Functional identification of *ygiP* as a positive regulator of the *ttdA-ttdB-ygjE* operon

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Received 9 December 2005
Revised 8 March 2006
Accepted 13 March 2006

Functional characterization of unknown genes is currently a major task in biology. The search for gene function involves a combination of various *in silico*, *in vitro* and *in vivo* approaches. Available knowledge from the study of more than 21 LysR-type regulators in *Escherichia coli* has facilitated the classification of new members of the family. From sequence similarities and its location on the *E. coli* chromosome, it is suggested that *ygiP* encodes a *lysR* regulator controlling the expression of a neighbouring operon; this operon encodes the two subunits of tartrate dehydratase (TtdA, TtdB) and YgiE, an integral inner-membrane protein possibly involved in tartrate uptake. Expression of tartrate dehydratase, which converts tartrate to oxaloacetate, is required for anaerobic growth on glycerol as carbon source in the presence of tartrate. Here, it has been demonstrated that disruption of *ygiP*, *ttdA* or *ygjE* abolishes tartrate-dependent anaerobic growth on glycerol. It has also been shown that tartrate-dependent induction of the *ttdA-ttdB-ygjE* operon requires a functional YgiP.

INTRODUCTION

Analysis of the *Escherichia coli* complete genomic sequence has revealed more than 900 coding sequences for which no function has yet been identified (www.shigen.nig.ac.jp/ecoli/pec/index.jsp). Genes of unknown function identified by sequencing programs include non-functional pseudo-genes, genes that are not expressed under standard laboratory conditions and genes involved in unknown metabolic pathways. The search for the function of such unknown genes involves studying their sequence, location on the chromosome and regulatory sequences, and the detection of physiological effects of their disruption in terms of morphology, growth ability, stress response and transcriptome modifications.

Regulators of the LysR family are present in many bacteria and are involved in the control of various processes, including virulence, stress responses and metabolism (Schell, 1993). LysR family regulators can be easily identified by sequence comparisons because they all contain a characteristic helix–turn–helix DNA-binding motif in the N-terminal part (Schell, 1993). The DNA-binding sites of LysR family members are generally 80–35 bp upstream from the transcription start site (Schell, 1993). No consensus sequence has been identified for the binding sites of LysR family members. More than half of the 21 characterized LysR regulators in *E. coli* regulate genes involved in catabolic or anabolic processes (Schell, 1993). The structural genes of most of these LysR type regulators are neighbours of their target genes.

Other characterized LysR family members, which control various process like transport, stress responses and DNA replication, do not map close to their targets (Schell, 1993). There are about 24 putative LysR regulators in *E. coli*. One, *ygiP*, is transcribed divergently from an operon containing two characterized genes (*ttdA* and *ttdB*) encoding the two subunits of a tartrate dehydratase (Reaney *et al.*, 1993). Tartrate dehydratase converts tartrate to oxaloacetate. *E. coli* can use L-, D- and *meso*-tartrate as reducible substrates for anaerobic growth on glycerol (Reaney *et al.*, 1993). Tartrate dehydratase activity is induced by L- and *meso*-tartrate during anaerobic growth with glycerol and may be repressed by glucose and oxygen (Reaney *et al.*, 1993). The sequence of the last gene of the operon, *ygiE*, is consistent with it encoding an integral inner-membrane protein potentially involved in tartrate uptake (Pos *et al.*, 1998). Here, we report the functional identification of *ygiP* as a positive regulator required for the L-tartrate-dependent induction of the *ttdA-ttdB-ygjE* operon. Inactivation of *ygiP*, *ttdA* or *ygjE* abolishes L-tartrate-dependent anaerobic growth on glycerol. In a *ygiP* mutant, the expression of the *ttdA-ttdB-ygjE* operon is not induced by L-tartrate.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Bacteria were grown aerobically at 37 °C in LB rich medium or in M63 (Miller, 1972) supplemented

Table 1. Stains and plasmids used in this study

Strains	Relevant characteristics	Source or reference
JOE SM551 (λ InCh1)	SM551 (λ InCh1) (Km ^R)	Boyd <i>et al.</i> (2000)
MG 1655	F ⁻	Blattner <i>et al.</i> (1997)
POP3	<i>araD139 ΔlacU169 rpsL relA thi</i>	Raibaud <i>et al.</i> (1985)
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46</i> <i>thi relA1 lac⁻ F'[proAB⁻ lacI^q</i> <i>lacZΔM15 Tn10 (tet^R)]</i>	Bullock <i>et al.</i> (1987)
FB85	F ⁻ <i>hcaR::cat</i> (Cm ^R)	Turlin <i>et al.</i> (2001)
FB827	F ⁻ <i>ΔlacX74 hcaR::lacZ arcA::Km</i>	Turlin <i>et al.</i> (2005)
BW25113	<i>lacI^q rrnB_{T14} lacZ_{WJ16} hsdR514</i> <i>araBAD_{AH33} rhaBAD_{LD78}</i>	Datsenko & Wanner (2000)
BW25113 <i>fnr::Km</i>	<i>lacI^q rrnB_{T14} lacZ_{WJ16} hsdR514</i> <i>araBAD_{AH33} rhaBAD_{LD78} fnr::Km</i>	This study
JD24079	KP7600 F ⁻ <i>lacI^q lacZΔM15 galK2</i> <i>galT22 λ^- in (rrnD-rrnE)1 ygiE::Km</i> <i>miniTn10</i>	NIG collection (transposon insertion disruptant: http://shigen.lab.nig.ac.jp/ ecoli/strain/nbrp/resource.jsp)
MG 1655 <i>ygiP</i>	F ⁻ <i>ygiP::cat</i> (Cm ^R)	This study
MG 1655 <i>ttdA</i>	F ⁻ <i>ttdA::cat</i> (Cm ^R)	This study
MG 1655 <i>ygiE</i>	F ⁻ <i>ygiE::Km miniTn10</i> (Km ^R)	This study
POP3	POP3	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R)	
POP3 <i>ygiP</i>	POP3 <i>ygiP::cat</i>	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R , Cm ^R)	
POP3 <i>ttdA</i>	POP3 <i>ttdA::cat</i>	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R , Cm ^R)	
POP3 <i>ygiE</i>	POP3 <i>ygiE::Km</i>	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R , Km ^R)	
POP3 <i>fnr</i>	POP3 <i>fnr::Km</i>	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R , Km ^R)	
POP3 <i>arcA</i>	POP3 <i>arcA::Km</i>	This study
<i>attλ::ttdA-lacZ</i>	<i>attλ::ttdA-lacZ</i> (Ap ^R , Km ^R)	
Plasmids		
pBR322	Ap ^R , Tc ^R	Sambrook <i>et al.</i> (1989)
pAM239	Sp ^R	Letoffe <i>et al.</i> (1994)
pHP 45 Ω - <i>cat</i>	Ap ^R , Cm ^R	Fellay <i>et al.</i> (1987)
pGP/ <i>lacZ</i> t	Ap ^R	Rossi <i>et al.</i> (2003)
pKD13	Ap ^R , Cm ^R	Datsenko & Wanner (2000)
pGP/ <i>ttdA-lacZ</i>	Ap ^R	This study
pBR322 <i>ttdA-lacZ</i>	Ap ^R	This study
pAM239:: <i>ygiP</i>	Sp ^R	This study
pAM239:: <i>ttdA-B</i>	Sp ^R	This study

with glucose (0.4%), glycerol (0.4%) or gluconate (0.4%), as carbon sources. When specified, tartrate was added to a final concentration of 0.4%. Anaerobic cultures were grown in Erlenmeyer flasks with rubber stoppers and with shaking. Anoxia was achieved by bubbling with N₂ for 2 min. Antibiotics were added to the following final concentrations (μ g ml⁻¹): ampicillin (Ap), 100; spectinomycin (Sp), 100; chloramphenicol (Cm), 20; kanamycin (Km), 25. Solid medium contained 1.5% Difco agar. Deep agar growth tests were performed in 63 B1 minimal medium containing 0.3% agar supplemented with various carbon sources. Fifteen millilitres of the growth test medium heated at 50 °C were introduced into a screw-topped tube. One hundred microlitres of the cell suspension (OD₆₀₀ = 1) was added and gently mixed with a pipette into the test

medium. The test tubes were then closed with a screw cap and incubated at 37 °C. After 72 h, the OD₆₀₀ of the samples in the test tubes was measured in a Spectronic 20D spectrophotometer.

Genetic techniques. P1 lysates and transductions were performed as described by Miller (1972). Cells were transformed by the calcium chloride method (Maniatis *et al.*, 1982) or electroporation (Dower *et al.*, 1988).

DNA manipulations. Chromosomal DNA was isolated according to the method of Marmur (1961). Large-scale plasmid DNA preparations were made using the Plasmid Maxi Kit (Qiagen) as recommended by the manufacturer. Small-scale plasmid DNA preparations were done as described by Birnboim & Doly (1979). Restriction,

modification and ligation were carried out according to the manufacturer's recommendations. DNA fragments of interest were separated by gel electrophoresis and isolated by using the QIAquick Gel Extraction Kit (Qiagen). DNA fragments were amplified in a Hybaid PCR thermocycler, using the Expand High Fidelity PCR System (Boehringer).

Disruption of *ygiP* and *ttdA*. Non-polar mutations that delete the entire *ygiP* or *ttdA* gene were created by allelic exchange with the non-polar *cat* gene cassette from a derivative of pHP45Ω (Fellay *et al.*, 1987). A three-step PCR procedure was used to produce a PCR product in which the Cm gene cassette is flanked by arms of about 900 bp corresponding to the sequences upstream from the start codon and downstream from the stop codon of the gene of interest.

The *cat* gene cassette (0.9 kb) was amplified from *E. coli* FB85 (see Table 1) using the primers cat3 and cat5 (Table 2). The primers used for *ygiP* were *ygiPav1AV* and *casav1am* for the left 900 bp homology arm, and *casamtav* and *Amtam* for the right 900 bp homology arm. (Table 2). For *ttdA*, the primers used were *ygiPamamt* and *ygiPamtavl* for the left 900 bp homology arm, and *ttdABavlamt* and *ttdABavl1* for the right 900 bp homology arm (Table 2).

The 2.7 kb PCR products resulting from the three-step procedure and in which the *ygiP* or *ttdA* gene had been substituted with a Cm cassette were introduced into *E. coli recD* strain V355 by electroporation (Russell *et al.*, 1989). Correct chromosomal insertion was checked by PCR amplification using *cat* primers cat3 and cat 5 in combination with *ygiP* external primers *ygiPav1av* and *Amtam*, or in combination with *ttdA* external primers *ygiPamamt* and *ttdABavl1* (Table 2). Mutations were then introduced into MG1655 and POP3 *attλ::ttdA-lacZ* by P1 transduction.

Disruption of *fnr*. The *fnr*-disrupted strain was constructed using PCR products and a standard one-step gene inactivation protocol for disruption of chromosomal genes that is based on the high efficiency of the phage λ Red recombinase using BW25113 (Datsenko & Wanner, 2000). The PCR product is amplified from pKD13 as a template with primers PO309 and PO310 (Table 2). To check the mutation, PCR was performed with an upstream-specific primer for the *fnr* gene, PO312, and a Km-cassette-specific primer, K1 (Datsenko & Wanner, 2000) (Table 2). The *fnr* mutation was then introduced into MG1655 and POP3 *attλ::ttdA-lacZ* by P1 transduction.

Plasmid constructions

pGPttdA-lacZ. A DNA fragment corresponding to 390 bp upstream from the *E. coli* MG1655 *ttdA* gene was amplified with primers *fttdAAMT* and *fttdAAVL* (Table 2). The PCR products were digested with *EcoRI* and *SmaI* and ligated to pGPlacZtt (Rossi *et al.*, 2003) digested with *EcoRI* and *SmaI* to create pGPttdA-lacZ.

pAM239::ygiP. *ygiP* was amplified from chromosomal MG1655 DNA using primers *ygiPamt* and *ygiPavl* (Table 2). After filling-in by T4 polymerase, the PCR fragment was inserted into the *HincII* site of pAM239 giving pAM239::ygiP.

pAM239::ttdA-B. A *ttdA-B* DNA fragment was amplified from chromosomal MG1655 DNA with primers *ttdA-Bamt* and *ttdA-Bavl* (Table 2). After filling-in by T4 polymerase, the PCR fragment was inserted into the *HincII* site of pAM239 giving pAM239::ttdAB.

Insertion of *ttdA-lacZ* into the *E. coli* chromosome. The *EcoRI*–*HindIII* fragment from pGPttdA-lacZ, containing the *ttdA-lacZ* transcriptional fusion, was inserted between the *EcoRI* and *HindIII* sites of pBR322 to give pBR322ttdA-lacZ. The fusion was

Table 2. Primers used in this study

Fragment	Forward primer (5'–3')	Reverse primer (5'–3')
<i>cat</i> cassette	AATTTCTGCCATTTCATCCGC	TTGGATCGGCACGTAAGAGGT
<i>ygiP</i> left	GTCAAGCGTAACCTGAGGCAATCCG	ACCTCTTACGTGCGCATCAAAATCCACCAGAAATCCTCGGG
<i>ygiP</i> right	GCGGATGAATGGCAGAAATTATTATCCGCAGTTGTGATAAGC	AGTTCTGCCGCTTTGGATTGG
<i>ttdA</i> left	CTACATGACCTGATAGCTTCATCG	GCGGATGAATGGCAGAAATTTTGTCTGTGGAAAAATTCACCGCC
<i>ttdA</i> right	ACCTCTTACGTGCGCATCAAAATCCTGACAAACCCGATCAAAGC	AAGGAGAGGGGTGAGCCTCTCCGG
Pr <i>ttdA</i>	AAAGAAATCCAACGCGATGTTCTGTAACAACGTTGGC	AAACCCGGGTTTTTGTATTGTCTGTGGAAAAATTCACCC
<i>ygiP</i>	AATACACTGGCTTTATCAACAACATG	AGATCCCCAGTGTATTCTGGTGG
<i>ttdA-B</i>	AAACTGGAGTTGCCATGATGAGC	AAGGAGAGGGGTGAGCCTCTCCGG
<i>fnr::Km</i>	TGCTTAGACTTACTTGCTCCCTAAAAGATGTTAAATTGACAA- ATATCAATTACGGCTTGAGCAGACCTATGATTCGGGGGATCCG- TCGACC	TCTGCCAGATCAATAAATCAGAAAAATTTAATGATATGACAGAA- GGATAGTGAGTTATGCGGAAAAATCAGGCAACGGTGTAGGCT- GGAGCTGCTTCG
<i>kmr</i> control	TTTGTGCCAGCTTTGTTACAC	CAGTCATAGCCGAATAGCCT
	PO312	K1

transferred by homologous recombination into λ InCh (http://beck2.med.harvard.edu/resources/InCh/lambda_InCh_frames.htm) (Boyd *et al.*, 2000). The recombinant phage was used to lysogenize strain POP3. After integration and stabilization of the recombinant lysogens, integration of the transcriptional fusions at the *att* site in the *E. coli* chromosome was confirmed by PCR.

Preparation of labelled cDNA, array hybridization and capture of data

RNA isolation. RNA was isolated from bacteria cultured anaerobically in 50 ml M63B1 supplemented with gluconate as carbon source in the presence and absence of tartrate. A sample of each culture was taken after 6 h incubation at 37 °C ($OD_{600}=0.2-0.3$). RNA was isolated using the Qiagen RNeasy Protect, RNeasy Mini Kit and RNeasy-free DNase sets, according to the manufacturer's instructions.

Oligonucleotide microarray construction and transcriptomic experiments. *E. coli* Oligo Library (Sigma-Genosys) was printed by a Lucidea microarray spotter (Amersham Biosciences) on Corning UltraGAPS slides (Corning) and treated with a DNA cross-linking reagent (Amersham Biosciences) diluted 1:2. Microarray experiments were performed as described by Kershaw *et al.* (2005). For each experiment, 20 µg total RNA was used to produce Cy3- and Cy5-labelled cDNA using the CyScribe Post-Labeling Kit (Amersham Biosciences/GE Healthcare), according to the manufacturer's instructions. The array slides were prehybridized for 4 h in filtered prehybridization solution (25 % formamide, $5 \times$ SSC, 10 mg BSA l⁻¹, 0.1 % SDS) at 42 °C, and then briefly washed in ethanol and dried by centrifugation at 1000 g for 5 min. The probe in hybridization solution [25 % formamide, $5 \times$ SSC, 0.1 % SDS, 0.1 µg poly(A) ml⁻¹, $1 \times$ Denhardt's solution and 80 pmol Cy3 and Cy5 combined probe] was heated to 95 °C for 3 min and hybridized with an ArrayBooster (Adveritix), according to the manufacturer's instructions. After hybridization, slides were washed in wash buffer I ($2 \times$ SSC/0.1 % SDS) for 2 min at 42 °C, wash buffer II ($0.2 \times$ SSC) for 2 min at room temperature and then twice in wash buffer III ($0.05 \times$ SSC) for 2 min at room temperature. All washes included vigorous shaking of the microarray slides. The slides were dried by centrifugation at 1000 g for 5 min and then analysed using an FLA8000 scanner (Fuji film).

Microarray data analysis. The 4289 individual genes on the microarrays were classified into three groups. To compare the wild-type strains and their derivatives, we measured Cy3 signal intensities for the reference strain and Cy5 signal intensities for the mutant. To analyse the effect of the addition of tartrate, we measured Cy3 signal intensities for the strain grown in the absence of tartrate and Cy5 signal intensities for the strain grown in the presence of tartrate. Spots were classified into three groups. In group 1, both Cy3 and Cy5 signal intensities were higher than 0.5 (the value of 0.5 is low, but nevertheless significant in this experiment: the negative control oligo spot, an O157-specific oligo, gave intensities below 0). In group 2, either the Cy3 or Cy5 signal intensity was more than 1 SD higher than the negative intensity value. In group 3, both signals were lower than the negative intensity value. Initially, all spots of group 1 were normalized by defining the mean of the ratios (Cy5/Cy3) of all spots as 1.0. The ratios of group 2 spots could not be determined by this method due to absence of significant Cy3 or Cy5 fluorescence. Group 2 spots with high Cy3 or Cy5 intensity values (>1000) were selected. Spots in group 3 were not considered further, being undetectable. Genes that were significantly differently expressed in different conditions were then selected according to the following criteria, provided that similar values were seen in two independent experiments. From group 1, we selected genes that showed relative ratios less than 0.33 or greater than 3.0 as being down- or upregulated, respectively. For genes classified into group 1

in one experiment and group 2 in another identical experiment, those that showed a relative ratio less than 0.33 or higher than 3.0 in one hybridization and high intensity values (>1000) in the other were scored as being down- or upregulated, respectively. Genes classified into group 2 in both hybridizations were recognized as genes with altered regulation. Complete microarray datasets, in compliance with MIAME guidelines (<http://www.mged.org/miame>), are available at <http://genome.naist.jp/array/> (raw and processed data).

β -Galactosidase assay. β -Galactosidase was assayed by the method described by Pardee *et al.* (1959).

RESULTS AND DISCUSSION

Disruption of *ygiP*, *ttdA* or *ygiE* abolishes L-tartrate-dependent anaerobic growth on glycerol

Because of its position on the *E. coli* chromosome, *ygiP* may be a positive regulator for the neighbouring *ttdA*-*ttdB*-*ygiE* operon. We therefore investigated the effect of *ygiP* disruption on tartrate-dependent anaerobic growth on glycerol. As a control, the effect of *ttdA* disruption was also investigated. The disrupted *ygiP* and *ttdA* genes (see Methods) were recombined into the chromosome of *E. coli* *recD* strain V355 and then transferred to strain MG1655 by P1 transduction. Mutation *ygiE::Km* was transferred from strain JD24079 to strain MG1655 using P1 transduction (see Methods). Strain MG1655 and its *ygiP*, *ttdA* and *ygiE* derivatives were tested for aerobic growth in LB and M63 B1 medium with glucose as carbon source. The growth of each of the *ygiP*, *ttdA* and *ygiE* mutants was similar to that of the parental wild-type strain (data not shown). The *ygiP*, *ttdA* and *ygiE* mutants and the parental wild-type strain were then tested for aerobic and anaerobic growth on M63 B1 in deep 0.3 % agar tubes containing various carbon sources (see Methods). Neither *ygiP*, nor *ttdA*, nor *ygiE* disruption had any effect on aerobic or anaerobic growth with glucose as carbon source (data not shown). With succinate as carbon source, growth of wild-type and mutant strains was visible only at the top of the agar (data not shown). Thus the strictly aerobic catabolism of succinate does not occur inside the deep agar, suggesting anaerobic growth conditions. With tartrate as carbon source, only the wild-type strain grew anaerobically, but did so poorly. Growth of all three strains was strictly aerobic with glycerol as carbon source. When tartrate was added to glycerol, the wild-type strain grew both aerobically and anaerobically ($OD_{600}=0.35$ at the bottom of the test tube). In contrast, the *ygiP*, *ttdA* and *ygiE* mutants did not grow anaerobically in the presence of glycerol and tartrate ($OD_{600}=0.035$ at the bottom of the test tube). Thus, the disruption of *ygiP* generates a *TtdA*⁻ phenotype for tartrate-dependent anaerobic growth on glycerol. This is consistent with *YgiP* regulating the neighbouring *ttdA*-*ttdB*-*ygiE* operon. The tartrate-dependent anaerobic growth of the *ygiP* strain on glycerol was restored by introducing a plasmid expressing either *ygiP* or *ttdA* and *ttdB* under the control of a *lac* promoter (data not shown). In contrast, restoration of anaerobic growth on glycerol to the *ttdA* mutant strain required a plasmid containing *ttdA* and *ttdB*

Table 3. Regulation of the expression of the *ttdA-lacZ* transcriptional fusion

Strain POP3 *attλ::ttdA-lacZ* was grown aerobically or anaerobically in M63 minimal medium with glucose (Glu), glycerol (Gly) or gluconate (Gln) as carbon sources in the presence or absence of L-tartrate. For each experiment, the β -galactosidase activity was assayed, as described in Methods, three times (OD₆₀₀ 0.2, 0.3 and 0.4) during growth. The β -galactosidase specific activities reported are means of at least three experiments; the error range represents 1 SD.

Carbon source	β -Galactosidase specific activity	
	+ O ₂	-O ₂
Glu	6 ± 0.6	5 ± 0.5
Glu + tartrate	5 ± 0.5	7 ± 0.7
Gly	6 ± 0.6	8 ± 0.8
Gly + tartrate	4 ± 0.4	40 ± 4
Gln	6 ± 0.6	6 ± 0.6
Gln + tartrate	7 ± 0.7	45 ± 4.5

under the control of the *lac* promoter; *ygiP* did not complement the deficiency (data not shown).

Regulation of *ttdA* expression level

The inability to use tartrate for anaerobic growth could be a consequence of a loss of tartrate dehydratase activity (Reaney *et al.*, 1993), possibly due to insufficient expression of the *ttdA-ttdB-ygjE* operon. We constructed a *ttdA-lacZ* transcriptional fusion and inserted it into the chromosome of strain POP3 (POP3 *attλ::ttdA-lacZ*) to study the regulation of the operon. We tested growth conditions to identify those allowing better expression of the *ttdA-lacZ* fusion (Table 3). Cells grown aerobically with glucose or glycerol as carbon source expressed a low basal level of β -galactosidase activity (about 4–7 units). This low level of expression of the *ttdA-lacZ* transcriptional fusion agrees with the absence of tartrate dehydratase activity in *E. coli* grown in the presence of oxygen (Reaney *et al.*, 1993). Strain

POP3 *attλ::ttdA-lacZ* grown with glucose or glycerol as carbon source expressed *ttdA-lacZ* fusion similarly in the presence and absence of oxygen. For bacteria grown anaerobically with glycerol as carbon source, the presence of tartrate increased *ttdA-lacZ* expression about fivefold. In contrast, when glucose was used as carbon source, the addition of tartrate had no effect (Table 3). These results are consistent with those showing that, with glycerol as carbon source, tartrate dehydratase activity is induced by L-tartrate but repressed by glucose and oxygen (Reaney *et al.*, 1993). As *ygiP* and *ttdA* mutants are impaired for anaerobic growth with glycerol (see above) we used gluconate, which does not provoke catabolic repression of *ttdA-ttdB-ygjE* expression. We observed L-tartrate-dependent induction of the *ttdA-lacZ* fusion with gluconate as carbon source (Table 3).

YgiP is required for *ttdA-ttdB-ygjE* expression

To investigate the effect of *ygiP* disruption on expression of the *ttdA-ttdB-ygjE* operon, we constructed strain POP3 *ygiP attλ::ttdA-lacZ*. Strains POP3 *attλ::ttdA-lacZ* and POP3 *ygiP attλ::ttdA-lacZ* were grown in conditions inducing and non-inducing for *ttdA-lacZ* expression and then assayed for β -galactosidase activity. Disruption of *ygiP* had no effect on expression of the *ttdA-lacZ* fusion during aerobic growth on gluconate in either the presence or absence of tartrate (Table 4). In contrast, during anaerobic growth on gluconate, *ygiP* disruption completely abolished the tartrate-dependent induction of the *ttdA-lacZ* fusion (Table 4). Thus YgiP may be a positive regulator responsible for the tartrate-dependent induction of the *ttdA-ttdB-ygjE* operon.

Tartrate acts as the co-inducer of *ttdA-ttdB-ygjE* expression

During anaerobic growth, addition of L-tartrate increases *ttdA-lacZ* expression fivefold. This effect may be due to tartrate itself, or to its conversion to oxaloacetate by tartrate dehydratase. We therefore constructed strains POP3 *ygiE attλ::ttdA-lacZ* and POP3 *ttdA attλ::ttdA-lacZ*. For each strain, we measured β -galactosidase activity under conditions inducing and non-inducing for *ttdA-ttdB-ygjE* operon

Table 4. Effect of *ygiP*, *ttdA* or *ygiE* disruption on expression of the *ttdA-lacZ* fusion

Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate (Gln) as carbon source in the presence or absence of L-tartrate. For each experiment, the β -galactosidase activity was assayed, as described in Methods, three times (OD₆₀₀ 0.2, 0.3 and 0.4) during growth. The β -galactosidase specific activities reported are means of at least three experiments; the error range represents 1 SD.

Carbon source	O ₂	β -Galactosidase specific activity			
		POP3 <i>attλ::ttdA-lacZ</i>	POP3 <i>ygiP</i> <i>attλ::ttdA-lacZ</i>	POP3 <i>ttdA</i> <i>attλ::ttdA-lacZ</i>	POP3 <i>ygiE</i> <i>attλ::ttdA-lacZ</i>
Gln	+	4 ± 0.4	7 ± 0.7	9 ± 0.9	8 ± 0.8
Gln	–	6 ± 0.6	5 ± 0.5	12 ± 1.2	12 ± 1.3
Gln + tartrate	+	6 ± 0.6	6 ± 0.6	18 ± 1.8	8 ± 0.8
Gln + tartrate	–	45 ± 4.5	7 ± 0.7	2800 ± 280	14 ± 1.4

expression. Disruption of *ygiE* and *ttdA* had no effect on the expression of the *ttdA-lacZ* fusion in the absence of tartrate (Table 4). Disruption of *ygiE* abolished the tartrate-dependent induction of the *ttdA-lacZ* fusion (Table 4). This shows that YgiE is necessary for the entry of tartrate, which is required for induction of the *ttdA-ttdB-ygiE* operon. In contrast, disruption of *ttdA* substantially increased tartrate induction of the *ttdA-lacZ* fusion (Table 4). This latter result suggests that when tartrate is not degraded, in this case due to a *ttdA* mutant, it accumulates inside the cell resulting in very strong induction of the *ttdA-ttdB-ygiE* operon. Thus, tartrate is a co-inducer of the *ttdA-ttdB-ygiE* operon.

Genome-wide transcriptome analysis of target genes for YgiP

Both its position on the chromosome and genetic analysis indicate that YgiP is a positive regulator required for L-tartrate-dependent anaerobic growth on glycerol. The effect of *ygiP* disruption could be the consequence of YgiP having a direct and specific effect on *ttdA-ttdB-ygiE* operon expression, or of pleiotropic regulatory activity. We used microarray analysis to investigate the possible pleiotropic activity of YgiP, using bacteria grown anaerobically with gluconate in the presence or absence of tartrate. Three different transcriptome profiles were determined and compared: the transcriptome profiles of strain MG1655 grown in the presence and absence of tartrate; MG1655 wild-type and *ttdA* mutant; and MG1655 and its *ygiP* mutant. In the wild-type strain, tartrate strongly induced only the expression of the *ttdA-ttdB-ygiE* operon. Thus, inducing conditions for tartrate dehydratase activity induce the expression of the *ttdA-ttdB-ygiE* operon but have little effect on the rest of the transcriptome. The transcription product of the *ttdA-ttdB-ygiE* operon is absent from the *ttdA* mutant even when bacteria are grown under inducing conditions. The *ttdA* disruption did not significantly modify the expression of other genes in either the presence or absence of tartrate. Thus, the absence of tartrate dehydratase activity does not cause significant alteration of the transcriptome. The comparison of MG1655 and its *ygiP* derivative showed that tartrate-dependent induction of the *ttdA-ttdB-ygiE* operon is abolished by *ygiP* disruption. Furthermore, the effect of *ygiP* disruption on transcriptome expression was mainly restricted to the *ttdA-ttdB-ygiE* operon, consistent with YgiP being a specific regulator. The physiological effects of *ygiP* disruption could all be consequences of the downregulation of the expression of only *ttdA*, *ttdB* and *ygiE*. All transcriptome data and the experimental information are available at <http://genome.naist.jp/array/>, in compliance with the MIAME guidelines (<http://www.mged.org/miame>).

Conclusion

In this report we have predicted, using *in silico* analysis, and confirmed, using a physiological approach, the function of YgiP. Like other regulators of the LysR family involved in the control of metabolic processes, the structural gene for YgiP is adjacent to its target genes. Moreover, transcriptome

analysis clearly showed that the regulatory activity of YgiP, now renamed TtdR, is mainly restricted to the adjacent *ttdA-ttdB-ygiE* operon. Thus, TtdR is essential for anaerobic growth on glycerol as carbon source in the presence of tartrate through its activation of the *ttdA-ttdB-ygiE* operon. Induction of *ttdA-ttdB-ygiE* operon expression, which requires tartrate addition, is strongly enhanced in a *ttdA* strain unable to convert tartrate to oxaloacetate and thus accumulates tartrate. Tartrate is thus the co-inducer of TtdR for induction of the *ttdA-ttdB-ygiE* operon. Tartrate dehydratase activity is induced by L- and meso-tartrate during anaerobic growth with glycerol and is repressed by glucose and aerobiosis (Reaney *et al.*, 1993). In *E. coli*, many transitions from aerobic to anaerobic metabolism are controlled at the transcriptional level by the activity of the dual global regulator FNR (Kang *et al.*, 2005), and by the two-component regulatory system ArcAB (Liu & De Wulf, 2004). The activity of these two regulators is differentially modulated in the presence or in the absence of oxygen. A canonical FNR-binding site had been identified in the promoter of the *ttdA-ttdB-ygiE* operon (Reaney *et al.*, 1993). We first investigated the effect of *fnr* disruption on *ttdA-lacZ* fusion expression in bacteria grown with gluconate and tartrate in the absence or in the presence of oxygen. Disruption of *fnr* had no effect on *ttdA-lacZ* expression in both the absence and presence of oxygen (Table 5). Also, disruption of *arcA* only slightly decreased the tartrate-dependent induction of the *ttdA-lacZ* fusion when bacteria were grown in the absence of oxygen (Table 5). As a consequence, we hypothesize that most of the regulatory effect of oxygen on the *ttdA-ttdB-ygiE* operon expression is mediated by TtdR. TtdR, which shares 44 % identity with OxyR (data not shown), contains two cysteines, cysteine²⁸⁸ and cysteine²⁹⁶, which might participate in the constitution of a redox sensor as has been shown for cysteine¹⁹⁹ and

Table 5. Effect of *fnr* or *arcA* disruption on expression of the *ttdA-lacZ* fusion

Bacteria were grown aerobically or anaerobically in M63 minimal medium with gluconate (Gln) as carbon source in the presence of L-tartrate. For each experiment, the β -galactosidase activity was assayed, as described in Methods, three times (OD₆₀₀ 0.2, 0.3 and 0.4) during growth. The β -galactosidase specific activities reported are means of at least three experiments; the error range represents 1 SD.

Strain	β -Galactosidase specific activity	
	−O ₂	+O ₂
POP3 <i>attλ::ttdA-lacZ</i>	6 ± 0.6	45 ± 4.5
POP3 <i>fnr attλ::ttdA-lacZ</i>	7 ± 0.7	50 ± 5
POP3 <i>arcA attλ::ttdA-lacZ</i>	6 ± 0.6	26 ± 2.6
POP3 <i>ttdA attλ::ttdA-lacZ</i>	18 ± 1.8	2800 ± 280
POP3 <i>ttdA fnr attλ::ttdA-lacZ</i>	20 ± 2	2700 ± 270
POP3 <i>ttdA arcA attλ::ttdA-lacZ</i>	17 ± 1.7	1600 ± 160

cysteine²⁰⁸ of OxyR (Zheng *et al.*, 1998). Sequence analysis of TtdR orthologues deposited in the MBGD database (<http://mbgd.genome.ad.jp/>) showed that these two cysteines are found only in *E. coli* TtdR. In addition, two highly conserved cysteines, cysteine¹⁷⁰ and cysteine¹⁹³, are found in many TtdR orthologues. There are several well characterized cysteines in many redox-sensing proteins, such as SoxS, OxyR, Fnr, ArcB and BagS (Bauer *et al.*, 1999; Malpica *et al.*, 2004; Swem *et al.*, 2003). Cysteines in these proteins exist under two different states, oxidized and reduced. By changes in these states, proteins can sense changes in the cellular redox environment. In the case of TtdR, since there was no induction of *ttdA-lacZ* transcriptional fusion in the presence of tartrate and oxygen, we propose that the redox states of cysteine¹⁷⁰ and cysteine¹⁹³, on one hand, and cysteine²⁸⁸ and cysteine²⁹⁶ on the other hand, change during a transition from anaerobic to aerobic conditions. This change would lead to inactivation of TtdR which, in turn, would not be able to activate the transcription of the *ttdA-B-ygiE* target operon. To test this hypothesis, a deeper analysis of the importance of cysteines for TtdR regulatory function is required.

ACKNOWLEDGEMENTS

We gratefully acknowledge Cécile Wandersman, Elie Dassa and Philippe Delepelaire for critical reading of the manuscript.

REFERENCES

- Bauer, C. E., Elsen, S. & Bird, T. H. (1999). Mechanisms for redox control of gene expression. *Annu Rev Microbiol* **53**, 495–523.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Blattner, F., Plunkett, G., Bloch, C. & 14 other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Boyd, D., Weiss, D. S., Chen, J. C. & Beckwith, J. (2000). Towards single-copy gene expression systems making gene cloning physiologically relevant: *lambda* *InCh*, a simple *Escherichia coli* plasmid-chromosome shuttle system. *J Bacteriol* **182**, 842–847.
- Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. *Biotechniques* **5**, 376–378.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Dower, W. J., Miller, J. F. & Radgale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**, 6127–6145.
- Fellay, R., Frey, J. & Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. *Gene* **52**, 147–154.
- Kang, Y., Weber, K. D., Qiu, Y., Kiley, P. J. & Blattner, F. R. (2005). Genome-wide expression analysis indicates that FNR of *Escherichia coli* K-12 regulates a large number of genes of unknown function. *J Bacteriol* **187**, 1135–1160.
- Kershaw, C. J., Brown, N. L., Constantinidou, C., Patel, M. D. & Hobman, J. L. (2005). The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* **151**, 1187–1198.
- Letoffe, S., Ghigo, J. M. & Wandersman, C. (1994). Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. *J Bacteriol* **176**, 5372–5377.
- Liu, X. & De Wulf, P. (2004). Probing the ArcA-P modulon of *Escherichia coli* by whole genome transcriptional analysis and sequence recognition profiling. *J Biol Chem* **279**, 12588–12597.
- Malpica, R., Franco, B., Rodriguez, C., Kwon, O. & Georgellis, D. (2004). Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proc Natl Acad Sci U S A* **101**, 13318–13323.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pardee, A. B., Jacob, F. & Monod, J. (1959). The genetic control and cytoplasmic expression of inducibility in the synthesis of β -galactosidase of *Escherichia coli*. *J Mol Biol* **1**, 165–178.
- Pos, K. M., Dimroth, P. & Bott, M. (1998). The *Escherichia coli* citrate carrier CitT: a member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts. *J Bacteriol* **180**, 4160–4165.
- Raibaud, O., Gutierrez, C. & Schwartz, M. (1985). Essential and nonessential sequences in malPp, a positively controlled promoter in *Escherichia coli*. *J Bacteriol* **161**, 1201–1208.
- Reaney, S. K., Begg, C., Bungard, S. J. & Guest, J. R. (1993). Identification of the L-tartrate dehydratase genes (*ttdA* and *ttdB*) of *Escherichia coli* and evolutionary relationship with class I fumarase genes. *J Gen Microbiol* **139**, 1523–1530.
- Rossi, M. S., Paquelin, A., Ghigo, J. M. & Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: the inducer and the transported substrate are different molecules. *Mol Microbiol* **48**, 1467–1480.
- Russell, C. B., Thaler, D. S. & Dahlquist, F. W. (1989). Chromosomal transformation of *Escherichia coli* *recD* strains with linearized plasmids. *J Bacteriol* **171**, 2609–2613.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* **47**, 597–626.
- Swem, L. R., Kraft, B. J., Swem, D. L., Setterdahl, A. T., Masuda, S., Knaff, D. B., Zaleski, J. M. & Bauer, C. E. (2003). Signal transduction by the global regulator RegB is mediated by a redox-active cysteine. *EMBO J* **22**, 4699–4708.
- Turlin, E., Perrotte-piquemal, M., Danchin, A. & Biville, F. (2001). Regulation of the early steps of 3-phenylpropionate catabolism in *Escherichia coli*. *J Mol Microbiol Biotechnol* **3**, 127–133.
- Turlin, E., Sismeiro, O., Le Caer, J. P., Labas, V., Danchin, A. & Biville, F. (2005). 3-Phenylpropionate catabolism and the *Escherichia coli* oxidative stress response. *Res Microbiol* **156**, 312–321.
- Zheng, M., Aslund, F. & Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**, 1718–1721.