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Identification of the *Escherichia coli* K-12 *ybhE* Gene as *pgl*, Encoding 6-Phosphogluconolactonase

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We report identification of the *Escherichia coli ybhE* gene as the *pgl* gene that encodes 6-phosphoglucono-lactonase. A tentative identification was first made based on the known approximate location of the *pgl* gene and the similarity of the presumptive ybhE-encoded protein sequence to a known Pgl enzyme. To test this notion, the *ybhE* gene was deleted and replaced with a drug marker. Like previously characterized *pgl* mutants, the *ybhE* deletion mutant had a Blu$^-$ phenotype (dark-blue staining with iodine due to accumulation of starch after growth on minimal maltose) and demonstrated impaired growth on minimal glucose medium when combined with a *pgi* mutation. Biochemical assay of crude extracts for 6-phosphogluconolactonase enzymatic activity showed that *ybhE* encodes this activity. The *ybhE* gene was transferred from the *E. coli* chromosome to an expression vector. This *ybhE* clone complemented both the precise deletion of the *ybhE* gene and a larger deletion, *pgl*Δ*8*, for the Blu$^-$ phenotype and for phosphogluconolactonase activity, confirming that *ybhE* is the *pgl* gene. A newly observed phenotype of *pgl* strains is a lowered frequency of appearance of Bgl$^+$ mutants that can utilize the β-glucoside salicin. This is likely due to poor growth of Bgl$^+$ *pgl* strains on salicin due to the accumulation of 6-phosphogluconolactone.

In *Escherichia coli*, the pentose phosphate (pentose-P) pathway is used to create ribose molecules for biosynthesis, to increase reducing power in the cell, and to metabolize some sugars. It can also be used to metabolize glucose if the primary glycolytic pathway, the Embden-Meyerhof pathway, is blocked, e.g., by mutation of the phosphoglucoisomerase (*pgi*) gene (for a review, see reference 10). A key enzyme of the pentose-P pathway is the product of the *pgl* gene, 6-phosphogluconolactonase (6-P-gluconolactonase, or Pgl), which catalyzes the hydrolysis of 6-phosphogluconolactone (6-P-gluconolactone, the product of glucose 6-phosphate dehydrogenase [G6PD], encoded by *zwf*) to 6-phospho-gluconate (6-P-gluconate) (Fig. 1A). Though this reaction can proceed spontaneously in vitro, cells carrying both *pgi* and *pgl* mutations grow very poorly on glucose as a sole carbon source, indicating a requirement for *pgl* in vivo. Although Pgl enzymes are predicted to be present in many organisms, a *pgl* gene has not been identified in most microbial genomes (7). In both mice and paramecia, G6PD and Pgl activities are contained in a single bifunctional protein (5, 6).

In *E. coli*, *pgl* mutations have been localized by conjugation experiments and deletion analysis to the region between *att*λ and *modC* (previously known as *chlD*), at approximately 17 min on the *E. coli* genetic map (9, 15). For reasons that are not entirely clear, *pgl* mutants accumulate starch when grown on maltose and are stained dark blue by iodine (1, 15). The maltose blue or Blu$^-$ phenotype (1) has been used as an indicator of the *pgl* phenotype.

In this paper, we report the identification of the *E. coli ybhE* gene as *pgl*. This identification is confirmed by the Blu$^-$ phenotype and loss of 6-P-gluconolactonase activity in crude extracts of *ybhE* mutants. Interestingly, the predicted Pgl/YbhE protein has been categorized as a 3-carboxy-*cis,cis*-muconate lactonizing enzyme (14), members of which catalyze the reversible formation of a lactone from 3-carboxy-*cis,cis*-muconates (Fig. 1B). Unexpectedly, we observed that Pgl mutants are deficient in growth on the β-glucoside salicin, perhaps because of the accumulation of 6-P-gluconolactone.

MATERIALS AND METHODS

Bacteria and plasmids. Bacterial strains and plasmids are listed in Table 1.

Primers. Sequences of primers used to make and confirm mutations are available upon request.

Construction of a *ybhE* deletion, *ybhE*<>*tet* and other mutant strains. A "precise" deletion of the *ybhE* open reading frame was constructed with recombineering (in vivo genetic engineering) technology (22). Briefly, hybrid primers with approximately 50 bases of 5′ homology to DNA sequences flanking the *ybhE* gene were used with the PCR to amplify a drug cassette encoding tetracycline resistance. This PCR product was transformed into electrocompetent DY378 cells induced for the phage λ Red recombination system. Samples of the electroporation mixture were plated on Luria-Bertani plates containing 12.5 μg of tetracycline/ml. Drug-resistant colonies were purified, and the novel junctions between the drug marker and the *ybhE*-flanking DNA were confirmed by a secondary PCR analysis that could also confirm that the wild-type copy of *ybhE* was absent (see reference 23 for details). The cojoined symbols (<>) indicate the removal of a gene and its replacement with a drug marker by recombineering. The *ybhE*<>*tet* and other mutations generated by recombineering were introduced into MG1655 by P1 transduction. To ensure that the tightly linked defective λ prophage used for recombineering was not cotransduced, the transductants were plated at 42°C to select against the temperature-sensitive λ lysogens. Loss of λ immunity and the ability to plate the T4*r*II phage, both being indicators

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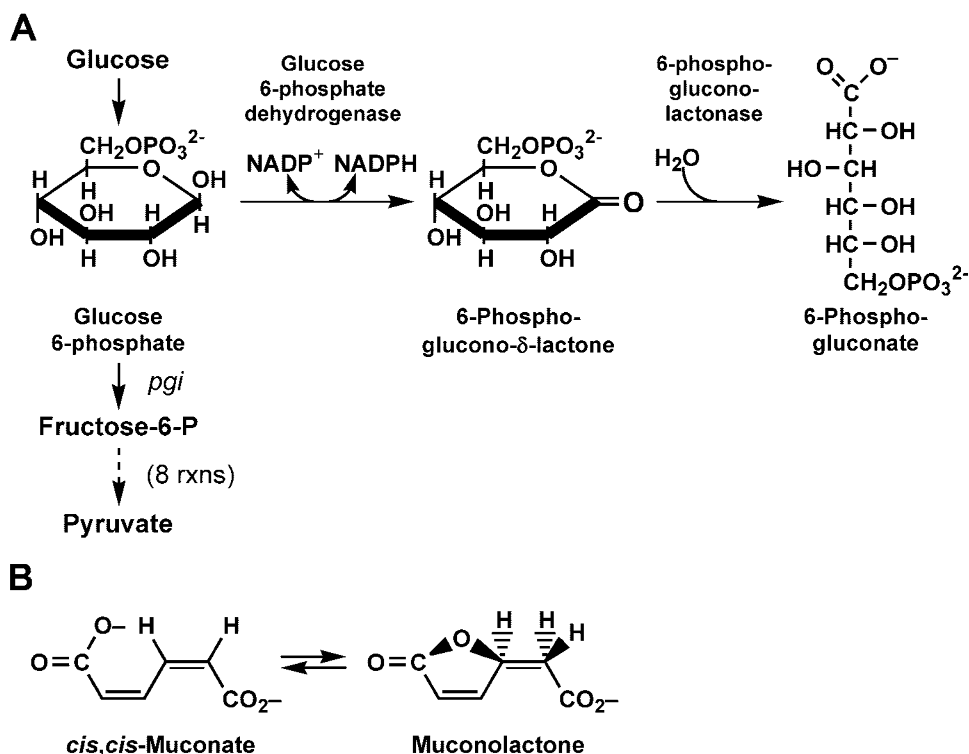

FIG. 1. (A) Early steps of glycolysis and the pentose-P pathway, detailing reactions catalyzed by the G6PD and Pgl enzymes. G6PD and Pgl are encoded by the *zwf* and *pgl* genes, respectively. (B) The predicted activity of the YbhE/Pgl enzyme based on its annotation as a 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

of the absence of the prophage, were confirmed. A *ybhE<>amp* construct was also generated from the *ybhE<>tet* cassette for some experiments. A similar recombineering strategy was used to delete the *zwf* gene and replace it with a chloramphenicol (*cam*) or kanamycin (*kan*) cassette and to replace the *yieK* gene with a *cam* cassette. All of the deletions made by recombineering removed the entire structural gene.

Assay of the Blu phenotype. Bacteria were streaked on minimal maltose agar petri plates and incubated at 32 or 37°C until colonies appeared. The plates were then inverted over plastic 100- by 15-mm petri dishes containing solid I_2 crystals for 2 min, allowing iodine vapor to stain the colonies. Mutant *pgl* colonies stain dark blue-black, whereas wild-type colonies are not stained (1).

Isolation of Bgl$^+$ mutants. Strain MG1655 (like other K-12 strains) cannot use salicin as a C source and is designated Bgl^o (cryptic). When spread on MacConkey plates containing 1% salicin (Mac-SLC) and incubated at 30°C, MG1655 forms pale colonies but red papillae eventually appear, due to spontaneous mutations to a Bgl$^+$ phenotype. Papillae were picked and purified on Mac-SLC plates. The frequency of Bgl$^+$ mutations was estimated from Mac-SLC plates spread with ~50 to 500 cells and incubated at 30°C. After 2 to 4 days, the colonies

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Source or reference	Relevant genotype	Background
DY378	Court laboratory	(λ*c*I*857*Δ*cro-bio*)	W3110
MG1655	B. Bochner	Wild type	*E. coli* K-12
M1924	This study	*ybhE<>tet* (λ*c*I*857*Δ*cro-bio*)	MG1655
M1925-29	This study	Spontaneous Bgl$^+$	MG1655
M1951-55	This study	*ybhE<>tet* transductants	M1925-29
M1971	This study	*ybhE<>tet*	MG1655
M2523	This study	*ybhE<>amp*	MG1655
M2524	This study	*ybhE<>tet* (λ*c*I*857*Δ*cro-bio*) [pJL6], Amp^r	M1924
M2526	This study	*ybhE<>tet* (λ*c*I*857*Δ*cro-bio*) [pJL6-*ybhE/pgl*], Amp^r	M1924
M2527	This study	*c*I*857* allele at *lac* [pJL6] Amp^r	M2537
M2528	This study	*c*I*857* allele at *lac* [pJL6-*ybhE/pgl*] Amp^r	M2537
M2537	This study	*c*I*857* allele at *lac*	MG1655
M2531	This study	*ybhE<>amp pgi*::Tn*10*	MG1655
M2532	This study	*ybhE<>amp pgi*::Tn*10 zwf<>kan*	MG1655
M2534	This study	*zwf<>kan pgi*::Tn*10*	MG1655
M2536	This study	*ybhE<>amp zwf<>kan*	MG1655
M2537	Court laboratory	*c*I*857* allele at *lac*	MG1655
pJL6	20	p_L expression vector under *c*I*857* control; Amp^r	
pJL6-*ybhE/pgl*	This study	pJL6 expressing *ybhE/pgl*, Amp^r	

FIG. 2. Genetic map of the *E. coli* chromosome in the region between *modC* and *att*λ. The genes and putative gene products listed in the annotated *E. coli* genome sequence are as follows: *ybhA*, phosphatase; *ybhE* (shown here to be *pgl*), isomerase; *ybhD*, LysR-type transcriptional regulator; *ybhH*, unknown; *ybhI*, membrane pump protein; *ybhJ*, unknown; and *ybhC*, pectinesterase.

were examined under a binocular microscope and the number of salicin-fermenting papillae per colony was recorded. The Poisson distribution was used to estimate the average number of papillae per colony (8).

Retrieval of the *ybhE* gene in a gap repair reaction using recombineering. The expression vector pJL6 is an ampicillin-resistant pBR322 derivative containing the phage λ p_L promoter for use in gene expression (20). A portion of the plasmid containing the origin of replication, the *bla* gene, and the p_L promoter was amplified by PCR with primers with flanking homology to the *ybhE*/*pgl* gene, such that recombination of the gene onto the plasmid would result in its expression from the p_L promoter (17). The PCR-derived linear vector was used in a recombineering reaction in strain DY378, and colonies resistant to 100 μg of ampicillin/ml were selected. When the prophage in DY378 was not induced for recombineering, no Amp^r colonies appeared, because the linear DNA cannot circularize. Induction of the recombination system generated approximately 160 Amp^r colonies from one electroporation mixture. Sixteen drug-resistant colonies were purified and plasmid DNA was isolated; 14 of 16 colonies contained the correct insert as defined by restriction analysis. The plasmid from one correct clone was designated pJL6-*ybhE*/*pgl*.

6-P-gluconolactonase activity assay. The assay of Kupor and Fraenkel (15) was used. The substrate, 6-P-gluconolactone, is unstable and thus not commercially available but was made by lyophilization of 6-P-gluconic acid (Sigma P-7627) with a predicted yield of approximately 25% (15). Prior to lyophilization, 200 mg of 6-P-gluconic acid was dissolved in 100 ml of distilled water, equilibrated with 5 g of AG 50W-X8 Resin Hydrogen form (Bio-Rad) for 1 h at room temperature, and filtered to remove the resin. Bacteria to be assayed were grown overnight at 37°C in 5 ml of Luria-Bertani broth with aeration (30°C for temperature-sensitive strains). Cells were collected by centrifugation, washed once with 0.85% NaCl, and resuspended in 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. Cells were broken by sonication, the debris was pelleted by centrifugation, and the supernatant (~2.5 mg/ml protein) was assayed for Pgl activity. The lactone (2.0 μmol in ~40 μl of lyophilized product resuspended in 5 ml of H_2O) was added to 1.25 mg of protein extract in 1 ml of 0.1 M K-phosphate buffer (pH 7.0) and incubated at 30°C. At 0, 15, and 30 min, 0.2-ml portions were transferred to microcentrifuge tubes containing 0.2 ml of 3.5 M NaOH and 0.2 ml of 3 M NH_2OH. After 2 min, 0.2 ml of 4 M HCl was added, the tubes were mixed and centrifuged to remove precipitated protein, and 0.2 ml of 0.37 M $FeCl_3$ in 0.1 M HCl was added to each supernatant fraction. The absorbance of the ferric hydroxamate was read at 540 nm.

RESULTS

Tentative identification of *ybhE* as *pgl*. The *pgl* gene was previously mapped between *modC* (formerly *chlD*) and the bacteriophage λ attachment site (*att*λ); mutation of *pgl* gives a Blu$^-$ phenotype (9, 15). According to the annotated *E. coli* K-12 sequence (4), there are seven genes between *modC* and *att*λ (Fig. 2). The predicted protein sequences of these seven genes were subjected to BLAST analysis (2, 3). The only sequence in this region that showed significant homology to known Pgl enzymes was the product of the *ybhE* gene, annotated as a putative isomerase with a predicted length of 331 amino acids. A BLAST comparison of the YbhE protein sequence against the entire nonredundant database indicated homologs in a number of other bacteria and produced one alignment to a putative 6-P-gluconolactonase from *Bacillus cereus* (12). The alignment shows 27% identical residues and 43% conserved residues over a region of 294 amino acids. The BLAST analysis also identified a conserved domain, COG2706, defining a 3-carboxymuconate cyclase enzyme. The proposed activity of this enzyme is conversion of *cis,cis* muconates to muconolactones (14) (Fig. 1B). This activity is not that expected for the Pgl reaction but the similarity encouraged us to analyze the *ybhE* gene.

Another *E. coli* protein gave repeated hits when the *E. coli* K-12 database was searched with some known protein sequences annotated as 6-P-gluconolactonase. This was the product of the *yieK* gene, also a putative isomerase. The YieK sequence, used in a BLAST search against the entire nonredundant database, gave a conserved domain hit to NagB, a glucosamine-6-phosphate isomerase/deaminase, and highly significant alignments to several enzymes indicated to be both 6-P-gluconolactonase and glucosamine-6-phosphate isomerase/deaminase, such as those from *Thermoanaerobacter tengcongensis* and *Vibrio vulnificus* CMCP6.

Deletion of *ybhE* and other genes by recombineering. Phage λ Red recombination (22) was used to remove the *ybhE* open reading frame and replace it with a tetracycline resistance cassette. This recombineering was also used to delete the *yieK* and the *zwf* genes and replace them with drug cassettes. The *zwf* gene encodes glucose-6-phosphate dehydrogenase, which acts prior to 6-P-gluconolactonase to create the 6-P-gluconolactone hydrolyzed by Pgl (14) (Fig. 1A). All mutations generated by recombineering were moved into MG1655 by P1 transduction; the absence of the λ prophage from the final constructs was confirmed.

Blu$^-$ phenotype of the *ybhE* mutant strain. Bacteria deleted for *ybhE* were incubated on minimal maltose agar plates and tested for the Blu phenotype. The *ybhE* deletion strains stained dark blue and thus were Blu$^-$, a phenotype diagnostic of *pgl* mutants. In contrast, wild-type MG1655 colonies turned slightly yellow when subjected to the same regimen. The presence or absence of the closely linked λ prophage did not affect the Blu phenotype. Strains defective for both the *ybhE* and *zwf* genes turned brown but not blue-black; this indicates that *zwf* is required for the Blu$^-$ phenotype. Mutants that lacked only the *yieK* or *zwf* genes had a Blu$^+$ phenotype.

Growth of *ybhE* mutants on minimal glucose plates. We assayed growth of the *ybhE* mutant on minimal glucose agar at 37°C, alone and in combination with mutations in *zwf* and/or in *pgi*, which encodes phosphoglucose isomerase. Since Pgi is required in the primary trunk of the Embden Meyerhoff glycolysis pathway just beyond the pentose-P branch pathway, mutants blocked in both the pentose-P and Embden-Myerhoff pathways should not grow on glucose as a sole carbon source; the growth of such mutants is actually inhibited due to accumulated high levels of glucose-6-P (13). We also assayed a *pgi zwf* double mutant. Whereas the *ybhE* and *zwf* single mutants grew well on minimal glucose, the *pgi* single mutant grew more slowly and never achieved the same large colony size. The *pgi zwf* double mutant was largely unable to grow using glucose as a sole carbon source, with only a faint suggestion of colony formation, whereas the *pgi ybhE* double mutant grew slowly, forming very small colonies after several days. The *ybhE zwf pgi* triple mutant did not grow on minimal glucose, forming only microcolonies after several days. Our observations confirm those of Kupor and Fraenkel (15, 16) and suggest that elimi-

FIG. 3. Two phenotypes of *E. coli pgl* mutants. (Left) *pgl* mutants accumulate starch and stain blue-black with iodine vapors when grown on minimal maltose plates, not seen for wild-type strains. (Right) Wild-type strains cannot utilize salicin as a C source but give rise to Bgl^+ mutants that appear as dark red papillae on MacConkey-salicin plates, not seen in *pgl* mutants. Streaks of three different strains are shown on each plate. From the top and proceeding clockwise: wt (MG1655), a *pgl* mutant containing the control plasmid pJL6 (M2524), the *pgl* mutant containing the pgl^+ plasmid pJL6-*ybhE*/*pgl* (M2526), MG1655, M2524, and M2526.

nation of only the *ybhE*/*pgl* gene by mutation is not sufficient to eliminate carbon flow through the pentose-P pathway. The growth of strains with both *pgi* and *zwf* mutations may also be inhibited by glucose-6-P accumulation.

Retrieval of the *ybhE* gene onto an expression vector. The *ybhE* gene was directly cloned from the *E. coli* chromosome to the expression vector, pJL6 (20), under the control of the phage λ p_L promoter using λ Red to catalyze the recombination (17). The $ybhE^+$ clone and the vector lacking an insert were introduced by electroporation into M1924 and M2537. These strains carry a chromosomal copy of the phage λ *c*I*857* encoding a temperature-sensitive repressor in order to repress the p_L promoter. Purified transformants were grown on minimal maltose plates at 30°C, and the Blu phenotype was tested. The p_L promoter on the pJL6 plasmid is somewhat derepressed even at 30°C (20), and the pJL6 plasmid with the *ybhE* insert (pJL6-*ybhE*/*pgl*) was found to complement the Blu^- phenotype at this temperature (Fig. 3). The *ybhE* clone also complemented the other Pgl phenotypes we monitored: 6-P-gluconolactonase activity and Bgl^+ papillation (see below).

Assay for 6-P-gluconolactonase activity. Extracts of overnight cultures of *ybhE* mutants and wild-type cells were assayed for 6-P-gluconolactonase activity (Fig. 4A) as described in Materials and Methods. The assay is not entirely quantitative as the initial concentration of the substrate, 6-P-gluconolactone, is unknown. However, the assay demonstrates that the absorbance (monitored at 540 nm) of the ferric hydroxamate compound formed by conjugation with the lactone ester decreased substantially over the time monitored for $ybhE^+$ strains, but displayed only a slight decrease over the same time period for *ybhE* mutant strains. Extracts of the pJL6-*ybhE*/*pgl* plasmid-containing strain expressing YbhE/Pgl function complemented the *ybhE*<>*tet* strain in the 6-P-gluconolactonase activity assay (Fig. 4B).

Feeding of *pgi zwf* and *pgi zwf pgl* mutant strains by *pgl* mutants. To minimize confusion, we will henceforth refer to the *ybhE* open reading frame as *pgl* and the YbhE enzyme as Pgl. The pentose-P pathway intermediate 6-P-gluconolactone accumulates in *pgl* mutants, especially when *pgi*-defective. Kupor and Fraenkel (16) have characterized a bypass reaction for metabolism of this compound in *pgl* mutants in which 6-P-gluconolactone is converted to gluconate 6-P. We tested whether bacteria which cannot grow on glucose due to mutations in both *pgi* and *zwf* or *pgi zwf* and *pgl* would be "fed" by *pgl* mutants when spread side by side on minimal glucose plates. Figure 5 shows a composite picture from three minimal glucose plates, illustrating that the triple-mutant strain *pgl pgi zwf* (on top in all cases) can be fed by a *pgl pgi* double mutant, less well by a *pgl* single mutant, and not at all by a *pgl zwf* double mutant. The *pgi zwf* double mutant is fed in a similar fashion by the *pgl pgi* double mutant and the *pgl* single mutant but not by the *pgl zwf* double mutant (Table 2). We interpret these results to mean that 6-P-gluconolactone (or its breakdown product) accumulates in a *pgl* mutant (and even more so when *pgi* is also defective) and that this compound can be used to feed the *pgi zwf* cells, in accordance with the model of Kupor and Fraenkel (16).

Fewer Bgl^+ mutants from *pgl* strains. *E. coli* does not normally ferment β-glucosides such as salicin because of silencing by H-NS and transcription termination within the *bgl* operon (Bgl^o cryptic phenotype). However, spontaneous Bgl^+ variants arise frequently, mostly due to transposition of IS*1* or IS*5* to the *bgl* region (19). Colonies of wild-type cells spread on MacConkey-salicin (Mac-SLC) plates are initially pale, since the

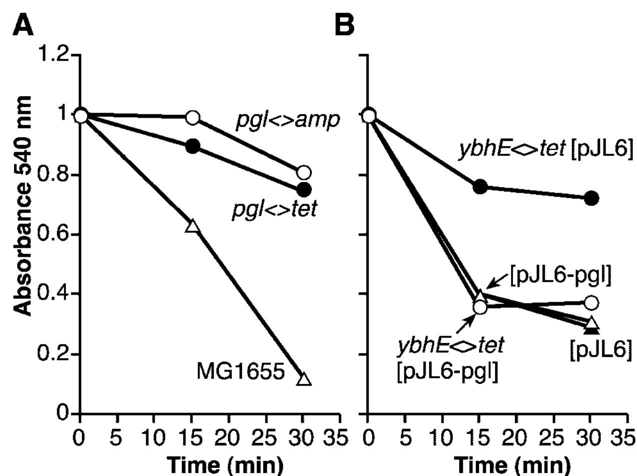

FIG. 4. 6-P-gluconolactonase assays of *ybhE*/*pgl* mutant and wild type *E. coli*, and of bacteria containing the YbhE/Pgl expression vector and control plasmid. (A) △, Assay of MG1655 containing an intact *ybhE*/*pgl* gene; ○ and ●, M2523 (*ybhE*<>*amp*) and M1971(*ybhE*<>*tet*), respectively. (B) ●, M2524 (*ybhE*<>*tet* [pJL6]); ○, M2526 (*ybhE*<>*tet* with *ybhE* expression plasmid [pJL6-*ybhE*/*pgl*]); ▲, M2527 (*c*I*857* [pJL6]); △, M2528 (*c*I*857* [pJL6-*ybhE*/*pgl*]). Details of the assay are described in Materials and Methods.

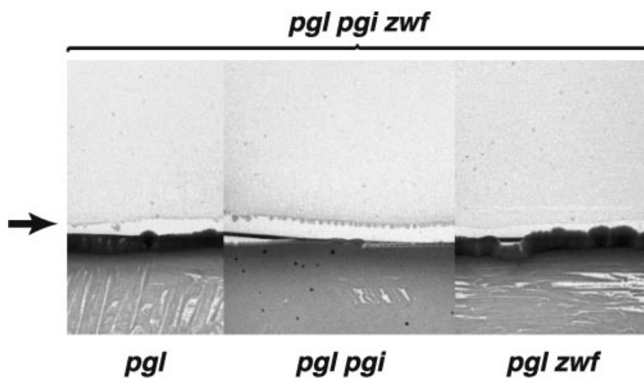

FIG. 5. Detail of strains grown side by side on minimal glucose agar demonstrating feeding of M2532, the *pgl<>amp pgi*::Tn*10 zwf<>kan* triple mutant, by both M2523, the *pgl<>amp* single mutant (at lower left) and M2531, the double mutant *pgl<>amp pgi*::Tn*10* (lower center). In all cases, the upper strain is the triple mutant and is being fed. M2536, the *pgl<>amp zwf<>kan* strain (lower right), cannot feed the triple mutant, presumably because 6-P-gluconolactone is not made in the absence of G6PD. The arrow indicates the edge of the streak of the triple mutant, where small colonies arise due to feeding. See Table 2 for interpretations.

salicin is not utilized. After 2 days at 30°C, however, red papillae of salicin-utilizing (Bgl$^+$ phenotype) mutants are readily detected within many colonies. We noticed that very few Bgl$^+$ mutants arose from the *pgl<>tet* mutant and these only after additional days of incubation at 30°C (Fig. 3). We considered two possibilities: either the *pgl* defect interferes with the mutational event itself or it interferes with the ability of the Bgl$^+$ mutants to utilize salicin. To distinguish between these possibilities, five independent Bgl$^+$ (M1925-M1929) mutants were isolated from wild-type MG1655 cells and then transduced to *pgl* with P1 grown on *pgl<>tet* donors (M1951-M1955). We reasoned that if the defect was in the production of the mutants, these Bgl$^+$ *pgl* cells should now normally express the Bgl$^+$ phenotype. We observed, however, that the Bgl$^+$ *pgl* cells did not express the Bgl$^+$ phenotype fully. When Bgl$^+$ cells were spread on Mac-SLC plates at 30°C, the red color due to salicin-utilization was much fainter and the colony size was smaller for the *pgl* transductants than for the parental strains. Furthermore, when growing in log-phase on minimal medium with salicin as sole C source, the doubling time of Bgl$^+$ *pgl*$^+$ cells was 150 to 165 min, whereas that of the Bgl$^+$ *pgl<>tet* transductants was 270 min. Similar results were obtained for the rare Bgl$^+$ mutants isolated directly from *pgl* cells; when plated on either Mac-SLC plates or minimal-salicin plates, the growth on salicin was reduced. These results show that *pgl* cells grow poorly on salicin.

The normal substrate for Pgl, 6-P-gluconolactone, accumulates in *pgl* mutants. Others have indicated (21) that the lactone may inhibit Bgl$^+$ activity. Therefore, we tested a strain that carried a mutation for both *pgl* and *zwf*. The *zwf* mutation prevents 6-P-gluconolactone production. If 6-P-gluconolactone accumulation is the basis for the poor expression of the *bgl* operon, a doubly mutant *pgl zwf* strain should express *bgl* normally. Indeed, this was observed. The frequency of salicin papillation in a *pgl zwf* strain was similar to that of the wild type, as was the color reaction on Mac-SLC plates and the growth on minimal-salicin plates. Thus, the accumulation of 6-P-gluconolactone appears to be the basis for the poor growth of Bgl$^+$ *pgl* cells on salicin.

Thompson and coworkers (21) have shown that the β-glucosidase of *Fusobacterium mortiferum* is active on salicin and similar β-glucosides and have observed that 6-P-gluconolactone binds this β-glucosidase and severely inhibits its activity. We examined the possibility that 6-P-gluconolactone inhibited BglB by assaying BglB (18) in Bgl$^+$ cells growing on salicin as a sole C source. No significant difference was observed in BglB activity for Bgl$^+$ strains whether *pgl*$^+$ or lacking *pgl* (data not shown). Thus, the accumulation of 6-P-gluconolactone does not seem to affect β-glucosidase activity and must contribute to the poor utilization of salicin in some other manner.

DISCUSSION

The availability of completely sequenced and annotated genomes of many organisms has expedited comparative and functional genomic analyses. Using the approximate location of the *E. coli pgl* gene and an easily scored phenotype (Blu), we have demonstrated that the *E. coli ybhE* gene, predicted to encode an isomerase, actually encodes the 6-P-gluconolactonase enzyme and thus is *pgl*. The identification of *ybhE* as *pgl* was confirmed by three independent tests: (i) the Blu$^-$ phenotype of the *ybhE* mutant and its complementation by the cloned *ybhE* gene, (ii) the behavior of the mutant strain on minimal glucose when combined with a *pgi* mutation, and (iii) a biochemical assay for 6-P-gluconolactonase activity. Our functional analysis of *pgl* was greatly facilitated by another emerging technology, recombineering (22). Recombineering was used to create most of the mutations described in this paper, and was also used to retrieve the *pgl* gene from the *E. coli* chromosome into an expression vector without the necessity of amplifying the gene with PCR.

6-P-gluconolactonase, encoded by *pgl*, is the second enzyme in the pentose-P pathway and converts 6-P-gluconolactone to gluconate-6-P. Gluconate-6-P is used as a substrate for both the pentose-P pathway and the inducible pathway for catabolism of gluconate, the Entner-Doudoroff pathway. Thus, the Pgl enzyme has the potential to contribute a metabolite to two pathways. Double mutants in *pgl* and *pgi* form small colonies on minimal glucose agar, however, demonstrating that Pgl is not absolutely required for some carbon flow through either

TABLE 2. Ability of *pgl* mutant strains to feed various glycolysis mutants on minimal glucose

Feeder strain	Strain being fed	Effect and interpretation
pgl	*pgi zwf*	+; Confirms that the *pgl* mutant supplies either the 6-P-gluconolactone or other metabolite that can feed the *pgi zwf* strain
pgl pgi	*pgi zwf*	++; Enhanced ability to feed the *pgi zwf* strain results from more carbon being forced through the pentose-P pathway of the feeder strain
pgl zwf	*pgi zwf*	−; Confirms that 6-P-gluconolactone is needed for feeding
pgl pgi	*pgl pgi zwf*	++; Because this strain can feed the triple mutant, the 6-P-gluconolactone must be converted to some other compound, presumably gluconate-6-P, either enzymatically or by spontaneous hydrolysis

the pentose-P pathway or the Entner-Doudoroff pathway utilizing gluconate. This leakiness may be due to the reaction characterized by Kupor and Fraenkel (16) in which 6-P-gluconolactone is dephosphorylated, secreted, converted to gluconate, and phosphorylated upon uptake, yielding gluconate-6-P. However, spontaneous hydrolysis of 6-P-gluconolactone can also account for these results, and either interpretation would explain the feeding results described here.

The secondary structure of Pgl is apparently not well conserved among different organisms. The *E. coli* Pgl/YbhE protein was previously categorized as a 3-carboxy-*cis,cis*-muconate lactonizing enzyme (Fig. 1B) and predicted to have a beta propeller cycloisomerase structure (14). We have not checked this enzymatic activity, since our results demonstrate a 6-P-gluconolactonase activity for Pgl (Fig. 1A and 4). It remains theoretically possible that Pgl is a bifunctional enzyme. BLAST alignment of the *Pseudomonas aeruginosa* Pgl enzyme (11) with the *E. coli* Pgl protein reveals no significant homology, although the *P. aeruginosa* Pgl has significant homology to NagB, a glucosamine-6-phosphate deaminase, as does the *E. coli* YieK protein. YieK shows homology to some Pgl enzymes but the *yieK* mutant did not display a Blu$^-$ phenotype in our assays, did not reduce growth on minimal glucose when combined with both *pgl* and *pgi* mutations, and did not decrease the frequency of appearance of spontaneous Bgl$^+$ mutants on Mac-SLC plates. In mice and malarial parasites, Pgl and G6PD activities are both present in a single bifunctional enzyme (5, 6). The gene encoding Pgl remains unidentified in a number of microbes (7). Our results suggest that some genes annotated as encoding a 3-carboxy-*cis,cis*-muconate lactonizing enzyme may actually encode a 6-P-gluconolactonase.

Transposition of IS*1* or IS*5* to the *bgl* region of the chromosome is mainly responsible for the genesis of Bgl$^+$ mutants (19). It was previously observed that *rho* mutants of *E. coli* give rise to Bgl$^+$ mutants at a lower frequency than the wild type, and this was traced to defective transposition in *rho* mutants (8). However, the lower frequency of appearance of Bgl$^+$ mutants from *pgl* parents observed here has a different explanation, since the Bgl$^+$ mutants that arose in wild-type cells (and found to be due to IS*1* and IS*5* transpositions) displayed poor growth on salicin after they were transduced to *pgl*<>*tet*. This seems related to the accumulation of 6-P-gluconolactone, since the frequency of appearance of Bgl$^+$ mutants from *zwf pgl* mutants is like that from wild-type cells. Reconstruction experiments showed that when *pgl* cells were mixed with a very low proportion of Bgl$^+$ *pgl*<>*tet* cells and streaked on Mac-SLC plates, the Bgl$^+$ cells did not produce the red color due to fermentation of salicin that is seen when they are streaked by themselves (data not shown). However, the precise defect that is responsible for this remains unknown.

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