Cytochrome c_{550} is an essential component of the quinoprotein ethanol oxidation system in Pseudomonas aeruginosa: cloning and sequencing of the genes encoding cytochrome c_{550} and an adjacent acetaldehyde dehydrogenase

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INTRODUCTION

When growing on alcohols, a number of Gram-negative bacteria induce enzymes with pyrroloquinoline quinone (PQQ) as prosthetic group. In contrast to NAD\(^+\)-dependent alcohol dehydrogenases, which occur in the cytoplasm, these PQQ-dependent quinoproteins are located in the periplasm. PQQ-dependent alcohol dehydrogenases can be divided into two groups; those enzymes which transfer electrons to a separate soluble c-type cytochrome and, in contrast, the quinohaemoproteins containing a c-type cytochrome haem domain at the C-terminus of the alcohol dehydrogenase polypeptide chain (Anthony, 1992).

Pseudomonas aeruginosa grown aerobically on ethanol produces an ethanol oxidation system which consists of a periplasmic, soluble quinoprotein alcohol dehydrogenase (QEDH) linked to an electron transport chain (Rupp & G"{O}risch, 1988). Two components of this...
electron transport chain, a periplasmic, soluble cytochrome c550 and the co-type cytochrome oxidase, have been identified (Reichmann & Górisch, 1993; Matsushita et al., 1982). Cytochrome c550 mediates electron transfer between the QEDH and the cytochrome oxidase via an unknown membrane component (Reichmann & Górisch, 1993).

A similar alcohol oxidation system is found in methylotrophic bacteria during growth on methanol and more than 25 genes have been identified in Methylobacterium extorquens AM1, Paracoccus denitrificans and Methylobacterium organophilum (Lidstrom et al., 1994). The soluble quinoprotein methanol dehydrogenase shows similar catalytic and molecular properties as the QEDH (Górisch & Rupp, 1989). Since an NAD+-dependent methanol dehydrogenase is also known (Arfman et al., 1989), it is suggested to use the abbreviation QMDH for the NAD+-dependent alcohol dehydrogenase and MDH for the NAD+-dependent methanol dehydrogenase.

The QMDH is connected to an electron transport chain that, in contrast to P. aeruginosa, consists of the two soluble cytochromes cL and cH, and a cytochrome oxidase (Goodwin & Anthony, 1995). In M. extorquens, cytochrome cL is the direct electron acceptor of QMDH; it shows little similarity to other known cytochromes (Nunn & Anthony, 1988). The gene encoding cytochrome cL (mxaG) is located in an operon mxaFJGl (Harms et al., 1987; Anderson et al., 1990; van Spanning et al., 1991) together with the structural genes of QMDH (mxaF, mxaA). The genetic organization of the mxaFJGl operon seems to be conserved among methylotrophic bacteria and it would be interesting to see if the gene encoding cytochrome c550 in P. aeruginosa is located in a similar operon and if its amino acid sequence reveals similarity to cytochrome cL.

With the exception of methylotrophic bacteria, little is known about the gene organization of quinoprotein alcohol oxidation systems in Gram-negative bacteria. This paper describes the organization of a cluster with five genes encoding components of the quinoprotein ethanol oxidation system in P. aeruginosa and the properties of a mutant with an interrupted cytochrome c550 gene.

METHODS

Bacterial strains and culture conditions. Strains and plasmids are listed in Table 1. Escherichia coli was grown in LB medium in the presence of antibiotics as described by Sambrook et al. (1989). P. aeruginosa ATCC 17933 was grown in LB or in minimal medium with ethanol or sucrose as carbon and energy source as described previously (Rupp & Górisch, 1988). Antibiotics were added at the following concentrations: 20 μg tetracycline ml⁻¹, 50 μg kanamycin ml⁻¹, 100 μg carbencillin ml⁻¹. All growth tests were repeated three times.

Recombinant DNA work and construction of a gene library. Standard DNA techniques were performed as described by Sambrook et al. (1989). Total DNA from P. aeruginosa was isolated according to Wilson (1994) and a gene library in E. coli S17-1λpir was constructed using the cosmids pLAIR3 and the strategy described by Staskawicz et al. (1987). The 17-mer 5′-digoxigenin-labelled mixed oligonucleotides (5′ AAG GAR TGG CGS GAY AC 3′) were synthesized by TIB MOLBIOL (Berlin). Southern blotting and hybridization experiments were done with the DIG DNA labelling and detection kit (Boehringer Mannheim). For DNA sequencing, a 3.2 kb hybridizing fragment was cloned in pUC18, resulting in plasmid pTB3070. A set of nested deletions were generated using exonuclease III as described by Sambrook et al. (1989). The Sequenase kit and 32P-labelled ATP (Amersham) were used for sequencing both strands.

Database searches were made using the program BLAST or gapped BLAST (Altschul et al., 1997). Pattern searches with protein sequences were performed with the PROSITE database (Bairoch et al., 1997). For protein sequence alignment the program CLUSTAL w was used (Thompson et al., 1994).

Isolation of mutants. Mutagenesis of P. aeruginosa with N-methyl-N'-nitro-N-nitrosoguanidine (MNGG) was performed as described by Miller (1992). Mutagenized cells were spread on plates with succinate minimal medium to restrict growth of auxotrophs. Colonies were transferred by replica printing onto ethanol minimal medium and again on succinate minimal medium. Mutants unable to grow at all or growing slowly on ethanol and with a reversion rate below 10⁻⁶ were characterized biochemically.

Biochemical characterization of mutants. Mutants were grown overnight in LB medium, washed twice and diluted fourfold in ethanol minimal medium. After shaking for 6 h at 37 °C to induce synthesis of the ethanol oxidation system, cells were harvested, washed twice with ice-cold 50 mM Tris buffer (pH 7.9) containing 10 mM CaCl₂, and immediately used for enzyme assays. QEDH activity was determined as described previously (Rupp & Górisch, 1988) using whole-cell suspensions and adding 12.5 mM KCN to the assay buffer. All mutants defective in QEDH activity showed less than 10% activity compared to wild-type cells. The presence of apo-QEDH was detected by measuring QEDH activity after incubating cells with 3 μg PQQ ml⁻¹ for 30 min at 25 °C (Murzel & Górisch, 1991). PQQ was determined using the assay described by Geiger & Górisch (1987). All mutants defective in PQQ biosynthesis produced maximally 10% of wild-type PQQ concentration levels.

Genetic techniques. Diparental and triparental matings between E. coli and P. aeruginosa were carried out by mixing aliquots of overnight cultures on LB agar. After 6 h at 37 °C, cells were resuspended and spread on selective medium. E. coli S17-1λpir was used in diparental matings; E. coli HB101 carrying pRK2013 was used as helper strain in triparental matings. To check if complementation occurred in trans, plasmid DNA of complemented P. aeruginosa mutants was isolated and used to transform E. coli JM109. Transformants were again used as donor in a triparental mating with the uncomplemented P. aeruginosa mutant as recipient.

Expression and detection of cytochrome c550 in E. coli JM109. Expression of cytochrome c550 in E. coli JM109 and periplasmic disruption were carried out as described by Ubbink et al. (1992). Cytochrome c550 was quantified spectrophotometrically as described previously (Reichmann & Górisch, 1993). The cytochrome c550-dependent ferricyanide reductase activity of QEDH was measured as described by Reichmann & Górisch (1993). SDS-PAGE was performed as described by Laemmli (1970) and peroxidase staining as described by Francis & Becker (1984).

Expression and detection of aldehyde dehydrogenase in E. coli JM109. E. coli JM109 containing pTB3071 was grown
**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong>&lt;sup&gt;P. aeruginosa&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 17933</td>
<td>Wild-type</td>
<td>Cetin &lt;i&gt;et al.&lt;/i&gt; (1965)</td>
</tr>
<tr>
<td>MS1, 4, 5, 6, 7, 8, 17, 20</td>
<td>ATCC 17933 derivative, mutant class I</td>
<td>This study</td>
</tr>
<tr>
<td>MS9, 11, 12, 13, 16, 18</td>
<td>ATCC 17933 derivative, mutant class II</td>
<td>This study</td>
</tr>
<tr>
<td>MS2, 10, 14, 19</td>
<td>ATCC 17933 derivative, mutant class III</td>
<td>This study</td>
</tr>
<tr>
<td>MS3, 15, 21</td>
<td>ATCC 17933 derivative, &lt;i&gt;exaB&lt;/i&gt;:&lt;i&gt;Km&lt;/i&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-12 pir</td>
<td>Tp&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; recA thi pro &lt;i&gt;hsdR&lt;/i&gt;:&lt;i&gt;M&lt;/i&gt;·&lt;i&gt;Rp&lt;/i&gt;:&lt;i&gt;2&lt;/i&gt;-&lt;i&gt;Tc&lt;/i&gt;:&lt;i&gt;Mu&lt;/i&gt;:&lt;i&gt;Km&lt;/i&gt; &lt;i&gt;Tn&lt;/i&gt;7·&lt;i&gt;pir&lt;/i&gt;</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
</tr>
<tr>
<td>JM109</td>
<td>F&lt;i&gt;traD36 lac&lt;/i&gt;&lt;sup&gt;+&lt;/sup&gt; Δ&lt;i&gt;(lacZ)&lt;/i&gt; M15 proA·&lt;i&gt;B&lt;/i&gt;′/&lt;i&gt;recA1&lt;/i&gt; endA1 gyrA96 thi &lt;i&gt;hsdR17 supE&lt;/i&gt;44 relA1 Δ&lt;i&gt;(lac-proAB)&lt;/i&gt;</td>
<td>Yanisch-Perron &lt;i&gt;et al.&lt;/i&gt; (1985)</td>
</tr>
<tr>
<td>HB101</td>
<td>&lt;i&gt;supE&lt;/i&gt;44 &lt;i&gt;hsdS&lt;/i&gt;220(&lt;i&gt;rec&lt;/i&gt;-&lt;i&gt;m&lt;/i&gt;·&lt;i&gt;<em>&lt;/i&gt;·&lt;i&gt;</em>&lt;/i&gt;) &lt;i&gt;recA13 ara-14&lt;/i&gt; &lt;i&gt;proA2 lacY&lt;/i&gt;1 &lt;i&gt;galK2 rpsL&lt;/i&gt;20 &lt;i&gt;xyl-5 mtl-1&lt;/i&gt;</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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</tr>
<tr>
<td>pLAFR3</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;; broad-host-range cosmid</td>
<td>Staskawicz &lt;i&gt;et al.&lt;/i&gt; (1987)</td>
</tr>
<tr>
<td>pUC18, pUC19</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; cloning and expression vector</td>
<td>Yanisch-Perron &lt;i&gt;et al.&lt;/i&gt; (1985)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;; helper plasmid for triparental mating</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pUCP202T</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; broad-host-range plasmid</td>
<td>Schweizer &lt;i&gt;et al.&lt;/i&gt; (1996)</td>
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<tr>
<td>pSUP1021</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;; suicide vector containing Tn5</td>
<td>Simon &lt;i&gt;et al.&lt;/i&gt; (1986)</td>
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<tr>
<td>pTB3001</td>
<td>Tc&lt;sup&gt;r&lt;/sup&gt;; ~ 25 kb genomic DNA partially digested with &lt;i&gt;Sau3A1&lt;/i&gt; from &lt;i&gt;P. aeruginosa&lt;/i&gt; cloned in &lt;i&gt;BamHI&lt;/i&gt; site of pLAFR3</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3070</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; fragment from pTB3001 cloned between &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; sites of pUC18 (&lt;i&gt;exaBC&lt;/i&gt; orientation opposite &lt;i&gt;Plac&lt;/i&gt; of pUC18)</td>
<td>This study</td>
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<tr>
<td>pTB3071</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; fragment from pTB3001 cloned between &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; sites of pUC19 (&lt;i&gt;exaBC&lt;/i&gt; orientation same as &lt;i&gt;Plac&lt;/i&gt; of pUC19)</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3081</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; ~ 1 kb insert after exonuclease III treatment of pTB3070 (&lt;i&gt;exaB&lt;/i&gt; opposite &lt;i&gt;Plac&lt;/i&gt; of pUC18)</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3106</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;; 2-4 kb &lt;i&gt;XhoI&lt;/i&gt;–&lt;i&gt;XhoI&lt;/i&gt; fragment containing Km&lt;sup&gt;r&lt;/sup&gt; gene of Tn5 cloned between &lt;i&gt;XhoI&lt;/i&gt;–&lt;i&gt;XhoI&lt;/i&gt; sites of pTB4003</td>
<td>This study</td>
</tr>
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<td>pTB3109</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; fragment from pTB3070 with complete &lt;i&gt;exaBC&lt;/i&gt; and &lt;i&gt;pqqA&lt;/i&gt; genes cloned between &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;PstI&lt;/i&gt; sites of pUCP202T</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3110</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; 2-7 kb &lt;i&gt;XhoI&lt;/i&gt;–&lt;i&gt;BamHI&lt;/i&gt; fragment from pTB3070 with complete &lt;i&gt;exaC&lt;/i&gt; and &lt;i&gt;pqqA&lt;/i&gt; genes cloned between &lt;i&gt;XhoI&lt;/i&gt;–&lt;i&gt;BamHI&lt;/i&gt; sites of pUCP202T</td>
<td>This study</td>
</tr>
<tr>
<td>pTB3111</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; ~ 1 kb EcoRI–&lt;i&gt;PstI&lt;/i&gt; fragment from pTB3081 with complete &lt;i&gt;exaC&lt;/i&gt; gene cloned between EcoRI–&lt;i&gt;PstI&lt;/i&gt; sites of pUCP202T</td>
<td>This study</td>
</tr>
<tr>
<td>pTB4003</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; 67 kb &lt;i&gt;BamHI&lt;/i&gt;–&lt;i&gt;BamHI&lt;/i&gt; fragment from pTB3001 cloned in the &lt;i&gt;BamHI&lt;/i&gt; site of pUC18</td>
<td>Diehl &lt;i&gt;et al.&lt;/i&gt; (1998)</td>
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</tbody>
</table>
overnight in LB medium, collected by centrifugation, re-
suspended in 1/50 volume of 100 mM Tris/HCl buffer, pH 8:0
and disrupted in a French pressure cell. Aldehyde dehydro-
genase activity was assayed as described by von Tigerstrom &
Razzel (1966).

Site-directed mutagenesis. A pUC18 derivative, pTB3106,
was constructed by cloning a 2-4 kb XhoI fragment with the
kanamycin-resistance gene of transposon Tn5 from pSUP1021
in the cytochrome c550 gene. This plasmid was used to
transform P. aeruginosa via electroporation (Smith &
Iglewski, 1989). Potential site-directed mutants with a Km'
Cb' phenotype were selected for Southern blot hybridization.

RESULTS
Isolation and characterization of mutants unable to
grow on ethanol

After mutagenesis with MNNG, a total of 21 mutants
unable to grow at all or growing slowly on ethanol were
isolated from 5000 colonies. The mutants were classified
into four groups (Table 2). Eight mutants contained an
active QEDH and secreted PQQ in the culture super-
natants, which might indicate a defect in a component of
the electron-transport chain or the metabolism of
acetaldehyde (class I). The mutants were also grown
anaerobically on LB media containing KNO₃ or NaNO₂
as described by Vijgenboom et al. (1997). Under these
conditions the soluble cytochrome c₅₅₀ is induced instead
of cytochrome c₅₅₀ and a mutant with a defect in cytochrome c maturation should grow more slowly. Seven mutants were able to grow on LB anaerobically
like the wild-type. Mutant MS6 grew more slowly and
did not reach the maximum OD₆₅₀ of the wild-type,
indicating a defect in cytochrome c maturation. Another
six mutants produced PQQ but showed no QEDH
activity and therefore might contain a mutation in a
gene encoding QEDH or in a regulatory gene involved in
QEDH expression (class II). A further four mutants
were presumably blocked in the biosynthesis of PQQ or
its transport to the periplasm, because they did not
secrete PQQ in the culture supernatants, but QEDH
activity was detected after reconstitution with external
PQQ (class III). Finally, three mutants were found
which did not produce PQQ and did not show QEDH
activity, even after reconstitution with external PQQ
(class IV).

Complementation of the mutants with a gene library
of P. aeruginosa

All mutants could be complemented with the cosmid
gene library of P. aeruginosa. Ten cosmids were isolated,
of which cosmid pTB3001 could complement ten
mutants comprising members of all four classes (Table
2). None of the other cosmids was able to complement
any of the ten mutants complemented by pTB3001.

Table 2. Properties of P. aeruginosa mutants defective in ethanol oxidation

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Possible mutation in</th>
<th>Mutant no.</th>
<th>Complementation by pTB3001</th>
</tr>
</thead>
<tbody>
<tr>
<td>I PQQ production; active QEDH</td>
<td>Structural or regulatory gene</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>formed</td>
<td>of the electron transport</td>
<td>4</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>chain or metabolism of acetaldehyde</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>−</td>
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<tr>
<td></td>
<td></td>
<td>8</td>
<td>−</td>
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<tr>
<td></td>
<td></td>
<td>17</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>−</td>
</tr>
<tr>
<td>II PQQ production; no QEDH</td>
<td>Structural or regulatory gene</td>
<td>9</td>
<td>−</td>
</tr>
<tr>
<td>formed</td>
<td>for expression of QEDH</td>
<td>11</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>+</td>
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<td></td>
<td></td>
<td>16</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>III No PQQ production; apo-QEDH</td>
<td>Structural or regulatory gene</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>formed</td>
<td>of PQQ biosynthesis</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>holoenzyme activity</td>
<td></td>
<td>14</td>
<td>−</td>
</tr>
<tr>
<td>after addition of PQQ</td>
<td></td>
<td>19</td>
<td>+</td>
</tr>
<tr>
<td>IV No PQQ production; no apo-QEDH</td>
<td>Regulation?</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>formed</td>
<td></td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>−</td>
</tr>
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</table>
Cloning of cytochrome c\textsubscript{550} from \textit{P. aeruginosa}

Complementation of mutants:

\begin{tabular}{|c|c|}
\hline
#17 & + \\
#25 & + \\
\hline
\end{tabular}

\textbf{Fig. 1.} Physical and restriction map of the cloned genomic DNA fragments. \textit{exaA}, QEDH gene; \textit{exaB}, cytochrome c\textsubscript{550} gene; \textit{exaC}, acetaldehyde dehydrogenase gene; \textit{pqqA}, encoding putative precursor peptide for PQQ biosynthesis; \textit{pqqB}, encoding a protein assumed to be involved in PQQ transport; P, PstI; B, BamHI; X, Xhol; E, EcoRI. (a) Map of the sequenced 3.2 kb genomic DNA insert of pTB3070 and of the fragments used to complement mutants M517 and M525. The insert of pTB3111 was obtained by digesting pTB3070 with exonuclease III. The EcoRI site is derived from the polycloning site of pUC18. (b) Position of the 2.4 kb DNA fragment containing the kanamycin-resistance cassette of transposon Tn5 in plasmid pTB3106. The position of \textit{exaA} and the positions of the restriction sites downstream of \textit{exaA} were determined by Diehl \textit{et al.} (1998).

Hybridization and subcloning of the cytochrome c\textsubscript{550} gene

The N-terminal amino acid sequence of cytochrome c\textsubscript{550} from \textit{P. aeruginosa} was determined as HGDVT PQAVD TKGLE PLGKE WRDTN PYRKP YAK. This sequence is identical with the one reported by Schrover \textit{et al.} (1993) except for the first amino acid: AGDVT PQAVD TKGLE PLGK. The sequence KEWRDT was chosen for the design of mixed oligonucleotides. Based on the codon usage of \textit{P. aeruginosa} (West \& Iglewski, 1988) a mixed 17-mer oligonucleotide was deduced (see Methods). Southern blotting and hybridization with all complementing cosmids led to the identification of a 3.2 kb BamHI–PstI fragment from cosmid pTB3001. The fragment was subcloned into pUC18 and the insert of the resulting plasmid pTB3070 was sequenced.

Nucleotide sequence of the 3.2 kb fragment

The sequence of the pTB3070 insert revealed three complete and two truncated ORFs with different orientations as shown in Fig. 1(a). The first ORF is only partially present. It is in reverse orientation to all other ORFs and encodes 157 bp of the QEDH N-terminus (Diehl \textit{et al.}, 1998). This gene was named \textit{exaA}.

The second ORF encodes a protein of 145 amino acids. The deduced protein sequence contains the stretch of 33 amino acids which was determined for the N-terminus of cytochrome c\textsubscript{550}, indicating that this gene, \textit{exaB}, encodes cytochrome c\textsubscript{550}.

The third ORF encodes a putative product of 506 amino acid residues, which shows a high similarity with bacterial NAD\textsuperscript{+}-dependent aldehyde dehydrogenases.
Fig. 2. Alignment of the deduced amino acid sequence of P. aeruginosa acetaldehyde dehydrogenase (p.aeru.) with other microbial aldehyde dehydrogenases. a.eutr, acetaldehyde dehydrogenase of A. eutrophus (Prieffert et al., 1992); r.sp., chloroacetaldehyde dehydrogenase of Rhodococcus sp. strain N186/21 (Nagy et al., 1995); v.chol., aldehyde dehydrogenase of V. cholerae (Parsot & Mekalanos, 1991); e.coli, aldehyde dehydrogenase B of E. coli (Xu & Johnson, 1995).

Residues identical with the sequence of p.aeru. are in black boxes. For the p.aeru. sequence the NAD+-binding site is marked by crosses; the PROSITE motifs are marked with asterisks (glutamic acid active site) or a dashed line (cysteine active site).

Identities of around 64% and similarities of about 75% were found with the enzymes from Alcaligenes eutrophus (Prieffert et al., 1992), Rhodococcus sp. strain N186/21 (Nagy et al., 1995), V. cholerae (Parsot & Mekalanos, 1991) and E. coli (Xu & Johnson, 1995) (Fig. 2). A PROSITE database search revealed the presence of two motifs, which were also present in all the bacterial aldehyde dehydrogenases mentioned above.
Cloning of cytochrome c\textsubscript{550} from P. aeruginosa

\textbf{Fig. 3.} Alignment of the deduced amino acid sequence of \textit{P. aeruginosa} cytochrome c\textsubscript{550} (cytc550) with cytochrome c\textsubscript{6} from \textit{C. reinhardtii} (cytc6) and the haem domain of the quinohaemoprotein ethanol dehydrogenase from \textit{G. suboxydans} (qh-edh). Residues identical with the sequence of cytc550 are shown in black boxes. The signal peptide of cytochrome c\textsubscript{550} is marked by a dotted line, the haem binding motif by crosses and the putative sixth ligand of the haem iron by an asterisk.

(Fig. 2). The first motif is a consensus sequence containing a glutamic acid [LIVMFGA]-E-[LIMSTAC]-[GS]-G-[KNLM]-[SADN]-[TAPFV] that is implicated in the catalytic activity of the aldehyde dehydrogenases. In the presumed \textit{P. aeruginosa} aldehyde dehydrogenase this motif VELGGKSP starts at amino acid position 261. The consensus sequence of the second motif contains a cysteine residue necessary for activity [FYLVA]-X\textsubscript{3}-G-[QE]-X-C-[LIVMFGA]-[AGCN]-X-[GSTADNERK]. This motif FFNQGEVCTCPS was found starting at amino acid position 294. The active site cysteine is the amino acid residue 301. There is also a putative NAD-binding motif GXGX\textsubscript{2}G (Hidalgo et al., 1991), which starts at amino acid position 218 and reads GFGREAG. The high sequence similarity to other bacterial aldehyde dehydrogenases suggests that this gene, \textit{exaC}, encodes an aldehyde dehydrogenase. A typical intrinsic terminator structure with a downstream stretch of 8 thymidine nucleotides is found 113 bp downstream of \textit{exaC}.

The fourth ORF encodes a peptide of 23 amino acids, which shows high similarity to the \textit{pqaq}A gene products of several Gram-negative organisms. It shares 72% identity and 99% similarity with the respective peptide from \textit{Pseudomonas fluorescens} (Schnider et al., 1995). The \textit{pqaq}A gene encodes a small peptide believed to serve as the precursor for the biosynthesis of the cofactor PQQ (Goosen et al., 1992; Meulenberg et al., 1992). However, recently Toyama & Lidström (1998) showed that inactivation of this gene does not prevent PQQ biosynthesis in \textit{M. extorquens}.

A fifth ORF downstream of \textit{pqaq}A is truncated and the stretch of 52 amino acid residues of the partial sequence shows 75% identity and 84% similarity with the \textit{pqaq}B gene product of \textit{P. fluorescens} (Schnider et al., 1995). The \textit{pqaq}B gene product is assumed to be involved in the transport of PQQ across the cytoplasmic membrane (Velterop et al., 1995).

Sequencing of the genome of \textit{P. aeruginosa} PAO1 is in progress and preliminary DNA sequence data are available via the internet. Part of the sequenced 3-2 kb genomic DNA fragment from \textit{P. aeruginosa} ATCC 17933 was found in the genome database of \textit{P. aeruginosa} PAO1 with only minor sequence differences.

\textbf{Sequence analysis of the cytochrome c\textsubscript{550} gene}

The amino acid sequence derived from \textit{exaB} identifies the gene product as the \textit{P. aeruginosa} preprotein of the periplasmic cytochrome c\textsubscript{550} (Fig. 3). It carries a typical bacterial signal peptide of 24 amino acids with the signal peptidase recognition site ALA. This is followed by a stretch of 33 amino acids, the sequence of which is identical with the determined N-terminus of cytochrome c\textsubscript{550}. A typical haem-binding motif CXXCH is found at position 49 and methionine M95 or M98 could serve as the sixth ligand of the haem iron. The mature cytochrome c\textsubscript{550} consists of 121 amino acids and the predicted molecular mass of 13985 Da is in close agreement with the previously reported molecular mass of 15000 Da (Reichmann & Görisch, 1993).

A database search revealed no high similarity to other soluble bacterial c-type cytochromes. The highest similarity exists to cytochrome c\textsubscript{6} of the green alga \textit{Chlamydomonas reinhardtii} (Hill et al., 1991), with 34% identities over 77 amino acids. A slightly lower similarity is also found with the C-termini of several quinohaemoprotein alcohol dehydrogenases of the acetic acid bacteria \textit{Glucobacter suboxydans} (Kondo & Horinouchi, 1997), \textit{Acetobacter pasteurianus} (Takehara et al., 1993) and \textit{Acetobacter aceti} (Inoue et al., 1989) (Fig. 3).

\textbf{Expression of cytochrome c\textsubscript{550} and aldehyde dehydrogenase in \textit{E. coli}}

To check the biological activity of the \textit{exaB} gene product, we examined its expression in \textit{E. coli}. The 3-2 kb insert of pTB3070 was cloned into pUC19, resulting in plasmid pTB3071, with the cytochrome c gene located downstream of the lac promoter. Formation of holoprotein cytochrome c\textsubscript{550} in \textit{E. coli} JM109 containing pTB3071 was only observed under oxygen-
heterologously expressed cytochrome was shown to be identical with that of cytochrome c₅₅₀ isolated from P. aeruginosa. No activity was detected in the periplasmic fraction of E. coli JM109 carrying pUC19. These data demonstrate that the cloned gene encodes cytochrome c₅₅₀ and the heterologously expressed protein is functionally active with QEDH. Expression of the aldehyde dehydrogenase in E. coli was investigated with plasmids pTB3070, pTB3071 and pUC19 as control. Aldehyde dehydrogenase activity with acetaldehyde as substrate was only present in E. coli JM109 containing plasmid pTB3071 and a specific activity of 1.0 U (mg protein)⁻¹ was determined in cell-free extract. SDS-PAGE revealed an extra protein band at 56000 Da, which corresponded to the predicted molecular mass of the acetaldehyde dehydrogenase gene product (Fig. 5).

Complementation experiments with mutants defective in ethanol oxidation

To investigate which of the ten mutants is complemented by the 3.2 kb PstI–BamHI fragment, it was cloned into the broad-host-range vector pUCP20T, creating plasmid pTB3109 (Fig. 1a). This plasmid was able to complement mutant MS17. Mutant MS17 was able to grow, albeit slowly, on ethanol. To investigate if either the acetaldehyde dehydrogenase or the cytochrome c₅₅₀ gene is defective, two additional plasmids were constructed with pUCP20T. Plasmid pTB3110 contained the acetaldehyde dehydrogenase gene followed by pqqA and the truncated pqqB and pTB3111 contained only the gene encoding cytochrome c₅₅₀ and the upstream promoter sequence (Fig. 1a). The empty vector, pTB3110 and pTB3111 were transferred into mutant MS17 via triparental mating. Only pTB3111 containing the cytochrome c₅₅₀ gene complemented the mutant and enhanced the growth rate on ethanol to wild-type level.

Inactivation of the cytochrome c₅₅₀ gene

To investigate if cytochrome c₅₅₀ is essential for growth on ethanol, the exab gene was inactivated by site-directed mutagenesis using pTB3106 (Fig. 1b). A Km³ Ch³ mutant, MS25, was obtained as described in Methods. MS25 was unable to grow on ethanol and Southern blotting of the genomic DNA confirmed the presence of the kanamycin-resistance cassette in the cytochrome c gene (data not shown). In complementation experiments with MS25, pTB3109 with the complete 3.2 kb insert and pTB3111 carrying only the cytochrome c₅₅₀ gene restored growth on ethanol. We also confirmed that the complementation occurred in trans and was not a result of a homologous recombination. Growth tests in liquid culture revealed that MS25 containing pTB3109 or pTB3111 showed similar limiting conditions as described by Ubbink et al. (1992). The specific concentration in the periplasmic fraction was 1.9 nmol cytochrome c₅₅₀ (mg protein)⁻¹. In control experiments with E. coli JM109 carrying pTB3070 or pUC19, no cytochrome c₅₅₀ was detected (Fig. 4). The reduced-minus-oxidized difference spectrum of the heterologously expressed cytochrome was shown to be identical with that of cytochrome c₅₅₀ isolated from P. aeruginosa. The molecular mass, determined by SDS-PAGE with haem staining, is also identical (data not shown). QEDH does not transfer electrons directly to ferricyanide. However, ethanol-dependent ferricyanide reductase activity of QEDH can be detected in the presence of cytochrome c₅₅₀ (Reichmann & Görisch, 1993). This ferricyanide activity of QEDH was observed using the periplasmic fraction of E. coli JM109 carrying pTB3071. The activity was the same when compared with equivalent amounts of native cytochrome c₅₅₀ from P. aeruginosa. No activity was detected in the periplasmic fraction of E. coli JM109 carrying pUC19. These data demonstrate that the cloned gene encodes cytochrome c₅₅₀ and the heterologously expressed protein is functionally active with QEDH. Expression of the aldehyde dehydrogenase in E. coli was investigated with plasmids pTB3070, pTB3071 and pUC19 as control. Aldehyde dehydrogenase activity with acetaldehyde as substrate was only present in E. coli JM109 containing plasmid pTB3071 and a specific activity of 1.0 U (mg protein)⁻¹ was determined in cell-free extract. SDS-PAGE revealed an extra protein band at 56000 Da, which corresponded to the predicted molecular mass of the acetaldehyde dehydrogenase gene product (Fig. 5).
doubling times and reached a similar maximum OD₅₇₀ as the wild-type *P. aeruginosa* containing one or the other plasmid.

**DISCUSSION**

Until now only three components involved in the ethanol oxidation system of *P. aeruginosa* have been identified: QEDH, a soluble cytochrome *c₅₅₀* and the final oxidase (Reichmann & Görisch, 1993; Matsushita et al., 1982). In addition, the involvement of a membrane component has been suggested (Reichmann & Görisch, 1993) and the participation of azurin, accepting electrons of cytochrome *c₅₅₀*, has been proposed (Duine, 1995). Recently, however, Vijgenboom et al. (1997) showed that azurin is not an essential component of the ethanol oxidation system in *P. aeruginosa*. Our study aimed to identify other proteins essential for growth on ethanol and to obtain information about the genetic organization of the genes involved. Four classes of mutants were isolated (Table 2). Classes I–III correspond to mutant phenotypes defective in the methanol oxidation system described for methylotrophic bacteria (Nunn & Lidstrom, 1986; Springer et al., 1995). Class IV mutants unable to produce either PQQ or apo-QEDH have not been described among methylotrophs. In the present work we focused on a 3.2 kb fragment containing the cytochrome *c₅₅₀* gene *exaB*.

The deduced amino acid sequence of the identified cytochrome *c₅₅₀* gene shows all characteristics of c-type cytochromes and encodes a signal peptide for transport to the periplasm. Interruption of the cytochrome *c₅₅₀* gene in *P. aeruginosa* impaired growth on ethanol completely and showed unambiguously that cytochrome *c₅₅₀* is an essential component of the ethanol oxidation system in this organism. The alignment of the amino acid sequences of cytochrome *c₅₅₀* and cytochrome *c₆* from the alga *Chlamydomonas reinhardii* (Hill et al., 1995) revealed two conserved residues towards the C-terminus: M95 and P96. Both residues are conserved in cytochromes *c₆* of algae, where methionine serves as the sixth ligand of the haem iron (Moore & Pettigrew, 1990). We suggest that in cytochrome *c₅₅₀* M95 also serves as the sixth ligand.

QEDH and cytochrome *c₅₅₀* from *P. aeruginosa* are proteins with functions equivalent to QMDH and cytochrome *c₅* in methylotrophic bacteria. In both systems the small, soluble cytochrome accepts electrons from the respective PQQ-dependent alcohol dehydrogenase. Surprisingly, only 20% identity was observed with cytochrome *c₅* from *Methylobacterium extorquens* (Nunn & Anthony, 1988). By contrast, sequence alignments of cytochrome *c₅₅₀* from *P. aeruginosa* showed a significant, albeit low, similarity of 31–33% identity to the C-terminal haem domain of the membrane-bound quinohaemoprotein alcohol dehydrogenases of several acetic acid bacteria. The similarity to the haem domain of the soluble quinohaemoprotein ethanol dehydrogenase of *Comamonas testosteroni* with 26% identity was even lower (Stoorvogel et al., 1996).

Another gene (exaC) was located downstream of the cytochrome *c₅₅₀* gene and in the same orientation. The deduced amino acid sequence of *exaC* exhibits high similarity to aldehyde dehydrogenases of Gram-negative bacteria. The high similarity to the acetaldehyde dehydrogenase of *Alcaligenes eutrophus* (Priefert et al., 1992), together with the close proximity of the aldehyde dehydrogenase and the cytochrome *c₅₅₀* genes in *P. aeruginosa*, suggest that the enzyme is a component of the ethanol oxidation system in this organism. An NAD⁺-dependent acetaldehyde dehydrogenase induced on ethanol has already been described in *P. aeruginosa* ATCC 9027 (von Tigerstrom & Razzel, 1966), and two constitutive acetaldehyde dehydrogenases have also been purified (Guerrillot & Van de Casteele, 1977). Interestingly, in *C. testosteroni* an aldehyde dehydrogenase gene is reported to be located downstream of the quinohaemoprotein ethanol dehydrogenase, but no sequence information is available (Stoorvogel et al., 1996).

Expression of the acetaldehyde dehydrogenase and cytochrome *c₅₅₀* in *E. coli* was only achieved with pTB3071, where both genes are in the same orientation as the lac promoter of the pUC vector. The acetaldehyde dehydrogenase and the cytochrome *c₅₅₀* gene are cotranscribed in *E. coli*, which presumably is also the case in *P. aeruginosa*. We found an intrinsic transcriptional termination signal downstream of the acetaldehyde dehydrogenase gene followed by part of the PQQ biosynthesis operon. The presence of a promoter in front of the cytochrome *c₅₅₀* gene is demonstrated by the successful complementation in trans of the *P. aeruginosa* mutant MS25, using pTB3109 and pTB3111.

The gene encoding cytochrome *c₅₅₀* in *P. aeruginosa* is clustered together with other genes of the ethanol oxidation system. When naming the gene cluster in *P. aeruginosa*, we followed the suggestions given by Lidstrom et al. (1994) for the methanol oxidation system in methylotrophs. The genes involved in ethanol oxidation in *P. aeruginosa* discussed in the present communication form the linkage group *a*. This linkage group *exaABC* contains three genes, with *exaA* encoding the QEDH, *exaB* the soluble cytochrome *c₅₅₀* and *exaC* an acetaldehyde dehydrogenase. The *pqq* operon required for the biosynthesis of the PQQ cofactor of QEDH is downstream of this gene cluster. The organization of the three genes forming the *exaABC* gene cluster in *P. aeruginosa* is quite different from the respective *mxaFJGl* operon encoding QMDH and cytochrome *c₅* in methylotrophic bacteria. Furthermore, in methylotrophs the *pqq* operon does not follow the *mxaFJGl* operon. It would be interesting to compare the two quinoprotein alcohol oxidation systems in these different Gram-negative bacteria with respect to their regulation and other components involved.

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