A complex regulatory network controls aerobic ethanol oxidation in *Pseudomonas aeruginosa*: indication of four levels of sensor kinases and response regulators

Demissew S. Mern,¹ Seung-Wook Ha,² Viola Khodaverdi,³ Nicole Gliese⁴ and Helmut Görisch³

Correspondence

Helmut Görisch
helmut.goerisch@tu-berlin.de

¹Helmholtz-University Group Molecular Epidemiology, German Cancer Research Center, Im Neuenheimer Feld 581, D-69120 Heidelberg, Germany

²The Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA

³Fachgebiet Angewandte Biochemie, Institut für Biotechnologie, Technische Universität Berlin, Seestrasse 13, D-13353 Berlin, Germany

⁴BioGenes GmbH, Koepenicker Strasse 325, D-12555 Berlin, Germany

In addition to the known response regulator ErbR (former AgmR) and the two-component regulatory system EraSR (former ExaDE), three additional regulatory proteins have been identified as being involved in controlling transcription of the aerobic ethanol oxidation system in *Pseudomonas aeruginosa*. Two putative sensor kinases, ErcS and ErcS', and a response regulator, ErdR, were found, all of which show significant similarity to the two-component *flhSR* system that controls methanol and formaldehyde metabolism in *Paracoccus denitrificans*. All three identified response regulators, EraR (formerly ExaE), ErbR (formerly AgmR) and ErdR, are members of the *luxR* family. The three sensor kinases EraS (formerly ExaD), ErcS and ErcS' do not contain a membrane domain. Apparently, they are localized in the cytoplasm and recognize cytoplasmic signals. Inactivation of gene *ercS* caused an extended lag phase on ethanol. Inactivation of both genes, *ercS* and *ercS'*, resulted in no growth at all on ethanol, as did inactivation of *erdR*. Of the three sensor kinases and three response regulators identified thus far, only the EraSR (formerly ExaDE) system forms a corresponding kinase/regulator pair. Using reporter gene constructs of all identified regulatory genes in different mutants allowed the hierarchy of a hypothetical complex regulatory network to be established. Probably, two additional sensor kinases and two additional response regulators, which are hidden among the numerous regulatory genes annotated in the genome of *P. aeruginosa*, remain to be identified.

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INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* ATCC 17933 is able to grow aerobically on ethanol as the sole source of carbon and energy. The initial step, converting ethanol to acetaldehyde, is catalysed by a periplasmic quinoprotein ethanol dehydrogenase (QEDH), with 2,7,9-tricarboxypyrroloquinoline quinone (PQQ) as prosthetic group (Rupp & Görisch, 1988). QEDH is linked to an electron transport chain that includes soluble cytochrome *c*₅₅₀ and a *co*-type cytochrome oxidase (Reichmann & Görisch, 1993; Matsushita *et al.*, 1982). The PQQ cofactor can be removed reversibly, and incubation of the apoform

with PQQ and Ca²⁺ leads to a fully active enzyme (Mutzel & Görisch, 1992). The X-ray structure has been solved at 2.6 Å resolution and reveals that the backbone folding and the active site of QEDH are very similar to those of the large subunits of the PQQ-dependent quinoprotein methanol dehydrogenases (QMDHs) of *Methylobacterium extorquens* and *Methylophilus* W3A1 (Keitel *et al.*, 2000; Ghosh *et al.*, 1995; Xia *et al.* 1996). Recently, we demonstrated that the unusual disulfide ring formed by adjacent cysteine residues present in QEDH is essential for efficient electron transfer to cytochrome *c*₅₅₀ (Mennenga *et al.*, 2009).

Studies with a number of regulatory mutants have revealed that ethanol oxidation in *P. aeruginosa* is controlled by at least seven genes organized in a complex network, where the different components act in a hierarchical manner

Abbreviations: MU, Miller units; PQQ, pyrroloquinoline quinone; QEDH, quinoprotein ethanol dehydrogenase.

(Görisch, 2003). Three of these genes have been identified so far: a two-component regulatory system, EraSR (formerly ExaDE), which encodes a sensor kinase and the respective response regulator, controlling transcription of the *exaA* gene, encoding QEDH (Schobert & Görisch, 2001). The response regulator ErbR (formerly AgmR) controls transcription of the *eraSR* (formerly *exaDE*) two-component system, the *exaBC* operon encoding cytochrome *c*₅₅₀ and an aldehyde dehydrogenase, as well as the *pqq* operon encoding the enzymes needed for PQQ biosynthesis (Gliese *et al.*, 2004). As shown in Fig. 1, the operons of the *exa* and *pqq* genes are clustered together on the genome of *P. aeruginosa* PAO1 (Stover *et al.*, 2000).

Here we report the identification of two additional sensor kinases, ErcS and ErcS', encoded by genes PA1992 and PA1976, and a response regulator, ErdR, encoded by gene PA3604, all of which are involved in the regulation of the ethanol oxidation system in *P. aeruginosa*. The three genes show significant similarity to the *Paracoccus denitrificans* *flhSR* two-component system, which controls methanol and formaldehyde metabolism in that organism (Harms *et al.*, 2001). Genes PA1976 and PA1992 were studied because they are located in close proximity to the *era* and *pqq* gene clusters (Fig. 1). Gene PA3604 was identified by searching the genome of *P. aeruginosa* for further response regulators with significant similarity to the *Paracoccus denitrificans* *flhSR* system. The regulatory genes discussed in the present study are listed in Table 1 with their current names and annotation together with their new suggested names.

METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium; *P. aeruginosa* ATCC 17933 and mutants were cultivated in LB or minimal medium supplemented with different carbon sources (Rupp & Görisch, 1988). Glucose (40 mM), succinate (40 mM), acetate (20 mM) and 0.5 % (v/v) of alcohols were used. When appropriate, the following antibiotics were added to the media: 100 µg carbenicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 50 µg gentamicin ml⁻¹ and 20 µg tetracycline ml⁻¹.

General genetic techniques. DNA manipulations were performed according to the protocols described by Sambrook *et al.* (1989) and

Ausubel *et al.* (2002). For PCRs, genomic DNA isolated from *P. aeruginosa* ATCC 17933 was used as template, and for primer design, the sequence of *P. aeruginosa* strain PAO1 was used, as the nucleotide identity between PAO1 and ATCC 17933 is 99 % (Schobert, 1999; Stover *et al.*, 2000). For amplification of gene PA3604, primers with restriction sites (indicated in bold, below) for *Xba*I and *Hind*III were used. The forward primer was 5'-AGCTCTAGAGCAGGC-CGGTATAGAGGAAC-3' and the reverse primer was 5'-TCA-GAAGCTTGACTGACGTCGAGGAGGAAG-3'. The 1.18 kb PCR product containing PA3604 was cloned into *Xba*I-*Hind*III sites of pUC18 and pUCP20T, resulting in plasmids pTB7076 and pTB7106. For amplification of gene PA1976, primers with restriction sites for *Hind*III were used. The forward primer was 5'-CAGGAAGCTT-CACCGCGATGAGCACCAGGT-3' and the reverse primer was 5'-CAGGAAGCTTCGGCTACGCCGTCTGGTAC-3'. The 3.07 kb PCR product containing PA1976 was cloned into the *Hind*III site of pUC19, resulting in plasmid pTB7029. For amplification of gene PA1992, primers with restriction sites for *Eco*RI and *Sph*I were used. The forward primer was 5'-AGCGAATTCACCTGATCGCCTTCAAGC-AC-3' and the reverse primer was 5'-ACTGAGCATGCTCCGCTC-GATGTTCTCTCTTC-3'. The 1.98 kb PCR product containing PA1992 was cloned into *Eco*RI-*Sph*I sites of pUC18 and pUCP20T, resulting in plasmids pTB7100 and pTB7101. Electroporation of *P. aeruginosa* ATCC 17933 was performed as described by Smith & Iglewski (1989). Triparental matings were performed as described previously (Kretzschmar *et al.*, 2001).

Site-directed inactivation of genes. Site-directed inactivation of genes of *P. aeruginosa* ATCC 17933 by a kanamycin- or gentamicin-resistance cassette was done by the *sacB*-based strategy with the suicide vector pEX18Ap or pEX18Tc (Hoang *et al.*, 1998). Sucrose-resistant colonies were obtained by streaking *P. aeruginosa* merodiploids on LB plates supplemented with 5 % sucrose. For inactivation of gene PA3604, the *Sma*I-digested kanamycin-resistance cassette was ligated into the blunted *Apal*-*Pst*I sites of pTB7076, resulting in pTB7077, and the insert was cloned into the *Xba*I-*Hind*III sites of pEX18Ap, resulting in pTB7078. The Km^r gene is transcribed in the same orientation as the PA3604 gene. For inactivation of gene PA1976, the kanamycin-resistance cassette was ligated into the *Bsa*I site of pTB7029, resulting in pTB7035, and the insert was cloned into the *Hind*III site of pEX18Tc, resulting in pTB7040. The same orientation of both genes was verified. For inactivation of gene PA1992, the gentamicin-resistance cassette was ligated into the *Bam*HI site of pTB7100, resulting in pTB7103, and the insert was cloned into the *Eco*RI-*Xba*I sites of pEX18Ap, resulting in pTB7104. The Gm^r gene is transcribed in the same orientation as the PA1992 gene. The suicide vectors were introduced into *P. aeruginosa* via 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.

Fig. 1. Arrangement of the *exa* and *era* to *erc* genes on the chromosome of *P. aeruginosa* PAO1 and ATCC 17933. Genes *exa*, encoding components of ethanol oxidation metabolism, are shown in black; *pqq* genes are indicated by horizontal hatching. Regulatory genes *eraSR* (*exaDE*), *erbR* (*agmR*), *ercS* and *ercS'*, controlling operons *exa* and *pqq*, are shown with diagonal hatching; gene *erdR* is not shown. Mutants discussed in the text are indicated by their names.

Table 1. Renaming of the regulatory genes of the ethanol oxidation system in *P. aeruginosa* PAO1

S, sensor kinase of two-component regulatory system; R, response regulator. *exa*, Ethanol oxidation genes cluster a, encode proteins needed for ethanol metabolism: *exaA* (QEDH), *exaBC* (cytochrome c_{550} , NAD⁺-dependent aldehyde dehydrogenase); *era* genes encode regulatory proteins controlling transcription of operon *exaA*: first level of two-component regulatory systems; *erb* genes encode regulatory proteins controlling transcription of operons *eraSR*, *exaBC* and *pqqABCDE*: second level of two-component regulatory systems; *erc* genes encode regulatory proteins controlling transcription of *erb* genes: third level of two-component regulatory systems; *erd* genes encode regulatory proteins controlling transcription of *erc* genes: fourth level of two-component regulatory systems.

PAO1 gene	Annotation (Stover <i>et al.</i> , 2000)	Current name	Reference	Suggested name
PA1982	PQQ-dependent alcohol dehydrogenase	<i>exaA</i>	Schobert & Görisch (1999)	<i>exaA</i>
PA1983	Cytochrome <i>c</i>	<i>exaB</i>	Schobert & Görisch (1999)	<i>exaB</i>
PA1984	Probable aldehyde dehydrogenase	<i>exaC</i>	Schobert & Görisch (1999)	<i>exaC</i>
PA1976	Sensor kinase (<i>flhS</i> -like)			<i>ercS'</i>
PA1978	Response regulator	<i>agmR</i>	Schweizer (1991); Gliese <i>et al.</i> (2004)	<i>erbR</i>
PA1979	Sensor kinase	<i>exaD</i>	Schobert & Görisch (2001)	<i>eraS</i>
PA1980	Response regulator	<i>exaE</i>	Schobert & Görisch (2001)	<i>eraR</i>
PA1992	Sensor kinase (<i>flhS</i> -like)			<i>ercS</i>
PA3604	Response regulator (<i>flhR</i> -like)			<i>erdR</i>

Promoter-probe vectors. Promoter-probe vectors were constructed to study the transcriptional regulation of genes PA3604 and *erbR* (*agmR*). A 280 bp promoter region of PA3604 was cloned into the *XbaI*–*HindIII* sites of pQF50 upstream of the *lacZ* gene (Farinha & Kropinski, 1990), and a 1.0 kb promoter region of *agmR* was cloned into the *KpnI*–*HindIII* sites of pQF50, resulting in promoter-probe vectors pTB7107 and pTB7108, respectively. For amplification of the promoter region of PA3604, primers with restriction sites (indicated in bold, below) for *XbaI* and *HindIII* were used. The forward primer was 5'-AGCTCTAGATGATCAGCATCAGGACATCG-3' and the reverse primer was 5'-TCAGAAAGCTTGGCGATCAGGATCTCG-TAAG-3'. For amplification of the promoter region of *erbR* (*agmR*), primers with restriction sites for *KpnI*–*HindIII* were used. The forward primer was 5'-CTAGGTACCAGCAGCCAGCGGTA-CTCC-3' and the reverse primer was 5'-TCTGAAGCTTCGTAG-GTGATGGCCTGTAGC-3'.

To study transcription of the operon PA1991/PA1992, three different promoter-probe vectors were constructed. A 0.53 kb promoter region of PA1991, a 0.62 kb putative promoter region in front of PA1992, and a 1.7 kb region containing the promoter region of PA1991, gene PA1991 and the putative promoter of PA1992, were amplified by PCR and cloned into the *XbaI*–*HindIII* sites of pQF50 upstream of the *lacZ* gene, resulting in promoter-probe vectors pTB7082, pTB7083 and pTB7084, respectively. The primers used for these amplifications contained restriction sites for *XbaI* and *HindIII*. For amplification of the promoter region of PA1991, the forward primer was 5'-TAGTCTAGAGCCAGTCTCTATGGGGTCAG-3' and the reverse primer was 5'-TCAGAAAGCTTGTAGTTGGCGACGCTGTG-3'. For amplification of the putative promoter region of PA1992, the forward primer was 5'-TAGTCTAGACTGATCGATGGCAATCTGG-3' and the reverse primer was 5'-TCAGAAAGCTTACAGCCATTGTAGC-GGTTG-3'. The 1.7 kb fragment containing the promoter of PA1991, gene PA1991 and the putative promoter region of PA1992 was amplified using the forward primer 5'-TAGTCTAGAGCCAGTC-TCTATGGGGTCAG-3' and the reverse primer 5'-TCAGAAAGCTTA-CAGCCATTTGTAGCGGTTG-3'.

Promoter-probe vectors of the genes *exaA* (pTB3138), *exaBC* (pTB3139), *eraSR* (*exaDE*) (pTB7074) and *pqqABCDE* (pTB7023) were as described previously (Schobert & Görisch, 1999; Gliese *et al.*, 2004). pTB3138, pTB3139 and pTB7023 are derivatives of pEDY305

(Schwartz *et al.*, 1998); pTB7074 is a derivative of pQF50 (Farinha & Kropinski, 1990).

β -Galactosidase assay. β -Galactosidase activity was determined in toluene-treated cells according to the procedure of Miller (1992). Determination of β -galactosidase activity in mutants unable to grow on ethanol was performed after induction on ethanol, as described previously (Schobert & Görisch, 2001).

Internet tools. The *Pseudomonas* Genome Database (<http://www.pseudomonas.com>; Winsor *et al.*, 2009), was used to obtain DNA sequences of *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000). BLAST was used for DNA or protein database searches (Altschul *et al.*, 1997).

RESULTS AND DISCUSSION

Naming and renaming genes involved in ethanol oxidation and its regulation in *P. aeruginosa*

When naming the *exa* gene cluster we followed the suggestions given by Lidstrom *et al.* (1994). The three genes involved in ethanol oxidation form the linkage group a (Schobert & Görisch, 1999). Later we found genes PA1979 and PA1980, separated from the linkage group *exaABC* by a gene encoding a small protein of unknown function, and which encode a two-component regulatory system. These genes control transcription of the *exaA* operon and were named *exaDE* (Schobert & Görisch, 2001). In 2004 we identified AgmR as controlling transcription of the *exaDE* regulatory system and also transcription of operons *exaBC* and *pqq* (Gliese *et al.*, 2004). AgmR, a response regulator encoded by PA1978, has been described as involved in glycerol metabolism (Schweizer, 1991). Inactivation of the *agmR* gene was later shown not to affect glycerol metabolism in *P. aeruginosa* PAO1 (Schweizer, 1992; Schweizer & Po, 1996) and ATCC 17933 (Gliese *et al.*, 2004). However, AgmR is an important

Table 2. Strains and plasmids used in this study

In promoter-probe vectors, the putative promoter region and the truncated gene *X* are indicated by *P_X'-lacZ*.

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>P. aeruginosa</i> strains		
ATCC 17933	Wild-type	Cetin <i>et al.</i> (1965)
MD1	ATCC 17933 derivative, PA3604::Km ^r	This study
MD2	SH1 derivative, PA3604::Km ^r , PA1992::Gm ^r	This study
NG2	ATCC 17933 derivative, PA1976::Km ^r	This study
NG3	ATCC 17933 derivative, <i>agmR</i> ::Km ^r	Gliese <i>et al.</i> (2004)
SH1	ATCC 17933 derivative, PA1992::Gm ^r	This study
SH2	NG2 derivative, PA1992::Gm ^r , PA1976::Km ^r	This study
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Hanahan (1983)
HB101	<i>supE44</i> <i>hsdS20</i> (<i>r_Bm_B</i>) <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacYl</i> <i>galK2</i> <i>rpsL20</i> <i>xyl-5</i> <i>mtl-1</i>	Boyer & Roulland-Dussoix (1969)
Plasmids		
pAC Ω Gm	Gm ^r ; cloning vector with Gm ^r cassette and Ω fragments	Schweizer (1993)
pEDY305	Tc ^r , <i>lacZ</i> promoter-probe vector	Schwartz <i>et al.</i> (1998)
pEX18Ap	Ap ^r ; oriT ⁺ <i>sacB</i> ⁺ , gene replacement vector with multiple cloning site (MCS) from pUC18	Hoang <i>et al.</i> (1998)
pEX18Tc	Tc ^r ; oriT ⁺ <i>sacB</i> ⁺ , gene replacement vector with MCS from pUC18	Hoang <i>et al.</i> (1998)
pQF50	Ap ^r ; <i>lacZ</i> promoter-probe vector	Farinha & Kropinski (1990)
pRK2013	Km ^r ; helper plasmid for triparental mating	Figurski & Helinski (1979)
pUC18, pUC19	Ap ^r ; cloning and expression vector	Yanisch-Perron <i>et al.</i> (1985)
pUCP20T	Ap ^r ; broad-host-range vector	Schweizer <i>et al.</i> (1996)
pTB3001	Tc ^r ; 25 kb genomic DNA partially digested with <i>Sau3AI</i> from <i>P. aeruginosa</i> cloned in <i>Bam</i> HI site of pLAFR3	Schobert & Görisch (1999)
pTB3070	Ap ^r ; 3.2 kb <i>Bam</i> HI– <i>Pst</i> I fragment from pTB3001 cloned into <i>Bam</i> HI– <i>Pst</i> I sites of pUC18	Schobert & Görisch (1999)
pTB3131	Ap ^r Km ^r ; 1.0 kb PCR product with promoter and Km ^r cassette of Tn5 cloned into <i>Eco</i> RI– <i>Bam</i> HI sites of pUC19	Kretzschmar <i>et al.</i> (2001)
pTB3138	Tc ^r ; (<i>P_{exaA'}-lacZ</i>) 0.89 kb <i>Pst</i> I– <i>Sal</i> I fragment from pTB3070 cloned into <i>Pst</i> I– <i>Xho</i> I sites of pEDY305	Schobert & Görisch (2001)
pTB3139	Tc ^r ; (<i>P_{exaB'}-lacZ</i>) 0.89 kb <i>Pst</i> I– <i>Xba</i> I fragment from pTB3135 cloned into <i>Pst</i> I– <i>Xba</i> I sites of pEDY305	Schobert & Görisch (2001)
pTB7023	Tc ^r ; (<i>P_{pqqAB'-lacZ}</i>) 2.5 kb <i>Xho</i> I– <i>Bam</i> HI fragment from pTB3070 cloned into <i>Xho</i> I– <i>Bgl</i> II sites of pEDY305	Gliese <i>et al.</i> (2004)
pTB7029	Ap ^r ; 3.07 kb PCR product of gene PA1976 cloned into <i>Hind</i> III site of pUC19	This study
pTB7035	Ap ^r Km ^r ; 4.07 kb Km ^r cassette cloned into <i>Bsa</i> I site of PA1976 in pTB7029	This study
pTB7037	Ap ^r ; (<i>P_{pqqAB'-lacZ}</i>) 2.2 kb <i>Sal</i> I– <i>Bam</i> HI fragment from pTB3070 with promoter region of <i>pqqABCDE</i> cloned into <i>Sal</i> I– <i>Bam</i> HI sites of pQF50	This study
pTB7040	Tc ^r Km ^r ; 4.07 kb PCR product of gene PA1976 with Km ^r cassette cloned into <i>Hind</i> III site of pEX18Tc	This study
pTB7060	Ap ^r ; 1.67 kb PCR product containing gene <i>agmR</i> cloned into the <i>Pst</i> I– <i>Bam</i> HI sites of pUCP20T	Gliese <i>et al.</i> (2004)
pTB7070	Ap ^r Gm ^r ; 1.04 kb Gm ^r cassette from pAC Ω Gm cloned into <i>Eco</i> RI site of pUC18	This study
pTB7074	Ap ^r ; (<i>P_{exaDE'}-lacZ</i>) 1.16 kb promoter region of <i>exaDE</i> cloned into <i>Bgl</i> II– <i>Xba</i> I sites of pQF50	Gliese <i>et al.</i> (2004)
pTB7076	Ap ^r ; 1.18 kb PCR product of gene PA3604 cloned into <i>Xba</i> I– <i>Hind</i> III sites of pUC18	This study
pTB7077	Ap ^r Km ^r ; 1.0 kb <i>Sma</i> I– <i>Sma</i> I fragment of Km ^r cassette cloned into <i>Apal</i> – <i>Pst</i> I sites of pTB7076	This study
pTB7078	Ap ^r Km ^r ; 1.94 kb <i>Xba</i> I– <i>Hind</i> III fragment from pTB7077 cloned into <i>Xba</i> I– <i>Hind</i> III sites of pEX18Ap	This study
pTB7082	Ap ^r ; (<i>P_{PA1991'-lacZ}</i>) 0.53 kb PCR product containing the promoter region of PA1991 cloned into the <i>Xba</i> I– <i>Hind</i> III sites of pQF50 (pPA1991)	This study

Table 2. cont.

Strain or plasmid	Relevant genotype or phenotype	Source or reference
pTB7083	Ap ^r ; (<i>P</i> _{PA1992'} - <i>lacZ</i>) 0.62 kb PCR product containing the promoter region of PA1992 cloned into the <i>Xba</i> I– <i>Hind</i> III sites of pQF50 (pPA1992)	This study
pTB7084	Ap ^r ; (<i>P</i> _{PA1991,1992'} - <i>lacZ</i>) 1.7 kb PCR product containing the promoter region of PA1991, gene PA1991 and the putative promoter region of PA1992 cloned into the <i>Xba</i> I– <i>Hind</i> III sites of pQF50 (pPA1991 + 1992)	This study
pTB7100	Ap ^r ; 1.98 kb PCR product of gene PA1992 cloned into <i>Eco</i> RI– <i>Sph</i> I sites of pUC18	This study
pTB7101	Ap ^r ; 1.98 kb PCR product of gene PA1992 cloned into <i>Eco</i> RI– <i>Sph</i> I site of pUCP20T	This study
pTB7103	Ap ^r Gm ^r ; 1.02 kb <i>Bam</i> HI fragment of Gm ^r cassette cloned into <i>Bam</i> HI site of pTB7100	This study
pTB7104	Ap ^r Gm ^r ; 2.23 kb <i>Eco</i> RI– <i>Xba</i> I fragment from pTB7103 cloned into pEX18Ap	This study
pTB7106	Ap ^r ; 1.18 kb <i>Xba</i> I– <i>Hind</i> III fragment from pTB7076 cloned into <i>Xba</i> I– <i>Hind</i> III sites of pUCP20T	This study
pTB7107	Ap ^r ; (<i>P</i> _{PA3604'} - <i>lacZ</i>) 280 bp promoter region of PA3604 cloned into <i>Xba</i> I– <i>Hind</i> III sites of pQF50	This study
pTB7108	Ap ^r ; (<i>P</i> _{agmR'} - <i>lacZ</i>) 1.0 kb promoter region of <i>agmR</i> cloned into <i>Kpn</i> I– <i>Hind</i> III sites of pQF50	This study

regulatory element in ethanol metabolism of *P. aeruginosa* ATCC 17933. It controls transcription of a regulon that comprises the operons *exaBC* and *pqq* and the two-component regulatory system *exaDE*. With the identification of genes PA1976, PA1992 and PA3604 as further two-component regulatory systems, we find apparently four levels of regulation. Here we introduce for all proteins involved in regulating the operons *exa* and *pqq* the name ethanol metabolism regulatory factors, indicating different levels, a, b, etc. for a hierarchical organization of two-component systems. The type of factor is indicated by S for sensor kinase and R for response regulator.

The *exaDE* two-component regulatory system controls transcription of the *exaA* operon and will be renamed. It represents the first level of regulation (Schobert & Görisch, 2001), and is called *eraSR*. The response regulator AgmR represents the second level of regulation (Gliese *et al.*, 2004), and is renamed ErbR. The two FlhS-like sensor kinases encoded by PA1976 and PA1992 are required for ErbR (AgmR) expression and can partially compensate for each other. These sensor kinases are named ErcS (PA1992) and ErcS' (PA1976). The FlhR-like response regulator encoded by gene PA3604 is named ErdR (fourth level of regulation). It is required for expression of ErcS and ErbR. The *exa* genes encoding components involved in ethanol oxidation and the different regulatory genes *era* to *erd* controlling the *exa* and *pqq* operons are listed in Table 1.

Identification of genes possibly involved in regulating ethanol utilization

Genes encoding components of the ethanol oxidation pathway (*exa*) and its regulation are clustered in the genome of *P. aeruginosa*. Fig. 1 shows the arrangement of the *exaA*, *exaBC*, *eraSR* (*exaDE*) and *pqq* operons as found in *P. aeruginosa* ATCC 17933 by sequencing a fragment of

about 13 kb from cosmid pTB3001. An identical arrangement of these genes has been reported in *P. aeruginosa* PAO1 (Stover *et al.*, 2000; *Pseudomonas* Genome Database; Winsor *et al.*, 2009). Therefore, we use the gene numbers of *P. aeruginosa* PAO1 to identify genes in the present paper. Gene *ercS'* (PA1976), upstream of the *exa* gene cluster, and gene *ercS* (PA1992), downstream of the *pqq* genes, were found to encode two-component sensor kinases with significant amino acid sequence similarity in their C-terminal parts to FlhS in *Paracoccus denitrificans*. The two-component regulatory system FlhSR controls methanol, formaldehyde and methylamine oxidation in *Paracoccus denitrificans* (Harms *et al.*, 2001). Gene *erdR* (PA3604) of *P. aeruginosa* encodes a putative response regulator and was identified by its 60 % similarity to FlhR of *Paracoccus denitrificans*. In addition, the product of gene *erdR* also shows a 61 % similarity in amino acid sequence with response regulator ErbR (AgmR), which controls transcription of the *exa* and *pqq* operons in *P. aeruginosa* (Gliese *et al.*, 2004).

Generation of regulatory mutants

Mutants of *P. aeruginosa* ATCC 17933 were generated by inactivating the response regulator gene *erdR* and the two sensor kinase genes *ercS'* and *ercS*. The *flhS*-like gene *ercS'* was interrupted by a Km^r cassette, resulting in mutant NG2; the second *flhS*-like gene *ercS* was inactivated by a combination of Gm^r cassette insertion and deletion of a 640 bp fragment, resulting in mutant SH1. By interrupting gene *ercS* in mutant NG2, double mutant SH2 was generated, with both *flhS*-like genes inactivated. The *flhR*-like gene *erdR* was inactivated by a combination of Km^r cassette insertion and deletion of a 262 bp fragment, resulting in mutant MD1. A double mutant with both genes inactivated, the *flhS*-like *ercS* and the *flhR*-like *erdR*, was generated from mutant SH1

by Km^r cassette insertion and deletion of a 262 bp fragment in gene *erdR*, resulting in mutant MD2. The correct insertion of resistance cassettes and construction of mutants was verified by PCR product analysis (data not shown). The different mutants with their genotypes are listed in Table 2.

Growth of regulatory mutants

Growth behaviour of the mutants was determined on various substrates (Table 3). Mutant NG2 grew on all substrates tested like the wild-type. Inactivation of the FlhS-like sensor kinase *ErcS'* apparently did not influence growth of the organism. However, inactivation of the FlhS-like sensor kinase *ErcS*, generating mutant SH1, caused a significant extension of the lag phase on ethanol, while the generation time was unaltered with respect to the wild-type. On glucose and succinate, mutant SH1 showed an increase in generation time. When gene *ercS'* was inactivated in the background of mutant SH1, generating double mutant SH2 with both *flhS*-like genes, *ercS'* and *ercS*, inactivated, no growth at all occurred on ethanol for up to 50 h (Table 3). Both sensor kinases are therefore involved in regulation of ethanol utilization. Growth of mutant SH2 on glucose was retarded, while no effect was found on succinate or acetate.

Mutant MD1, with the *flhR*-like gene *erdR* inactivated, did not grow on ethanol for up to 80 h. Therefore the intact response regulator *ErdR* is essential for ethanol utilization. Mutant MD1 did grow on glucose or succinate like the wild-type, but unlike mutant SH2, no growth was observed on acetate (Table 3). Double mutant MD2, in which the *flhS*-like gene *ercS* and the *flhR*-like gene *erdR* were both inactivated, behaved like Mutant MD1 on ethanol, glucose and succinate (data not shown). Upon transformation *in trans* by plasmids carrying an intact copy of the inactivated chromosomal gene, all mutants showed wild-type behaviour again (data not shown).

Transcription of the ethanol oxidation system in regulatory mutants

Transcription of the operons *exaA*, *eraSR* (*exaDE*), *exaBC* and *pqq* was studied using reporter gene constructs, with the respective promoter regions in vectors pEDY305 and pQF50. Transcription of the four operons of the ethanol oxidation system was reduced in all generated putative regulatory mutants. In mutant NG2, which did not show any difference in growth behaviour when compared with the wild-type, transcription of all four operons was reduced to 60–80 % of that of the wild-type. In mutant SH1, transcription was reduced even further to 25–45 % (Fig. 2). In double mutant SH2, with both *flhS*-like sensor kinase genes inactivated, and in mutant MD1, where an *flhR*-like gene was inactivated, transcription was reduced to a value below 2 % with respect to the wild-type (data not shown). The results demonstrate that all three *flh*-like gene products are required for expression of the *exa*, *eraSR* (*exaDE*) and *pqq* operons in *P. aeruginosa*.

We previously reported (Gliese *et al.*, 2004) that in mutant NG3, where gene *erbR* (*agmR*) is inactivated, the *exaA*, *eraSR* (*exaDE*), *exaBC* and *pqq* operons are not transcribed. This situation is identical to that found here with mutants SH2 and MD1. It appears that the FlhS-like sensor kinases *ErcS'* and *ErcS* as well as the FlhR-like response regulator *ErdR* are required for *ErbR* (*AgmR*) expression.

Transcription of regulatory factors in the different regulatory mutants

Reporter gene constructs with the promoter regions of *erbR* (*agmR*; pTB7108) and *erdR* (pTB7107) cloned into vector pQF50 allowed the determination of their transcription in the different mutants.

Table 3. Growth of *P. aeruginosa* mutants on various carbon sources

Results represent mean values of at least three independent experiments. SK, two-component regulatory system sensor kinase; RR, response regulator. Gen, generation time; Lag, lag phase; wt, wild-type value; >, generation time or lag phase greater than 1.5 times the wild-type value; >>, extreme lag phase of 20 h; NG, no growth for at least 50 h.

<i>P. aeruginosa</i> ATCC 17933 strain	Gene inactivated	Carbon source			
		Ethanol	Glucose	Succinate	Acetate
Mutant NG2 (PA1976::Km ^r)	SK	Gen/Lag wt/wt	Gen/Lag wt/wt	Gen/Lag wt/wt	Gen/Lag wt/wt
Mutant SH1 (PA1992::Gm ^r)	SK	wt/>>	>/wt	>/wt	wt/>
Mutant SH2 (PA1976::Km ^r ; PA1992::Gm ^r)	SK SK	NG	>/>	wt/wt	wt/wt
Mutant MD1 (PA3604::Km ^r)	RR	NG	wt/wt	wt/wt	NG*

*U. Kretzschmar, personal communication.

Fig. 2. Promoter activities of the *exaA*, *eraSR* (*exaDE*), *exaBC* and *pqqABCDE* operons in wild-type and mutant strains of *P. aeruginosa* after growth or induction on ethanol. (a) Promoter activities of *exaA* (pTB3138), *exaBC* (pTB3139) and *pqqABCDE* (pTB7023) using reporter constructs of pEDY305. Black bars, *exaA*; horizontally hatched bars, *exaBC*; white bars, *pqqABCDE*. (b) Promoter activities of *eraSR* (*exaDE*, pTB7074) and *pqqABCDE* (pTB7037) using reporter constructs of pQF50. Hatched bars, *eraSR* (*exaDE*); white bars, *pqqABCDE*. The promoter activities of *exaA*, *eraSR* (*exaDE*), *exaBC* and *pqqABCDE* in mutants SH2, MD1 and NG3 were less than 2% of the activities in the wild-type (not shown). Results represent mean values of at least three independent experiments. ND, Not determined.

Transcription of *erbR* (*agmR*) was reduced, albeit to different levels, in all the mutants with defective *flhS*- or *flhR*-like genes (Table 4). Therefore, sensor kinases ErcS' and ErcS and the response regulator ErdR all participate in controlling ErbR (AgmR) expression. The ErbR (AgmR) response regulator in turn controls transcription of the *exa* and *pqq* operons (Gliese *et al.*, 2004).

Transcription of gene *erdR*, encoding an FlhR-like response regulator, was reduced only slightly in the respective defective mutants (Table 4). Apparently, its transcription does not depend on the FlhS-like sensor kinases ErcS' and ErcS or the response regulator ErbR (AgmR). ErdR appears to represent the highest level of regulatory components identified so far (Fig. 3).

The gene sequence of *ercS*, encoding an FlhS-like sensor kinase, overlaps with the upstream gene PA1991 by 17 bp. Presumably, the two genes form a transcriptional unit. To study the transcriptional regulation of *ercS* (PA1992), reporter gene constructs were generated with different putative promoter sequences. The putative promoter region of PA1991 together with part of gene PA1991 was amplified and cloned in the reporter gene vector pQF50, resulting in plasmid pTB7082. The construct pTB7082 records the transcriptional activity of a putative PA1991 promoter. By amplifying a sequence in front of *ercS*, which belongs to the coding sequence of PA1991, together with part of *ercS*, and cloning it into vector pQF50, plasmid pTB7083 was generated, which records the transcriptional activity of a putative promoter in front of *ercS*. Construct

Table 4. Promoter activities of genes *erbR* (*agmR*) and *erdR* in wild-type and mutant strains of *P. aeruginosa* after growth or induction on ethanol

I, after induction; G, after growth. Results represent mean $\pm$ SD of at least three independent experiments.

<i>P. aeruginosa</i> ATCC 17933 strain	Gene inactivated	Promoter activity (MU)		Condition
		<i>P_{erbR}</i> (<i>agmR</i>) pTB7108*	<i>P_{erdR}</i> (PA3604) pTB7107*	
Wild-type		1400 $\pm$ 30	290 $\pm$ 13	G or I
NG2 (PA1976::Km ^r)	<i>ercS'</i>	1200 $\pm$ 110	300 $\pm$ 10	G
SH1 (PA1992::Gm ^r)	<i>ercS</i>	170 $\pm$ 14	250 $\pm$ 5	G
SH2 (PA1976::Km ^r); (PA1992::Gm ^r)	<i>ercS'</i> <i>ercS</i>	78 $\pm$ 11	250 $\pm$ 11	I
MD1 (PA3604::Km ^r)	<i>erdR</i>	65 $\pm$ 2	—	I
NG3 (PA1978::Km ^r)	<i>erbR</i> (<i>agmR</i>)	—	260 $\pm$ 20	I

*pQF50 reporter constructs.

Fig. 3. Hypothetical regulatory scheme of a complex regulatory network controlling transcription of operons *exa* and *pqq* of the quinoprotein ethanol oxidation system in *P. aeruginosa* ATCC 17933. Solid arrows indicate demonstrated positive control of transcription, broken arrows indicate assumed positive control of transcription. The identified genes encoding sensor kinases S and response regulators R are shown in larger type than the corresponding two-component system genes not yet identified. //, Response regulator–sensor kinase pairing.

pTB7084 was generated by amplifying the sequence encompassing the putative promoter region of PA1991, the whole of gene PA1991, and part of gene *ercS*. This reporter records transcription of gene *ercS* as a function of the promoter in front of gene PA1991. The three constructs were transferred into *P. aeruginosa* wild-type and mutant MD1. After growth or induction on ethanol the activities of the reporter genes were determined (Table 5). The highest activity in the wild-type was found with pTB7082, where the putative PA1991 promoter is used and the reporter gene resides in PA1991. With pTB7083, the lowest activity was found, indicating that in front of *ercS* there is no strong promoter or no promoter at all. This conclusion is supported by the higher activity found with pTB7084, about 23 % of the activity found with pTB7082. The two genes PA1991 and *ercS* (PA1992) form a transcriptional unit with a promoter in front of PA1991. The activity of *ercS*, however, seems to be downregulated with respect to that of PA1991.

To test whether transcription of the operon PA1991/*ercS* (PA1992) is impaired in mutant MD1, the activities of pTB7082 and pTB7084 were determined in the background of this mutant. As shown in Table 5, the activities of both reporter constructs were reduced significantly in mutant MD1. The promoter activity of gene PA1991 was only about 20 % of that found in the wild-type, while the transcriptional activity of the sensor kinase encoded by *ercS* (PA1992) was even lower at about 12 % of that found in the wild-type. Therefore, ErdR is required for expression of ErcS.

Transcription of genes *erbR* (*agmR*), *ercS* and *erdR* on different substrates

Transcription of gene *ercS*, encoding an FlhS-like sensor kinase, and genes *erbR* (*agmR*) and *erdR*, encoding FlhR-like response regulators, was studied in wild-type *P. aeruginosa* grown on different substrates. The promoter activities of gene *erbR* (*agmR*) varied with the carbon source supplied for growth. With ethanol, the highest promoter activity of 1260 Miller units (MU) ($\pm 7\%$) was

found, while on glucose and succinate, only 5–10 % of this value was detected. On butanol, 40 %, and on glycerol, 60 % of the activity on ethanol was found. Therefore, transcription of gene *erbR* (*agmR*) is induced on ethanol, which then leads to induction of the *exa* and *pqq* operons.

The promoter activities of the *ercS* gene studied with pTB7084 also varied with the carbon source. With ethanol, the highest activity of 250 MU was found, but on glucose, only 14 %, and on succinate, only 22 % of this value was detected. Thus, transcription of gene *ercS* was also induced on ethanol.

Transcription of gene *erdR* did not change when *P. aeruginosa* was grown on different carbon sources. On ethanol, butanol, glycerol, glucose and succinate, promoter activities of 360 ± 30 MU were found. Apparently, gene *erdR* is not induced by ethanol but is transcribed constitutively. The fact that transcription of *erdR* is not reduced in any of the regulatory mutants described so far, together with the observation that it is not induced by ethanol, suggests again that it forms the highest level of a hierarchical organization of two-component regulatory systems (Fig. 3).

Constitutive expression of the FlhS-like sensor kinase ErcS does not restore transcription of operons *exa* and *pqq* in mutant MD1

The FlhR-like response regulator ErdR is required for transcription of the FlhS-like sensor kinase encoded by *ercS* (Table 5). As can be seen in Fig. 2, mutant NG2, with a defect in sensor kinase ErcS' but an intact sensor kinase ErcS, showed appreciable expression of the operons *exa* and *pqq*. Expression vector pTB7101 carries gene *ercS* under the control of the *lac* promoter, which is recognized in *P. aeruginosa* and leads to constitutive expression of ErcS. Vector pTB7101 was transferred to mutant MD1 and expression of the *exa* and *pqq* operons was determined using promoter-probe vectors pTB3138, pTB3139 and pTB7023. Constitutive expression of gene *ercS*, however, did not restore expression of the *exa* and *pqq* operons (data

Table 5. Transcription of *ercS* in *P. aeruginosa* wild-type and mutant MD1 after growth or induction on ethanol

I, after induction; G, after growth. Results represent mean $\pm$ SD of at least three independent experiments.

<i>P. aeruginosa</i> ATCC 17933 strain	Promoter-probe vector*	Promoter activity (MU)	Condition
Wild-type	pTB7082	1082 $\pm$ 190	G or I
Wild-type	pTB7083	78 $\pm$ 26	G
Wild-type	pTB7084	251 $\pm$ 25	G
MD1 <i>erdR</i> (PA3604::Km ^r)	pTB7082	240 $\pm$ 35	I
MD1 <i>erdR</i> (PA3604::Km ^r)	pTB7084	32 $\pm$ 3.5	I

*pQF50 reporter constructs with the following putative promoter regions: pTB7082, *P*_{PA1991'}-*lacZ*; pTB7083, *P*_{PA1992'}-*lacZ*; pTB7084, *P*_{PA1991/1992'}-*lacZ*.

not shown). This result suggests that ErdR controls transcription of sensor kinase ErcS and a so far unknown corresponding response regulator ErcR. Constitutive expression of ErcS is not sufficient to start the signal cascade, because in mutant MD1 the corresponding response regulator ErcR will still be missing.

However, reduction of *ercS* transcription in mutant MD1 could also be explained by assuming that ErcS and ErdR constitute a single two-component regulatory system that is controlled by autoregulation. Inactivation of the response regulator gene *erdR* would cause lower transcription of the system. However, a similarly reduced transcription of *erdR* was not observed in mutants SH1 and SH2 (Table 4), which may indicate that autoregulation operates only on gene *ercS*. Mutant SH2, in which sensor kinase genes *ercS* and *ercS'* are inactivated, does not grow on ethanol but grows on acetate, while mutant MD1, with response regulator gene *erdR* inactivated, does not grow on ethanol and also not on acetate (Table 3). The different properties of the two mutants may indicate that ErcS' and ErcS are not cognate to ErdR. However, mutants showing different phenotypes may also be found upon inactivation of a sensor kinase or its cognate response regulator of a single two-component system. If the response regulator under consideration shows activation of activity in both its phosphorylated and its unphosphorylated state, the response regulator mutant will show a larger number of defects compared with the mutant with the inactivated cognate sensor kinase. Such a situation is observed with response regulator mutant MD1, which is unable to grow on ethanol or acetate, while sensor kinase mutant SH2 still grows on acetate. The observed behaviour might suggest that the response regulator ErdR supports growth on ethanol in its phosphorylated state but supports growth on acetate in its unphosphorylated form. Since upon growth on ethanol, acetate utilization is also needed, this seems to be an improbable scenario, indicating that sensor kinase ErcS and response regulator ErdR may not be cognate members of a single two-component regulatory system. The *erd* system then will be the higher-level one that controls transcription of the *erc* system (Fig. 3).

Constitutive expression of ErbR (AgmR) restores expression of the ethanol oxidation system in mutant SH2 but not in mutant MD1

Transcription of gene *erbR* (*agmR*) depends on the FlhS-like sensor kinases ErcS' and ErcS (Table 4). In mutant SH2, with both sensor kinases inactivated, gene *erbR* (*agmR*) was not transcribed and the activity of the *exaA*, *exaBC* and *pqqAB* promoters was less than 2 % of that found in the wild-type (see legend to Fig. 2). Expression vector pTB7060 carries gene *erbR* (*agmR*) under the control of the *lac* promoter, which causes constitutive expression of ErbR (AgmR) in *P. aeruginosa*. Mutant SH2 was transformed by vector pTB7060, and constitutive expression of the response regulator ErbR (AgmR) partially restored the promoter activity of the *exa* and *pqq* operons. Compared with the wild-type, the activity found for *exaA* was 41 %, that for *exaBC* 22 %, and that for *pqqAB* 69 %. The results confirm that transcription of the ethanol oxidation system is controlled by ErbR (AgmR).

The data suggest either that an active sensor kinase cognate to ErbR is expressed in mutant SH2 or that unphosphorylated ErbR (AgmR) has low transcriptional activity. Vector pTB7060 leads to constitutive expression of ErbR (AgmR), and even though the level of expression is not known, the concentration of ErbR (AgmR) may be sufficient to allow transcription of the *exa* and *pqq* operons.

However, when vector pTB7060 was transferred to mutant MD1, no transcription of the *exa* and *pqq* operons was observed (data not shown). This result is not in agreement with the assumption of unphosphorylated ErbR (AgmR) having low transcriptional activity, since the expression level of ErbR with pTB7060 is expected to be the same in both mutants, MD1 and SH2.

From the experimental results with mutant SH2, in which by constitutive expression of ErbR (AgmR), transcription of *exaA*, *exaBC* and *pqqAB* was found, it must be concluded that a sensor kinase corresponding to ErbR (AgmR) is present in the mutant. This sensor kinase activating ErbR (AgmR) cannot be ErcS' or ErcS, because both are inactivated in mutant SH2. Therefore, sensor kinases ErcS' or ErcS and response regulator ErbR do not

form a cognate pair, but rather are members of two different regulatory systems. The *ercSR* system is the higher-level one, which controls transcription of response regulator ErbR (AgmR) (Fig. 3). Transcription of the so far unknown sensor kinase ErbS cognate to ErbR (AgmR), however, seems not to be controlled by the *ercSR* system, because it is present in mutant SH2.

The gene encoding response regulator ErbR (AgmR) is located on the genome next to the two-component system EraSR (ExaDE); however, with the opposite orientation (Fig. 1). It could be argued that sensor kinase EraS (ExaD) might be responsible for phosphorylating ErbR (AgmR) also. As described in Schobert & Görisch (2001), an inactive sensor kinase EraS (ExaD) in mutant MS13 or an inactive response regulator EraR (ExaE) in mutant MS12 both prevent transcription of gene *exaA* encoding QEDH, but not transcription of gene *exaB* encoding cytochrome *c*₅₅₀. Inactivation (Gliese *et al.*, 2004) of response regulator ErbR (AgmR) in mutant NG3 prevents transcription of three different operons: *eraSR* (*exaDE*), *exaBC* and *pqq*. In the case of EraS (ExaD) being able to phosphorylate the response regulator ErbR (AgmR) in addition to EraR (ExaE), the differences observed with mutant MS13, defective in EraS (ExaD), and mutant NG3, defective in ErbR (AgmR), must be caused by the response regulator ErbR, whether phosphorylated or unphosphorylated, supporting the transcription of different genes. Such a scenario with respect to ErbR does not seem reasonable, because all functions impaired in mutant NG3 are needed for ethanol metabolism and are expected to be controlled by the phosphorylated form of ErbR. The differences observed with mutants MS13 and NG3 therefore indicate that EraS and ErbR are probably not cognate members of a two-component regulatory system.

In mutant MD1, the response regulator ErdR is inactivated, and constitutive expression of ErbR (AgmR) by vector pTB7060 does not lead to transcription of the *exa* and *pqq* operons. It appears that ErdR is required for expression of the so far unknown sensor kinase ErbS cognate to ErbR (AgmR) (Fig. 3).

Hypothetical complex network with five two-component regulatory systems organized hierarchically to control aerobic ethanol oxidation in *P. aeruginosa*

Previously, based on studies with a limited number of regulatory mutants, it was concluded that at least seven genes are involved in regulating ethanol metabolism in *P. aeruginosa* (Görisch, 2003). The response regulator ErbR (AgmR) controls the transcription of operons *exaBC*, *pqqABCDE* and *eraSR* (*exaDE*), the last two-component system controlling the transcription of operon *exaA* (Gliese *et al.*, 2004). The observation that transcription of the operons *exaA*, *eraSR* (*exaDE*), *exaBC* and *pqqABCDE*, and the response regulator gene *erbR* (*agmR*), is reduced or completely prevented in mutants NG2, SH1, SH2 and MD1

indicates that the genes inactivated in these mutants are required for transcription of the operons *exa*, *pqq* and *era* (Fig. 2, Table 4) by directing transcription of gene *erbR* (*agmR*) (Fig. 3). In the present study we identified two further sensor kinases and an additional response regulator involved in controlling transcription of the ethanol oxidation system. Of the three known sensor kinases, EraS (*exaD*), ErcS and ErcS', and the three known response regulators, EraR (ExaE), ErbR (AgmR) and ErdR, only EraSR are known to form a cognate pair (Schobert & Görisch, 2001).

The present report demonstrates that several two-component regulatory systems are involved in regulating ethanol metabolism in *P. aeruginosa*. We provide sufficient evidence for a hierarchical organization of a number of sensor kinases and response regulators. An attempt to pair the known sensor kinases and the known response regulators to cognate members of two-component regulatory systems showed that a number of assumptions have to be made to achieve a satisfactory explanation for all the experimental data. Those assumptions include that the systems are autoregulated and that autoregulation is exerted only on the sensor kinase, while the response regulators involved must show different activating activities whether in the phosphorylated or unphosphorylated state. Improbable regulatory properties of the response regulators involved have to be assumed.

Such difficulties do not arise when making the simple assumption that there exist two additional sensor kinases cognate to ErdR and ErbR (AgmR) and two additional response regulators cognate to ErcS' and ErcS, as shown in Fig. 3. The systems are hierarchically organized, with four levels of sensor kinases and response regulators, and at least 10 genes organized in five different two-component systems are involved in regulating aerobic ethanol metabolism in *P. aeruginosa* (Fig. 3). This hypothetical regulatory scheme is the simplest one that explains all the experimental results without making further complicating assumptions. The model is, however, complex in the sense that five different two-component regulatory systems are involved. The hypothetical model depicted in Fig. 3 could be disproved by demonstrating direct phosphorylation of response regulators ErdR and ErbR (AgmR) by one of the known sensor kinases, or could be verified by identification of the so far unknown sensor kinase genes *erdS* and *erbS* and the unknown response regulator genes *ercR'* and *ercR*.

According to our hypothetical model, ErdR is a global response regulator which directs transcription of a regulon comprising the operons PA1991/*ercS*, *ercS'*, the unknown *erbS*, and the genes encoding the unknown response regulators ErcR and ErcR' (Fig. 3). This global response regulator ErdR appears to be constitutively expressed, as was reported for response regulator FlhR, controlling metabolism of methanol and formaldehyde in *Paracoccus denitrificans*. The transcription of the response regulator gene *erbR* is controlled by the *erc* system.

Gene *eraR* (*exaE*) encodes a response regulator of 225 aa, *erbR* (*agmR*) encodes one of 221 aa, and the ErdR protein is a 217 aa polypeptide. All three response regulators are members of the LuxR family and contain a CheY-type receiver domain and a helix–turn–helix DNA-binding motif.

Gene *eraS* (*exaD*) encodes a sensor kinase of 272 aa, which shows within the transmitter domain in the C-terminal portion a 33% similarity to histidine-type sensor kinases (Schobert & Görisch, 2001). The EraS (ExaD) protein contains an internal methionine residue. In annotating the *P. aeruginosa* PAO1 sequence, the internal ATG codon was regarded as the actual start codon of a shorter putative sensor kinase, resulting in a predicted protein of 216 aa (*Pseudomonas* Genome Database; Winsor *et al.*, 2009). However, in both cases, within the N-terminal domain of the putative sensor kinases neither a signal peptide nor transmembrane helices can be found. It appears that EraS (ExaD) is a soluble protein localized in the cytoplasm. Gene *ercS* encodes a hybrid sensor kinase of 564 aa and gene *ercS'* one of 881 aa. Both ErcS and ErcS' show in their C-terminal portions 56% similarity to the sensor kinase FlhS of *Paracoccus denitrificans*. The FlhSR system in *Paracoccus* directs methanol and formaldehyde oxidation (Harms *et al.*, 2001). Both sensor kinases, ErcS and ErcS', again do not contain in their N-terminal domain a signal peptide or transmembrane helices. They are soluble proteins localized in the cytoplasm.

It is of interest to note that all three sensor kinases identified so far as involved in the regulation of aerobic ethanol metabolism in *P. aeruginosa* appear to be soluble cytoplasmic proteins. This implies that signals 2, 3 and 5 in Fig. 3 must be metabolites in the cytoplasm. This argument is in accord with the observation that several mutants with defects in genes encoding components of intermediary metabolism show impaired regulation of the ethanol oxidation system. Such is the case in mutants with inactivated genes *proB* (PA4565), encoding glutamate kinase, and gene *glcB* (PA0482), encoding malate synthase (unpublished data), and in a mutant with a defect in the *aceA* gene, encoding isocitrate lyase (Kretzschmar *et al.*, 2008). So far, however, the data do not allow any metabolite to be pinpointed which might serve as an internal signal for one of the sensor kinases.

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