could provide information that would help us to understand the function of YciF in the cell.

To test the possibility that the P*rhaB*-*yciGFE* Δ*yciGFE*::Kan mutant had a wild-type growth phenotype in the absence of the inducer was because of continued expression of *yciE* from an internal promoter, we decided to transfer the Δ*yciGFE*::Kan mutation into strains with and without the P_{rhaB}-*yciGFE* construct. A selectable marker linked to the *yciGFE* locus was used to select transductants that had received the *yciGFE* chromosomal region from the donor strain. The transductants were then tested for the presence or absence of the unselected Δ*yciGFE*::Kan mutation. If the operon is required for normal growth, we would expect to see decreased linkage between the selected marker and the Δ*yciGFE*::Kan mutation in the strain lacking the P_{rhaB}-*yciGFE* construct. If deletion of the *yciGFE* operon has no effect on growth, we would expect to find the same linkage between the Δ*yciGFE*::Kan mutation and the selected marker in

TATGAGA<u>**TTGCCA**</u>GTTACGGGACATTAGC<u>**GACGCT**</u>GGCTGAACAATTAGGTTTACC

GTAAAGCAGCGAAGCTTCTGAAAGAAACCCTGGAAGAAGAAAAGGCCACCGACATCAA

ACTGAC<u>TGA</u>TCTGGCCATTAATAACGTAAATAAGAAAGCCGAAAATAAAGCCTGAAAT

ATGAATTTTAACTTTTAGTCATTTTATAAAGAGGACATTTTC**ATG**AATC

FIG. 12. Putative internal promoter for *yciE* located in the *yciF* coding region. The sequences underlined and in bold indicate the predicted –35 and –10 regions of the promoter. The stop codon at the end of the *yciF* coding sequence is underlined. The start codon for the *yciE* coding sequence is shown in bold.

the two strains. This, in fact, is what was found (Table 5, column 4). The cotransduction frequency between the selected marker and the Δ*yciGFE*::Kan was 82% in both recipient strains. The same strategy was used for each of the other deletion/replacement mutations: Δy*ciG*::Kan, Δ*yciF*::Kan, and Δ*yciE*::Kan. In every case, there was the same linkage between the selected marker and the unselected mutation whether or not the recipient strain carried the P_{rhaB}-*yciGFE* construct.

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