A soluble two-component regulatory system controls expression of quinoprotein ethanol dehydrogenase (QEDH) but not expression of cytochrome c₅₅₀ of the ethanol-oxidation system in Pseudomonas aeruginosa

Max Schobert and Helmut Görisch

The regulation of the divergent promoters of the exaAB genes in Pseudomonas aeruginosa ATCC 17933, in which exaA encodes a quinoprotein ethanol dehydrogenase and exaB codes for a cytochrome c₅₅₀, was studied. Using transcriptional lacZ fusions, promoter activity during growth on several substrates was measured. These promoter-probe vectors were also used to identify regulatory mutants defective in exaAB induction. Transcription from both exaA and exaB was reduced significantly in four mutants. Two other mutants showed transcription from exaA that was reduced, but higher than wild-type transcription from exaB. The genes that are needed for exaA promoter induction were sequenced and found to encode a two-component regulatory system: a histidine sensor kinase, which lacks a transmembrane helical N-terminus and is presumably located in the cytoplasm, and a response regulator. The phenotypic characterization and restoration of the wild-type behaviour of the different regulatory mutants produced by different cosmids and subclones indicate that six different genes may be involved in regulating ethanol oxidation in P. aeruginosa.

Keywords: exaAB, regulation, sensor kinase, response regulator, divergent promoter

INTRODUCTION

Pseudomonas aeruginosa grows aerobically on ethanol by using a pyrroloquinoline quinone (PQQ)-dependent quinoprotein ethanol dehydrogenase (QEDH) (Rupp & Görisch, 1988). The QEDH is located in the periplasm and transfers electrons to an endoxidase via a soluble cytochrome c₅₅₀ (Reichmann & Görisch, 1993). In the past, it was speculated that the ethanol-oxidation system in P. aeruginosa might be quite similar to the methanol-oxidation system of Methylobacterium extorquens AM1. At the biochemical level, QEDH and cytochrome c₅₅₀ are very similar to the methanol-oxidation system of Methylobacterium extorquens AM1. At the biochemical level, QEDH and cytochrome c₅₅₀ share some features with their counterparts in methylotrophs, i.e. quinoprotein methanol dehydrogenase (QMDH) and cytochrome c₅₅₀ (Anthony, 1996; Goodwin & Anthony, 1998). However, a second cytochrome c is not present in P. aeruginosa. Recently, we cloned and sequenced several genes of P. aeruginosa involved in ethanol oxidation and demonstrated that this system differs from the methanol-oxidation system in terms of the participating genes and their organization (Diehl et al., 1998; Schobert & Görisch, 1999). The QMDH of M. extorquens AM1 is a heterotetramer consisting of a large subunit and a small subunit (Nunn et al., 1989), whereas the QEDH of P. aeruginosa is a homodimer lacking a small subunit (Diehl et al., 1998; Keitel et al., 2000). The amino acid similarity observed between QEDH and several QMDHs is about 30%, but no significant similarity exists between cytochrome c₅₅₀ and cytochrome c₅₅₀. Nevertheless, cytochrome c₅₅₀ of P. aeruginosa accepts electrons from QEDH (A. Diehl &

**Abbreviations:** Cb, carbenicillin; MM, minimal medium; POQ, pyrroloquinoline quinone; QEDH, quinoprotein ethanol dehydrogenase; QMDH, quinoprotein methanol dehydrogenase; Tc, tetracycline.

The GenBank accession number for the sequence reported in this work is AJ009858.2 (= CAB 95009.1).
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METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this work are listed in Table 1. Escherichia coli and P. aeruginosa were cultivated as described previously (Schobert & Görisch, 1999). The minimal media for P. aeruginosa (Rupp & Görisch, 1988) contained different carbon sources: alcohols were added at 0.5% (v/v), the acetate concentration was 20 mM and the succinate concentration was 40 mM. Antibiotics were added at the following concentrations: 20 µg tetracycline (Tc) ml⁻¹, 50 µg kanamycin ml⁻¹, 100 µg carbenicillin (Cb) ml⁻¹.

General genetic techniques and recombinant DNA work. Triparental matings and complementation experiments were performed as described previously (Schobert & Görisch, 1999). Routine recombinant DNA work was performed according to the protocols described by Sambrook et al. (1989). DNA sequencing of both strands was done by DMBC, Berlin.

Database searches were made using the programs BLAST or gapped BLAST (Altschul et al., 1997). For DNA editing and protein-sequence alignment, the program DNAMAN (Lynnon Biosoft) was used. Protein sequence alignment was done using CLUSTAL W (Thompson et al., 1994). Searches for trans-membrane helices were done using the SOSUI system described by Hirokawa et al. (1998). Prosite profiles were searched using the ProfileScan server at the Swiss Institute for Experimental Cancer Research (http://www.isrec.isb-sib.ch/software/PSFSCAN_form.html).

β-Galactosidase assay. β-Galactosidase activity was determined with cells treated with toluene, according to the procedure of Miller (1992). P. aeruginosa containing promoter-probe vectors was grown on different media containing Tc. Cultures were harvested at an OD₆₃₀ of 0.5 and cooled on ice for 5 min. Cells were washed and resuspended in the same volume of 20 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA (buffer A). Prior to the assay, cells were diluted in Z-buffer (Miller, 1992).

Determination of β-galactosidase activity in P. aeruginosa mutants unable to grow on ethanol was performed after induction on ethanol: the constructs containing pTB3139 or pTB3139 were grown overnight in LB-Tc medium. An aliquot of 0.5 ml was washed and diluted fourfold in minimal medium (MM)-ethanol-Tc medium. After shaking of the cultures for 6 h at 37 °C, the β-galactosidase activity induced was determined. The OD₆₃₀ was measured at the beginning and the end of the induction period, to detect any growth of revertants.

Specific activity of QEDH. The QEDH activity was measured as described by Rupp & Görisch (1988). The protein concentration was determined according to Bradford (1976).

RESULTS

Construction of promoter-probe vectors

To study the activity and regulation of the divergent exaA and exaB promoters with transcriptional lacZ fusions, we used the promoter-probe vector pEDY305. To monitor exaA promoter activity, the 0.89 kb PstI–SalI fragment of pTB3070 was cloned between the PstI–Xbol sites of pEDY305. For cloning of this small fragment into the large plasmid, we followed a simple
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 17933</td>
<td>Wild-type</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>MS1, -4, -5, -6, -7, -8, -17, -20</td>
<td>ATCC 17933 derivative, mutant class I</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>MS9, -11, -12, -13, -16, -18</td>
<td>ATCC 17933 derivative, mutant class II</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>MS2, -10, -14, -19</td>
<td>ATCC 17933 derivative, mutant class III</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>MS3, -15, -21</td>
<td>ATCC 17933 derivative, mutant class IV</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>F′traD36 lacP Δ(lacZ)M15 proA+B′/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac–proAB)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>HB101</td>
<td>supE44 hsdR20(r−m−) recA13 ara-14 proA2lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript</td>
<td>Ap′; cloning and expression vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>Tc′; broad-host-range cosmid</td>
<td>Staskawicz et al. (1987)</td>
</tr>
<tr>
<td>pEDY305</td>
<td>Tc′; lacZ promoter-probe vector</td>
<td>Schwartz et al. (1998)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km′; helper plasmid for triparental mating</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pUC18, pUC19</td>
<td>Ap′; cloning and expression vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUCP20T</td>
<td>Ap′; broad-host-range plasmid</td>
<td>Schweizer et al. (1996)</td>
</tr>
<tr>
<td>pTB3001</td>
<td>Tc′; 25 kb genomic DNA partially digested with Sau3AI from P. aeruginosa cloned in BamHI site of pLAFR3</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>pTB3035, -3040, -3052, -3057</td>
<td>Tc′; 25 kb genomic DNA partially digested with Sau3AI from P. aeruginosa cloned in BamHI site of pLAFR3</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3070</td>
<td>Ap′; 3.2 kb BamHI–PstI fragment from pTB3001 cloned between BamHI–PstI sites of pUC18 (exaBC orientation opposite that of Plac of pUC18)</td>
<td>Schobert &amp; Girsch (1999)</td>
</tr>
<tr>
<td>pTB3112</td>
<td>Ap′; 6.2 kb PstI–PstI fragment from pTB3001 cloned in PstI site of pUCP20T</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3134</td>
<td>Ap′; 1.7 kb HindIII–AgeI fragment from pTB3070 cloned in HindIII–SmaI-digested pBluescript</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3135</td>
<td>Ap′; 0.89 kb HindIII–SalI fragment from pTB3070 cloned in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3138</td>
<td>Tc′; 0.89 kb PstI–SalI fragment from pTB3070 cloned in PstI–Xhol sites of pEDY305</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3139</td>
<td>Tc′; 0.89 kb PstI–XbaI fragment from pTB3135 cloned in PstI–XbaI sites of pEDY305</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3140</td>
<td>Tc′; 1.1 kb XhoI–XbaI fragment from pTB3134 cloned in XhoI–XbaI sites of pEDY305</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3141</td>
<td>Tc′; 1.1 kb PstI–XbaI fragment from pTB3134 cloned in PstI–XbaI sites of pEDY305</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3142</td>
<td>Ap′; 3 kb HindIII–NcoI fragment of pTB3112 removed, ends filled in and vector religated</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3143</td>
<td>Ap′; 2.5 kb SacI fragment of pTB3112 removed, and vector religated</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3144</td>
<td>Ap′; 3 kb MluI–SmaI fragment of pTB3112 removed, ends filled in and vector religated</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3145</td>
<td>Ap′; 6.7 kb BamHI–BamHI fragment from pTB4003 cloned in the BamHI site of pUCP20T</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3146</td>
<td>Ap′; 1.1 kb HindIII–NcoI fragment of pTB3145 removed, ends filled in and vector religated</td>
<td>This study</td>
</tr>
<tr>
<td>pTB4003</td>
<td>Ap′; 6.7 kb BamHI–BamHI fragment from pTB3001 cloned in the BamHI site of pUC18</td>
<td>Diehl et al. (1998)</td>
</tr>
</tbody>
</table>
two-step cloning procedure: pTB3070 was digested with SalI and ligated with the Xhol-digested pEDY305. Positive transformants were easily detected on LB plates containing Cb and Tc. The correct orientation of the exaA promoter and the lacZ gene was confirmed by restriction analysis. The plasmid DNA of a positive clone was digested with PstI to remove the unwanted part of the pTB3070 vector. The resulting pEDY305 vector containing only the 0–89 kb PstI–SalI fragment of pTB3070 was eluted from the agarose gel, religated, and named pTB3138 (Fig. 2).

To construct an exaB promoter–lacZ fusion, the 0–89 kb PstI–SalI fragment of pTB3070 was first cloned into a HindIII–SmaI-digested pBluescript vector. In this vector (pTB3134), the partial exaC gene ends at the former AgeI site, which is followed by a XbaI site. This vector is fused to pEDY305 via the XbaI site. To generate a promoter-probe vector containing a transcriptional lacZ fusion with the exaC gene, including the complete exaB promoter region, the pBluescript vector was removed by digestion with PstI. The resulting promoter-probe vector was religated and named pTB3141 (Fig. 2).

To generate a promoter-probe vector for the exaC gene, without the exaB promoter region, the fusion plasmid between pTB3134 and pEDY305 was digested with Xhol to eliminate the pBluescript vector together with a 1 kb fragment carrying the exaA–exaB promoter region. The resulting promoter-probe vector, pTB3140, contains the partial exaC gene with a 0.4 kb upstream sequence to the Xhol site within the exaB gene, but no exaB promoter (Fig. 2).

Establishing the β-galactosidase assay

The vectors pTB3138, pTB3139, pTB3140, pTB3141 and pEDY305 were transferred into wild-type *P. aeruginosa* ATCC 17933 by triparental mating. To obtain reproducible β-galactosidase transcription levels, it was essential, in the expression experiments, to inoculate with precultures grown on MM-succinate. To
Table 2. Promoter activities of P. aeruginosa constructs on different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pTB3138*</th>
<th>pTB3139*</th>
<th>pTB3140b†</th>
<th>pTB3141*</th>
<th>PEDY305†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>71.5 ± 10.1</td>
<td>50.0 ± 5.4</td>
<td>0.14</td>
<td>50.6 ± 16.4</td>
<td>0.04</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>59.9 ± 1.8</td>
<td>32.1 ± 0.8</td>
<td>0.21</td>
<td>31.6 ± 3.7</td>
<td>0.05</td>
</tr>
<tr>
<td>1,2-Propanediol</td>
<td>52.0 ± 6.7</td>
<td>44.8 ± 9.0</td>
<td>0.12</td>
<td>34.8 ± 3.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Butanol</td>
<td>42.0 ± 4.2</td>
<td>22.7 ± 2.6</td>
<td>0.13</td>
<td>19.9 ± 0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Succinate</td>
<td>34.0 ± 0.02</td>
<td>36.0 ± 0.01</td>
<td>0.81</td>
<td>23.0 ± 0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Acetate</td>
<td>30.0 ± 3.4</td>
<td>23.0 ± 2.4</td>
<td>0.13</td>
<td>19.0 ± 1.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* The activity (Miller kU) and standard deviation were obtained from three independent experiments.
† Each activity value (Miller kU) is the mean of two independent experiments.

determine if the specific β-galactosidase activity during growth on ethanol depends on the growth phase, P. aeruginosa pTB3138 was grown on MM-ethanol-Tc medium and then β-galactosidase activity was measured as a function of growth. The activity (Miller units) was constant between OD_{620} values of 0.2 and 0.7. In all subsequent experiments, β-galactosidase activity was measured with cultures at an OD_{620} of 0.5.

A positive factor is needed for transcription of exaA

To investigate the effect of an additional exaA promoter copy on the specific enzymic activity of the QEDH, P. aeruginosa strains containing the following plasmids were grown on MM-ethanol-Tc until an OD_{620} of 0.5 was reached: pTB3138, containing the 0.89 kb PstI–SalI fragment of pTB3070 with the complete exaA–exaB promoter region; pTB3001, a pLAFR3 derivative containing a 20 kb genomic DNA fragment with the exaA–exaB promoter region and the complete exaA gene; and pLAFR3, the empty vector, which served as a control. All three vectors are derivatives of pRK290, Tc resistant and larger than 20 kb. As an additional control, the P. aeruginosa wild-type was grown on MM-ethanol medium. The specific activity of QEDH was the same in the P. aeruginosa wild-type without any plasmid as it was in P. aeruginosa containing pLAFR3 or pTB3001. Thus, neither the presence of Tc nor a second copy of the exaA gene and its promoter influences the specific activity of QEDH. In P. aeruginosa containing pTB3138, with the exaA promoter followed by a truncated exaA gene, the specific QEDH activity dropped to 50% of that of the wild-type. Since the native form of QEDH is a homodimer, the polypeptide product produced by the truncated exaA gene might interfere with subunit association, thereby reducing the enzyme activity. This, however, is unlikely, because it is known from the QEDH X-ray structure that none of the 18 N-terminal amino acids of the truncated exaA gene product is involved in subunit association (Keitel et al., 2000). The results suggest that a positive factor, needed for starting transcription at the exaA promoter, is limiting.

Transcription levels of exaA, exaB and exaC

β-Galactosidase activities were determined with P. aeruginosa strains containing the various lacZ fusion vectors, after growth on different carbon sources (Table 2). The promoter activities of exaA and exaB were monitored with wild-type P. aeruginosa containing pTB3138 or pTB3139. Induction of both promoters was observed with the four alcohols tested. The β-galactosidase activities during growth on MM-succinate-Tc and MM-acetate-Tc were low. The highest induction of both promoters was observed after growth on ethanol. Plasmids pTB3140 and pTB3141 were used to study transcription of the exaC gene in P. aeruginosa. Whilst the induction pattern with P. aeruginosa/pTB3141 obtained on the different substrates was similar compared to the results with P. aeruginosa/pTB3139, very low β-galactosidase activity was measured with P. aeruginosa/pTB3140 (Table 2). The latter plasmid lacks the exaB promoter and part of the exaB gene, so the result demonstrates that an additional strong promoter is not present in front of exaC. With P. aeruginosa carrying the empty vector PEDY305, constant, low β-galactosidase activities were obtained, indicating that plasmid copy numbers did not change significantly on the different substrates used (Table 2).

Identification of regulatory mutants in P. aeruginosa

Recently, we isolated and characterized 21 mutants of P. aeruginosa unable to grow on ethanol. These mutants were grouped into four classes by biochemical characterization (Schobert & Görisch, 1999). Plasmids pTB3138 (exaA::lacZ fusion) and pTB3139 (exaB::lacZ fusion) were used to identify regulatory mutants with a diminished transcription rate for exaA and/or exaB by transferring the plasmids separately into all mutants by triparental mating. Since the mutants are unable to grow on ethanol, β-galactosidase activity was determined after induction on MM-ethanol-Tc medium. Six mutants (MS9, MS11, MS12, MS13, MS16 and MS21) showed significantly reduced transcription rates for the exaA promoter, which amounted to about 10% of the wild-type level (Fig. 3). These mutants were
that of the wild-type.

† Mutants using succinate as the carbon source was unable to produce PQQ. The generation time of the regulatory mutants was determined, by sequence similarity, to be located at position 194 and a conserved region near the C-terminus of the protein.

Phenotypic characterization of the identified regulatory mutants

All of the P. aeruginosa mutants unable to grow on ethanol were previously complemented with a cosmids library (Schobert & Görisch, 1999). Table 3 lists the complementing cosmids for the six identified regulatory mutants, together with their regulatory defects and their ability to produce PQQ. The generation time of the regulatory mutants using succinate as the carbon source was determined along with their ability to use acetate or glucose for growth. None of the six regulatory mutants produce active QEDH or the apo-form of the enzyme.

Subcloning the genes restoring growth of MS12 and MS13

Cosmid pTB3001 restored growth on ethanol for mutants MS12 and MS13. Constructs of both mutants containing subclone pTB3112, carrying a 6·2 kb PstI–PstI fragment of pTB3001, also showed wild-type behaviour. Using preliminary sequence data obtained from the P. aeruginosa genome project we constructed three further subclones: pTB3142, pTB3143 and pTB3144 (Fig. 4). Mutants MS12 and MS13 both showed growth on ethanol after transformation by plasmids pTB3143 and pTB3144. Restriction analysis of cosmids pTB3001 showed that pTB3144 has an overlapping region with pTB4003, a plasmid cloned and partially sequenced previously (Diehl et al., 1998). The overlapping parts of both plasmids contain the complete gene encoding a response regulator. The insert of pTB4003 was cloned in the broad-host-range vector pUCP20T. This plasmid pTB3145, restored wild-type behaviour only in mutant MS12. Another subclone of this plasmid, pTB3146, contained the insert of pTB3145 without the response regulator (Fig. 4) and was unable to restore growth in MS12.

Regulatory two-component system

Sequence analysis of the 2029 bp insert DNA from pTB3144 showed the presence of two putative ORFs. The first ORF starts at position 354 and ends at position 1182. It encodes a protein of 272 aa; this gene was named exaD. A BLAST search revealed similarities of between 31 and 33% to the C-terminal domain of several sensor kinases of the histidine type (Fig. 5). Within this transmitter domain, several conserved amino acids are present (Stock et al., 1989). The putative histidine residue involved in phosphorylation was determined, by sequence similarity, to be located at position 87. A conserved asparagine residue is located at position 194 and a conserved region near the C-terminus

Table 3. Properties of regulatory mutants of the ethanol-oxidation system in P. aeruginosa

<table>
<thead>
<tr>
<th>Mutant class</th>
<th>Mutant</th>
<th>Altered regulation</th>
<th>Complementing cosmid</th>
<th>PQQ biosynthesis*</th>
<th>Growth on:†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Succinate</td>
</tr>
<tr>
<td>II</td>
<td>MS9</td>
<td>exaA/exaB</td>
<td>pTB3035</td>
<td>+</td>
<td>&gt;WT</td>
</tr>
<tr>
<td></td>
<td>MS11</td>
<td>exaA/exaA</td>
<td>pTB3040</td>
<td>+</td>
<td>&gt;WT</td>
</tr>
<tr>
<td></td>
<td>MS16</td>
<td>exaA/exaA</td>
<td>pTB3052</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>MS12</td>
<td>exaA</td>
<td>pTB3001</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>MS13</td>
<td>exaA</td>
<td>pTB3001</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td>IV</td>
<td>MS21</td>
<td>exaA/exaB</td>
<td>pTB3052</td>
<td>–</td>
<td>WT</td>
</tr>
</tbody>
</table>

* Data from Schobert & Görisch (1999).
† NG, no growth; WT, growth like that of the wild-type; >WT, mutants show generation times more than 50% longer than that of the wild-type.
Regulation of the *P. aeruginosa* exaAB promoters

**Fig. 4.** Position of pTB3001 subclones complementing mutants M512 and M513. Part of the pTB3001 insert DNA and several identified genes are also shown. Numbers above the subclones indicate complementation of mutant 12 or mutant 13. B, BamHI; M, MluI; N, NotI; P, PstI; S, SacI.

**Fig. 5.** Alignment of the deduced amino acid sequence of ExaD with the C-terminal domain of several sensor kinases of the histidine type. Identical residues are shown against a black background. Pa_ExaD, the ExaD sequence of *P. aeruginosa* ATCC 17933; Ec_UhpB, the UhpB sequence of *E. coli* (Friedrich & Kadner, 1987); St_UhpB, the UhpB sequence of *S. typhimurium* (Island et al., 1992); Ea_HrpX, the HrpX sequence of *Erwinia amylovora* (accession no. AAD24682). The putative histidine residue involved in phosphorylation is marked by an asterisk; other conserved residues presumably involved in ATP binding are indicated by crosses.

contains a DXGXG motif followed by GXGL. These sequences are known to contribute to ATP binding (Bilwes et al., 1999). In *exaD*, this motif starts at position 221 and reads DDGRG; it is followed by GFG, starting at position 233 (Fig. 5).

Two putative sensor kinases (accession nos CAB76008 and CAA22397) of *Streptomyces coelicolor* show 38% similarity to ExaD, but no further information about these kinases and their function is available. The *uhpB* gene products of *E. coli* (Friedrich & Kadner, 1987) and *Salmonella typhimurium* (Island et al., 1992) showed 33% identity over 199 aa, and the *hrpX* gene product of *Erwinia amylovora* (accession no. AAD24682) showed 31% identity over 202 aa. There is also an identity of 28%, over 225 aa, to the MxcQ protein of *M. organo-
and ends at position 1872. The accession number AJ009858.2. It starts at position 1195.

The ExaE protein shows identities of 40–41% to the ExaD protein of P. aeruginosa ATCC 17933; St. UhpA, the UhpA sequence of S. typhimurium (Island et al., 1992); Ec. UhpA, the UhpA sequence of E. coli (Friedrich & Kadner, 1987); Ea. HrpY, the HrpY sequence of Erwinia amylovora (accession no. AAD24683). The putative receiving aspartate residue is located at position 53. Other highly conserved residues in the histidine-receiving aspartate residue are located at position 53. Other highly conserved residues in the histidine-receiving module of response regulators described by Henikoff et al. (1990) are also found, i.e. aspartate 7 and lysine 103.

The ExaE protein shows identities of 40–41% to the uhpA gene products of S. typhimurium (Island et al., 1992) and E. coli (Friedrich & Kadner, 1987), and 34% identity to HrpY of Erwinia amylovora (accession no. AAD24683). With the introduction of four gaps, the ExaE sequence shows identities of about 36% to the mxeE gene product of M. organophilum (Xu et al., 1995) and to MxaB of M. extorquens (Springer et al., 1998). The exaD and exaE genes are separated only by 15 bp and form a typical bacterial two-component system consisting of a sensor kinase and a response regulator. Fig. 4 shows the localization of the exaDE genes and their relative orientation with respect to the exaABC genes. Comparison of the exaDE genes of P. aeruginosa ATCC 17933 with the corresponding genes of the P. aeruginosa PAO1 genome project revealed only negligible differences, i.e. two different amino acids at non-conserved positions in ExaE (and no differences in the ExaD amino acid sequence). Using the P. aeruginosa PAO1 genome project, we looked for sequences near exaD. Upstream and in the opposite orientation is another two-component system (gphRS) involved in glycerol metabolism (Schweizer, 1991). Beyond these genes, there are two ORFs showing homology to flhS and ORF2 of Paracoccus denitrificans. The flhS gene product seems to be a protein histidine kinase involved in global control of C1 metabolism in P. denitrificans (van Spanning et al., 2000).

DISCUSSION

In wild-type P. aeruginosa, the divergent promoters of genes exaA and exaB are both induced by various alcohols, with ethanol causing the highest transcription rate. The transcription rates of the exaB and the downstream-located exaC gene were found to be equal. This demonstrates that the two adjacent exaBC genes are cotranscribed from the exaB promoter. An additional, albeit weak, promoter might be present in front of exaC, but the standard deviations associated with the
reporter-gene measurements do not allow a definite conclusion.

The physiological characterization of six mutants and the results of transformation experiments restoring growth by different cosmids and various subclones derived from cosmid pTB3001 allowed us to estimate the number of regulatory genes involved. Mutants MS16 and MS21 acquired wild-type behaviour after transformation by the same two cosmids and were unable to grow on acetate, but they differ in their ability to produce PQQ. Mutants MS9 and MS11 show identical phenotypes. They produce PQQ, grow poorly on succinate and not on acetate or glucose, but different cosmids restore growth on ethanol. In all four mutants (MS9, MS11, MS16 and MS21), no activity of exaA and exaB promoters was found (Table 3). The properties of these four mutants indicate that four different regulatory genes might be involved. However, in mutants MS12 and MS13, only induction of the exaA promoter was impaired, indicating that the two divergent promoters are regulated differently. The two mutants show identical phenotypes and both are converted to wild-type behaviour by cosmid pTB3001 and by different subclones thereof. Subclone pTB3144 restores growth on ethanol with both mutants, whereas pTB3145 restores growth on ethanol only for mutant MS12.

Plasmid pTB3145 carries a gene encoding a previously sequenced response regulator (Diehl et al., 1998). At that time, it was unknown if this gene was involved in the regulation of the ethanol-oxidation system. Sequencing of the part of pTB3144 that also restores the growth of MS13 led to the identification of a gene encoding a histidine sensor kinase. This gene ends only 15 bp upstream of the response-regulator gene and shares the same orientation. Both genes form a typical bacterial two-component system and were named exaDE. The sensor kinase shares 30–35% identity with other bacterial sensor kinases. Although the typical histidine kinase motif is present, no transmembrane helices were found, indicating that ExaD is a soluble, cytoplasmic sensor kinase.

The cytoplasmic localization of the soluble ExaD sensor kinase raises the question of the nature of the signal molecule activating this enzyme. So far, there is no indication that ExaD might be activated in a manner similar to CheA, a soluble kinase of E. coli involved in chemotaxis, the autophosphorylation of which is controlled by methyl-accepting chemotaxis proteins. These methyl-accepting chemotaxis proteins are membrane-bound chemoreceptors that detect chemical stimuli in the periplasm.

In contrast, in methylotrophic bacteria, both sensor kinases essential for growth on methanol (MxbD, MxcQ) are membrane bound, with a periplasmic loop that is assumed to be the receptor domain. In M. extorquens, a hierarchy of the different regulatory genes is presumed (Springer et al., 1997): the two-component system mxcQE controls the expression of mxbDM, which regulates expression of the mxaF promoter and the pqq biosynthesis genes. There is also a single response-regulator gene, mxaB, without an adjacent sensor kinase; mxaB is essential for mxaF induction and is also involved in induction of the pqq biosynthesis genes (Morris & Lidstrom, 1992; Ramamoorthy & Lidstrom, 1995).

Our data clearly show that the response regulator ExaE is necessary to induce transcription from the exaA promoter. The divergent promoter region between exaA and exaB does not contain sequences that can be unambiguously identified as known σ70, σ44 or other consensus sequences known for Pseudomonas (Ronald et al., 1992). However, we found a 23 bp sequence motif with dyad symmetry (CGTCCGGAA-N17-TTCCCGGACG) between exaA and exaB. It is located 113 bp upstream of exaA and 177 bp upstream of exaB and might constitute a binding motif for the response regulator ExaE.

Concluding remarks

We have investigated the regulatory network controlling the ethanol-oxidation system in P. aeruginosa. Our results with the six regulatory mutants studied show that six different genes might be involved. In one group of regulatory mutants, the induction of both exaA and exaB is impaired, whereas in another group only induction of exaA is impaired. The observed regulatory pattern presumably allows P. aeruginosa to use cytochrome c550 also as an electron acceptor with enzymes for other carbon sources besides ethanol. Experiments designed to identify the signal molecule of the exaD sensor kinase and to verify binding of the exaE response regulator in the exaA promoter region are under way. In addition, cloning and characterization of the other regulatory genes involved will be performed to establish whether or not there is a hierarchical organization of the different regulatory factors.

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REFERENCES


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