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Cloning and Characterization of the *Pseudomonas aeruginosa zwf* Gene Encoding Glucose-6-Phosphate Dehydrogenase, an Enzyme Important in Resistance to Methyl Viologen (Paraquat)

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In this study, we cloned the *Pseudomonas aeruginosa zwf* gene, encoding glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that catalyzes the NAD^+- or $NADP^+$-dependent conversion of glucose-6-phosphate to 6-phosphogluconate. The predicted *zwf* gene product is 490 residues, which could form a tetramer with a molecular mass of ~220 kDa. G6PDH activity and *zwf* transcription were maximal in early logarithmic phase when inducing substrates such as glycerol, glucose, or gluconate were abundant. In contrast, both G6PDH activity and *zwf* transcription plummeted dramatically when bacteria approached stationary phase, when inducing substrate was limiting, or when the organisms were grown in a citrate-, succinate-, or acetate-containing basal salts medium. G6PDH was purified to homogeneity, and its molecular mass was estimated to be ~220 kDa by size exclusion chromatography. Estimated K_m values of purified G6PDH acting on glucose-6-phosphate, $NADP^+$, and NAD^+ were 530, 57, and 333 μM, respectively. The specific activities with NAD^+ and $NADP^+$ were calculated to be 176 and 69 μmol/min/mg. An isogenic *zwf* mutant was unable to grow on minimal medium supplemented with mannitol. The mutant also demonstrated increased sensitivity to the redox-active superoxide-generating agent methyl viologen (paraquat). Since one by-product of G6PDH activity is NADPH, the latter data suggest that this cofactor is essential for the activity of enzymes critical in defense against paraquat toxicity.

Pseudomonas aeruginosa is a gram-negative bacillus that is virtually ubiquitous throughout nature. It is also an opportunistic pathogen of humans, most notably those who have cystic fibrosis or whose immune systems have been compromised (e.g., as a result of burns or cancer chemotherapy) (11, 16, 55). The organism has a remarkable capacity to utilize a wide range of carbon sources for growth under a variety of environmental conditions. Although tricarboxylic acid (TCA) cycle intermediates such as succinate are preferentially utilized by *P. aeruginosa*, the organism readily catabolizes glucose. In contrast to the facultative organism *Escherichia coli*, *P. aeruginosa* does not metabolize glucose via the Embden-Meyerhof pathway because it does not possess 6-phosphofructokinase (36). Thus, the catabolism of glucose by *P. aeruginosa* requires its conversion to glyceraldehyde-3-phosphate and pyruvate via the Enter-Doudoroff enzymes 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda) (36). Depending on the physiological conditions, glucose is converted to 6-phosphogluconate by one of two routes, one of which is oxidative and the other of which is phosphorylative. The direct oxidative route involves oxidation of glucose to gluconate and 2-ketogluconate in the periplasm via membrane-bound glucose and gluconate dehydrogenases (29). Recently both oxidative routes have been shown to be physiologically significant with the isolation of mutants blocked in either gluconate or 2-ketogluconate utilization (67a). Alternatively, the phosphorylative route involves uptake of glucose by an inducible transport system where, once inside the organism, it is phosphorylated by glucokinase and next converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), the product of the *zwf* gene (29).

Interestingly, a *zwf* mutant of *E. coli* was reported to be more sensitive to the redox-active, superoxide (O_2^-)-generating agent methyl viologen (paraquat) (19). It was then postulated by Liochev and Fridovich (38) that this sensitivity was attributed to a reduced level of NADPH, a cofactor necessary for the activity of glutathione reductase (3) and alkylhydroperoxide reductase (32), enzymes which combat paraquat-mediated oxidative stress.

In this study, we describe the cloning and characterization of the *zwf* gene of *P. aeruginosa* PAO1. We demonstrate that inactivation of the *zwf* gene does not allow mutant organisms to grow on mannitol as the sole carbon source. In addition, we demonstrate that the *zwf* gene is under tight control, being highly transcribed in the presence of inducing agents such as glycerol and glucose and weakly transcribed in the presence of the TCA cycle intermediate succinate. In addition, we demonstrate that G6PDH activity is important in resistance to the O_2^--generating agent paraquat.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All *P. aeruginosa* and *E. coli* strains used in this study are listed in Table 1 and were maintained on Luria (L)-agar plates containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of Bacto Agar per liter. Frozen stocks were stored indefinitely at −80°C in a 1:1 mixture of 25% glycerol and stationary-phase culture grown in L broth.

Growth conditions. All bacteria were grown from single-colony isolates in either L broth or a basal salts medium (69) supplemented with 20 to 60 mM

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Source or reference
E. coli		
DH5α-MCR	F$^-$ *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*)	Bethesda Research Laboratories
SM10	Km^r, mobilizer strain	63
P. aeruginosa		
PAO1	Wild type	27
PAO9010	Gm^r *zwf*::Gm^r	This study
PAO9011	Gm^r *zwf*::*lacZ*-Gm^r (sense orientation)	This study
PAO9012	Gm^r *zwf*::*lacZ*-Gm^r (antisense orientation)	This study
Plasmids		
pBluescript KS−/+	Extended polylinker pUC derivative	Stratagene
pNOT19	Ap^r, pUC19 + *Not*I site	59
pMOB3	Ap^r Cm^r Km^r *sacB oriT mob*	59
pUCGM	Ap^r Gm^r, pUC19 + 850-bp Gm^r cassette	60
pZ1918	Ap^r, pUC19/18 with 3.2-kb *lacZ* cassette	61
pZ1918G	Ap^r Gm^r, pZ1918 with Gm^r cassette from pUCGM immediately downstream of the *lacZ* gene	H. P. Schweizer
pEX30	Ap^r, broad-host-range expression vector	
pEX100T	Ap^r, mobilizable *oriT sacB* vector for construction of mutants	62
pUCP22	Ap^r, broad-host-range cloning vector	68
pPZ523	Ap^r, pUCP22 with 2.5-kb *Sac*II fragment, complements *P. aeruginosa* PFB98 (*zwf-1*)	This study
pJFM1	Ap^r, pBluescript SK− with 2.5-kb *Sac*II fragment containing *zwf*	This study
pJFM2	Ap^r, pBluescript SK− with 1.6 kb *zwf* PCR product	This study
pJFM3	Ap^r, *Afl*III-*Eco*RI fragment of 1.6-kb *zwf* PCR product in *Nco*I and *Eco*RI sites of pEX30	This study
pJFM4	Ap^r, 1.7-kb *Pvu*II *zwf* fragment in *Sma*I site of pEX100T	This study
pJFM5	Ap^r Gm^r, pJFM4 with 4.0-kb *lacZ*-Gm^r cassette in *Bam*HI site of *zwf* in sense orientation	This study
pJFM6	Ap^r Gm^r, pJFM4 with 4.0-kb *lacZ*-Gm^r cassette in *Bam*HI site of *zwf* in antisense orientation	This study
pJFM7	Ap^r, pUCP22 with 1.0-kb *Pst*I-*Bam*HI fragment of *zwf*	This study
pJFM8	Ap^r, pJFM7 with 3.2-kb *lacZ* in *Stu*I site of *zwf*	This study
pJFM9	Ap^r, pNOT19 with ~1-kb *Pst*I-*Bam*HI fragment of *zwf*	This study
pJFM10	Ap^r, pJFM1 with ~850-bp Gm^r cassette in *Hinc*II site of *zwf*	This study
pJFM11	pJFM2 with 5.8-kb *Not*I *oriT sacB* fragment from pMOB3	This study

[a] Abbreviations used for genetic markers are as described by Holloway et al. (27). *mob*, mobilization site (ColE1); Tra$^+$, conjugative phenotype; *oriT*, origin of transfer (RK2); Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance.

selected carbon sources. Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel at 70 rpm unless otherwise indicated. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure proper aeration. All agar media were solidified with 1.5% Bacto Agar.

Cloning and sequence analysis of *P. aeruginosa* PAO1 *zwf*. Steps involved in the cloning of the *P. aeruginosa* PAO1 *zwf* gene are described in Results. All DNA sequencing was performed by the dideoxy method on double-stranded DNA (Sequenase 2.0; U.S. Biochemical, Cleveland, Ohio). Sequence obtained by this method was confirmed on both strands by using a PRISM Dye Deoxy terminator cycle sequencing kit and analyzed on an ABI model 373A DNA sequencer. Additional DNA sequencing was provided by the Biotechnology Program of the East Carolina University School of Medicine. Oligonucleotides for DNA sequencing reactions and PCR analysis were synthesized in the DNA Core Facilities, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, or were provided by the Biotechnology Program, East Carolina School of Medicine. Sequence analysis was performed with Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.), MacVector 4.1.1 (Eastman Chemical Co., New Haven, Conn.), or Gene Runner (Hastings Software, Inc.). Amino acid alignments were performed by using either the BLASTP program provided by the National Center for Biotechnology Information (1) or the Align Plus 3 global alignment program (Sci-Ed Software, Durham, N.C.). Potential transcription start sites were identified by using the Neural Network Promoter Prediction program (http://www-hgc.lbl.gov/projects/promoter.html [54]).

Manipulation of recombinant DNA. Plasmid DNA was transformed into either *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, Md.) or *E. coli* SM10 (63). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) was added to agar medium to detect the presence of insert DNA via blue/white selection. Restriction endonucleases, alkaline phosphatase, and T4 DNA ligase were used as specified by the vendor (Gibco-BRL); Plasmid DNA was isolated on a small scale by the alkaline lysis method described by Maniatis et al. (43) or on a large scale by using a plasmid isolation kit (Qiagen). Restriction fragments were recovered from agarose gels by using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, Maine).

Construction of the G6PDH overexpression vector, pJFM3. PCR primers ZWF-START (5′-GTAACAACACATGTCTGATGTCCGCGTTCT-3′) upstream of the *P. aeruginosa zwf* gene and KS (5′-TCGAGGTCGACGGTATC-3′) of pBluescript KS− were used to PCR amplify the *zwf* gene from pJFM1, and this fragment was cloned into the *Eco*RV site of pBluescript KS−. This plasmid, designated pJFM2, was digested with *Afl*III and *Eco*RI, and the resulting ~1.6-kb *zwf* fragment was ligated into the *Nco*I/*Eco*RI-cut expression vector pEX30 (61a), forming pJFM3. Plasmid pJFM3 was then used for overproduction and purification of G6PDH as described below.

Purification of *P. aeruginosa* G6PDH. Overproduction of G6PDH in *P. aeruginosa* PAO1 was accomplished after the following steps. Bacteria harboring the *zwf* overexpression vector pJFM3 were grown in 4 liters of L broth containing carbenicillin (400 μg/ml) to an optical density at 600 nm (OD_{600}) of 0.3. The synthesis of T7 polymerase was then induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the bacteria were allowed to grow for an additional 3 h at 37°C. The bacteria were pelleted by centrifugation at 10,000 × *g* for 15 min, washed in 0.9% saline, and resuspended in 50 mM Tris-HCl (pH 7.8) containing mercaptoethanol (0.02%), lysozyme (0.02%), and the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 μM), and pepstatin (0.5 μM). The suspension was incubated on ice for 1 h and subjected to three freeze (−80°C)-thaw (37°C) cycles to aid in breakage of the cells and further disrupted in a French pressure cell at 12,000 lb/in^2 at 4°C. Unbroken cells and cell debris were clarified by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. The clarified extract was brought to 38% saturation with ammonium sulfate and allowed to incubate at 4°C for 17 h, and the precipitated protein was clarified by centrifugation at 10,000 × *g* for 20 min. The precipitate was dissolved in 20 mM potassium phosphate buffer (KP_i), pH 6.8, and dialyzed against six 1-liter changes of the same buffer at 4°C. This solution was filtered

through a 0.22-μm-pore-size filter (Nalgene) and concentrated with an Amicon YM-100 membrane. The retentate, containing G6PDH, was passed over a 2- by 18-cm DE-52 column (Whatman International Ltd., Kent, England) and eluted with a 20 to 400 mM gradient of KP_i (pH 6.8). After concentration of the G6PDH-containing fractions as described above, sample was loaded on a 1.5- by 6-cm hydroxyapatite column and eluted with a 20 to 150 mM gradient of KP_i. Finally, G6PDH was purified from two smaller contaminating proteins by passage through a 3.5- by 100-cm Bio-Gel 150 gel filtration column equilibrated with 20 mM KP_i (pH 6.8) at 4°C while maintaining a flow rate of 0.2 ml/min. Purified G6PDH was then stored on ice at 0°C.

Construction of a *P. aeruginosa zwf* mutant. The strategy for insertional mutagenesis of the *zwf* gene was based on the sucrose counterselection technique (59). To accomplish this, a ~1-kb *Pst*I-*Bam*HI fragment from pJFM1 was ligated into pNOT19 (59), forming pJFM9. This plasmid was linearized with *Hin*cII, a unique site within the *zwf* gene, and ligated to a *Sma*I-cut ~850-bp gentamicin resistance (Gm^r) cassette from pUCGM (60), creating pJFM10. This plasmid was linearized with *Not*I and ligated to the 5.8-kb *oriT sacB*-containing fragment of pMOB3 (59), creating pJFM11. After biparental mating of *E. coli* SM10 harboring pJFM11 and *P. aeruginosa* PAO1, plasmid integration into the genome by homologous recombination was assessed by selection on *Pseudomonas* isolation agar (PIA)-gentamicin plates. Isolated Gm^r colonies were picked and grown in L broth until mid-log phase, and serial dilutions were plated on PIA-gentamicin plates containing 5% sucrose. Candidate *zwf* mutants were confirmed by Southern blot and G6PDH activity gel analyses.

Paraquat sensitivity assays. Bacteria were grown for 17 h at 37°C under aerobic conditions in L broth without NaCl containing 400 μg of carbenicillin per ml and 0.2 mM IPTG. Aliquots (2.5 μl) of these suspensions were added to 2.5 ml of the same medium containing increasing concentrations of paraquat and incubated on a roller wheel at 90 rpm for 17 h at 37°C. The final absorbance of the appropriately diluted suspensions was recorded on a Beckman DU 20 (Fullerton, CA) spectrophotometer at 600 nm.

Cell extract preparation, nondenaturing gel electrophoresis, and biochemical assays. Cell extracts of mid-logarithmic-phase organisms or overnight-grown bacteria were prepared from cultures harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM sodium phosphate buffer (pH 7.0) and sonicated in an ice water bath for 10 s with a model W-225 sonicator (Heat-Systems, Inc., Farmington, N.Y.) at setting 5. The sonicate was then clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. Cell extract for native gel electrophoresis was prepared as described above except that 50 mM Tris-HCl (pH 7.8) was used as the diluent. G6PDH activity was monitored by the production of NADPH at 340 nm in 1-ml reaction mixtures containing 1 mM glucose-6-phosphate, 0.4 mM $NADP^+$, and cell extract, using a Beckman DUP spectrophotometer equipped with an NGI (Elk Grove Village, Ill.) Servogor chart recorder unless otherwise indicated. G6PDH activity staining in 7.5% nondenaturing gels was performed with the same reagents as in the spectrophotometric assay described above except for the addition of 0.16 mM phenazine methosulfate and 0.18 mM nitroblue tetrazolium (37). Paraquat: NAD(P)H oxidoreductase activity was assayed by two methods. The first involved following the oxidation of NADH or NADPH spectrophotometrically at 340 nm (39). The second involved activity staining in 10% nondenaturing gels soaked in a mixture of 50 mM Tris-HCl (pH 7.5), 4 mM paraquat, 1 mM nitroblue tetrazolium, and either 0.2 mM NADH or NADPH (40). β-Galactosidase assays were performed on either chloroform-sodium dodecyl sulfate (SDS)-treated bacteria or cell extracts, using *o*-nitrophenyl-β-D-thiogalactopyranoside (ONPG) as the substrate; the results were expressed as international units (micromoles of ONPG hydrolyzed/minute/milligram of protein), using a millimolar extinction coefficient for ONPG of 3.1 (45). Catalase activity was monitored by following the decomposition of 18 mM H_2O_2 at 240 nm (4, 6, 25). Superoxide dismutase (SOD) activity was monitored by following the autoxidation of pyrogallol at 320 nm (52), a modification of the original method described by Marklund and Marklund (44). Protein concentrations were estimated by the method of Bradford (5), using bovine serum albumin fraction V (Sigma) as the standard.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession no. AF029673.

RESULTS

DNA sequence analysis of the *P. aeruginosa zwf* gene. The *P. aeruginosa* PAO1 *zwf* gene was initially cloned on an 11-kb *Bam*HI fragment that also contained the *eda* gene, encoding keto-3-deoxy-6-phosphogluconate aldolase (64). The *zwf* gene was localized to a ~2.5-kb *Sac*II fragment. The predicted *zwf* gene product is 490 residues, which could form a tetramer with an molecular mass of ~220 kDa (Fig. 1). A putative ribosome binding (Shine-Dalgarno) site (GGttGG) was identified 9 bp upstream of the *zwf* ATG start codon. The estimated size of the translated G6PDH monomer was ~55.6 kDa, with a pI of 12.6.

Amino acid similarity with other G6PDH proteins. The *P. aeruginosa* G6PDH was aligned with seven other G6PDH proteins (identified by GenBank accession number and, in brackets, reference) from *E. coli* (M55005 [57]), *Mycobacterium tuberculosis* [unpublished]), (Z95844). *Chlamydia trachomatis* (U83195 [unpublished]), *Haemophilus influenzae* (U32737 or L42023 [15]), *Erwinia chrysanthemi* (X74866 [28]), *Anabaena* sp. strain PCC 7120 (U33282 [48]), and *Synechococcus* sp. strain PCC 7942 (U33285 or X64768 [58]), using the Align Plus 3 multiple protein alignment program (Fig. 2). The *P. aeruginosa* G6PDH revealed the greatest identity with G6PDH proteins from *E. chrysanthemi* (55% identity) and *E. coli* (54% identity). The weakest homology was demonstrated with the G6PDH of *H. influenzae* (19% identity). The remaining G6PDH proteins were >40% identical.

Construction of an isogenic *zwf* mutant of *P. aeruginosa* PAO1. G6PDH-deficient *P. aeruginosa* PAO1 strains called PFB103 (*zwf-2*) and PFB98 (*zwf-1*) were initially constructed by chemical mutagenesis (50, 56). These strains are incapable of utilizing mannitol as the sole carbon source and cannot catabolize glucose via the phosphorylative pathway because of a deficiency in G6PDH activity (50, 56). Here we elected to construct an isogenic *zwf* mutant of wild-type strain PAO1 containing a selectable antibiotic resistance marker, the details of which are given in Materials and Methods. Insertional inactivation of *zwf* was first confirmed by Southern analysis on a $sucrose^r$ Gm^r *zwf* mutant called PAO9010. Genomic DNA from the wild-type strain and PAO9010 was cut with *Pst*I/*Bam*HI, transferred to nitrocellulose, and probed with a ~1-kb *Pst*I-*Bam*HI *zwf* fragment. Insertional inactivation of the *zwf* gene was confirmed by demonstration of a band of the predicted size (1.85 kb [Fig. 3A, lane 2]) relative to wild-type DNA (~1.0 kb [Fig. 3A, lane 1]). Insertional inactivation of the *zwf* gene was also confirmed by monitoring G6PDH activity in nondenaturing gels. Figure 3B (lane 2) demonstrates an absence of G6PDH activity band in the mutant organism, while activity is clearly evident in the wild-type strain (lane 1).

Transcription of the *zwf* gene and G6PDH activity are maximal in logarithmic phase. To examine the regulation of *zwf* in *P. aeruginosa*, we used both transcriptional and translational approaches. To monitor *zwf* transcription, a 1.7-kb *Pvu*II *zwf*-containing fragment from pJFM1 was cloned into the unique *Sma*I site in pEX100T (62). This plasmid, pJFM4, was cut with *Bam*HI and ligated to a 4.0-kb *Bam*HI-cut promoterless *lacZ*-Gm^r cassette from pZ1918G (61a), resulting in either pJFM5 (sense orientation) or pJFM6 (antisense orientation). These plasmids were conjugated into *P. aeruginosa* PAO1 via biparental mating and selected on PIA containing gentamicin (300 μg/ml). Genetic confirmation of the *zwf*::*lacZ*-Gm^r fusion mutant strain PAO9011 was elucidated after sucrose counterselection (as with strain PAO9010) and Southern analysis (data not shown). As shown in Fig. 4A, *zwf* (sense) transcription rose dramatically in early to mid-logarithmic phase, followed by a marked drop in activity when the organisms entered late log to stationary phase. Antisense transcriptional activity throughout the entire growth phase was negligible (data not shown). G6PDH enzymatic activity paralleled that of the *zwf* transcriptional analysis in Fig. 4A, with activity rapidly rising in the early stages of growth and falling markedly at the later stages (Fig. 4B).

Effect of carbon source on *zwf* transcription. G6PDH activity is induced when organisms are grown in minimal medium supplemented with either glucose, gluconate, or glycerol, highly reduced substrates. In pseudomonads, G6PDH activity

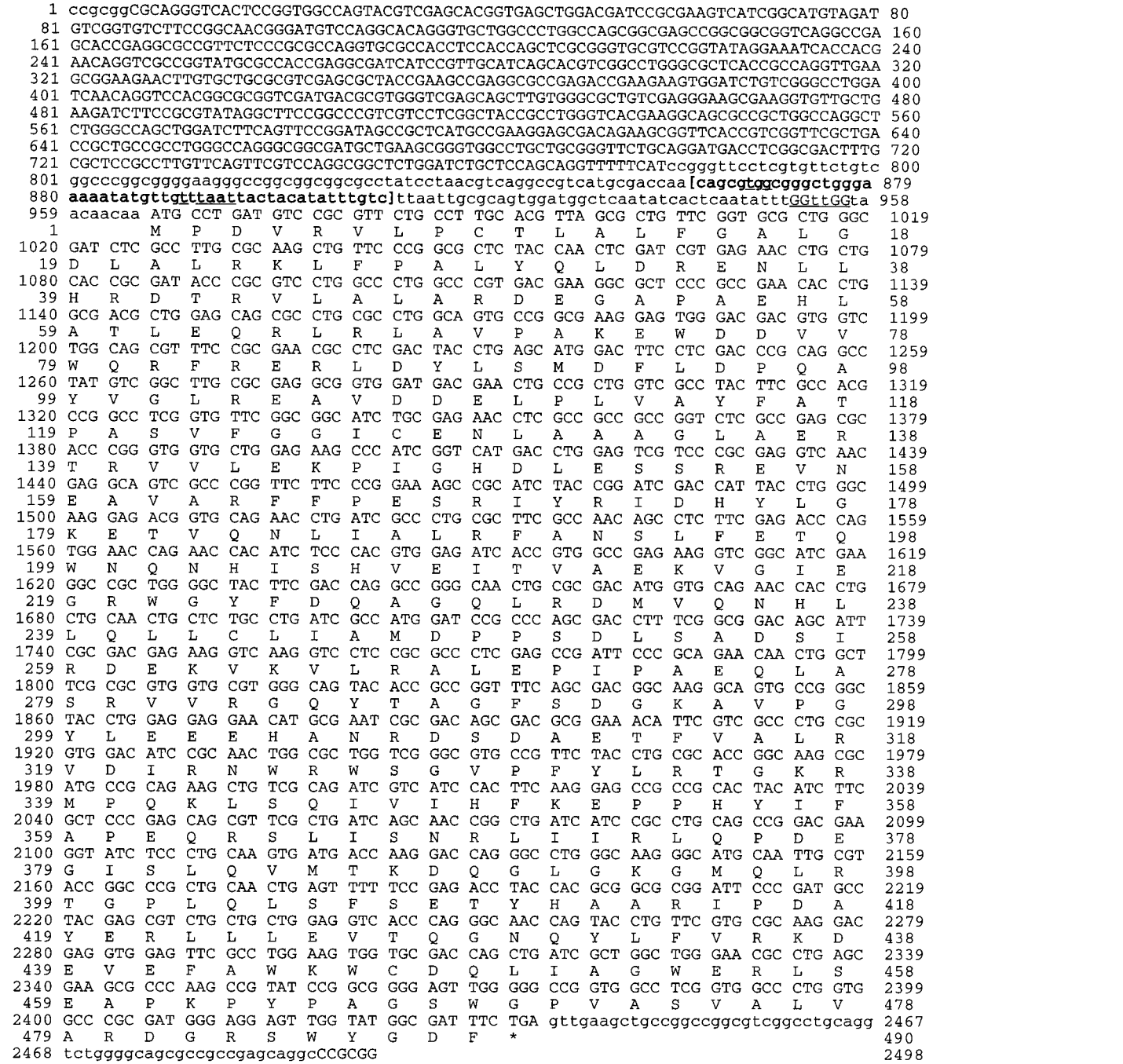

FIG. 1. DNA sequence of the *zwf* gene of *P. aeruginosa* PAO1. Coding sequences are uppercase, noncoding sequences are lowercase. The *zwf* coding sequence starts at position 966. The putative ribosome binding (Shine-Dalgarno) sequence is underlined prior to the ATG start codon. A predicted σ^{70}-like promoter is bracketed, with the −35 and −10 regions underlined, and is based on the Neural Network Promoter Prediction program (54). The asterisk indicates the *zwf* stop codon.

is repressed by organic acids via catabolite repression (39; for a review, see reference 10), and this effect has been assumed to be at the level of transcription. To test this assumption, we elected to determine the level of *zwf* transcription in *zwf*::*lacZ*-Gm^r fusion strain PAO9011 (constructed above) grown in media containing a variety of carbon sources. As shown in Fig. 5, there is a broad range of transcriptional control of the *zwf* gene. Growth on glycerol, gluconate, and glucose allowed for the highest levels of *zwf* transcription. In contrast, growth on organic acids reduced *zwf* transcription, with succinate and acetate evoking the strongest catabolite repression.

Purification of *P. aeruginosa* G6PDH and enzyme kinetics. To determine the kinetic parameters of the *P. aeruginosa* G6PDH, we purified G6PDH from wild-type bacteria overexpressing the enzyme. We used a four-step procedure involving (i) ammonium sulfate precipitation of cell extracts, (ii) DE-52 anion exchange, (iii) hydroxyapatite chromatography, and (iv)

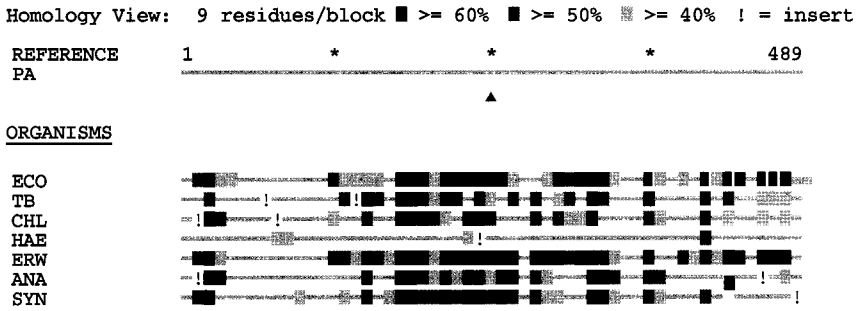

FIG. 2. Alignment of G6PDH proteins from various organisms. G6PDH proteins were retrieved from GenBank and aligned by using the global alignment program Align Plus 3. Abbreviations: PA, *P. aeruginosa*; ECO, *E. coli*; TB, *M. tuberculosis*; CHL, *C. trachomatis*; HAE, *H. influenzae*; ERW, *E. chrysanthemi*; ANA, *Anabaena* sp.; SYN, *Synechococcus* sp. (B) Homology blocks of each G6PDH protein.

Bio-Gel 150 gel filtration. Figure 6 demonstrates the stages of purification using SDS-polyacrylamide gel electrophoresis (PAGE). Clearly, the most significant purification step was DE-52 anion-exchange chromatography (Fig. 6, lane 4). Some proteins which bound to DE-52 did not bind to hydroxyapatite (Fig. 6, lane 5). The smaller contaminating proteins still present after hydroxyapatite chromatography were resolved easily with the Bio-Gel 150 matrix, while G6PDH, because of its large size (estimated at ~220 kDa), eluted from the column at the void volume (Fig. 6, lane 6). We obtained approximately 500 μg of purified G6PDH, a small portion of which was used for the kinetic analyses described below.

Many G6PDH enzymes have been purified from organisms or tissues from various phyla. Unlike the *P. aeruginosa* (35) and several other bacterial G6PDH enzymes, however, many G6PDH enzymes cannot use both NAD^+ and $NADP^+$ as cofactors for enzyme activity. To determine the specificity of the *P. aeruginosa* G6PDH toward G6P, NAD^+, and $NADP^+$, we performed a kinetic analysis using each compound. Double-reciprocal Lineweaver-Burk plots of enzymatic activity as a function of G6P, NAD^+, and $NADP^+$ concentration are shown in Fig. 7. The estimated K_m values for G6P (with $NADP^+$ as the cofactor), NAD^+, and $NADP^+$ were 530, 333, and 57 μM, respectively. The specific activities for NAD^+ and $NADP^+$ were calculated to be 176 and 69 μmol/min/mg.

An absence of G6PDH confers enhanced sensitivity to paraquat. The intracellular redox status of all living cells is governed in part by the levels of $NAD(P)^+$ relative to NAD(P)H. NADPH is an essential cofactor for glutathione reductase (3) and alkylhydroperoxide reductase (32), enzymes important in combating oxidative stress (9). Since by-products of G6PDH activity are NADH and NADPH, and reduced levels of these cofactors would limit the efficacy of such enzymes, we predicted that a *zwf* mutant would be more sensitive to paraquat. However, for paraquat to cause oxidative stress, it must first be reduced, followed by autoxidation of the paraquat monocation radical (PQ^*) by oxygen, creating O_2. Paraquat reduction within *E. coli* is catalyzed by three paraquat:NADPH oxidoreductases (39). It would be predicted that the activity of such an enzyme(s) under aerobic conditions would, in part,

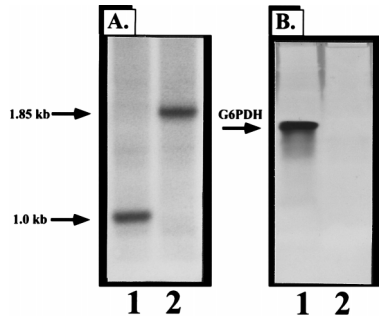

FIG. 3. Construction of a *P. aeruginosa* isogenic *zwf* mutant PAO9010. (A) Genomic DNA was digested with *Pst*I and *Bam*HI and transferred to nitrocellulose. Southern blot analysis was performed with a ^{32}P-labeled 1.0-kb *Pst*I/*Bam*HI *zwf* fragment. Lane 1, PAO1 (wild type); lane 2, PAO9010 (*zwf* mutant). (B) G6PDH activity gel (7.5%) of cell extracts. Lane 1, PAO1 (wild type); lane 2, PAO9010 (*zwf* mutant).

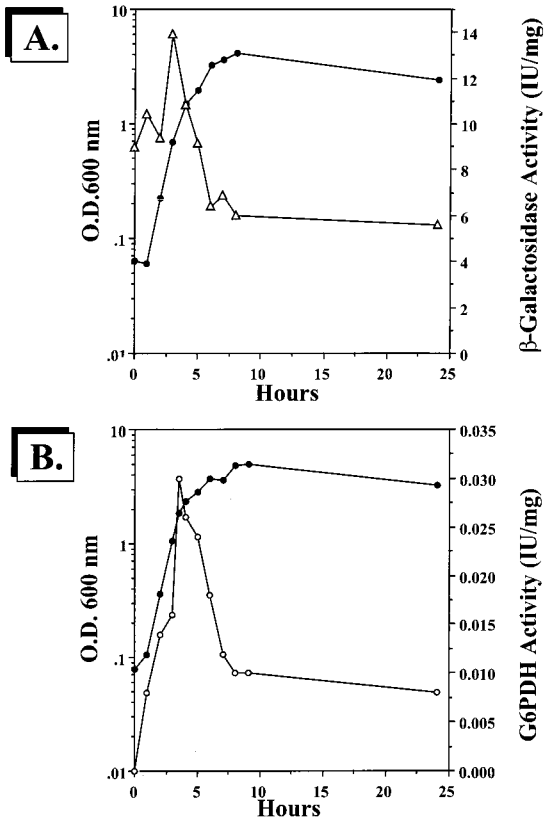

FIG. 4. Analysis of *zwf* transcription (A) and G6PDH activity (B) throughout a normal aerobic growth phase. Wild-type bacteria and strains PAO9011 (*zwf*::*lacZ*-Gm^r, sense orientation) and PAO9012 (*zwf*::*lacZ*-Gm^r, antisense orientation) were grown aerobically at 37°C in L broth. At intervals, organisms were harvested and specific activities for β-galactosidase and G6PDH were measured. ●, OD_{600}; △, β-galactosidase specific activity of strain PAO9011; ○, G6PDH activity.

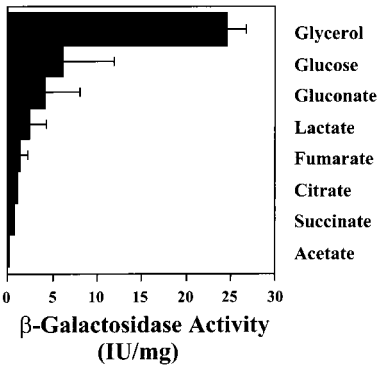

FIG. 5. Effect of carbon source of *zwf* transcription. *P. aeruginosa* PAO9011, with *zwf*::*lacZ* integrated into the chromosome (single copy), was grown aerobically at 37°C in minimal medium containing various carbon sources: glycerol (40 mM), glucose (20 mM), gluconate (20 mM), lactate (40 mM), fumarate (30 mM), citrate (20 mM), succinate (30 mM), and acetate (60 mM). Cell suspensions were assayed in early log phase for β-galactosidase activity as previously described (45).

dictate susceptibility or resistance to paraquat. Because the *P. aeruginosa* G6PDH enzyme also generates NADH (35), it is possible that paraquat can also be reduced by enzymes utilizing this cofactor that may not utilize NADPH. To monitor the activities of NADH- and NADPH-dependent paraquat oxidoreductases and to ensure that wild-type and *zwf* mutant levels of these enzymes were similar, cell extracts of both strains were subjected to nondenaturing PAGE followed by activity staining for both enzymes. As shown in Fig. 8, *P. aeruginosa* possesses both NADPH (Fig. 8A)- and NADH (Fig. 8B)-dependent paraquat oxidoreductases; there were two of the latter and at least five of the former. The addition of PQ (lanes 5 to 8 in Fig. 8A and B) triggered a 1.2-fold increase in oxidoreductase activity, which was measured spectrophotometrically (data not shown). Thus, based on the activity gel, spectrophotometric measurement, and linear scanning densitometry (data not shown), we conclude that wild-type and *zwf* mutant levels of both paraquat-reducing enzymes were similar. Furthermore, the activities of the important antioxidants SoD and catalase were identical in both wild-type and *zwf* mutant organisms (data not shown).

Finally, to test whether a G6PDH deficiency increases the susceptibility of *P. aeruginosa* to paraquat, wild-type and *zwf* mutant strains containing the *zwf* overexpression vector pJFM3 or the control vector pEX30 were grown in L broth in the absence of NaCl and exposed to increasing concentrations of paraquat. Our rationale for not supplementing L broth with NaCl in this experiment is that NaCl has been shown to interfere with paraquat for its receptor on the cell surface and thus restricts its antibiotic efficacy (34). As shown in Fig. 9, wild-type organisms were resistant to 60 μM paraquat whereas the *zwf* mutant demonstrated increased sensitivity at concentrations of 40 μM and greater. When the *zwf* gene was provided in *trans* in both the *zwf* mutant and wild-type strains, there was enhanced resistance to paraquat which exceeded that of wild-type organisms.

DISCUSSION

P. aeruginosa is capable of utilizing myriad carbon sources for growth. Although it prefers TCA cycle intermediates (e.g., succinate or citrate), it also readily metabolizes glucose. A key enzyme involved in glucose metabolism is G6PDH, which converts glucose-6-phosphate to 6-phosphogluconate. 6-Phosphogluconate is further metabolized by the obligatory Entner-Doudoroff pathway (29). In this study, we have cloned and characterized the *zwf* gene encoding G6PDH to better understand the physiological role of this enzyme in normal aerobic metabolism.

Regulation of the *P. aeruginosa zwf* gene was found to be under tight transcriptional control. The transcription of *zwf*, as well as G6PDH activity, was greatest during mid-logarithmic phase and then rapidly decreased to basal levels. This was not surprising since substrates triggering elevated *zwf* transcription (e.g., glucose) are likely depleted once organisms begin to enter the stationary phase (41). Furthermore, *P. aeruginosa* and other *Pseudomonas* species exhibit a strong catabolite repression control (10, 42), and this pattern of induction/repression is a hallmark of such an event. Catabolite repression control of glucose-6-phosphate activity is normally striking, as evidenced by an approximate 90 to 99% reduction in activity when cells are grown on organic acids (66). We observed a similarly wide range of transcriptional control for the chromosomal *zwf*::*lacZ* fusion and now conclude that catabolite repression control of the *zwf* gene is at the level of transcription. Interestingly, when the *zwf*::*lacZ* fusion was introduced on a multicopy plasmid (pUCP22 at ~30 copies/cell [61b]), β-galactosidase activity was reduced only ~50% during growth on organic acids compared to glucose, glycerol, or gluconate (data not shown). This is strikingly similar to the HexC phenotype (64), where a cloned promoter for the *hex* regulon genes *edd* and *gap* is proposed to titrate a *hex* regulon repressor, leading to increased basal activity of all five *hex* regulon enzymatic activities (Zwf, Edd, Eda, Gap, and Glk) (12, 64, 65). This result indicates the presence of a Hex repressor binding site upstream of the *zwf* gene, for which there is in vitro evidence (51).

Unlike eukaryotic G6PDH enzymes, which can utilize only $NADP^+$ as a cofactor, many bacterial enzymes, including those from *Leuconostoc mesenteroides* (13), *Acetobacter suboxydans* (8), *A. hansenii* (53), *Azotobacter vinelandii* (2), *P. aeruginosa* (this study and reference 35), *P. fluorescens* (37), *P.* (*Burkholderia*) *cepacia* (7), and *P. multivorans* (67), utilize both NAD^+ and $NADP^+$. Of particular interest was the remarkably low K_m of the *P. aeruginosa* G6PDH for its substrate, glucose-6-phosphate (530 μM). Estimated K_m values range from 2.3 to 2.7 mM for glucose-6-phosphate of purified or partially purified G6PDH enzymes or the NAD^+/$NADP^+$-cofactored G6PDH from the related organism *P. fluorescens* (37) to 5 mM in the other related species *P. multivorans* (67) and *Burkholderia* (formerly *Pseudomonas*) *cepacia* (7). Furthermore, the cofactor specificity for the *P. aeruginosa* G6PDH was nearly sixfold greater for $NADP^+$ (K_m = 57 μM) than for NAD^+ (K_m = 333

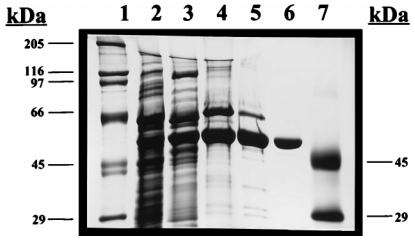

FIG. 6. Analysis of the purification of *P. aeruginosa* PAO1 G6PDH by SDS-PAGE (10% gel). Lane 1, high-molecular-weight protein standard; lane 2, crude cell extract (45 μg); lane 3, 50% ammonium sulfate cut (30 μg); lane 4, DE-52 column eluate (10 μg); lane 5, hydroxyapatite column eluate, (7 μg); lane 6, Bio-Gel 150 column eluate (2 μg); lane 7, low-molecular-weight protein standard.

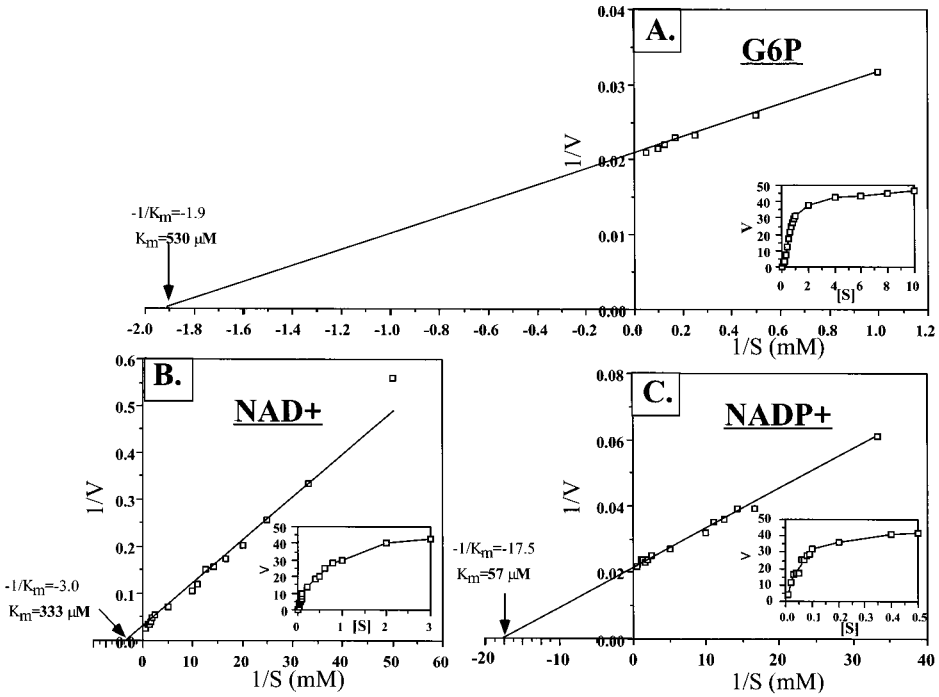

FIG. 7. Estimation of K_m for glucose-6-phosphate (G6P; A), NAD^+ (B), and $NADP^+$ (C) with purified *P. aeruginosa* G6PDH. Rates were measured in terms of an increase in absorbance at 340 nm at 22°C. Initial rates as a function of either glucose-6-phosphate, NAD^+, or $NADP^+$ are presented above on reciprocal coordinates, using 4.2 nM purified *P. aeruginosa* G6PDH.

μM) but the specific activity was greater with NAD (176 versus 69 U/mg). One particular advantage for possessing an enzyme with such superb efficiency is that the valuable cellular reducing equivalents NADH and NADPH, by-products of G6PDH activity, are essential cofactors for hundreds of enzymes involved in catabolic or anabolic processes and cellular defense and could be produced in environments where glucose is limiting.

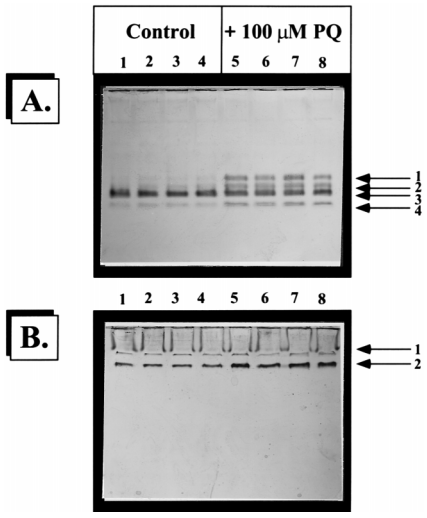

FIG. 8. Analysis of wild-type and *zwf* mutant cell extracts for paraquat:NADPH and paraquat:NADH oxidoreductase activity. *P. aeruginosa* PAO1 and PAO9010 containing either pEX30 (vector control) or the *zwf* expression vector pJFM3 were grown in L broth minus NaCl containing 400 μg of carbenicillin per ml and 0.2 mM IPTG until mid-logarithmic phase (OD_{600} = 0.6), and the culture was divided into two parts. One set was treated with 100 μM paraquat (PQ), and the other served as a control. These suspensions were incubated aerobically for an additional hour at 37°C prior to preparation of cell extracts. Extracts were separated by nondenaturing PAGE on 7.5% polyacrylamide gels and stained for paraquat:NADPH (A) and paraquat:NADH (B) oxidoreductase activities as previously described (40). Lanes 1 through 4, mid-logarithmic-phase organisms; lanes 5 through 8, mid-logarithmic-phase organisms plus 100 μM paraquat for 1 h. Lanes 1 and 5, PAO1(pEX30); lanes 2 and 6, PAO1(pJFM3); lanes 3 and 7, PAO1 *zwf*(pEX30); lanes 4 and 8, PAO1 *zwf*(pJFM3).

The role of G6PDH in susceptibility to paraquat was also dissected. Resistance to reactive oxygen intermediates in *P. aeruginosa* is governed in part by antioxidant enzymes, including iron- and manganese-cofactored SOD (21–26) and at least two heme catalases (6, 21). One compound which exacerbates the production of O_2^- and H_2O_2 within aerobic bacteria is paraquat (18, 20). In *E. coli*, transcriptional control of the *zwf* gene is governed by the *soxRS* regulon (for a review, see reference 14) and increased upon exposure to paraquat (18, 33). It was postulated that an increase in G6PDH activity is brought about because of the need for NADPH, required as a cofactor for glutathione reductase (3) and alkylhydroperoxide reductase (32) activities. The latter enzymes are important in combating paraquat-mediated oxidative stress (9). The sensitivity of our *P. aeruginosa zwf* mutant to paraquat is consistent with an unpublished observation in *E. coli*, where a *zwf* mutant demonstrated increased sensitivity to both the related redox-active compound menadione and H_2O_2 (17). Although the enhanced sensitivity of our *P. aeruginosa zwf* mutant was modest, enhanced resistance could be conferred by overexpression of intracellular G6PDH (Fig. 9). Still, sensitivity to paraquat is likely dependent on the presence or absence of multiple cellular factors, some of which include SOD (24), catalase (6), methionine sulfoxide reductase (46, 47), DNA repair systems (30, 31), and various regulatory proteins (e.g., SoxRS [14]).

Although the *P. aeruginosa gor* gene encoding glutathione reductase has been cloned (49), no mutants are currently available. Similarly, an *ahp* (alkylhydroperoxide reductase [32]) homolog has not been identified in this organism. It will be important to construct *gor* and, if possible, *ahp* mutants of *P.*

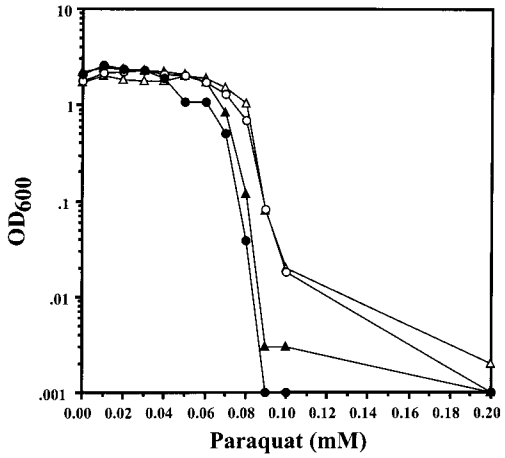

FIG. 9. Effect of paraquat on growth of wild-type and *zwf* mutant *P. aeruginosa. P. aeruginosa* PAO1 and PAO9010 harboring either pEX30 (control vector) or pJFM3 (*zwf* overexpression plasmid) were grown in L broth (without NaCl) containing 400 μg of carbenicillin per ml and 0.2 mM IPTG for 17 h at 37°C under aerobic conditions. Aliquots (2.5 μl) were added to 2.5 ml of the same media containing increasing concentrations of paraquat and incubated on a roller wheel at 90 rpm for 17 h at 37°C. The final OD_{600} nm was recorded. The data are representative of four different experiments. ▲, PAO1(pEX30); ●, PAO9010(pEX30); △, PAO1(pJFM3); ○, PAO9010(pJFM3).

aeruginosa to determine whether inactivation of these genes increases susceptibility to various forms of oxidative stress.

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J.-F.M. and P.W.H. contributed equally to completion of this project.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Anderson, B. M., and C. D. Anderson.** 1995. Purification and characterization of *Azotobacter vinelandii* glucose-6-phosphate dehydrogenase: dual coenzyme specificity. J. Bacteriol. **321:**94–100.
3. **Asnis, R. E.** 1955. A glutathione reductase from *Escherichia coli*. J. Biol. Chem. **213:**77–85.
4. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195:**133–140.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, S. M., M. L. Howell, M. L. Vasil, A. Anderson, and D. J. Hassett.** 1995. Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J. Bacteriol. **177:**6536–6544.
7. **Cacciapuoti, A. F., and T. G. Lessie.** 1977. Characterization of the fatty acid-sensitive glucose 6-phosphate dehydrogenase from *Pseudomonas cepacia*. J. Bacteriol. **132:**555–563.
8. **Cheldelin, V. H.** 1961. Metabolic pathways in microorganisms. John Wiley & Sons, New York, N.Y.
9. **Christman, M. F., R. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
10. **Collier, D. N., P. W. Hager, and P. V. Phibbs, Jr.** 1996. Catabolite repression control in the Pseudomonads. Res. Microbiol. **147:**551–561.
11. **Cross, A., J. R. Allen, J. Burke, G. Ducel, A. Harris, J. John, D. Johnson, M. Lew, B. MacMillan, R. Skalova, R. Wenzel, and J. Tenney.** 1983. Nosocomial infections due to *Pseudomonas aeruginosa*: review of recent trends. Rev. Infect. Dis. **5:**S837–S845.
12. **Cuskey, S. M., J. A. Wolff, P. V. Phibbs, Jr., and R. H. Olsen.** 1985. Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. J. Bacteriol. **162:**865–871.
13. **DeMoss, R. D.** 1955. Glucose 6-phosphate and 6-phosphogluconic dehydrogenases from *Leuconostoc mesenteroides*. Methods Enzymol. **1:**328–334.
14. **Demple, B.** 1996. Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon-a review. Gene **179:**53–57.
15. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
16. **Govan, J. R. W., and G. S. Harris.** 1986. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. Microbiol. Sci. **3:**302–308.
17. **Greenberg, J. T.** 1989. Adaptive response to oxidative stress in *Escherichia coli*. Ph.D. thesis. Harvard University, Cambridge, Mass.
18. **Greenberg, J. T., and B. Demple.** 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. J. Bacteriol. **171:**3933–3939.
19. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
20. **Hassan, H. M., and I. Fridovich.** 1979. Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. **196:**385–395.
21. **Hassett, D. J., L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen.** 1992. Antioxidant defense mechanisms in *Pseudomonas aeruginosa*: resistance to the redox-active antibiotic pyocyanin and demonstration of a manganese-cofactored superoxide dismutase. Infect. Immun. **60:**328–336.
22. **Hassett, D. J., M. L. Howell, U. Ochsner, Z. Johnson, M. Vasil, and G. E. Dean.** 1997. An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator (Fur) in *Pseudomonas aeruginosa*: *fur* mutants produce elevated alginate levels. J. Bacteriol. **179:**1452–1459.
23. **Hassett, D. J., M. L. Howell, P. A. Sokol, M. Vasil, and G. E. Dean.** 1997. Fumarase C activity is elevated in response to iron deprivation and in mucoid, alginate-producing *Pseudomonas aeruginosa*: cloning and characterization of *fumC* and purification of native FumC. J. Bacteriol. **179:**1442–1451.
24. **Hassett, D. J., H. P. Schweizer, and D. E. Ohman.** 1995. *Pseudomonas aeruginosa sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. J. Bacteriol. **177:**6330–6337.
25. **Hassett, D. J., P. Sokol, M. L. Howell, J.-F. Ma, H. P. Schweizer, U. Ochsner, and M. L. Vasil.** 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake and altered aerobic metabolism. J. Bacteriol. **178:**3996–4003.
26. **Hassett, D. J., W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman.** 1993. Cloning of the *sodA* and *sodB* genes encoding manganese and iron superoxide dismutase in *Pseudomonas aeruginosa*: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. J. Bacteriol. **175:**7658–7665.
27. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
28. **Hugouvieux-Cotte-Pattat, N., and J. Robert-Baudouy.** 1994. Molecular analysis of the *Erwinia chrysanthemi* region containing the *kdgA* and *zwf* genes. Mol. Microbiol. **11:**67–75.
29. **Hunt, J. C., and P. V. Phibbs, Jr.** 1983. Regulation of alternative peripheral pathways of glucose catabolism during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. J. Bacteriol. **154:**793–802.
30. **Imlay, J. A., and S. Linn.** 1986. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **166:**519–527.
31. **Imlay, J. A., and S. Linn.** 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **169:**2967–2976.
32. **Jacobson, F. S., R. W. Morgan, M. F. Christman, and B. N. Ames.** 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage: purification and properties. J. Biol. Chem. **264:**1488–1496.
33. **Kao, S. M., and H. M. Hassan.** 1985. Biochemical characterization of a paraquat tolerant mutant of *Escherichia coli*. J. Biol. Chem. **260:**10478–10481.
34. **Kitzler, J. W., and I. Fridovich.** 1986. Effects of salts on the lethality of

paraquat. J. Bacteriol. **167:**346–349.
35. **Lessie, T., and F. C. Neidhardt.** 1967. Adenosine triphosphate-linked control of *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase. J. Bacteriol. **93:**1337–1345.
36. **Lessie, T. G., and P. V. Phibbs, Jr.** 1984. Alternative pathway of carbohydrate utilization in pseudomonads. Annu. Rev. Microbiol. **38:**359–387.
37. **Lessmann, D., K.-L. Schmiz, and G. Kurz.** 1975. D-Glucose-6-phosphate dehydrogenase (Entner-Doudoroff enzyme) from *Pseudomonas fluorescens*. Eur. J. Biochem. **59:**545–559.
38. **Liochev, S. I., and I. Fridovich.** 1991. Effects of overproduction of superoxide dismutase on the toxicity of paraquat toward *Escherichia coli*. J. Biol. Chem. **266:**8747–8750.
39. **Liochev, S. I., and I. Fridovich.** 1994. Paraquat diaphorases in *Escherichia coli*. Free Radical Biol. Med. **16:**555–559.
40. **Liochev, S. I., A. Hausladen, W. F. Beyer, and I. Fridovich.** 1994. NADPH: ferredoxin oxidoreductase acts as a paraquat diaphorase and is a member of the *soxRS* regulon. Proc. Natl. Acad. Sci. USA **91:**1328–1331.
41. **Ma, J.-F., P. V. Phibbs, Jr., and D. J. Hassett.** 1997. Glucose stimulates alginate production and *algD* transcription in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **148:**217–221.
42. **MacGregor, C. H., J. A. Wolff, S. K. Arora, P. B. Hylemon, and P. V. Phibb, Jr.** 1992. Catabolite repression control in *Pseudomonas aeruginosa*, p. 198–206. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
43. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Marklund, S., and G. Marklund.** 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. **47:**469–474.
45. **Miller, J. H.** 1992. A short course in bacterial genetics, p. 72–74. Cold Spring Harbor Press, Plainview, N.Y.
46. **Moskovitz, J., B. S. Berlett, J. M. Poston, and E. R. Stadtman.** 1997. The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vivo. Proc. Natl. Acad. Sci. USA **94:**9585–9589.
47. **Moskovitz, J., M. A. Rahman, J. Strassman, S. O. Yancey, S. R. Kushner, N. Brot, and H. Weissbach.** 1995. *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. J. Bacteriol. **177:**502–507.
48. **Newman, J., H. Karakaya, D. J. Scanlan, and N. H. Mann.** 1995. A comparison of gene organization in the *zwf* region of the genomes of the cyanobacteria *Synechococcus* sp. PCC 7942 and *Anabaena* sp. PCC 7120. FEMS Microbiol. Lett. **133:**187–193.
49. **Perry, A. C., N. Ni Bhriain, N. L. Brown, and D. A. Rouch.** 1991. Molecular characterization of the *gor* gene encoding glutathione reductase from *Pseudomonas aeruginosa*: determinants of substrate specificity among pyridine nucleotide-disulphide oxidoreductases. Mol. Microbiol. **5:**63–71.
50. **Phibbs, P. V., Jr., S. M. McGowen, T. W. Feary, and W. T. Blevins.** 1978. Mannitol and fructose catabolic pathways of *Pseudomonas aeruginosa* carbohydrate-negative mutants and pleiotropic effects of certain enzyme deficiencies. J. Bacteriol. **133:**717–728.
51. **Proctor, W. D., S. K. Arora, P. W. Hager, and P. V. Phibbs, Jr.** 1997. Integration host factor and the putative repressor HexR bind the *hexC* locus of *Pseudomonas aeruginosa* PAO1, abstr. K-95. *In* Abstracts of the 97th General Meeting of the American Society for Microbiology 1997. American Society for Microbiology, Washington, D.C.
52. **Prohaska, J. R.** 1983. Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. J. Nutr. **113:**2048–2058.
53. **Ragunathan, S., and H. R. Levy.** 1994. Purification and characterization of the NAD-preferring glucose 6-phosphate dehydrogenase from *Acetobacter hansenii* (*Acetobacter xylinum*). Arch. Biochem. Biophys. **310:**360–366.
54. **Reese, M. G., and F. H. Eeckman.** 1995. Novel neural network algorithms for improved eukaryotic promoter site recognition. Presented at the Seventh International Genome Sequencing and Analysis Conference, Hilton Head, S.C.
55. **Reynolds, H. Y., A. S. Levine, R. E. Wood, C. H. Zierdt, D. C. Dale, and J. E. Pennington.** 1975. *Pseudomonas aeruginosa* infections: persisting problems and current research to find new therapies. Ann. Intern. Med. **82:**819–831.
56. **Roehl, R. A., T. W. Feary, and P. V. Phibbs, Jr.** 1983. Clustering of mutations affecting central pathway enzymes of carbohydrate catabolism in *Pseudomonas aeruginosa*. J. Bacteriol. **156:**1123–1129.
57. **Rowley, D. L., and R. E. J. Wolf.** 1991. Molecular characterization of the *Escherichia coli* K-12 *zwf* gene encoding glucose-6-phosphate dehydrogenase. J. Bacteriol. **173:**968–977.
58. **Scanlan, D. J., J. Newman, M. Sebaihia, N. H. Mann, and N. G. Carr.** 1992. Cloning and sequence analysis of the glucose-6-phosphate dehydrogenase gene from the cyanobacterium *Synechococcus* PCC 7942. Plant Mol. Biol. **19:**877–880.
59. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
60. **Schweizer, H. P.** 1993. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:**831–833.
61. **Schweizer, H. P.** 1993. Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. Gene **134:**89–91.
61a. **Schweizer, H. P.** Unpublished data.
61b. **Schweizer, H. P.** Personal communication.
62. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15–22.
63. **Simon, R., U. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
64. **Temple, L., S. M. Cuskey, R. E. Perkins, R. C. Bass, N. M. Morales, G. E. Christie, R. H. Olsen, and P. V. Phibbs, Jr.** 1990. Analysis of cloned structural genes and regulatory genes for carbohydrate utilization in *Pseudomonas aeruginosa* PAO. J. Bacteriol. **172:**6396–6402.
65. **Temple, L., A. Sage, G. E. Christie, and P. V. Phibbs, Jr.** 1994. Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**4700–4709.
66. **Tiwari, N. P., and J. J. R. Campbell.** 1969. Enzymatic control of the metabolic activity of *Pseudomonas aeruginosa* grown in glucose or succinate media. Biochim. Biophys. Acta **192:**395–401.
67. **Vander Wyk, J. C., and T. G. Lessie.** 1974. Purification and characterization of the *Pseudomonas aeruginosa* glucose-6-phosphate dehydrogenase active with nicotinamide adenine dinucleotide. J. Bacteriol. **120:**1033–1042.
67a. **Wallace, W. H., P. W. Hager, and P. V. Phibbs.** Unpublished data.
68. **West, S. E. H., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81–86.
69. **Wolff, J. A., C. H. MacGregor, R. C. Eisenberg, and P. V. Phibbs, Jr.** 1991. Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **173:**4700–4706.