

The *Pseudomonas aeruginosa devB/SOL* Homolog, *pgl*, Is a Member of the *hex* Regulon and Encodes 6-Phosphogluconolactonase

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A cyclic version of the Entner-Doudoroff pathway is used by *Pseudomonas aeruginosa* to metabolize carbohydrates. Genes encoding the enzymes that catabolize intracellular glucose to pyruvate and glyceraldehyde 3-phosphate are coordinately regulated, clustered at 39 min on the chromosome, and collectively form the *hex* regulon. Within the *hex* cluster is an open reading frame (ORF) with homology to the *devB/SOL* family of unidentified proteins. This ORF encodes a protein of either 243 or 238 amino acids; it overlaps the 5' end of *zwf* (encodes glucose-6-phosphate dehydrogenase) and is followed immediately by *eda* (encodes the Entner-Doudoroff aldolase). The *devB/SOL* homolog was inactivated in *P. aeruginosa* PAO1 by recombination with a suicide plasmid containing an interrupted copy of the gene, creating mutant strain PAO8029. PAO8029 grows at 9% of the wild-type rate using mannitol as the carbon source and at 50% of the wild-type rate using gluconate as the carbon source. Cell extracts of PAO8029 were specifically deficient in 6-phosphogluconolactonase (Pgl) activity. The cloned *devB/SOL* homolog complemented PAO8029 to restore normal growth on mannitol and gluconate and restored Pgl activity. Hence, we have identified this gene as *pgl* and propose that the *devB/SOL* family members encode 6-phosphogluconolactonases. Interestingly, three eukaryotic glucose-6-phosphate dehydrogenase (G6PDH) isozymes, from human, rabbit, and *Plasmodium falciparum*, contain Pgl domains, suggesting that the sequential reactions of G6PDH and Pgl are incorporated in a single protein. 6-Phosphogluconolactonase activity is induced in *P. aeruginosa* PAO1 by growth on mannitol and repressed by growth on succinate, and it is expressed constitutively in *P. aeruginosa* PAO8026 (*hexR*). Taken together, these results establish that Pgl is an essential enzyme of the cyclic Entner-Doudoroff pathway encoded by *pgl*, a structural gene of the *hex* regulon.

A cyclic version of the Entner-Doudoroff pathway is used by *Pseudomonas aeruginosa* to metabolize carbohydrates (Fig. 1) (9, 19, 36). The members of the pathway that are responsible for the metabolism of glucose to glyceraldehyde 3-phosphate and pyruvate are coordinately regulated and induced by growth on glycerol, fructose, mannitol, glucose, and gluconate. They are clustered in at least three operons near 39 min on the chromosome and are referred to as the *hex* regulon. They are under the control of the recently identified repressor *hexR* (29; W. D. Proctor, P. W. Hager, and P. V. Phibbs, Jr., Abstr. 98th Annu. Meet. Am. Soc. Microbiol. 1998, abstr. K-135).

The DNA sequence of the *P. aeruginosa zwf* gene encoding the unique glucose 6-phosphate dehydrogenase of *P. aeruginosa* was recently reported (22). *Zwf* catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone using either NAD or NADP as a cofactor. While 6-phosphogluconolactone can be hydrolyzed nonenzymatically to 6-phosphogluconate, the enzymatic activity (EC 3.1.1.31) was described some time ago (6). A phosphogluconolactonase activity has been identified and partially purified from *Pseudomonas fluorescens* (16) but, to our knowledge, has not been identified in *P. aeruginosa*. Purification of 6-phosphogluconolactonase has been achieved from *Zymomonas mobilis* (33), bovine erythrocytes (3), and bass liver (26), and in each case the enzyme appears to be a monomer of 26 to 30 kDa. *Escherichia coli* contains a 6-phosphogluconolactonase which is necessary for optimal growth when using the pentose phosphate shunt. Hence, *pgi pgl* double mutants, which cannot convert glucose-6-phosphate to fruc-

tose-6-phosphate, grow very slowly on glucose (18). The *pgl* gene locus was tightly linked by transductional analysis to *att-λ*, located at 15 min on the *E. coli* K-10 chromosome (17).

The isolation and molecular characterization of a *pgl* structural gene were first reported only recently (GenBank accession no. AF029673) (P. W. Hager, M. W. Calfee, and P. V. Phibbs, Abstr. 99th Annu. Meet. Am. Soc. Microbiol. 1999, abstr. K-148). An open reading frame with homology to *devB*, a putative “developmentally regulated” glucose-6-phosphate dehydrogenase, was identified immediately downstream of the *P. aeruginosa Zwf* coding sequence. Insertional inactivation of that *devB* homolog resulted in a slow-growth phenotype on mannitol and the loss of 6-phosphogluconolactonase activity. Normal growth on mannitol and 6-phosphogluconolactonase activity were restored by a plasmid containing the subcloned open reading frame, identifying the *P. aeruginosa devB* homolog as *pgl*. The expression of Pgl activity was positively regulated by growth on carbohydrates and negatively regulated by growth on succinate, a phenotype that is consistent with this gene’s being a member of the *hex* regulon. Subsequently, a human cDNA with homology to the *P. aeruginosa pgl* and other *devB* homologs was reported to encode Pgl (8).

MATERIALS AND METHODS

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

Genetic techniques. The *eda* gene was subcloned from plasmid pPZ300 in two steps. First, both orientations of a 2.9-kb *Bam*HI fragment from pPZ300 were cloned into pGEM-3Zf(+), creating pPZ474 and pPZ475. Second, a *Pst*I deletion of pPZ475 (removing 1.8 kb) resulted in pPZ505. The remaining 1.1-kb fragment containing *eda* from pPZ475 was cut out with *Pst*I and *Bam*HI and ligated into the *Pseudomonas*-compatible vector pUCP18, creating pPZ502, in which the *eda* gene is in the sense orientation following the *lac* promoter. The antisense orientation of the *eda* gene relative to the *lac* promoter was created by

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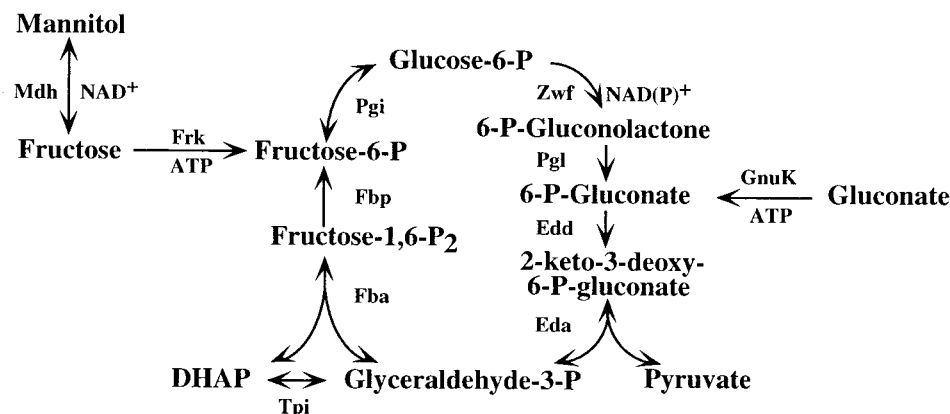


FIG. 1. Cyclic Entner-Doudoroff pathway of *P. aeruginosa*. In this pathway, the catabolism of mannitol occurs via glucose-6-phosphate and requires the activity of glucose-6-phosphate dehydrogenase (Zwf, EC 1.1.1.49), while the catabolism of gluconate does not require Zwf. Additional enzymatic activities are abbreviated as follows: Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14); Edd, 6-phosphogluconate dehydratase (EC 4.2.1.12); Fbp, fructose-1,6-bisphosphatase (EC 3.1.3.11); Fba, fructose biphosphate aldolase (EC 4.1.2.13); Frk, fructokinase (EC 2.7.1.4); GnuK, gluconokinase (EC 2.7.1.12); Mdh, mannitol dehydrogenase (EC 1.1.1.67); Pgi, phosphoglucoisomerase (EC 5.3.1.9); Pgl, 6-phosphogluconolactonase (EC 3.1.1.31); Tpi, triose phosphate isomerase (EC 5.3.1.1).

moving the 1.1-kb *eda* fragment into the *Pseudomonas*-compatible vector pPZ375, creating pPZ571.

The *pgl* knockout strain PAO8029 and the *pgl* merodiploid PAO8033 were constructed as follows. The 2-kb *XhoI*-*ApaI* fragment from plasmid pPZ303, containing the *pgl* gene, was cloned into the *XhoI* site of pGEM-7Z(+), creating pPZ594. The *Bam*HI site within the polylinker of pPZ594 was removed by

digestion of pPZ594 with *Eco*RI and *Sac*I with ligation of the product, creating pPZ595. The 0.9-kb *Sma*I fragment containing the gentamicin resistance cassette (*aacC1*) from pUCGM (32) was then cloned into the single remaining *Bam*HI site of pPZ595 (after making the *Bam*HI ends flush with T4 DNA polymerase), resulting in a disruption of the *pgl* coding sequence. Both orientations of *aacC1* into *pgl* were obtained as plasmids pPZ596 and pPZ602. DNA from pPZ596 or

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid ^a	Relevant characteristics ^b	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototroph	Holloway ^c (13)
PAO1838	<i>eda-900I met-9020</i>	Matsumoto ^d (31)
PAO8029	<i>pgl::aacC1</i>	This study
PAO8033	<i>aacC1</i> , Cb ^r	This study
PAO9010	<i>zwf::aacC1</i>	22
PAO8026	<i>hexR::aacC1</i>	29
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>	Clonetech
Plasmids		
pGEM-3Zf(+)	Ap ^r , cloning vector	Promega
pGEM-7Zf(+)	Ap ^r , cloning vector	Promega
pTZ19R	Ap ^r , cloning vector	PL-Pharmacia
pUCGM	Ap ^r , <i>aacC1</i>	Hassett ^e (32)
pUCP18	Cb ^r , oriV	37
pUCP22	Cb ^r , oriV	37
pPZ149	1.2-kb <i>Bam</i> HI fragment from pPZ300 in pTZ19R	This study
pPZ300	<i>zwf pgl eda</i> Cb ^r oriV	34
pPZ303	<i>zwf pgl eda</i> Cb ^r oriV	34
pPZ375	Cb ^r oriV	35
pPZ474	2.9-kb <i>Bam</i> HI fragment from pPZ300 in pGEM-3Zf(+), <i>eda</i>	This study
pPZ475	Opposite orientation of 2.9-kb <i>Bam</i> HI fragment in pGEM-3Zf(+), <i>eda</i>	This study
pPZ502	1.1-kb <i>Pst</i> I- <i>Bam</i> HI fragment from pPZ505 in pUCP18, <i>eda</i> oriV	This study
pPZ505	<i>Pst</i> I deletion of pPZ475, <i>eda</i>	This study
pPZ524	2.4-kb <i>Sst</i> II fragment, <i>zwf</i> , in pUCP22	This study
pPZ571	1.1-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pPZ502 in pPZ375, <i>eda</i> oriV	This study
pPZ594	2-kb <i>Xho</i> I- <i>Apa</i> I from pPZ303 in pGEM-7Zf(+), <i>pgl</i>	This study
pPZ595	<i>Eco</i> RI- <i>Sac</i> I deletion of pPZ594, creates unique <i>Bam</i> HI site in <i>pgl</i>	This study
pPZ596	0.86-kb <i>aacC1</i> cassette from pUCGM inserted into <i>Bam</i> HI site of pPZ595	This study
pPZ602	Identical to pPZ596 except <i>aacC1</i> cassette reversed	This study
pPZ603	2-kb <i>Sal</i> I- <i>Eco</i> RI from pPZ594 in pUCP18, <i>pgl</i> oriV	This study

^a *P. aeruginosa* gene designations are according to Holloway et al. (14)

^b Abbreviations: Cb^r, carbenicillin resistance; Ap^r, ampicillin resistance; *aacC1*, gentamicin resistance cassette.

^c B. W. Holloway, Monash University, Australia.

^d H. Matsumoto, Shinshu University, Matsumoto, Japan.

^e D. Hassett, University of Cincinnati, Ohio.

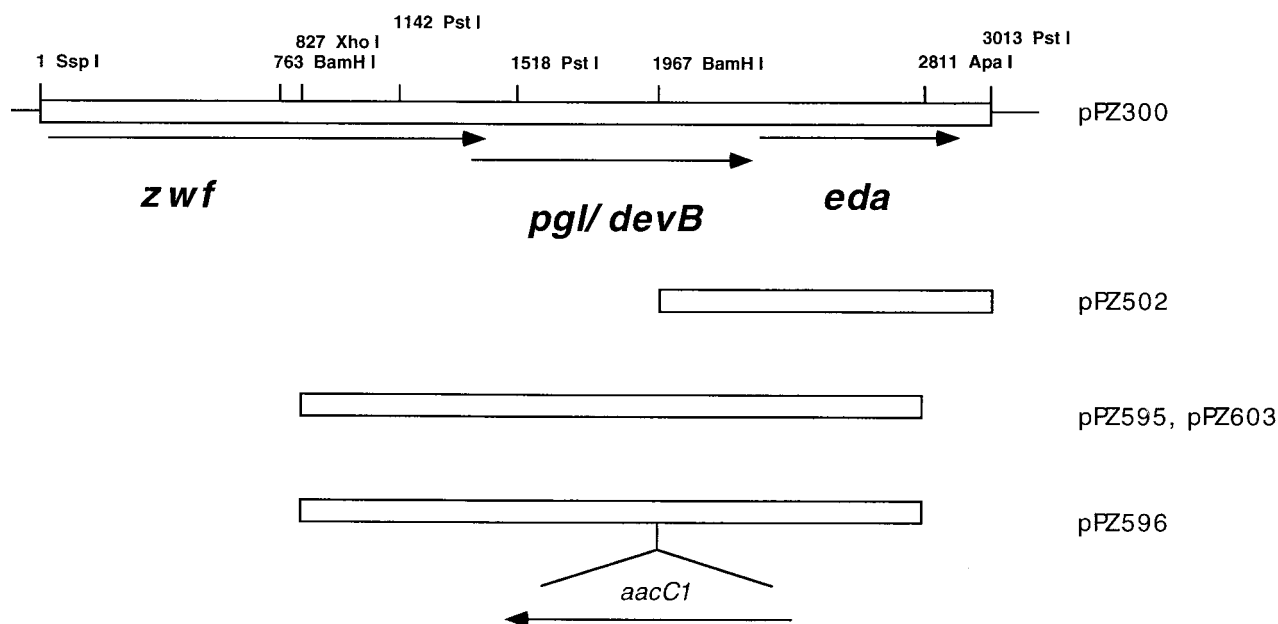


FIG. 2. Summary of the organization of the *zwf*, *pgl*, and *eda* genes and plasmids derived from pPZ300. Plasmid pPZ300 contains 11 kb of PAO1-derived DNA, of which only 3 kb is indicated here. Plasmid pPZ502 complements the *eda* mutant PAO1838. It was constructed by subcloning a 1-kb *Bam*HI-*Pst*I fragment from pPZ505 into the broad-host-range vector pUCP18. pPZ595 and pPZ603 contain the same 2-kb *Xho*I-*Sa*I fragment, and pPZ603 complements the *pgl* mutant PAO8029. pPZ596 is a *Pseudomonas* suicide vector, constructed from pPZ595. It contains a gentamicin resistance cassette, *aacC1*, inserted into the *Bam*HI site within *pgl/devB*. pPZ602 (not shown) is identical to pPZ596 except for the orientation of the *aacC1* cassette.

pPZ602 was electroporated into *P. aeruginosa* PAO1 using the method of Enderle and Farwell (11). Three putative recombinants were isolated on L agar containing gentamicin. The characterization of two of these mutants as PAO8029 and PAO8033 is described in the Results section.

Southern blots. Genomic DNA was isolated from strains PAO1, PAO8029, and PAO8033 using hexadecyltrimethyl ammonium bromide (CTAB) followed by equilibrium centrifugation in a CsCl gradient (2). The genomic DNA was digested with *Bam*HI, and 1 μ g of each digest was loaded on a 1% agarose gel, electrophoresed, and transferred to a nylon membrane by capillary action (2). Digestion of pPZ595 with *Apa*I and *Xho*I yielded DNA fragments of 2 and 3 kb, representing the *pgl* gene and the vector, respectively. The fragments were isolated from a 1% agarose gel using Qiaex resin (Qiagen, Chatsworth, Calif.) and labeled with [α - 32 P]dATP using the RadPrime DNA labeling system (Life Technologies, Rockville, Md.) as described by the manufacturers. Duplicate blots were probed with approximately 10^7 cpm of either the *pgl* or the vector-derived probe, washed, and exposed to X-ray film for 24 to 48 h.

Preparation of 6-phosphogluconolactone and assays of 6-phosphogluconolactonase and glucose-6-phosphate dehydrogenase activity. A qualitative assay of 6-phosphogluconolactonase, including the preparation of 6-phosphogluconolactone (by lyophilization of 6-phosphogluconate) and its quantitation using hydroxylamine and ferric chloride, has been previously described (17). The extinction coefficient of the ferric hydroxamate of 6-phosphogluconolactone was assumed to be equal to that determined for δ -gluconolactone ($E_{530} = 580 \text{ M}^{-1}$). For the determination of the specific activity of 6-phosphogluconolactonase, a method based on that of Beutler et al. was employed (4). Briefly, the production of 6-phosphogluconate was followed using 6-phosphogluconate dehydrogenase. Assays were done at 28°C in 0.1 M Tris-Cl buffer (pH 7.5) with 0.3 mM NADP, 0.1 mM 6-phosphogluconolactone, 1 mM dithiothreitol, and 0.5 U of yeast 6-phosphogluconate dehydrogenase. Glucose-6-phosphate dehydrogenase assays were done at 28°C; otherwise, the assay was as previously described (15). For the preparation of extracts for enzymatic assays, cells were harvested in mid-log-phase growth following growth in minimal medium (21) with a carbon source. Cells were washed with cold 0.9% NaCl, and the cell pellets were suspended in 5% of the original culture volume of 50 mM Tris-Cl-1 mM EDTA-1 mM dithiothreitol (pH 8) and broken in a French press at 16,000 lb/in 2 . Extracts were clarified by centrifugation at 175,000 $\times g$ for 30 min (Sorvall T-1270, 45,000 rpm). Protein was measured by the method of Bradford (5) using bovine serum albumin as the standard. Chemicals and coupling enzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.), and Bradford reagent was obtained from Bio-Rad (Hercules, Calif.).

Growth rates. Strains were initially isolated on selective Luria-Bertani agar plates and subsequently grown overnight in liquid minimal medium containing 10 mM succinate. Fresh minimal medium cultures containing 2 mM succinate and 20 mM gluconate or 2 mM succinate and 20 mM mannitol were started with a

10% (vol/vol) inoculum from the overnight culture. For the determination of growth rates, the turbidity was measured with a Klett-Summerson colorimeter with a red no. 66 filter. Under these conditions, *P. aeruginosa* utilizes the available succinate for growth before shifting to metabolism of the gluconate or mannitol (23). Hence, the gluconate- and mannitol-dependent growth rates were determined from the second phase of the growth curves.

DNA sequencing. The DNA sequences of the cloned fragments in pPZ505, pPZ149, and pPZ595 were determined by Commonwealth Biotechnologies, Inc. (Richmond, Va.). Plasmid pPZ149 contains a 1.2-kb *Bam*HI fragment derived from pPZ300 cloned into pTZ19R.

Computer analysis of sequences. Comparisons between individual sequences were carried out using the Genetics Computer Group software analysis package (Madison, Wis.), while comparisons to databases were carried out using the Basic Local Alignment Search Tool (BLAST) network service at the National Center for Biotechnology Information (NCBI), National Institutes of Health (1). Searches for promoter sequences were carried out using the program NNPP (www-hgc.lbl.gov/projects/promoter.html) (30).

Nucleotide sequence accession number. The DNA sequences of the *pgl* and *eda* genes were updated in GenBank (accession no. AF029673, 2 April 1999).

RESULTS AND DISCUSSION

Nucleotide sequence encoding *eda* and *pgl/devB*. The cloning and DNA sequence of the glucose-6-phosphate dehydrogenase gene (*zwf*) of *P. aeruginosa* were reported recently (22). Continuing downstream from *zwf*, two additional open reading frames were found (summarized in Fig. 2). Earlier work established that the Entner-Doudoroff aldolase gene (*eda*) was closely linked to *zwf* (10, 31), and both genes were subsequently cloned on an 11-kb fragment of *P. aeruginosa* DNA (34). The smallest subclones able to complement the *eda* mutant PAO1838 for growth on mannitol, glucose, and gluconate contained the two orientations of a 1.1-kb *Bam*HI-*Pst*I DNA fragment 3' to *zwf* (in plasmids pPZ502 and pPZ571). The DNA sequence of this region showed the *eda* gene to be 723 bp downstream of and in the same orientation as *zwf* (Fig. 3). The DNA sequence predicts that Eda contains 220 amino acids sharing 42% identity with its *E. coli* homolog. It is preceded by a Shine-Dalgarno sequence (GGAG) and followed by a po-

Zwf G S W G P V A S V A L V A R D G R S W Y
 ggg agt tgg ggg ccg gtg gcc tcg gtg gcc ctg gtg gcc cgc g ATG GGA GGA GTT GGT 75

G D F * Zwf end

Pgl ATG GCG ATT Tct gaG TTG AAG CTG CCG GCC GGC GTC GGC CTG CAG GTC TGG GGC AGC GCC 135
 M A I S E L K L P A G V G L Q V W G S A 20

GCC GAG CAG GCC CGC GGC CTG GCC GCC GAG GTC GCC GGC CGG TTG CGC TCG GCG CTG GCC 195
 A E Q A R G L A A E V A G R L R S A L A 40

GAG CAG GGC CAG GCG CTG CTG GTG GTG TCC GGT GGG CGC AGT CCG GTG GCC TTC CTC GAA 255
 E Q G Q A L L V V S G G R S P V A F L E 60

GCC TTG AGC GAG GAG CCG CTG GAC TGG TCG CGG ATC ACA GTC AGC CTG GCC GAC GAG CGC 315
 A L S E E P L D W S R I T V S L A D E R 80

TGG GTG CCG GAG TCG CAT GCC GAT AGC AAC GCC GGC CTG GTT CGC CGC CAC CTG CTC CGT 375
 W V P E S H A D S N A G L V R R H L L R 100

GGC GAG GCG GCG AAG GCG CGC TTC ATC GGC CTC TAC CAG CCG GCG GCG AGC CTG GAG GAA 435
 G E A A K A R F I G L Y Q P A A S L E E 120

GCG GCC GAG CTG GCC GAC CAT CAC CTG CAC GAG CTG CCA TTG CCG ATC GAC GTG CTG GTC 495
 A A E L A D H H L H E L P L P I D V L V 140

CTC GGC ATG GGC GAC GAC GGC CAT ACC GCC TCG CTG TTC CCG AAC AGC CCT GGC CTG GAC 555
 L G M G D D G H T A S L F P N S P G L D 160

CTG GCG ATG GAT CCC CAG GGG ACG CGC CGT TGC CTG CCG ATG TGG GCG CCG AGC GTG CCG 615
 L A M D P Q G T R R C L P M W A P S V P 180

CAC CAG CGC CTG ACC CTG CCG CGC GCC GTG CTG GCG GCG GCG AAG GTG CAG CTG CTG GCG 675
 H Q R L T L P R A V L A A A K V Q L L A 200

ATC CAG GGC CAG TCC AAG CTG GCC ACC CTG AAC GCC GCG CTG GCG GTC GAG GAC GAA CGG 735
 I Q G Q S K L A T L N A A L A V E D E R 220

CGG ATG CCG GTT CGC GCC TTC CTC CGC GCG CCG CTG ACG ATC CAT TGG TAC CCC TGA gtgg 796
 R M P V R A F L R A P L T I H W Y P *Pgl end 238

cggagctgtagcc ATG CAC AAC CTT GAA CAG AAG ACC GCC CGC ATC GAC ACG CTG TGC CGG 857

Eda M H N L E Q K T A R I D T L C R 16

GAG GCG CGC ATC CTC CCG GTG ATC ACC ATC GAC CGC GAG GCG GAC ATC CTG CCG ATG GCC 917
 E A R I L P V I T I D R E A D I L P M A 36

GAT GCC CTC GCC GCC GGC GGC CTG ACC GCC CTG GAG ATC ACC CTG CGC ACG GCG CAC GGG 977
 D A L A A G G L T A L E I T L R T A H G 56

CTG ACC GCC ATC CGG CGC CTC AGC GAG GAG CGC CCG CAC CTG CGC ATC GGC GCC GGC ACC 1037
 L T A I R R L S E E R P H L R I G A G T 76

GTG CTC GAC CCG CGG ACC TTC GCC GCC GCG GAA AAG GCC GGG GCG AGC TTC GTG GTC ACC 1097
 V L D P R T F A A A E K A G A S F V V T 96

CCG GGT TGC ACC GAC GAG TTG CTG CGC TTC GCC CTG GAC AGC GAA GTC CCG CTG TTG CCC 1157
 P G C T D E L L R F A L D S E V P L L P 116

GGC GTG GCC AGC GCT TCC GAG ATC ATG CTC GCC TAC CGC CAT GGC TAC CGC CGC TTC AAG 1217
 G V A S A S E I M L A Y R H G Y R R F K 136

CTG TTT CCC GCC GAA GTC AGC GGC GGC CCG GCG GCG CTG AAG GCG TTC TCG GGA CCA TTC 1277
 L F P A E V S G G P A A L K A F S G P F 156

CCC GAT ATC CGC TTC TGC CCC ACC GGA GGC GTC AGC CTG AAC AAT CTC GCC GAC TAC CTG 1337
 P D I R F C P T G G V S L N N L A D Y L 176

GCG GTA CCC AAC GTG ATG TGC GTC GGC GGC ACC TGG ATG CTG CCC AAG GCC GTG GTC GAC 1397
 A V P N V M C V G G T W M L P K A V V D 196

CGC GGC GAC TGG GCC CAG GTC GAG CGC CTC AGC CGC GAA GCC CTG GAG CGC TTC GCC GAG 1457
 R G D W A Q V E R L S R E A L E R F A E 216

CAC CGC AGA CAC TGA gactgcccgcctgggcatgcccggcttcggccgggttttttttatgccgtccggattatcc 1532
 H R R H * Eda end 220

ggggcatggaacggctaacgtcatcgcgcttattcccgatgcccggcggttcccgcagatagtcgccgagccgactctg 1612
 cag 1615

FIG. 3. Nucleotide and deduced amino acid sequences of the *pgl* and *eda* genes. The coding sequence of Pgl begins within the 3' end of the Zwf coding sequence at nucleotide 76. There is an alternative initiation codon for Pgl at nucleotide 61 (indicated in upper case). The coding sequence for Eda is separated from the end of Pgl by only 17 bp. Indicated are potential Shine-Dalgarno (wavy underlining) and rho-independent transcriptional termination sequences (underlined).

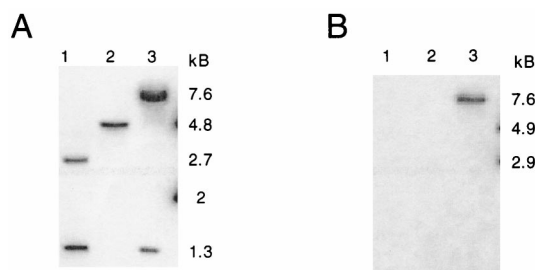


FIG. 4. Southern blots of genomic DNA digested with *Bam*HI. Duplicate blots were prepared as follows: lane 1, PAO1-derived DNA; lane 2, PAO8026-derived DNA; lane 3, PAO8033-derived DNA. (A) Blot hybridized with *pgl*-specific probe. (B) Blot hybridized with plasmid vector-specific probe.

tential transcription terminator. Although a promoter sequence between *zwf* and *eda* has not been identified, we presume that the 1.1-kb *eda* fragment does contain a promoter, since both orientations of the cloned *eda* gene are able to complement PAO1838.

The 723-bp region between *zwf* and *eda* has one open reading frame in the same orientation as *Zwf* and *Eda* with two potential ATG start codons located within the last eight codons of the *Zwf* sequence (Fig. 2). The potential initiation codons are in frame with each other, separated by only 5 codons, and both have potential Shine-Dalgarno sequences (GGUGG and GGAGG). The second initiation codon appears to be the true start site, as a *P. aeruginosa*-derived protein with the predicted N-terminal amino acid sequence (AISELK LPAGVGLQV) has been identified (Dennis Ohman, personal communication). This open reading frame encoding 238 amino acids has similarity to *devB* (from *Anabaena*) and *SOL* (from *Saccharomyces cerevisiae*) (see below). *DevB* has been proposed to be a "developmentally regulated" glucose-6-phosphate dehydrogenase (GenBank accession no. U14553). While *P. fluorescens* and *Pseudomonas cepacia* (reclassified as *Burkholderia cepacia*) have two glucose-6-phosphate dehydrogenases that function with either NAD^+ (in a catabolic role) or NADP^+ (in an anabolic role) (7, 20, 25), it is clear that *P. aeruginosa* has a single glucose-6-phosphate dehydrogenase activity (*Zwf*) capable of using either NAD^+ or NADP^+ (22, 28). One interpretation consistent with a single *zwf* gene is that the open reading frame with homology to *devB/SOL* represents a 6-phosphogluconolactonase.

To test this hypothesis, the *devB* open reading frame was interrupted by insertion of the *aacC1* gentamicin resistance cassette at the *Bam*HI site. The related suicide plasmids pPZ596 and pPZ602 contain the two orientations of *aacC1* (Fig. 2). After electroporation of pPZ596 and pPZ602 into *P. aeruginosa* PAO1, three gentamicin-resistant colonies were isolated. Two of these strains were gentamicin resistant but carbenicillin sensitive, suggesting gene replacement by recombination of the plasmid into the chromosome via double crossovers. One of these isolates was named PAO8029 and studied further. The third isolate, PAO8033, had both carbenicillin and gentamicin resistance, suggesting recombination via a single crossover. Southern blot analysis of *Bam*HI-digested DNA isolated from PAO1, PAO8029, and PAO8033 showed that these interpretations were correct (Fig. 4). *Bam*HI-digested PAO1 DNA contains two fragments (1.3 and 2.7 kb) that hybridized to the *devB*-derived probe. For PAO8029, the *Bam*HI site within the *devB* gene has been replaced with the 0.8-kb *aacC1* cassette, resulting in a single band of 4.8 kb. For PAO8033 there are two bands that hybridized to the *devB* probe, 1.3 kb

TABLE 2. Growth rates of *P. aeruginosa* strains with either gluconate or mannitol as the carbon source^a

Strain/plasmid	Doubling time (h)	
	Gluconate	Mannitol
PAO1	0.91 ± 0.01	2.75 ± 0.06
PAO8029	1.80 ± 0.02	28.4 ± 0.92
PAO8029/pPZ603	0.90 ± 0.05	3.02 ± 0.22
PAO9010 (<i>zwf</i>)	1.11 ± 0.10	No growth

^a Strains were grown at 37°C in minimal medium containing 2 mM succinate plus either 20 mM gluconate or 20 mM mannitol. Growth rates were determined from the gluconate- or mannitol-dependent phase of growth.

and 7.7 kb (Fig. 4A). When a duplicate blot was probed with DNA derived from the plasmid vector, only PAO8033 and the plasmid controls showed hybridization (Fig. 4B). Hence, for PAO8033, the results were consistent with a single crossover of the plasmid into the amino-terminal side of the gene.

Identification of *devB* as *pgl*. In *P. aeruginosa*, *Zwf* activity is absolutely required for growth on mannitol but not for growth on gluconate (Fig. 1). Compared to PAO1, PAO8029 grew at a reduced rate on gluconate (50%), as did the *zwf* mutant strain PAO9010 (82%). However, PAO8029 grew very slowly on mannitol, with a doubling time of approximately 30 h, or 9% of the wild-type rate (Table 2). While this is the expected phenotype of a *pgl* mutant (see below), it could represent a leaky *zwf* phenotype, so we tested for complementation using various plasmids. The phenotype of very slow growth on mannitol was not complemented by a plasmid vector (pPZ375) or the vector containing *zwf* (pPZ524) (data not shown), but was complemented by pPZ603 containing the *devB* homology region (Table 2). This confirmed our supposition that this *devB* homolog is not a glucose-6-phosphate dehydrogenase; instead, it represents a unique gene.

The phenotype of very slow growth on mannitol for

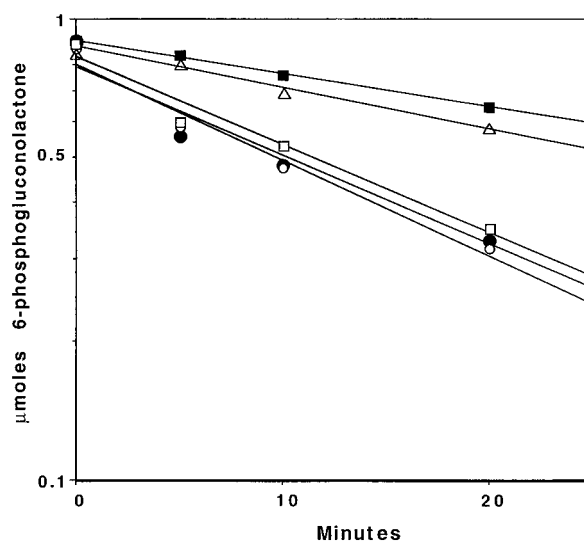


FIG. 5. Extracts of strain PAO8029 lack 6-phosphogluconolactonase. Assays contained 6-phosphogluconolactone and either water (Δ) or 100 μg of cell-free extract protein derived from strain PAO1 (\square), PAO8029 (\blacksquare), PAO8029 containing plasmid pPZ603 (\bullet), or PAO9010 (*zwf*) (\circ). At the indicated times, the reactions were terminated, and remaining 6-phosphogluconolactone was converted to a ferric hydroxymate and quantified colorimetrically. Strains were grown on minimal medium containing 20 mM gluconate as the sole carbon source, and cell extracts were prepared as described in the text.

Alignment of Pgl homologs

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PAO           ~~~~~ngvymai      selklpavq  lqwgssaeq  arglaaevag  rlrssalaegq  gallvsggr  spvafleals  eep...ldw  sritvsIade
H.pylori     ~~~~~mgyg         lfefnlkdc  hkalerfke  fhntalKkh  qsiATSGgr  spsIstqKls  vln...Lkw  heclisIvDe
Anabaena    ~~~~~mkt         vevlpdqtr  i.rslallt  kldtaikqgg  rftIALSGGS  TPkpllyeaIa  ...aqklpw  ekihvfwgDe
Syn.PCC     ~~~~~megkrki     hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
A.actin     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.infl      ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
T.pall.     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
SOL1        ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Consensus   ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe

PAO           ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.pylori     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Anabaena    ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Syn.PCC     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
A.actin     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.infl      ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
T.pall.     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
SOL1        ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Consensus   ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
    
```

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PAO           ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.pylori     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Anabaena    ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Syn.PCC     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
A.actin     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.infl      ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
T.pall.     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
SOL1        ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Consensus   ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
    
```

```

PAO           ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.pylori     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Anabaena    ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Syn.PCC     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
A.actin     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
H.infl      ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
T.pall.     ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
SOL1        ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
Consensus   ~~~~~             hspesvmap  vdvlinkqil  ieralvcvt  ritkalaerg  ggtIALSGGn  TPkpllyeaIa  ...rgalpw  ekihvfwgDe
    
```

FIG. 6. Alignment of amino acid sequences for representative homologs of the *P. aeruginosa* phosphoglucuronidactonase. The homologs were identified using the BLAST program and were aligned with Genetics Computer Group programs. The consensus sequence was generated using a plurality of five. The sequences include the PAO-derived sequence reported here, and ones from *Helicobacter pylori* (GenBank accession no. AE000616), *Anabaena* (Swiss-Prot accession no. P46016), *Synechocystis* sp. strain PCC6803 (DDBJ accession no. D90916), *Actinobacillus actinomycetemcomitans* (DDBJ accession no. D88189), *H. influenzae* (Swiss-Prot accession no. O57039), *Treponema pallidum* (GenBank accession no. AAC65464), and *S. cerevisiae* SOL1 (Swiss-Prot accession no. P50278). Sequence numbers refer to the *S. cerevisiae* SOL1 amino acid sequence (residues 1 to 30 not shown). Additional N-terminal sequence for the *H. influenzae* homolog (underlined) was deduced from the DNA sequence (12).

PAO8029 is reminiscent of *E. coli* phosphogluconolactonase mutants (17, 18). Hence, we assayed for 6-phosphogluconolactonase after growing *P. aeruginosa* PAO1, PAO9010 (*zwf*), PAO8029, and PAO8029 containing pPZ603 with gluconate as the carbon source. The ferric hydroxamate assay of 6-phosphogluconolactonase activity that measures the disappearance of the substrate is essentially qualitative because of a relatively high nonenzymatic rate and the presence of the γ and δ 6-phosphogluconolactonases in the substrate (17). Nevertheless, it is clear from the data in Fig. 5 that extracts from PAO1 and PAO9010 had phosphogluconolactonase activity, while the PAO8029 extract was essentially identical to the control without extract protein. An extract of PAO8029 containing plasmid pPZ603 also had phosphogluconolactonase activity, demonstrating complementation of the gene. Hence, the *P. aeruginosa devB* homolog was identified as *pgl*.

Given the nonenzymatic rate of hydrolysis for 6-phosphogluconolactone (with a $t_{1/2}$ of approximately 50 min; see Fig. 5), the dramatic reduction in growth rate for the *pgl* mutant strains grown on mannitol might seem surprising. However, this phenotype is consistent with the normally short metabolic lifetime of 6-phosphogluconolactone, as pointed out by Scopes (33). However, this would not account for the reduced rate of growth by strain PAO8029 on gluconate (compared to PAO1 and PAO9010 in Table 2). Since the cyclic Entner-Doudoroff pathway in *P. aeruginosa* would allow the synthesis of 6-phosphogluconolactone, we suggest that the substrate for Pgl, 6-phosphogluconolactone, is toxic.

It seems essential that there should be similar amounts of 6-phosphogluconolactonase and glucose-6-phosphate dehydrogenase activity in the cell in order to maintain a balanced flux through this metabolic pathway. A quantitative assay for 6-phosphogluconolactonase, in which the 6-phosphogluconolactonase is coupled to a 6-phosphogluconate dehydrogenase, showed that there are similar levels of these activities in extracts from PAO1 cells grown on gluconate (353 ± 26 mIU/mg for glucose-6-phosphate dehydrogenase and 388 ± 41 mIU/mg for 6-phosphogluconolactonase).

Conservation within the Pgl family. A search of the NCBI databases using the BLAST program (1) with the deduced amino acid sequence for Pgl as a query turned up a large number of family members, previously identified as DevB or SOL homologs. Alignment of several such members revealed important regions of conservation (motifs) that may include the active-site residues of 6-phosphogluconolactonase (Fig. 6). The two most conserved regions include IALSGGSTP and LGMG-DGHTASLFP. Note that the genomic sequence of the *Haemophilus influenzae* 6-phosphogluconolactonase homolog (*devB*; HIO556) is truncated prior to this first motif (12). However, the upstream sequence of *H. influenzae* does encode this motif with an appropriate ATG start codon (underlined in Fig. 6), as well as two internal stop codons (indicated as x in Fig. 6). We suggest either that the stop codons are sequencing artifacts or that *H. influenzae* has a mechanism for translating through them. A cDNA encoding a human cytosolic Pgl has recently been cloned and expressed in *E. coli* and shown to have Pgl activity (8). In addition to observing its homology with *P. aeruginosa* Pgl, the bacterial DevB, and the yeast SOL proteins, these authors also identified a less conserved homology with glucosamine-6-phosphate isomerase.

The overlapping nature of *Zwf* and *Pgl* in *P. aeruginosa* suggests a very tight translational control, perhaps necessary to balance their enzymatic activities. In general, the prokaryote-derived members of the *pgl* family are found next to *zwf* (24), with the notable exception of *E. coli*. Since *pgl* was originally identified and mapped in *E. coli* K-10 (an HfrC derivative of

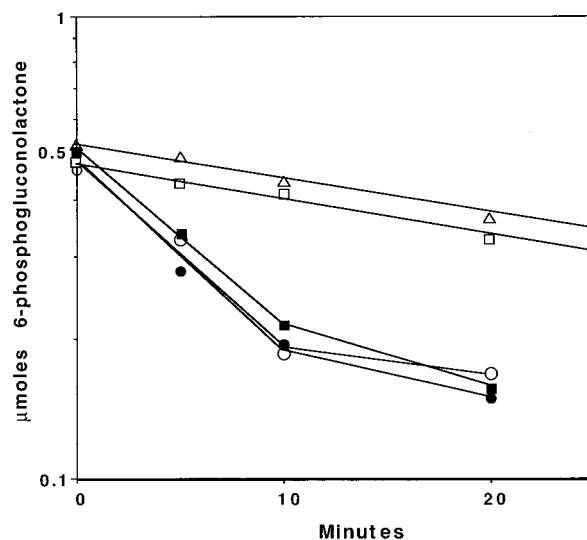


FIG. 7. *pgl* is a member of the *hex* regulon. Assays contained 6-phosphogluconolactone and either water (Δ) or 100 μ g of cell extract protein derived from strain PAO1 grown with succinate (\square) or gluconate (\blacksquare) as the sole carbon source or PAO8026 (*hexR*) grown with succinate (\circ) or gluconate (\bullet) as the sole carbon source. At the indicated times, reactions were terminated, and the remaining 6-phosphogluconolactone was quantified colorimetrically.

K-12), we were surprised when searches using BLAST failed to identify an obvious homolog in *E. coli*. The closest might be *nagB* (8) (GenBank accession no. M19284), whose map position is 1 min from the *E. coli pgl* gene.

In general, glucose-6-phosphate dehydrogenases are highly conserved proteins of about 500 amino acids. However, the rabbit microsomal glucose-6-phosphate dehydrogenase, derived from the lumen of the endoplasmic reticulum, contains 763 amino acids, with a "noncatalytic domain" in the middle of the enzyme (27). The "noncatalytic domain" contains the second Pgl motif (residues 399 to 411 of the rabbit microsomal glucose-6-phosphate dehydrogenase are GMGtDGHTAS LFP). Likewise, the amino acid sequence of the unusual glucose-6-phosphate dehydrogenase of the malaria parasite, *Plasmodium falciparum*, begins with a Pgl domain (33a). The recently described hexose-6-phosphate dehydrogenase from human bone marrow also contains a Pgl domain, located in the carboxy-terminal third of the gene (8, 24). Thus, it appears that these animals have evolved a simple solution for efficient metabolic flux through glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase by combining both activities into a single protein.

***pgl* is a member of the *hex* regulon.** Since *pgl* follows *zwf* directly, both in the arrangement of the genes and in metabolic pathway function, it seemed likely that they would be coordinately regulated. Growth with gluconate as the carbon source induced glucose-6-phosphate dehydrogenase (*Zwf*), while growth on succinate did not (340 ± 18 mIU/mg versus 3 ± 3 mIU/mg). Similarly, we observed that PAO1 had 6-phosphogluconolactonase activity when grown on gluconate but not when grown on succinate (Fig. 7). Recently, the repressor for the *hex* regulon has been identified (29; Procter et al., abstr. K-135). The HexR protein represses the expression of all the enzymes in the *hex* regulon by binding to DNA sequences upstream of each operon. Inactivation of HexR by insertion of a gentamicin resistance cassette into the chromosomal copy of *hexR* results in constitutive expression of the *hex* regulon (29; Procter et al., abstr.). In the *hexR* mutant strain PAO8026,

6-phosphogluconolactonase activity was present at equivalent levels when the strain was grown on either gluconate or succinate (Fig. 7). Thus, *pgl* is under the control of HexR and is demonstrated to be another member of the *hex* regulon.

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