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ORIGINAL PAPER

YqhC regulates transcription of the adjacent *Escherichia coli* genes *yqhD* and *dkgA* that are involved in furfural tolerance

Peter C. Turner · Elliot N. Miller · Laura R. Jarboe · Christy L. Baggett · K. T. Shanmugam · Lonnie O. Ingram

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Abstract Previous results have demonstrated that the silencing of adjacent genes encoding NADPH-dependent furfural oxidoreductases (*yqhD dkgA*) is responsible for increased furfural tolerance in an *E. coli* strain EMFR9 [Miller et al., Appl Environ Microbiol 75:4315–4323, 2009]. This gene silencing is now reported to result from the spontaneous insertion of an IS10 into the coding region of *yqhC*, an upstream gene. YqhC shares homology with transcriptional regulators belonging to the AraC/XylS family and was shown to act as a positive regulator of the adjacent operon encoding YqhD and DkgA. Regulation was demonstrated by constructing a chromosomal deletion of *yqhC*, a firefly luciferase reporter plasmid for *yqhC*, and by a direct comparison of furfural resistance and NADPH-dependent furfural reductase activity. Closely related bacteria contain *yqhC*, *yqhD*, and *dkgA* orthologs in the same arrangement as in *E. coli* LY180. Orthologs of *yqhC* are also present in more distantly related Gram-negative bacteria. Disruption of *yqhC* offers a useful approach to increase furfural tolerance in bacteria.

Keywords *Escherichia coli* · Furfural · *yqhC* · *yqhD* · *dkgA*

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P. C. Turner · E. N. Miller · L. R. Jarboe · C. L. Baggett · K. T. Shanmugam · L. O. Ingram (✉)
Department of Microbiology and Cell Science, University of Florida, Box 110700, Gainesville, FL 32611, USA
e-mail: Ingram@ufl.edu

Introduction

Lignocellulosic biomass (LCB) can be metabolized into a variety of fermentation products after the conversion of cellulose and hemicellulose polymers into soluble sugars [8, 10, 11]. Deconstruction of these carbohydrates requires a combination of chemical and enzymatic treatments. Side reactions during these treatments produce microbial inhibitors such as furans, organic acids, and phenolics that inhibit yeast and bacterial fermentation [19, 23]. Ethanologenic *Escherichia coli* that have been engineered to ferment all the sugars present in LCB polymers are also sensitive to these inhibitors [1].

Furfural, a dehydration product of pentose sugars, is one of the most important inhibitors derived from acid hydrolysis of biomass [15]. Toxicity of hemicellulose hydrolysates has been shown to correlate with furfural concentration. Removal of furfural from these hydrolysates by over-liming to pH 10 has been shown to render these syrups fermentable [15]. Supplementing over-limed hydrolysate with furfural restored toxicity.

Furfural is metabolized to furfuryl alcohol by NADPH-dependent oxidoreductases in yeast [9, 21, 24] and bacteria [2, 6, 26]. In *E. coli*, growth is inhibited by the addition of furfural at concentrations below 1.0 g l^{-1} but resumed following conversion of furfural to furfuryl alcohol [17, 18]. This transient inhibition resulted from the starvation of biosynthesis for NADPH during furfural reduction by YqhD and DkgA [17, 18]. Genes encoding these activities were induced by furfural in strain LY180. The *yqhD* and *dkgA* genes encode NADPH-dependent oxidoreductases with broad substrate ranges that include furfural [13, 17, 22, 25]. The low apparent K_m values for YqhD (8 μM NADPH) and DkgA (23 μM NADPH) [17] are similar to those of many biosynthetic enzymes. Paradoxically, increased expression

of oxidoreductases that reduce furfural to the less toxic furfuryl alcohol (*yqhD, dkgA*) is not beneficial for the bacterium but instead resulted in growth arrest by depleting NADPH needed for biosynthesis [18].

A furfural-tolerant mutant of LY180 (designated EMFR9) avoided furfural inhibition of growth by silencing the expression of *yqhD* and *dkgA* [17, 18]. No mutations were found in the coding regions or adjacent regulatory regions of *yqhD* or *dkgA* and the basis for gene silencing was unknown [17, 18]. In this study, we show that expression of *yqhD* and *dkgA* were silenced by an IS10 insertion sequence in *yqhC* and identify this gene as the positive regulator of the *yqhD dkgA* operon. Inactivation of *yqhC* offers a useful approach to increase furfural tolerance in bacterial biocatalysts.

Materials and methods

Strains, media, and growth conditions

Strain LY180 [17] is a derivative of *E. coli* KO11 [20], and its genotype is: Δ*frdBC::(Zm frg celYEc)* Δ*ldhA::(Zm* frg *casABKo) adhE::(Zm* frg *estZPp* FRT) Δ*ackA*::FRT *rrlE::(pdc adhA adhB* FRT) Δ*mgsA*::FRT. Strain EMFR9 [17] is a furfural-resistant mutant of LY180 selected by growth in the mineral salts medium containing xylose and furfural (1.0 g l^{-1}).

Strains were grown in AM1 mineral salts medium [16] supplemented with 20 g l^{-1} xylose in solid medium, with 50 g l^{-1} xylose for growth in most liquid cultures including in a growth curve analyzer, and with 140 g l^{-1} xylose in pH-controlled fermentation experiments.

BioScreen C growth curve analyzer

Seed cultures were grown in tubes (shaking water bath, 37°C) until the OD_{550} reached 0.4–0.6, diluted to an OD_{550} of 0.3 with fresh medium, and transferred (50 µl) to each well of a 100-well honeycomb plate containing 350 µl each of medium. Optical density was measured at 30-min intervals over a 65-h period (37°C) with 10 s shaking immediately before each reading (10 replicates per experiment; each experiment repeated twice).

Generation and sequencing of PCR products

Genomic DNAs were prepared from bacterial cultures using the Qiagen DNeasy Blood and Tissue kit. Regions of interest were PCR amplified with Qiagen Taq PCR master mix, purified with the QIAquick PCR purification kit, and submitted to the University of Florida sequencing core for analysis. Resulting data were assembled and compared by using Vector NTI software (Invitrogen).

Plasmid constructions

Plasmid pLOI4900 was constructed by transferring the firefly luciferase gene, originally from pGL3-Control (Promega), into pBAD24 [7], and then replacing the *araC* gene and pBAD promoter regions with a 151-bp region upstream from the *yqhD* ORF in LY180. This putative *yqhD* promoter region was PCR amplified using primers PCT6 and PCT7 (Table 1), and corresponds to bases 2,027–2,983 of the *E. coli* strain LY 180 *yqhC-yqhD-dkgA* region (GenBank GQ478251).

The *yqhC* gene was amplified from LY180 genomic DNA with primers PCT50 and PCT51, generating a PCR product containing the entire *yqhC* ORF plus a 354-bp region upstream (native promoter). This fragment (the complement of bases 1,566–3,336 of GenBank GQ478251) was cloned into pCC1 using the CopyControl PCR cloning kit (Epicentre) to produce pLOI4901. The pCC1 vector is a single-copy plasmid based on the *E. coli* F factor.

Construction of a *yqhC* deletion mutant

The *yqhC* coding region was replaced with the kanamycin resistance (*kan*) gene cassette from pKD4 [3] by homologous recombination. The 1.5-kb *kan* cassette was amplified from pKD4 with primers PCT46 and PCT47 (Table 1). The 418-bp region that lies at the upstream (5′) end of *yqhC* was amplified by using primers PCT48 and PCT49. Primers PCT 47 and PCT48 each contain a *Xho*I site (underlined in Table 1). These two PCR products were digested with *Xho*I and ligated. The resulting 1.9-kb fragment was amplified by using primers PCT49 and yqhC_ko_rev. Primer yqhC_ko_rev contains a 41-bp tail matching the 3′ end of the *yqhC* gene (downstream), and 19 bp homologous to the *kan* gene. The final 1.94-kb PCR product was used with Red recombinase-mediated recombination (Gene Bridges GmbH, Dresden, Germany) to delete the *yqhC* gene in LY180. The resulting strain LY180Δ*yqhC* was verified by PCR analysis and sequencing.

Furfural reduction in vivo

Cultures were grown to a cell density of 0.66 g DCW l^{-1} in fleakers containing 350 ml AM1 with 100 g l^{-1} xylose medium and sampled immediately before and 15, 30, and 60 min after addition of furfural (0.5 g l^{-1}) as previously described [17]. Residual furfural was measured in the culture broth after centrifugation using a spectrophotometric method [14].

Table 1 Primers

Primer name	Sequence (5′–3′)
PCT6	GCGTATGCATGCAATTTTGTAGCATTTCTCCAGC
PCT7	GCGGAATTCTACTTGCTCCCTTTGCTGGG
PCT46	ATGGTCCATATGAATATCCTCCTTAG
PCT47	GAGCTCGAGTAGGCTGGAGCTGCTTC
PCT48	GAGCTCGAGATGCGGCAATTTGATTGTGCGC
PCT49	GTTTCACGGCGTTCATCAGCG
PCT50	GTCTGGGCTGCTGGCTAAG
PCT51	TTTCATAAGCCGGGTTTGGCTC
YqhC_ko_rev	GACGATTTTCCCCGTTCCCGGCTGCTGTACCGGGAACGTATC ATATGAATATCCTCCTTA
birA_rtpcr_for	GTCTGGTTATCGGTATCGTGATG
birA_rtpcr_rev	CCAGTCAGCTCCACAAGAATGC
yqhC_rtpcr_for	CTTCATGCCGTCATGGATGATC
yqhC_rtpcr_rev	GTACTACGTGCTGACCGGAC
yqhD_rtpcr_for	GCTGACGCTAATCGAAGATG
yqhD_rtpcr_rev	GCAGGACGATAGCCAGTGTT
yqhD-dkgA_for	GGTCTGGACGGCAGCTCC
yqhD-dkgA_rev	CGGTATCAATCGAGCGATAACC
dkgA_rtpcr_for	TGTCGAAGCATGGAAAGGCATG
dkgA_rtpcr_rev	CAGATCGCGAATGACTTTCTGATC

*Xho*I site is underlined

Measurement of luciferase reporter activity in bacterial cells

Tube cultures of *E. coli* carrying appropriate reporter plasmids were grown in AM1 with 50 g l^{-1} xylose to an OD of 0.4. After sampling the cultures, furfural was added at 0, 0.1, 0.5, or 1 g l^{-1}, and incubation continued. Samples were removed for analysis 5, 15, and 30 min after addition of furfural. Untreated and furfural-treated cells were collected by centrifugation, resuspended in Qiagen Qproteome bacterial lysis buffer, and stored at −80°C. The lysates were thawed at 37°C for 2 min, and transferred to an opaque white 96-well plate. An equal volume (50 µl) of PerkinElmer BriteLite reagent was added to each well. Luminescence was measured by using a Promega Glomax 96-well luminometer.

Microarray analysis

For each strain, 4 replicate 350-ml cultures were grown to an OD of 1.5 (0.66 g DCW l^{-1}) and sampled. Furfural (0.5 g l^{-1}) was added and samples were removed after 15 min of incubation. Culture samples were rapidly cooled in a dry ice/ethanol bath for 40 s with agitation to avoid freezing. Cells were collected by centrifugation at 4°C, resuspended in RNA Later (Qiagen), and stored at −80°C. Cell pellets from the 4 fermentors were pooled and used for RNA isolation (Qiagen RNeasy Mini Kit). RNA was treated with DNase, re-purified, and assessed for quality using an Agilent Bioanalyzer. RNA samples were submitted to Nimblegen for conversion to cDNA, labeling with Cy3, and hybridization to the *E. coli* K12 TI8333 microarray chip. This chip contains 385,000 60-mer probes derived from *E. coli* K12 strain MG1655, and has 5 replicates of each probe with an average of 18 probes per gene. Normalized expression data from Nimblegen were imported into ArrayStar (DNA Star) for analysis.

qPCR methods

RNA prepared as described in the previous section was diluted to 50 ng/µl, and 50 ng RNA was converted to cDNA by treatment with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The RNA was incubated at 65°C for 5 min in the presence of random hexamers and dNTPs, chilled on ice, and then incubated with 1 µl (200 units) SuperScript II at 25°C for 10 min, at 42°C for 50 min, then at 70°C for 15 min to inactivate the enzyme. Control reactions without added reverse transcriptase were set up to assess the presence in the RNA of contaminating genomic DNA. Then, 2 µl of the cDNA was used as template in each 50-µl real-time PCR reaction, with iQ SYBR Green Supermix (BioRad), and each primer at 0.5 µM final concentration. The *birA* gene were used as an internal standard for each sample. The sequences of primers used for RT–PCR analysis

of *birA*, *yqhC*, *yqhD*, *dkgA*, and the intergenic *yqhD-dkgA* region are listed in Table 1. The number of cycles to reach the threshold (C_T value) was measured for each primer pair in duplicate samples of each cDNA. The $\Delta\Delta C_T$ method (the change following furfural addition in the difference between the C_T value for *birA* and the C_T value for gene of interest) was used to estimate changes in RNA levels after furfural treatment.

Accession numbers for nucleotide sequences and microarray data

The DNA sequences for the *yqhC-yqhD-dkgA* regions of both LY180 and EMFR9 have been deposited with GenBank (accession numbers GQ478251 and GQ478252 respectively). Microarray data was deposited with the Gene Expression Omnibus (GEO) at http://ncbi.nlm.nih.gov/geo with accession # GSE17786.

Results and discussion

The furfural-tolerant strain EMFR9 contains an IS10 insertion in *yqhC*

Silencing of two NADPH-dependent oxidoreductases (*yqhD* and *dkgA*) in a furfural-resistant mutant (EMFR9) of *E. coli* LY180 was previously shown to confer increased furfural tolerance [17]. However, sequencing (this work) of the furfural-resistant strain EMFR9 in the coding regions of these two genes or in the 500-bp regions immediately upstream and downstream did not reveal any mutations.

Immediately upstream of *yqhD* and transcribed in the opposite direction is *yqhC* (Fig. 1) encoding a putative regulatory protein belonging to the AraC/XylS family of DNA-binding proteins [4]. Many of these regulators are transcriptional activators, although some act as both activator and repressor. The divergent transcription (*yqhC*) with adjacent regulated genes (*yqhD dkgA*) is a pattern of organization found throughout the *E. coli* chromosome. PCR amplification of the *yqhC* gene from the genomic DNA of EMFR9 (this work) gave an unexpectedly large PCR product compared to the DNA from LY180. Sequencing this PCR product revealed the presence within *yqhC* of a 1.3-kb IS10 (Fig. 1), flanked by copies of a nine-base sequence TGCCAGGCT derived from *yqhC*. The observed lack of induction of the *yqhD* and *dkgA* genes in strain EMFR9 in response to furfural addition [17] could result from the IS10 insertion in *yqhC*, encoding the putative transcriptional regulator. Transcription of yqhC was inducible by furfural in LY180 but not in EMFR9 (Fig. 2a, microarray data from [17]), suggesting that YqhC may regulate its own expression.

Deletion of *yqhC* in LY180 increased furfural tolerance

To evaluate the role of YqhC in the regulation of *yqhD* and *dkgA*, the *yqhC* gene in LY180 was replaced with a kanamycin-resistance cassette to create LY180Δ*yqhC*. Furfural tolerance of LY180 and LY180Δ*yqhC* was compared and the results demonstrated that the mutation increased furfural resistance compared with the parent. Complementing the mutation by introducing a plasmid-borne copy (pLOI4901) of the wild type *yqhC* gene with its native promoter fully restored furfural sensitivity (Fig. 3c).

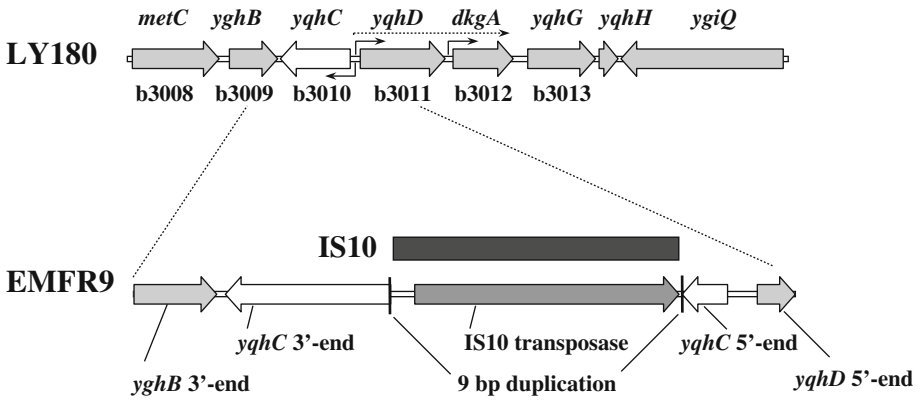

Fig. 1 Arrangement of the *yqhC-yqhD-dkgA* genes and surrounding regions in LY180 and EMFR9. The locations and directions of the coding regions for *yqhC, yqhD*, and *dkgA* are shown in the ethanologenic strain LY180 (*top line*) and in the furfural-resistant derivative EMFR9 (*lower line*). The flanking genes to the *left* of *yqhC* (*metC* and *yghB*) and to the *right* of *dkgA* (*yqhG*, *yqhH*, and *ygiQ*) are shown for LY180. Note the presence of IS10 within the *yqhC* gene of EMFR9. Promoters for *yqhD*, *yqhC*, and *dkgA* are shown by *arrows* (*solid lines*) based on this work (*yqhD* and *yqhC*) and for *dkgA* using information [5] available at EcoCyc [12]. Transcription from *yqhD* extending into *dkgA* is shown by an *arrow* with a *dotted line*

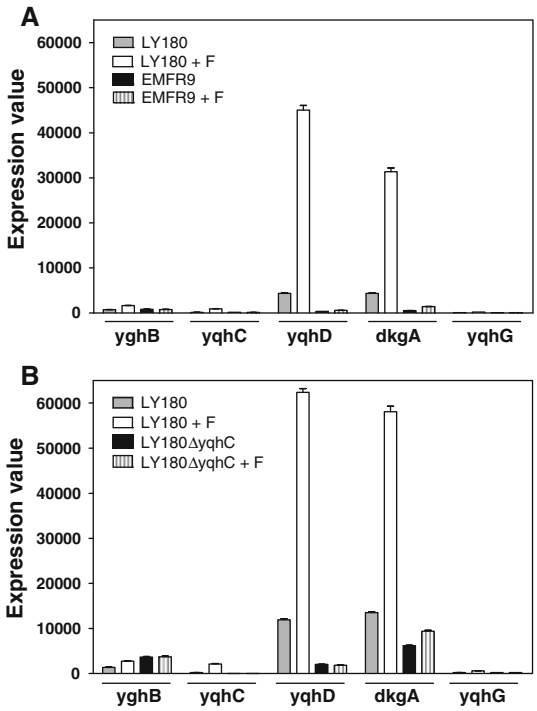

Fig. 2 Expression of transcripts in the *yqhC-yqhD-dkgA* region from LY180, EMFR9 and LY180Δ*yqhC* after addition of furfural. Transcript levels were determined by expression hybridization of total RNA against *E. coli* K12 microarrays. Cells were harvested either immediately before furfural addition, or 15 min after addition of 0.5 g l^{-1} furfural. The normalized expression values for selected genes are shown, with SEM error bars calculated from the 5 replicates of each probe present on the chip. **a** Expression levels for genes *yqhC*, *yqhD*, and *dkgA* with the flanking genes *yghB* and *yqhG* for strains LY180 and EMFR9 either untreated or treated for 15 min with 0.5 g l^{-1} furfural. These data are from Miller et al. [17]. **b** Expression levels for *yqhC*, *yqhD*, and *dkgA* plus flanking genes for strains LY180 and LY180Δ*yqhC* before and after treatment with furfural

The presence of empty vector (pCC1) in LY180Δ*yqhC* had no effect on furfural sensitivity (Fig. 3d). These results show that mutation of *yqhC* either by insertion of IS10 (in EMFR9) or by complete deletion (in LY180Δ*yqhC*) resulted in increased furfural tolerance.

The in vivo rate of furfural reduction by strain LY180 (0.042 ± 0.001 µmol min^{-1} mg DCW^{-1}) was significantly higher (68%; $P < 0.05$) than by strain LY180Δ*yqhC* (0.025 ± 0.005 µmol min^{-1} mg DCW^{-1}), consistent with induction of YqhD and DkgA in only the parental strain. These results also suggest that YqhC regulates the oxidoreductases encoded by *yqhD* and *dkgA*.

Transcriptional regulation of the *yqhD* promoter (luciferase reporter)

To evaluate the regulation of *yqhD*, plasmid pLOI4900 was constructed with the *yqhD* promoter region (151 bp) immediately upstream from a firefly luciferase reporter (Fig. 4a). This plasmid was used to investigate transcriptional regulation of the *yqhD* promoter in the parent LY180 and in LY180Δ*yqhC*. With LY180(pLOI4900), addition of furfural (0.1, 0.5, and 1.0 g l^{-1}, equivalent to 1, 5, and 10 mM) resulted in a dose-dependent increase in luciferase activity that was evident within 5 min (Fig. 4b). In the *yqhC* deletion strain harboring this plasmid, no furfural-dependent response was observed (Fig. 4c). Expression of luciferase in the absence of furfural was observed in both strains but was lower in LY180Δ*yqhC*.

EMFR9 carrying pLOI4900 gave similar results (not shown) to those with LY180Δ*yqhC*(pLOI4900). The basal level of luciferase activity in the absence of furfural was low, and not increased by the addition of furfural. No luciferase activity was detected in the absence of pLOI4900.

Other aldehydes known to be present in dilute acid hydrolysates of hemicellulose [21, 26] were also tested at various concentrations using LY180(pLOI4900) and LY180Δ*yqhC*(pLOI4900). All increased luciferase activity by 2- to 5-fold in LY180(pLOI4900) in comparison with untreated cells (data not shown). These included acetaldehyde (1 mM), propionaldehyde (1 mM), butyraldehyde (1 mM), 5-hydroxymethyl furfural (1 mM), and cinnamaldehyde (0.1 mM). Methylglyoxal (0.1 mM) was also found to increase luciferase activity in LY180(pLOI4900). None of these compounds increased activity of the *yqhD* promoter measured as luciferase activity in LY180*ΔyqhC*(pLOI4900).

Together, these results demonstrate that YqhC is a required, trans-active transcriptional activator for aldehyde-inducible transcription from the *yqhD* promoter. Mutations of *yqhC* (IS10 insertion or deletion) eliminated the aldehyde-induced increase in transcription. Considering the wide range of aldehyde structures that were active, it is possible that YqhC does not interact directly with inducers but responds to a physiological effect of aldehydes.

Effect of a *yqhC* deletion on transcript levels

Total RNA was prepared from strains LY180 and LY180Δ*yqhC* immediately before and 15 min after the addition of 0.5 g l^{-1} furfural. Expression results for the *yqhC-yqhD-dkgA* region together with flanking genes *yghB* (conserved inner membrane protein) and *yqhG* (unknown function) are shown in Fig. 2b. In LY180, expression of *yqhC*, *yqhD*, and *dkgA* transcripts was upregulated by the addition of furfural, as expected. This furfural response was absent in LY180Δ*yqhC*. Flanking genes were expressed at low levels and were less affected. The low level of apparent expression of *yqhC* in LY180Δ*yqhC* was similar to that observed for known gene deletions in LY180 such as *ldhA*, *adhE*, and *frdBC*, and reflected the background level of hybridization obtained with the *E. coli* K12 chip. These data are consistent with YqhC acting as a positive regulator of transcription from the *yqhD* promoter.

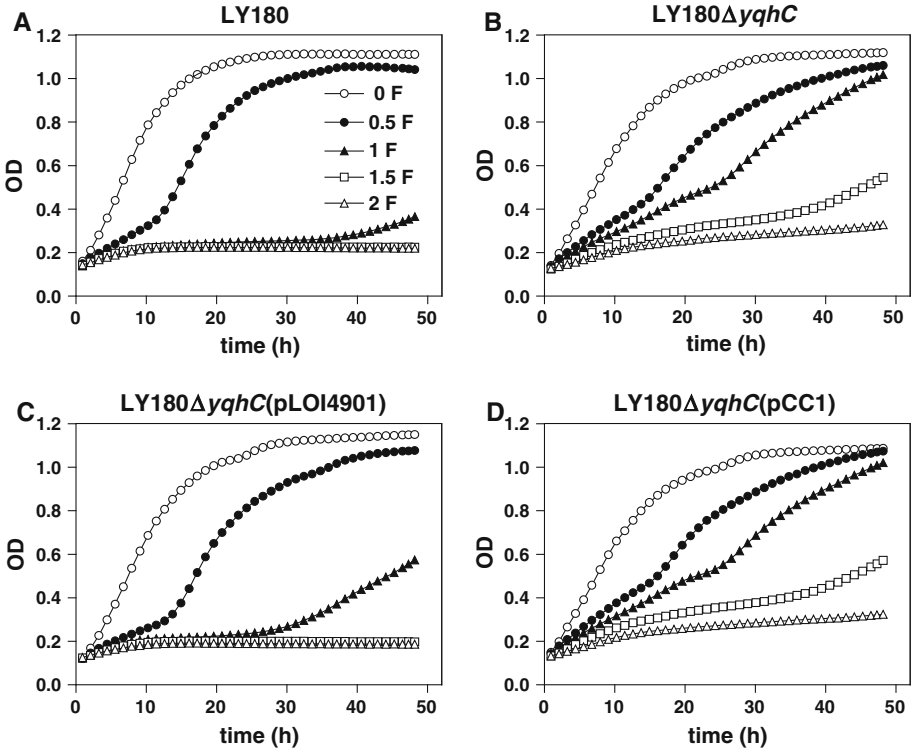

Fig. 3 The effect of *yqhC* deletion on growth in the presence of furfural. Strains were grown in AM1 medium with 50 g l^{-1} xylose, and containing 0, 0.5, 1, 1.5, or 2 g l^{-1} furfural. The optical density was monitored at 30-min intervals over a 48-h period. The strains tested were LY180 (**a**), LY180Δ*yqhC* (**b**), LY180Δ*yqhC* containing the single copy plasmid pLOI4901 carrying *yqhC*$^+$ (**c**), and LY180Δ*yqhC* containing the empty vector pCC1 (**d**)

The question of whether YqhC regulates other regions besides the one adjacent to the *yqhC* gene was addressed by analyzing all relevant microarray data from our laboratory. The data generated for LY180 and LY180*ΔyqhC* were combined with previous datasets [17, 18] which compared LY180 and EMFR9 (IS10 insertion in *yqhC*) in the presence and absence of furfural. We reasoned that IS10 inactivation of *yqhC* in EMFR9 should result in changes similar to those caused by the *yqhC* deletion (in LY180*ΔyqhC*). The expression data for the 4,237 genes represented on the TI8333 microarray chip were searched for perturbation in the absence of wild type *yqhC*. In all of the datasets examined, expression of *yqhD* and *dkgA* was consistently reduced in EMFR9 versus LY180, and in LY180*ΔyqhC* vs. LY180, both in the absence and presence of furfural. The fold reduction in *yqhD* expression with furfural for EMFR9 in comparison with LY180 was at least 8-fold and for *dkgA* at least 10-fold. However, we were unable to find other genes that were consistently up- or downregulated in the absence of a functional YqhC protein in all of the datasets. Thus *yqhD* and *dkgA* appear to be the only genes regulated by YqhC under these experimental conditions.

qPCR to assess transcription of the *yqhD-dkgA* intergenic region

We assessed the possibility that the observed increase in *dkgA* transcription following furfural addition could be due at least in part to readthrough transcription from the *yqhD* promoter. The *dkgA* gene has been reported to have its own promoter immediately upstream [5], but this promoter has not been characterized in detail other than the location of transcription initiation. The presence of transcripts extending through the intergenic region between *yqhD* and *dkgA* was investigated by qPCR, using primers flanking the *yqhD-dkgA* gap (Table 1). cDNA derived from RNA of LY180 cells grown both with and without furfural was used as template. Primer pairs for the *yqhC*, *yqhD*, and *dkgA* genes were also included (Table 1), together with a primer pair for the *birA* gene, which was used as a reference. The C_T values (number of PCR cycles to reach the threshold) was measured for each of the genes for RNA isolated before and after furfural treatment (Table 2). The ΔC_T value (number of PCR cycles relative to the *birA* reference) was obtained for each sample, and the $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T value for the minus furfural samples from the ΔC_T value for the plus furfural samples (Table 2). The C_T values for control cDNA template reactions without reverse transcriptase were greater than 31 (data not shown), indicating that gDNA contamination of the samples was not problematical.

The $\Delta\Delta C_T$ values for *yqhC*, *yqhD*, and *dkgA* were all negative (Table 2), i.e., fewer cycles were required to reach the threshold following furfural treatment compared with prior to treatment. These results are consistent with the microarray data in Fig. 2. For the *yqhD-dkgA* intergenic region, a PCR product of the expected size (372 bp) for bridging the gap between the genes was recovered (data not shown), and based on the $\Delta\Delta C_T$ value, the level of the

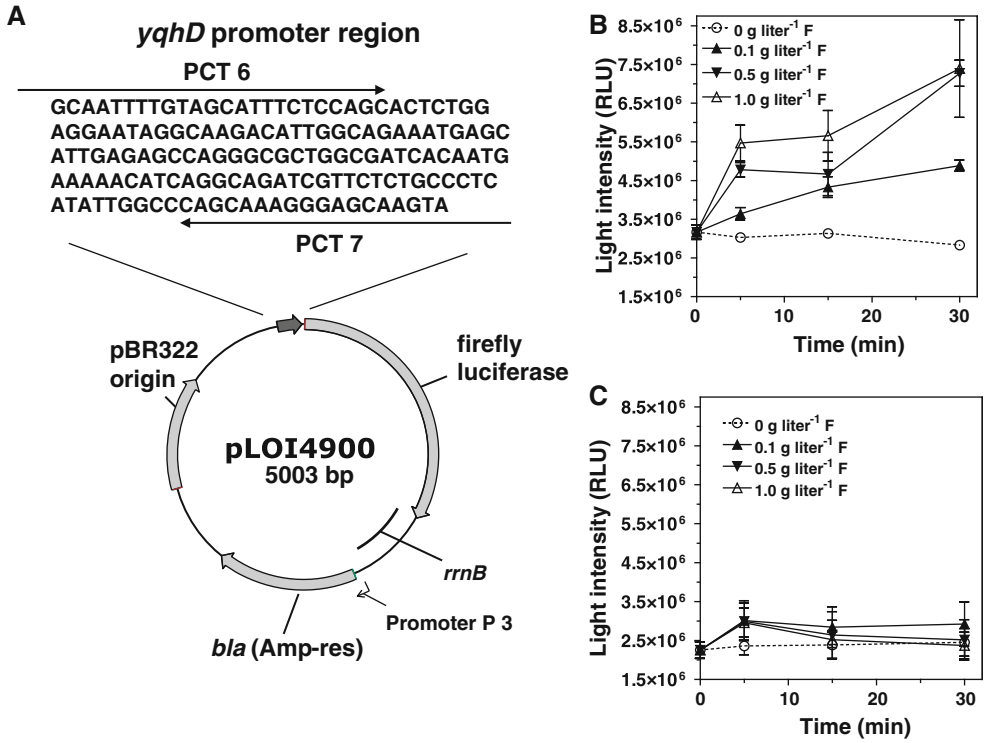

Fig. 4 Measurement of *yqhD* promoter activity using the firefly luciferase reporter. **a** The structure of the plasmid pLOI4900 carrying the firefly luciferase gene fused to the promoter region upstream from *yqhD*. The sequence of the 151-bp region immediately upstream from the *yqhD* ORF is indicated, together with the locations of the primers PCT 6 and PCT 7. **b** Effect of furfural addition on firefly luciferase expression from the *yqhD* promoter in LY180. Cultures of LY180 carrying pLOI4900 were grown in AM1 with 50 g l^{-1} xylose to OD_{550} = 0.4, and samples taken immediately before (t = 0) and at 5, 15, and 30 min after addition of 0, 0.1, 0.5, or 1 g l^{-1} furfural. Luciferase activity is expressed as relative luminescence units (RLU) per 0.4 OD_{550} units. *Error bars* indicate SEM. **c** Firefly luciferase expression from the yqhD promoter in LY180*ΔyqhC*(pLOI4900). Conditions and symbols as for Fig. 4b

Table 2 qPCR of RNA from LY180 cells isolated before and after treatment with furfural

Gene	C_T[a] LY180 − F	C_T LY180 + F	ΔC_T LY180 − F[b]	ΔC_T LY180 + F[b]	$\Delta\Delta C_T$[c]
birA	22.1	22.7	–	–	–
yqhC	23.6	21.3	1.5	−1.4	−2.9
yqhD	21.8	17.9	−0.3	−4.8	−4.5
yqhD-dkgA	23.8	19.0	1.7	−3.7	−5.4
dkgA	21.5	18.8	−0.6	−3.9	−3.3

[a] C_T values (the number of cycles to reach the threshold) are averages of two measurements

[b] $\Delta C_T = C_T$(gene) − C_T (*birA* ref)

[c] $\Delta\Delta C_T = (\Delta C_T$ LY180 + F) − (ΔC_T LY180 − F)

yqhD-dkgA intergenic transcript was increased following addition of furfural (Table 2). Transcription from the *yqhD* promoter therefore appears to extend into the adjacent *dkgA* gene, and is inducible by furfural.

Orthologs of *yqhC* and associated genes in other bacterial genera

The presence of *yqhC* orthologs and relatives of the *yqhD* and *dkgA* genes in bacteria other than *E. coli* was investigated by searching genomes available at EcoCyc [12]. Of the 46 genera containing orthologs of *E. coli yqhC*, 40 were Gram-negative organisms. The proximity of genes resembling *yqhD* and *dkgA* to *yqhC* orthologs was examined by using the EcoCyc multigenome browser (Fig. 5). Most genera (34 of 46 genera) did not contain any recognizable *yqhD* or *dkgA* orthologs near the *yqhC* ortholog (e.g., *Acinetobacter* sp. and *Xanthomonas campestris*, Fig. 5) including all of the Gram-positive organisms. However, 24 of these 34 genera did contain either a *yqhD* or *dkgA* ortholog elsewhere in the genome. Five of the 46 genera contained a nearby *yqhD* ortholog in addition to *yqhC* but no *dkgA* ortholog

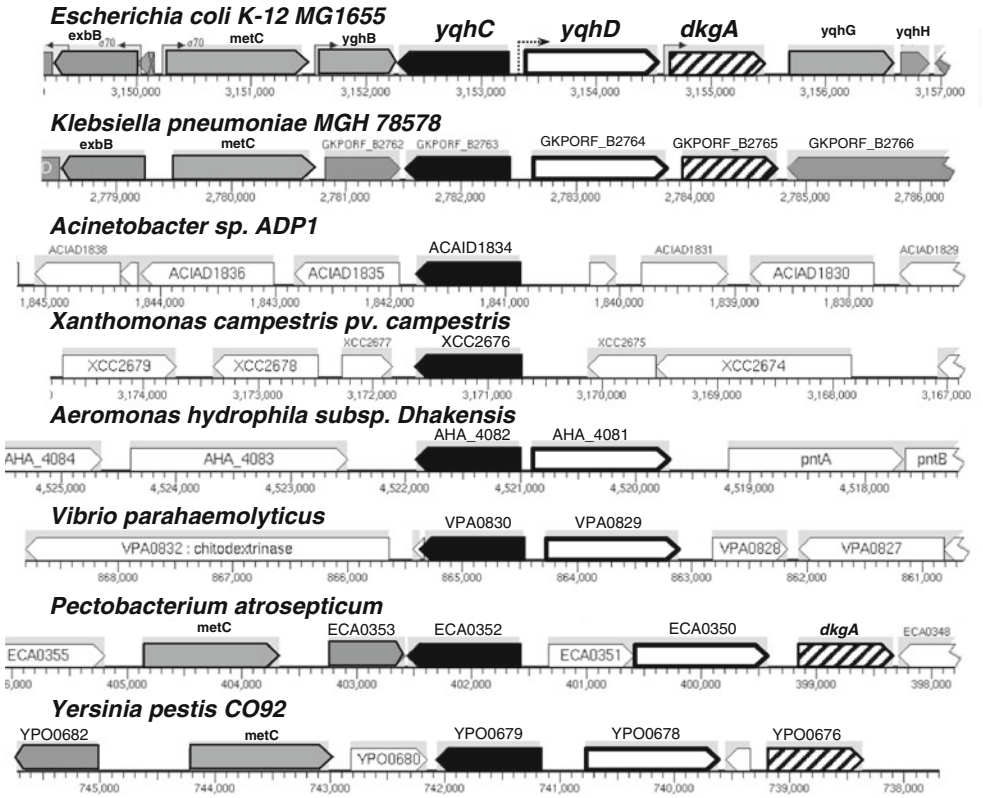

Fig. 5 Arrangement of *yqhC* orthologs in the genomes of selected bacteria. The arrangement of genes surrounding *yqhC* is shown for *Escherichia coli* K12 (*top line*), and for a selection of genera containing a *yqhC* ortholog. The *yqhC* ortholog in each genome is shown in *solid black* and is oriented leftward. *YqhD* orthologs where present are to the right of the *yqhC* ortholog, and indicated by *white arrows* with a *thick outline*. *DkgA* orthologs are shown by *cross-hatched arrows*. The numbers below the genes represent the coordinates in the genomes. The alignment of the genomes and detection of orthologs was created using the EcoCyc multigenome browser [12]

(*Aeromonas hydrophila* and *Vibrio parahaemolyticus* for example), and one (*Thermotoga maritima*, not shown) contained a nearby *dkgA* ortholog without a nearby *yqhD* ortholog. Five genera contained all three genes with an arrangement similar to *E. coli*. This group was limited to the Enterobacteriaceae and included: *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella, Pectobacterium*, and *Yersinia.* The arrangement of the genes in *P. atrosepticum* is unusual in that there is a probable nitroreductase gene, ECA0351, between the *yqhC* ortholog (ECA0352) and the *yqhD* ortholog (ECA0350).

The proximity of *yqhC* orthologs to orthologs of *yqhD* and in some cases *dkgA* suggests that the regulation of these orthologous genes may be like that in *E. coli*. The assembly of the *yqhC-yqhD-dkgA* cluster in evolution appears to have been a stepwise process, with association between *yqhC* and *yqhD* occurring first, followed by the addition of *dkgA* in the ancestor of the Enterobacteriaceae.

Conclusions

Experimental results presented here are consistent with YqhC acting as a positive transcriptional regulator of two NADPH-dependent oxidoreductases, *yqhD* and *dkgA*, that are involved in furfural reduction in *E. coli*. These results (Fig. 2) are also consistent with upregulation of *yqhC* transcription by the YqhC protein upon addition of furfural. This response is not limited to furfural. A diverse group of aldehydes including methylglyoxal and cinnamaldehyde also induced *yqhC*-dependent expression of *yqhD* and *dkgA*. Considering the structural diversity of these compounds, it is possible that induction represents a response to physiological effects of these aldehydes rather than direct binding to YqhC. In the furfural-resistant mutant EMFR9, silencing the expression of these genes was accompanied by insertion of IS10 into *yqhC*. Deletion of *yqhC* had a similar effect on expression and furfural tolerance. Both *yqhD* and *dkgA* encode furfural reductase activities that exhibit a low K_m for NADPH. Furfural reduction by these enzymes is proposed to inhibit growth and biosynthesis by depleting the pool of this essential cofactor.

The promoter upstream from *yqhD* is activated by furfural, and based on qPCR transcription extends into the *dkgA* gene downstream. *YqhG*, the gene immediately downstream from *dkgA*, is upregulated by furfural by 2.2-fold in

LY180, and like *yqhD* and *dkgA* is not expressed in EMFR9 even in the presence of furfural.

The level of tolerance to furfural in EMFR9 (MIC 2.0 g l^{-1} furfural) exceeds the level seen following inactivation of *yqhC* (MIC 1.5 g l^{-1} furfural), suggesting that other mutations may be involved in EMFR9. Future experiments are needed to identify additional mechanisms that contribute to furfural tolerance in EMFR9.

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