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Gene *ercA*, Encoding a Putative Iron-Containing Alcohol Dehydrogenase, Is Involved in Regulation of Ethanol Utilization in *Pseudomonas aeruginosa*

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Several two-component regulatory systems are known to be involved in the signal transduction pathway of the ethanol oxidation system in *Pseudomonas aeruginosa* ATCC 17933. These sensor kinases and response regulators are organized in a hierarchical manner. In addition, a cytoplasmic putative iron-containing alcohol dehydrogenase (Fe-ADH) encoded by *ercA* (PA1991) has been identified to play an essential role in this regulatory network. The gene *ercA* (PA1991) is located next to *ercS*, which encodes a sensor kinase. Inactivation of *ercA* (PA1991) by insertion of a kanamycin resistance cassette created mutant NH1. NH1 showed poor growth on various alcohols. On ethanol, NH1 grew only with an extremely extended lag phase. During the induction period on ethanol, transcription of structural genes *exa* and *pqqABCDEH*, encoding components of initial ethanol oxidation in *P. aeruginosa*, was drastically reduced in NH1, which indicates the regulatory function of *ercA* (PA1991). However, transcription in the extremely delayed logarithmic growth phase was comparable to that in the wild type. To date, the involvement of an Fe-ADH in signal transduction processes has not been reported.

Upon aerobic growth on ethanol, *Pseudomonas aeruginosa* ATCC 17933 expresses a periplasmic, soluble quinoprotein, ethanol dehydrogenase (QEDH), with pyrroloquinoline quinone (PQQ) as a cofactor (1). QEDH transfers electrons to soluble cytochrome c_{550} (2). After phenotypic characterization and complementation of chemical mutants that were unable to grow on ethanol, it was concluded that six or seven different genes might be involved in regulating ethanol oxidation (3, 4). These genes controlling the ethanol oxidation system in *Pseudomonas aeruginosa* are organized in a hierarchical manner (see Fig. 2, below). The response regulator ErbR controls transcription of structural genes encoding components necessary for ethanol oxidation (5). *exaB* codes for cytochrome c_{550}, *exaC* codes for an NAD^+-dependent acetaldehyde dehydrogenase (6), the operon *pqqABCDEH* encodes enzymes for PQQ biosynthesis (7), and *eraSR* code for a two-component regulatory system. EraSR activate transcription of *exaA* encoding quinoprotein ethanol dehydrogenase (QEDH) (3). In addition, two sensor kinases, ErcS (encoded by PA1992) and ErcS′ (encoded by PA1976) and the global response regulator ErdR (encoded by PA3604) have been identified as regulatory components of the ethanol oxidation system in *P. aeruginosa* (8).

In the present study, we tried to elucidate the function of *ercA* (PA1991), which is located adjacent to the sensor kinase ErcS (PA1992). PA1991 codes for a putative iron-containing NAD-dependent alcohol dehydrogenase (Fe-ADH). PA1146 and PA5186 are also annotated to code for putative Fe-ADHs in the *Pseudomonas* Genome Database (9, 10). Fe-ADHs form family III of the NAD(P)-dependent alcohol dehydrogenases (11). The three putative members of the Fe-ADH family found in the *P. aeruginosa* genome (encoded by PA1146, PA1991, and PA5186) show high sequence similarities to the 1,3-propanediol dehydrogenase of *Klebsiella pneumoniae* (12). While the majority of characterized Fe-ADH enzymes are involved in fermentation processes, a minority have been identified to play a major oxidative role: methanol dehydrogenase of *Bacillus methanolicus* and methanol:*N*,*N*-dimethyl-4-nitrosoaniline oxidoreductase from *Amycolatopsis methanolica* and *Mycobacterium gastri*, which contain a tightly bound NAD(P) cofactor (13), are active in oxidative pathways.

In this study, the putative Fe-ADH, which is encoded by PA1991, was identified as a regulatory component of the ethanol oxidation system in *P. aeruginosa* ATCC 17933. So far, the involvement of an Fe-ADH in signal transduction processes has not been reported. Thus, this study may contribute to further elucidate the complex regulation of the ethanol oxidation system in *P. aeruginosa*.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used in this work are listed in Table 1.

Escherichia coli was cultivated in Luria-Bertani (LB) medium, and *P. aeruginosa* was cultivated in LB or minimal medium (1) containing different carbon sources. Alcohols were added at 0.5% (vol/vol). Glucose and succinate were used at 40 mM, and acetate was used at 20 mM. Antibiotics were added as follows: tetracycline at 20 μg/ml, carbenicillin at 100 μg/ml, kanamycin at 50 μg/ml, and gentamicin at 50 μg/ml.

General genetic techniques and PCR. Routine recombinant DNA work was performed according to the protocols described previously by Sambrook et al. and Ausubel et al. (14, 15). Triparental matings were performed as described by Kretzschmar et al. (16). Electrotransformation of *P. aeruginosa* ATCC 17933 was performed as described by Smith and Iglewski (17).

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference
P. aeruginosa strains		
ATCC 17933	Wild type	29
MD1	ATCC 17933 derivative, ΔPA3604::Km^r	8
NH1	ATCC 17933 derivative, PA1991::Km^r	This study
NH2	SH1 derivative, PA1991::Km^r ΔPA1992::Gm^r	This study
NH3	ATCC 17933 derivative, ΔPA2572::Km^r	This study
NH5	ATCC 17933 derivative, PA5186::Km^r	This study
SH1	ATCC 17933 derivative, ΔPA1992::Gm^r	8
E. coli strains		
DH5α	*supE44* Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	30
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	31
Plasmids		
pACΩGm	Gm^r; cloning vector containing Gm^r cassette and Ω fragments	32
pEDY305	Tc^r; *lacZ* promoter-probe vector	22
pEX18Ap	Ap^r; *oriT*$^+$ *sacB*$^+$, gene replacement vector with multiple cloning site (MCS) from pUC18	20
pLAFR3	Tc^r; broad-host-range cosmid	33
pQF50	Ap^r; *lacZ* promoter-probe vector	21
pRK2013	Km^r; helper plasmid for triparental mating	34
pUC18, 19	Ap^r; cloning and expression vector	35
pUCP20T	Ap^r; broad-host-range vector	36
pTB3001	Tc^r; ~25-kbp genomic DNA partially digested with Sau3AI from *P. aeruginosa* cloned into BamHI site of pLAFR3	6
pTB3070	Ap^r; 3.2-kbp BamHI-PstI fragment from pTB3001 cloned into BamHI-PstI sites of pUC18	6
pTB3112	Ap^r; 6.2-kbp PstI-PstI fragment from pTB3001 cloned into PstI site of pUCP20T	3
pTB3131	Ap^r Km^r; 1.0-kbp PCR product with promoter and Km^r cassette of Tn*5* cloned into EcoRI-BamHI sites of pUC19	16
pTB3135	Ap^r; 0.89-kbp HindIII-SalI-fragment from pTB3070 cloned into HindIII-SalI sites of pUC19	3
pTB3138	Tc^r; *P_*exaA′*-lacZ*; 0.89-kbp PstI-SalI fragment from pTB3070 cloned into PstI-XhoI sites of pEDY305	3
pTB3139	Tc^r; *P_*exaB′*-lacZ*; 0.89-kbp PstI-XbaI fragment from pTB3135 cloned into PstI-XbaI sites of pEDY305	3
pTB3144	Ap^r; 3-kbp MluI-SmaI fragment of pTB3112 removed, ends filled in, and vector religated	3
pTB7023	Tc^r; *P_pqqAB́-lacZ*; 2.5-kbp XhoI-BamHI fragment of pTB3070 cloned into XhoI-BglII sites of pEDY305	5
pTB7060	Ap^r; 1.67-kbp PCR product of *erbR* cloned into PstI-BamHI sites of pUCP20T	5
pTB7074	Ap^r; *P_eraSR*(PA1979/1980)′*-lacZ*; 1.16-kbp promoter region of *exaDE* cloned into BglII-XbaI sites of pQF50	5
pTB7082	Ap^r; *P_ercA*(PA1991)′*-lacZ*; 0.53-kbp PCR product containing promoter region of PA1991 cloned into XbaI-HindIII sites of pQF50(pPA1991)	8
pTB7101	Ap^r; 1.98-kbp PCR product of PA1992 cloned into SphRI-EcoRI sites of pUCP20T	8
pTB7107	Ap^r; *P_erdR*(PA3604)′*-lacZ*; 280-bp promoter region of PA3604 cloned into XbaI-HindIII sites of pQF50	8
pTB7108	Ap^r; *P_erbR*(PA3604)′*-lacZ*; 1.0-kbp promoter region of *agmR* cloned into KpnI-HindIII sites of pQF50	8
pTB7200	Ap^r; 3.3-kbp PCR product containing *ercA-ercS* and its promoter cloned into EcoRI-HindIIII sites of pUCP20T	This study
pTB7212	Ap^r; 1.2-kbp PCR product of PA1991 cloned into EcoRI-HindIII sites of pUCP20T	This study
pTB7219	Ap^r; 1.2-kbp PCR product of PA1146 cloned into EcoRI-HindIII sites of pEX18Ap	This study
pTB7220	Ap^r; 1.2-kbp PCR product of PA1991 cloned into EcoRI-HindIIII sites of pEX18Ap	This study
pTB7222	Apr; 1.3-kbp PCR product of PA2572 cloned into EcoRI-HindIII sites of pEX18Ap	This study
pTB7224	Ap^r; 1.2-kbp PCR product of PA5186 cloned into EcoRI-KpnI sites of pEX18Ap	This study
pTB7225	Ap^r Km^r; 380-bp SmaI-SmaI fragment of pTB7219 removed and filled with PCR product of Km^r cassette	This study
pTB7226	Ap^r Km^r; 1.0-kbp PCR product of Km^r cassette cloned into SmaI site of pTB7220	This study
pTB7227	Ap^r Km^r; 500-bp PstI-PstI fragment of pTB7222 removed and filled with PCR product of Km^r cassette	This study
pTB7229	Ap^r Km^r; 1.0-kbp PCR product of Km^r cassette cloned into blunted NcoI site of pTB7224	This study
pTB7234	Ap^r; *P_*PA1146*-lacZ*; 500-bp PCR product containing promoter region of PA1146 cloned into NcoI-HindIIII sites of pQF50(pPA1146)	This study
pTB7235	Ap^r; P_PA2572′*-lacZ*; 500-bp PCR product containing promoter region of PA2572 cloned into NcoI-HindIIII sites of pQF50	This study
pTB7237	Ap^r; *P_*PA5186′*-lacZ*; 500-bp PCR product of promoter region of PA5186 cloned into NcoI-HindIIII sites of pQF50(pPA5186)	This study

Genomic DNA was isolated from *P. aeruginosa* ATCC 17933 as the template, and *Pfu* DNA polymerase (Promega) was used for PCR. For primer design, the sequence of *P. aeruginosa* strain PAO1 was used, since the nucleotide identity between PAO1 and ATCC 17933 has been reported to be 99% (18, 19). For amplification of most genes, oligonucleotides with restriction sites for EcoRI and HindIII, indicated in bold below, were used. For amplification of PA1146, the forward primer 5′-GC**GAATTC**ATGAGCGACCTGCATTACTGGA-3′ and the reverse primer 5′-TAT**AAGCTT**TCAGGCGAGAGTCCCGGCCAC-3′ were used. The 1.2-kbp PCR product containing PA1146 was cloned into EcoRI-HindIII sites of pEX18Ap, resulting in plasmid pTB7219. For PCR amplification of PA1991, the forward primer was 5′-CAT**GAATTC**CAGATGAGCCACG

ACCTCAG-3′ and the corresponding reverse primer was 5′-AAT**AAGC TT**TCAGAGGGCCTCGCCATAGAC-3′. The 1.2-kbp PCR product containing PA1991 was cloned into EcoRI-HindIII sites of pEX18Ap, resulting in plasmid pTB7220. For the PCR amplification of PA2572, the forward primer was 5′-GA**GAATTC**ATGAACGATAGCGCACCTCCT T-3′ and the corresponding reverse primer was 5′-TGT**AAGCTT**CTAG GTCGTCGACTCCGGGAG-3′. The 1.3-kbp PCR product containing PA2572 was cloned into EcoRI-HindIII sites of pEX18Ap, resulting in plasmid pTB7222.

For amplification of the promoter region of PA1991 plus PA1991 and PA1992, the forward primer was 5′-CA**GAATTC**CCAGTCTCTATGGG GTCAG-3′ and the reverse primer was 5′-ATC**AAGCTT**CCGCTCGATG TTCCTCTTC-3′. The 3.3-kbp PCR product containing the 445-bp region upstream of the start codon of PA1991 plus PA1991 and PA1992 with a possible terminator region was cloned into EcoRI-HindIII sites of pUCP20T, resulting in plasmid pTB7200. PA5186 was amplified using oligonucleotides with restriction sites for EcoRI and KpnI (shown in bold). The forward primer was 5′-CT**GAATTC**ATGCAAGCTTTCAGTT TCGCCA-3′, and the corresponding reverse primer was 5′-CT**GGTACC** TCAGTATGCCGCGCGATAGATC-3′. The 1.2-kbp PCR product containing PA5186 was cloned into the EcoRI-KpnI sites of pEX18Ap, resulting in plasmid pTB7224.

Gene inactivation. For inactivation of genes by a kanamycin resistance cassette, the *sacB*-based strategy with the suicide vector pEX18Ap (20) was employed. Sucrose-resistant colonies were obtained by streaking *P. aeruginosa* merodiploids on LB plates supplemented with 5% sucrose. For generation of mutants, the kanamycin resistance cassette of pTB3131 was amplified using primers with restriction sites (indicated in bold) for SmaI. The forward primer was 5′-AT**CCCGGG**GCAAAGAGAAAGCAG GTAGC-3′, and the corresponding reverse primer was 5′-CAT**CCCGGG** CTCAGAACTCGTCAAGAA-3′. For inactivation of PA1146, the SmaI-digested kanamycin resistance cassette was cloned into the SmaI site of pTB7219, resulting in pTB7225. PA1991 was inactivated by cloning the SmaI-digested kanamycin resistance cassette into the SmaI site of pTB7220, resulting in pTB7226. For inactivation of PA2572, the PstI-PstI fragment of the vector pTB7222 was removed, the ends were filled, and the SmaI-digested resistance cassette was cloned into blunted ends of pTB7222, resulting in pTB7227. PA5186 was inactivated by ligating the SmaI-digested kanamycin resistance cassette into the blunted NcoI site of pTB7224, resulting in pTB7229. The same orientation for the Km^r gene and interrupted gene was verified. The suicide vectors were introduced into *P. aeruginosa* by triparental mating, and after two independent homologous recombinations, potential site-directed double-crossover mutants with a Km^r (Gm^r) Cb^s Suc^r phenotype were selected.

Construction of promoter-probe vectors. Promoter-probe vectors were constructed to study the transcriptional regulation of PA1146, PA5186, and PA2572. Putative promoter regions (0.5 kbp upstream of the start codon) were amplified by PCR by using primers with restriction sites for NcoI and HindIII, indicated in bold. The PCR products were cloned into the NcoI-HindIII sites of the promoter-probe vector pQF50 upstream of the *lacZ* gene (21). For amplification of the promoter region of PA1146, the forward primer 5′-GC**GAATTC**ATGAGCGACCTGCATTA CTGGA-3′ and the corresponding reverse primer 5′-TAT**AAGCTT**TCA GGCGAGAGT-CCCGGCCAC-3′ were used, and the promoter region of PA5186 was amplified using the forward PCR primer 5′-ACTA**CCAT GG**ACCAATGGCATCCAGGCGCT-3′ and the corresponding reverse primer 5′-TGA**AAGCTT**GCATATCGGTCTCCTTGGGCG-3′. The forward PCR primer 5′-ACTA**CCATGG**TCCCTGCAAAGGCAGGCCG A-3′ and the corresponding reverse primer 5′-ACG**AAGCTT**CCATCTC CGTCTCGTTGGAAG-3′ were used. The corresponding promoter probe vectors pTB7234 (PA1146), pTB7237 (PA5186), and pTB7235 (PA2572) were constructed.

As previously described, the promoter-probe vectors for *exaA* (pTB3138), *exaBC* (pTB3139) (3), and *pqqABCDEH* (pTB7023) (5) are derivatives of pEDY305 (22). The promoter-probe vector for PA1991 (pTB7082) (8) is a derivative of pQF50 (21).

Demonstration of a common mRNA for *ercA* and *ercS*. Total RNA of wild-type cells was extracted using the RNeasy minikit (Qiagen). The resulting solution was incubated with RNase-free DNase (MBI Fermentas) according to the manufacturer's instructions. To generate cDNA, the RNA was incubated with reverse transcriptase (Hoffman-La Roche) according to instructions provided by the supplier. For amplification of a region spanning parts of both genes, which begins 150 bp upstream of and ends 150 bp downstream of the start codon of *ercS* (PA1992), the forward PCR primer 5′-CCTTCAAGCACGCCGTGGGTTTCCACGAGA-3′ and the corresponding reverse primer 5′-GCTCGAACAGCCATTTGTAGCG GTTGCGCT-3′ were used. The resulting DNA was visualized by agarose gel electrophoresis and ethidium bromide staining. The DNA product showed the expected length of 300 bp.

Enzyme assays and protein determination. The activity of β-galactosidase was determined with toluene-treated cells according to the procedure of Miller (23). Determination of β-galactosidase activity in NH1 was performed after induction on ethanol, as described by Schobert and Görisch (3) and after growth for 50 h to an optical density at 620 nm (OD_{620}) of 0.6. The empty promoter-probe vector pQF50 or pEDY305 without any promoter cloned into them was used as a baseline condition for measuring promoter activity. The presented values of promoter activities are the calculated mean values of the probes minus the baseline mean values.

Enzyme activity of the NAD^+-dependent Fe-ADH was determined with cell extracts. The method described for 1,3-propanediol oxidoreductase of *K. pneumoniae* was used (12). Cells were collected at an OD_{620} of 0.6 and disrupted by sonication, and cell debris was removed by centrifugation. Substrate-dependent NADH formation was determined spectrophotometrically at 340 nm. The assay mixtures contained potassium bicarbonate buffer (0.1 M; pH 9.0), ammonium sulfate (30 mM), NAD^+ (0.6 mM), various alcohols (0.01 M), and cell extract; 1,3-propanediol at a concentration of 0.1 M was used. Protein concentrations were determined using the method described by Groves et al. (24).

Bioinformatics tools. The *Pseudomonas aeruginosa* Genome Database (9, 10) was used to obtain DNA sequences of PAO1. Primers were designed using the Primer3 bioinformatics tool (25). Similarities between amino acid sequences of PA1991 and other protein sequences available in GenBank were assessed from BLAST searches (26). For determination of possible interactions between proteins, the program STRING (27) was used.

RESULTS AND DISCUSSION

The operon *ercA-ercS* and other genes possibly involved in regulating ethanol utilization. In *P. aeruginosa* PAO1, *ercA* (PA1991) and *ercS* (PA1992) are located downstream of and close to genes encoding components of the ethanol oxidation system (9, 10). This arrangement of genes is also found in *P. aeruginosa* ATCC 17933, the organism studied in the present report (Fig. 1).

The gene *ercS* overlaps with the upstream gene PA1991 by 17 bp. From transcriptional studies with different constructs of PA1991, encoding a putative Fe-ADH (9, 10), and *ercS*, it has been concluded that both genes are organized in an operon (8). In this study, we confirmed a common mRNA for the two genes by generating a 300-bp cDNA that spanned the end of PA1991 and the beginning of *ercS* (data not shown).

PA1991 shows high sequence similarity to the 1,3-propanediol dehydrogenase of *Klebsiella pneumoniae* (12). The NCBI BLAST analysis of the PA2572 nucleotide sequence showed high sequence similarity to a response regulator in *Pseudomonas* sp. M18 (complete CDS identity, 99%). The interaction between a putative response regulator encoded by PA2572 and the sensor kinase ErcS was predicted based on the program STRING (27) with a relatively

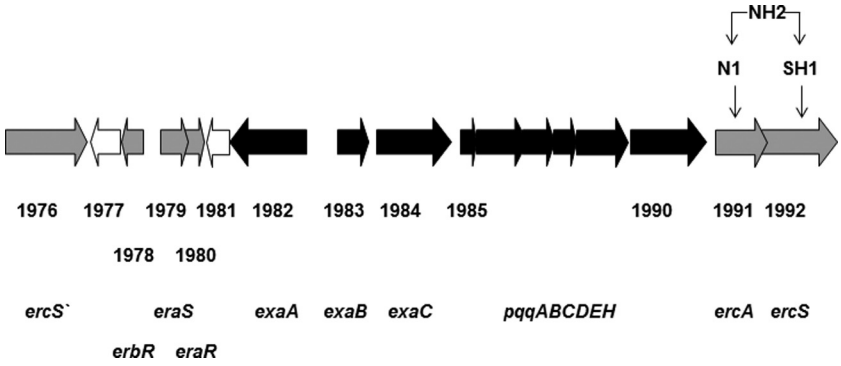

FIG 1 Arrangement of genes of the aerobic ethanol oxidation system and its regulation in *P. aeruginosa* ATCC 17933. Structural genes *exaA*, *exaBC*, and genes of the *pqq* operon are shown in black. Genes coding for regulatory components of the ethanol oxidation system, *eraSR*, *erbR*, *ercS*, and *ercS′*, as well as *ercA*, which encodes a putative Fe-ADH, are shown in gray. Downstream of *ercS* are genes encoding a probable major facilitator superfamily (MSF) transporter, two hypothetical proteins, and a peptidyl-prolyl *cis-trans* isomerase (PA1996). All these genes are transcribed in the opposite direction with respect to *ercS*. The gene *erdR* (PA3604) is not shown. Mutants discussed in the text are indicated by their names.

low confidence ($k = 0.6$). In contrast, interaction of the putative Fe-ADH encoded by PA1991 with the sensor kinase ErcS was predicted, surprisingly, with high confidence ($k = 0.9$). Two other genes of *P. aeruginosa*, PA1146 and PA5186, also encode putative Fe-ADHs (9, 10) with significant sequence similarities to the 1,3-propanediol dehydrogenase of *K. pneumoniae*. For the latter two Fe-ADHs, no interaction was predicted with the sensor kinase ErcS ($k < 0.15$).

Generation of putative regulatory mutants. PA2572, encoding a putative response regulator, was inactivated by combined deletion of 500 bp and insertion of a Km^r cassette (pTB7227), resulting in the mutant NH3. PA1991, encoding a putative Fe-ADH, was interrupted by insertion of a Km^r cassette (pTB7226), resulting in the mutant NH1. Double mutant NH2, with both genes of the operon PA1991-*ercS* inactivated, was generated by interrupting PA1991 in the mutant SH1 (8) by insertion of a Km^r cassette (pTB7226). PA1146(pTB7225) and PA5186(pTB7229), both encoding additional putative Fe-ADHs, were inactivated by insertion of a Km^r cassette. The mutant NH5 with an interrupted PA5186 was isolated, but we were unable to isolate a mutant with an inactivated PA1146. Presumably, such a defect is lethal. The correct insertion of resistance cassettes and construction of mutants was verified by PCR product analysis (data not shown). The mutants and their genotypes are listed in Table 1.

Growth of putative regulatory mutants. NH1 is unable to grow on 1,3-propanediol. Compared to the wild type, NH1 showed poor growth on butanol, with a significantly increased generation time. On ethanol, 1-propanol, and 1,2-propanediol, it showed an 8-fold-prolonged lag phase (Table 2). However, after the extended lag phase, NH1 grew on the three alcohols with the same generation time as the wild type did. To exclude reversion of NH1 during cultivation, it was reinoculated in fresh minimal medium with ethanol. This culture showed again the extreme 8-fold-prolonged lag phase. No differences in duration of lag phase or in generation time were found on glycerol, acetate, glucose, or succinate (Table 2). Growth of NH1 on the substrates tested was comparable to growth of SH1 with the inactivated sensor kinase ErcS (8). NH2, with both genes of operon PA1991-*ercS* inactivated, showed an identical growth pattern as NH1 (Table 2). Growth on ethanol was restored in NH1 by plasmid pTB7212, carrying PA1991, in SH1 by plasmid pTB7101, carrying PA1992, and in NH2 by plasmid pTB7200, carrying operon PA1991-*ercS*

TABLE 2 Growth parameters of different *P aeruginosa* mutants on various substrates

	Growth of mutant[a]							
	NH1 [*ercA* (PA1991)::Km^r], Fe-ADH gene inactivated		NH2 [*ercA* (PA1991)::Km^r Δ*ercS* (PA1992)::Gm^r], Fe-ADH and SK genes inactivated		NH3 [PA2572::Km^r], RR gene inactivated		NH5 [PA5186::Km^r], Fe-ADH gene inactivated	
Carbon source(s)	Gen	Lag	Gen	Lag	Gen	Lag	Gen	Lag
Ethanol, 1-propanol	wt	≫	wt	≫	wt	wt	wt	wt
1-Butanol	≫	wt	≫	wt	ND	ND	wt	wt
1,2-Propanediol	wt	≫	wt	≫	ND	ND	wt	wt
1,3-Propanediol	NG	NG	NG	NG	ND	ND	wt	wt
Acetate	wt	wt	wt	wt	wt	wt	wt	wt
Glucose	wt	wt	wt	wt	ND	ND	Wt	wt
Succinate	wt	wt	wt	wt	wt	wt	Wt	wt

[a] Gen, generation time; Lag, lag phase; SK, two-component regulatory system sensor kinase; RR, response regulator; wt, response similar to wild type; ≫, generation time or lag phase greater than 5 times the wild-type value; NG, no growth for at least 72 h; ND, not determined. Judgments of differences in generation times and lag phases were based on means ± standard deviations from at least three independent experiments.

TABLE 3 Growth comparison of *P. aeruginosa* complemented mutants versus the wild type on ethanol

Complementation of *P. aeruginosa* mutant	Growth of mutant[a]					
	NH1 (PA1991::Km)		SH1 (ΔPA1992::Gm^r)		NH2 (PA1991::Km^r ΔPA1992::Gm^r)	
	Gen	Lag	Gen	Lag	Gen	Lag
pUCP20T (empty vector)	wt	≫	wt	>	wt	≫
pTB7212 (PA1991)	wt	wt	wt	>	wt	>
pTB7101 (PA1992)	wt	≫	wt	wt	wt	≫
pTB7200 (PA1991, PA1992)	wt	wt	wt	wt	wt	wt

[a] Gen, generation time; Lag, lag phase; wt, response similar to wild type; >, lag phase greater than 2 times that of the wild type; ≫, lag phase greater than 5 times the wild-type value. Judgments of differences in generation times and lag phases were based on means ± standard deviations of at least three independent experiments.

(Table 3). NH3 with interrupted PA2572 and NH5 with interrupted PA5186 showed wild-type behavior on the substrates tested (Table 2). We assume that the products of both genes are not directly involved in aerobic ethanol oxidation or in its regulation.

Transcription of genes coding for the response regulator PA2572 and putative Fe-ADHs. To test promoter activity of the putative response regulator PA2572 and the different putative Fe-ADHs, promoter-probe vectors were generated. The respective promoter regions were cloned into the vector pQF50, resulting in plasmids pTB7235 (*P*_PA2572), pTB7234 (*P*_PA1146), and pTB7237 (*P*_PA5186). Transcription of the respective genes in wild-type cells was determined after growth on different carbon sources: ethanol, succinate, 1,2-propanediol, 1,3-propanediol, acetate, glycerol, and glucose. Transcription of PA1991 was determined using the promoter-probe vector pTB7082 (8). On ethanol or succinate, promoter activities of PA2572 of 94 ± 12 Miller units (MU) and 42 ± 6 MU were determined, respectively (means ± standard deviations). On ethanol or 1,2-propanediol, the highest promoter activity of PA1991, 703 ± 31 MU, was measured, while on glucose or succinate only 15% of this level was detected. On ethanol, promoter activities of PA1146 and PA5186 reached only 11 to 13% of the promoter activity of PA1991. PA1146 showed the highest activity, with 143 ± 16 MU on acetate, while on ethanol, 1,2-propanediol, or 1,3-propanediol, the activity was 74 ± 6 MU. PA5186 showed the highest activity, with 92 ± 6 MU on glycerol, while on ethanol and acetate the activity was 77 ± 7 MU. Upon growth on ethanol, the putative Fe-ADH encoded by PA1991 appeared to be the most prominent enzyme.

Promoter activities of operons *exa* and *pqq* in NH1. To determine transcriptional levels of the essential structural genes encoding QEDH (*exaA*), cytochrome c_{550} (*exaB*), and PQQ biosynthetic enzymes (*pqqABCDEH*), promoter-probe vectors pTB3138, pTB3139, and pTB7023 (3, 5) were transferred into wild-type *P. aeruginosa* or NH1 by triparental mating. The β-galactosidase activities were determined after induction of growth on minimal medium with ethanol as the carbon source.

Promoter activities of *exaA* and the operon *exaBC* in NH1 were reduced after induction by a factor of more than 100, compared to that of the wild type. Promoter activity of the operon *pqqABCDEH* was reduced by a factor of about 30 (Table 4). After complementation of NH1 by vector pTB7212, carrying PA1991, wild-type-like activities were detected again (Table 4). The results indicated that the putative Fe-ADH encoded by PA1991 is involved in the signal transduction process of the ethanol oxidation system. Because of this involvement, and the fact that PA1991 forms an operon with *ercS*, we named it *ercA*.

After an extremely extended lag phase, NH1 started to grow on ethanol with a generation time comparable to that of the wild type. The promoter activities of *exaA*, *exaBC*, and *pqqABCDEH* in NH1 reached values of about 50 to 75% of that of the wild type (Table 4). Since growth of NH1, after an extended lag phase, showed a generation time comparable to that of the wild type, the putative Fe-ADH encoded by *ercA* apparently does not catalyze a critical enzymatic step in the log phase of aerobic ethanol metabolism.

Activity of the NAD$^+$-dependent Fe-ADH. Wild-type *P. aeruginosa* ATCC 17933 was grown on ethanol for 6 h, while NH1 was grown for 50 h or was induced on ethanol for 6 h. In cell extracts, no NAD-dependent alcohol dehydrogenase activity could be detected in the absence of Mn^{2+} ions. By using the test described for 1,3-propanediol:NAD-oxidoreductase (12), extracts with or without membrane fragments showed identical en-

TABLE 4 Promoter activities of operons *exaA*, *exaBC*, and *pqqABCDEH* in *P. aeruginosa* wild type, mutant NH1, and NH1 complemented by vector pTB7212 (PA1991)[a]

Reporter gene construct in *P. aeruginosa* strain and condition	Promoter activity (kMU)			
	P_exaA (pTB3138)	P_exaBC (pTB3139)	P_pqq (pTB7023)	Empty vector (pEDY305)
WT, 6-h growth	45.5 ± 6.4	37.7 ± 5.3	26.7 ± 4.1	0.06 ± 0.02
WT(pTB7212) (PA1991), 6-h growth	43.3 ± 5.9	36.9 ± 5.1	27.7 ± 3.9	0.07 ± 0.02
NH1, 6-h induction	0.3 ± 0.03	0.3 ± 0.04	0.8 ± 0.07	ND
NH1, 50-h growth	24.8 ± 3.4	23.6 ± 4.4	20.5 ± 3.1	0.05 ± 0.02
NH1(pTB7212) (PA1991), 6-h growth	39.7 ± 4.4	36.7 ± 4.3	27.3 ± 3.2	0.06 ± 0.02

[a] The reporter gene constructs are derivatives of pEDY305. Activities with the wild type and complemented NH1 mutant were determined after growth on ethanol to an OD_{600} of 0.6 for about 6 h. Activities with NH1 were determined after induction on ethanol for 6 h and growth to an OD_{600} of 0.6 for about 50 h. The data represent means and standard deviations of three independent experiments. ND, not determined.

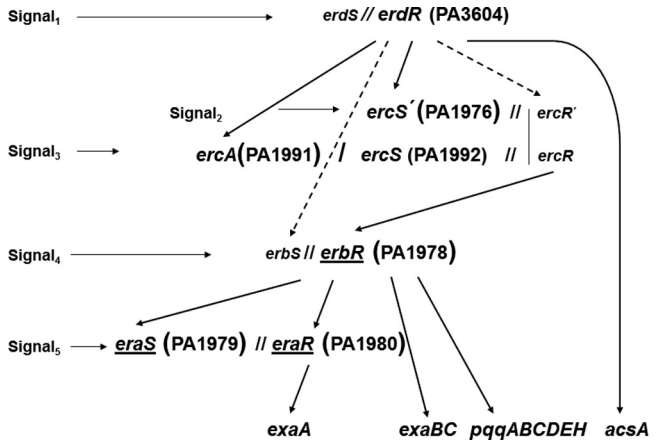

FIG 2 Hypothetical model of the complex regulatory network of the quinoprotein ethanol oxidation system in *P. aeruginosa*. In the present study, gene *ercA* (PA1991), which encodes a Fe-ADH, was identified to be essential for expression of the ethanol oxidation system. The hierarchical arrangement of sensor kinases and response regulators control the expression of QEDH, cytochrome c_{550}, and PQQ biosynthetic enzymes (8). Solid arrows indicate demonstrated positive control of transcription, and broken arrows indicate assumed positive control of transcription. The identified genes encoding sensor kinases S and response regulators R are shown in a larger font size with the respective gene numbers. The proposed corresponding two-component system genes not yet identified are shown in a smaller font size. Two slashes indicate response regulator-sensor kinase pairing. This figure is a modified version of Fig. 3 from our previous publication (8).

zyme activities for NAD^+-dependent Fe-ADH. The results indicated that the enzyme is soluble. After growth on ethanol, cell extracts of the wild type showed low Fe-ADH activities of about 18 MU/mg. Enzyme activity was also detected with propanol and 1,2-propanediol. The highest activity (82 to 92 MU/mg) was found with 100 mM 1,3-propanediol as the substrate. In extracts of wild-type cells grown on succinate, Fe-ADH activity with ethanol was below the detection limit, 5 MU/mg. Moreover, QEDH, the enzyme responsible for aerobic ethanol oxidation in *P. aeruginosa*, was not detected. After induction of NH1 for 6 h on ethanol, the activity of Fe-ADH was also below the detection limit, and again no QEDH activity was found. It appears that a functional Fe-ADH encoded by *ercA* (PA1991) is required for effective expression of QEDH. The Fe-ADH encoded by *ercA* (PA1991) might generate a signal for the sensor kinase ErcS. After the extremely long lag phase on ethanol, NH1 showed in the following logarithmic growth phase Fe-ADH activity of about 15 MU/mg and QEDH activity similar to the wild type. Under these conditions, the lost function of ErcA was probably compensated by another gene product that generated the signal needed for QEDH expression.

Transcription of *eraSR*, *erbR*, and *erdR* in NH1. Transcription of the two-component regulatory genes *eraSR*, *erbR*, and *erdR* was determined in NH1 in order to investigate the role of the *ercA* gene product in controlling the quinoprotein ethanol regulon (Fig. 2). The promoter-probe vectors pTB7074 (5), pTB7107, and pTB7108 (8) were used. After induction on ethanol for 6 h, transcription of the regulatory components EraSR and ErbR was reduced in NH1 to below 5% that of the wild type, indicating a direct influence of the *ercA* gene product (Table 5). Transcription of *erdR* was only slightly reduced, to about 75%, in NH1. These results were essentially the same as those found with SH1, in which *ercS* is inactivated (8). After 50 h, when NH1 resumed growth after the extremely long lag phase, transcription of the regulatory genes *erbR* and *erdR* reached the wild-type levels, while that of the *eraSR* operon reached only 25% (Table 5).

The data demonstrate transcriptional control of the quinoprotein ethanol regulon by the *ercA* gene product and lend further support for naming the gene *ercA*. Both gene products of the *ercA-ercS* operon are required for effective expression of the ethanol oxidation system. Presumably, the Fe-ADH encoded by *ercA* generates a signal that activates the sensor kinase ErcS. However, this signal remains unknown. Experiments to restore wild-type behavior of NH1 on ethanol by exogenous acetaldehyde were unsuccessful (data not shown).

Constitutive expression of the operon *ercA-ercS* does not restore expression of the ethanol oxidation system in MD1. The response regulator ErdR represents the highest level of the proposed signal cascade that regulates ethanol oxidation in *P. aeruginosa* (8). The cascade encompasses 4 levels of two-component systems (Fig. 2). This regulatory system is hypothetical and complex, but it is the simplest one to explain all of our former experimental results, without making further complicated and unlikely assumptions. ErdR regulates transcription of the one-level-lower operon *ercA-ercS*. In MD1, with an inactivated *erdR*, the promoter activity of the operon *ercA*-ercS was reduced to about 12%, and the promoter activity of the next-level-lower response regulator gene *erbR* was reduced to below 5% compared to transcription in the wild type. Constitutive expression of *ercS* in MD1 did not result in the expression of the *exa* and *pqq* operons, which encode the components of the ethanol oxidation system in *P. aeruginosa* (8). Furthermore, constitutive expression of *erbR* restored expression of the ethanol oxidation system in the mutant SH2, with an inactivated *ercS* (8). To study if transcription of the *exa* and *pqq* operons could be restored by constitutive expression of the complete *ercA-ercS* operon, the vector pTB7200 was constructed. This

TABLE 5 Activities of *eraSR*, *erbR*, and *erdR* promoters in *P. aeruginosa* wild-type and NH1 mutant strains[a]

P. aeruginosa strain and condition	Gene inactivated	Promoter activity (MU)			
		P_eraSR PA1979, PA1980 (pTB7074)	*P_erbR* PA1978 (pTB7108)	*P_erdR* PA3604 (pTB7107)	Empty vector (pQF50)
WT, 6-h growth		1,417 ± 153	1,649 ± 168	210 ± 4	23 ± 7
NH1, 6-h induction	PA1991 (Fe-ADH)	37 ± 5	55 ± 7	166 ± 7	25 ± 8
NH1, 50-h growth	PA1991 (Fe-ADH)	334 ± 29	1,409 ± 160	221 ± 14	27 ± 9

[a] The reporter gene constructs are derivatives of pQF50. Activities were determined after growth on ethanol to an OD_{620} of 0.6 for about 6 h with the wild type and for about 50 h with NH1. Promoter activities were also determined in NH1 after induction on ethanol for 6 h. The data represent means and standard deviations from three independent experiments.

TABLE 6 Promoter activities of *exaA*, *exaBC*, and *pqqABCDEH* operons in the wild type and mutants NH1 and SH1[a]

P. aeruginosa construct: strain/vector/gene(s)	Gene inactivated	Promoter activity (kMU)				Condition[b]
		P_exaA (pTB3138)	P_exaBC (pTB3139)	P_pqqAB (pTB7023)	Vector (pEDY305)	
Wild type/pUCP20T		41.4 ± 5.7	37.7 ± 5.3	26.7 ± 4.1	0.06 ± 0.02	G
NH1/pTB7060/*erbR*	*ercA* (PA1991)	12.9 ± 2.3	10.0 ± 1.4	11.6 ± 2.3	0.07 ± 0.02	I
NH1/pTB3144/*eraSR*	*ercA* (PA1991)	1.8 ± 0.2	0.42 ± 0.15	0.9 ± 0.15	0.06 ± 0.02	I
NH1/pUCP20T	*ercA* (PA1991)	0.28 ± 0.03	0.32 ± 0.04	0.8 ± 0.08	ND	I
SH1/pTB7060/*erbR*	*ercS* (PA1992)	10.5 ± 1.6	9.4 ± 1.4	14.3 ± 1.7	0.06 ± 0.02	I
SH1/pTB3144/*eraSR*	*ercS* (PA1992)	3.7 ± 0.5	0.46 ± 0.1	1.3 ± 0.2	0.05 ± 0.02	I
SH1/pUCP20T	*ercS* (PA1992)	0.52 ± 0.14	0.9 ± 0.19	1.5 ± 0.17	ND	I

[a] Activities were determined in mutants with vector pTB7060 constitutively expressing the response regulator ErbR and vector pTB3144 constitutively expressing the two-component system EraSR. Reporter constructs of pEDY305 were used. Activities were determined after induction of mutants for 6 h on ethanol or after growth of the wild type to an OD_{620} of 0.6 for about 6 h. Results represent means ± standard deviations of at least three independent experiments. ND, not determined.
[b] G, after growth; I, after induction.

vector carried the operon *ercA-ercS* under the control of the *lac* promoter, which is recognized in *P. aeruginosa* and leads to constitutive expression of both genes. Vector pTB7200 was transferred into MD1. After induction on ethanol, expression levels of *exaA* and operons *exaBC* and *pqqABCDEH* were determined with promoter-probe vectors pTB3138, pTB3139, and pTB7023. Under these conditions, the transcription levels of *exaA*, *exaBC*, and *pqqABCDEH* were below 5% that of the wild type and comparable to that in MD1 without the vector pTB7200 (data not shown). This result is in agreement with our previously proposed regulatory scheme (8). In MD1, transcription of the so-far-unknown sensor kinase ErbS, corresponding to the response regulator ErbR, is assumed to depend also on the response regulator ErdR, which is missing in MD1 (Fig. 2).

Constitutive expression of EraSR and ErbR partially restores expression of the ethanol oxidation system in NH1 and SH1. The response regulator ErbR controls transcription of the *exaBC*, the *pqq* and the *eraSR* operons (5). The two-component regulatory system EraSR controls transcription of *exaA*, which encodes QEDH (3). We previously showed that transcription of *erbR* depends on the sensor kinase ErcS (8) and, as shown in the present report, it also depends on the gene product of *ercA*. As shown in Fig. 2, in NH1, with a defective *ercA*, and in SH1, with a defective *ercS*, it is to be expected that constitutive expression of the one-level-lower ErbR will lead to the expression of the *exa* and *pqq* operons. Furthermore, constitutive expression of the next-level-lower EraSR will lead to the expression of *exaA*, but not of *exaBC* and *pqq*. The vector pTB7060 and vector pTB3144 were used for constitutive expression of *erbR* and the two-component system *eraSR*, respectively. Both vectors carry the respective genes under the control of a *lac* promoter, which is recognized in *P. aeruginosa*. The vectors were transferred into NH1 and SH1. Constitutive expression of ErbR led to induction of operons *exaA*, *exaBC*, and *pqq* (Table 6). The relative expression levels were about 25 to 40% in NH1 and about 25 to 50% in SH1 compared to that in the wild type. As expected, constitutive expression of EraSR in both mutants did not induce the *exaBC* or *pqq* operons. However, in both mutants, *exaA*, encoding QEDH, was induced, but to a rather low level of 5 to 9% of the wild-type level (Table 6). The reason for this low expression level is not clear. The hypothetical model shown in Fig. 2 does explain the basic experimental results, but it may need to be modified to explain the low expression levels described.

Conclusions. In the present study, we confirmed that PA1991 and *ercS* (PA1992) form an operon. Inactivation of the respective genes generated mutants NH1 and SH1, with similar properties (Table 2). Both genes are essential for effective expression of the ethanol oxidation system in *P. aeruginosa*. Therefore, we named PA1991, which forms an operon with *ercS*, *ercA*. The *ercA-ercS* operon regulates transcription of the response regulator ErbR, which controls the operons *eraSR*, *exaBC*, and *pqq*. The results generated with NH1 provided further support to the hierarchical complex regulatory network (Fig. 2), comprising possibly five different two-component systems that regulate ethanol oxidation in *P. aeruginosa*. It has been recently reported that the global response regulator ErdR is required for expression of the gene *acsA*, which encodes acetyl coenzyme A synthetase (28). Thus, ErdR controls, in addition to ethanol, acetate metabolism in *P. aeruginosa*. This observation is incorporated in Fig. 2.

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