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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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***Pseudomonas aeruginosa* ATCC 17933 grown aerobically on ethanol produces a soluble cytochrome c_{550} together with a quinoprotein ethanol dehydrogenase. A 3·2 kb genomic DNA fragment containing the gene encoding cytochrome c_{550} was cloned and sequenced. Two other complete and two truncated ORFs were also identified. A truncated ORF encoding the quinoprotein ethanol dehydrogenase (*exaA*) was found upstream of the cytochrome c_{550} gene (*exaB*) and in reverse orientation. An ORF encoding a NAD^+-dependent acetaldehyde dehydrogenase (*exaC*) was located downstream of the cytochrome c_{550} gene and in the same orientation. Another ORF showed similarity to the *pqqA* gene and a truncated ORF similarity to the *pqqB* gene, both involved in the biosynthesis of the prosthetic group PQQ. The organization of these genes was found to be different from the well-studied methanol oxidation system in methylotrophic bacteria. The deduced amino acid sequence of cytochrome c_{550} from *P. aeruginosa* showed some similarity to cytochrome c_6 of the alga *Chlamydomonas reinhardtii* and the haem domain of quinohaemoprotein alcohol dehydrogenases of acetic acid bacteria, but no similarity to the soluble cytochrome c_L of the quinoprotein methanol oxidation system of methylotrophs could be detected. A mutant of *P. aeruginosa* with an interrupted cytochrome c_{550} gene was unable to grow on ethanol, which proves that cytochrome c_{550} is an essential component of the ethanol oxidation system in this organism.**

Keywords: ethanol oxidation, quinoprotein, electron transport, cytochrome *c*, aldehyde dehydrogenase

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 ethanol dehydrogenase (QEDH) linked to an electron transport chain (Rupp & Görisch, 1988). Two components of this

Abbreviations: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PQQ, pyrroloquinoline quinone; QEDH, quinoprotein ethanol dehydrogenase; QMDH, quinoprotein methanol dehydrogenase.

The GenBank accession number for the sequence reported in this paper is AF068264.

electron transport chain, a periplasmic, soluble cytochrome c_{550} and the *co*-type cytochrome oxidase, have been identified (Reichmann & Görisch, 1993; Matsushita *et al*., 1982). Cytochrome c_{550} mediates electron transfer between the QEDH and the cytochrome oxidase via an unknown membrane component (Reichmann & Görisch, 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 (Görisch & 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 PQQ-dependent 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 c_L and c_H, and a cytochrome oxidase (Goodwin & Anthony, 1995). In *M. extorquens*, cytochrome c_L is the direct electron acceptor of QMDH; it shows little similarity to other known cytochromes (Nunn & Anthony, 1988). The gene encoding cytochrome c_L (*mxaG*) is located in an operon *mxaFJGI* (Harms *et al*., 1987; Anderson *et al*., 1990; van Spanning *et al*., 1991) together with the structural genes of QMDH (*mxaF*, *mxaI*). The genetic organization of the *mxaFJGI* operon seems to be conserved among methylotrophic bacteria and it would be interesting to see if the gene encoding cytochrome c_{550} in *P. aeruginosa* is located in a similar operon and if its amino acid sequence reveals similarity to cytochrome c_L.

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 c_{550} 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 succinate as carbon and energy source as described previously (Rupp & Görisch, 1988). Antibiotics were added at the following concentrations: 20 μg tetracycline ml^{-1}, 50 μg kanamycin ml^{-1}, 100 μg carbenicillin 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* S17-1λ *pir* was constructed using the cosmid pLAFR3 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 ^{35}S-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 (MNNG) 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 $CaCl_2$, and immediately used for enzyme assays. QEDH activity was determined as described previously (Rupp & Görisch, 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 (Mutzel & Görisch, 1991). PQQ was determined using the assay described by Geiger & Görisch (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* S17-1λ *pir* 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 c_{550} in *E. coli* JM109. Expression of cytochrome c_{550} in *E. coli* JM109 and periplasmic disruption were carried out as described by Ubbink *et al*. (1992). Cytochrome c_{550} was quantified spectrophotometrically as described previously (Reichmann & Görisch, 1993). The cytochrome c_{550}-dependent ferricyanide reductase activity of QEDH was measured as described by Reichmann & Görisch (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

Strain or plasmid	Relevant genotype or phenotype	Reference
Strains		
P. aeruginosa		
ATCC 17933	Wild-type	Cetin *et al.* (1965)
MS1, 4, 5, 6, 7, 8, 17, 20	ATCC 17933 derivative, mutant class I	This study
MS9, 11, 12, 13, 16, 18	ATCC 17933 derivative, mutant class II	This study
MS2, 10, 14, 19	ATCC 17933 derivative, mutant class III	This study
MS3, 15, 21	ATCC 17933 derivative, mutant class IV	This study
MS25	ATCC 17933 derivative, *exaB*::Km^r	This study
E. coli		
S17-1λ *pir*	Tp^r Sm^r *recA thi pro hsdR*$^-$*M*$^+$ RP4:2-Tc:Mu:Km Tn*7* λ *pir*	de Lorenzo & Timmis (1994)
JM109	F′*traD36 lacI*^{q} Δ(*lacZ*) M15 *pro*A$^+$B$^+$/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac–proAB*)	Yanisch-Perron *et al.* (1985)
HB101	*supE44 hsdS20*(r^-_B m^-_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Boyer & Roulland-Dussoix (1969)
Plasmids		
pLAFR3	Tc^r; broad-host-range cosmid	Staskawicz *et al.* (1987)
pUC18, pUC19	Ap^r; cloning and expression vector	Yanisch-Perron *et al.* (1985)
pRK2013	Km^r; helper plasmid for triparental mating	Figurski & Helinski (1979)
pUCP20T	Ap^r; broad-host-range plasmid	Schweizer *et al.* (1996)
pSUP1021	Tc^r Cm^r Km^r; suicide vector containing Tn*5*	Simon *et al.* (1986)
pTB3001	Tc^r; ~ 25 kb genomic DNA partially digested with *Sau*3AI from *P. aeruginosa* cloned in *Bam*HI site of pLAFR3	This study
pTB3070	Ap^r; 3·2 kb *Bam*HI–*Pst*I fragment from pTB3001 cloned between *Bam*HI–*Pst*I sites of pUC18 (*exaBC* orientation opposite P*lac* of pUC18)	This study
pTB3071	Ap^r; 3·2 kb *Bam*HI–*Pst*I fragment from pTB3001 cloned between *Bam*HI–*Pst*I sites of pUC19 (*exaBC* orientation same as P*lac* of pUC19)	This study
pTB3081	Ap^r; ~ 1 kb insert after exonuclease III treatment of pTB3070 (*exaB* opposite P*lac* of pUC18)	This study
pTB3106	Ap^r Km^r; 2·4 kb *Xho*I–*Xho*I fragment containing Km^r gene of Tn*5* cloned between *Xho*I–*Xho*I sites of pTB4003	This study
pTB3109	Ap^r; 3·2 kb *Bam*HI–*Pst*I fragment from pTB3070 with complete *exaBC* and *pqqA* genes cloned between *Bam*HI–*Pst*I sites of pUCP20T	This study
pTB3110	Ap^r; 2·7 kb *Xho*I–*Bam*HI fragment from pTB3070 with complete *exaC* and *pqqA* genes cloned between *Xho*I–*Bam*HI sites of pUCP20T	This study
pTB3111	Ap^r; ~ 1 kb *Eco*RI–*Pst*I fragment from pTB3081 with complete *exaC* gene cloned between *Eco*RI–*Pst*I sites of pUCP20T	This study
pTB4003	Ap^r; 6·7 kb *Bam*HI–*Bam*HI fragment from pTB3001 cloned in the *Bam*HI site of pUC18	Diehl *et al.* (1998)

overnight in LB medium, collected by centrifugation, resuspended in 1/50 volume of 100 mM Tris/HCl buffer, pH 8·0 and disrupted in a French pressure cell. Aldehyde dehydrogenase 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 *Xho*I fragment with the kanamycin-resistance gene of transposon Tn5 from pSUP1021 in the cytochrome c_{550} gene. This plasmid was used to transform *P. aeruginosa* via electroporation (Smith & Iglewski, 1989). Potential site-directed mutants with a Km^r Cb^s 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 supernatants, 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 KNO_3 or $NaNO_2$ as described by Vijgenboom *et al.* (1997). Under these conditions the soluble cytochrome c_{551} is induced instead of cytochrome c_{550} 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 OD_{620} 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

	Phenotype	Possible mutation in	Mutant no.	Complementation by pTB3001
I	PQQ production; active QEDH formed	Structural or regulatory gene of the electron transport chain or metabolism of acetaldehyde	1	−
			4	−
			5	+
			6	−
			7	−
			8	−
			17	+
			20	−
II	PQQ production; no active QEDH formed	Structural or regulatory gene for expression of QEDH	9	−
			11	−
			12	+
			13	+
			16	−
			18	+
III	No PQQ production; apo-QEDH formed; holoenzyme activity after addition of PQQ	Structural or regulatory gene of PQQ biosynthesis	2	+
			10	+
			14	−
			19	+
IV	No PQQ production; no apo-QEDH formed	Regulation?	3	+
			15	+
			21	−

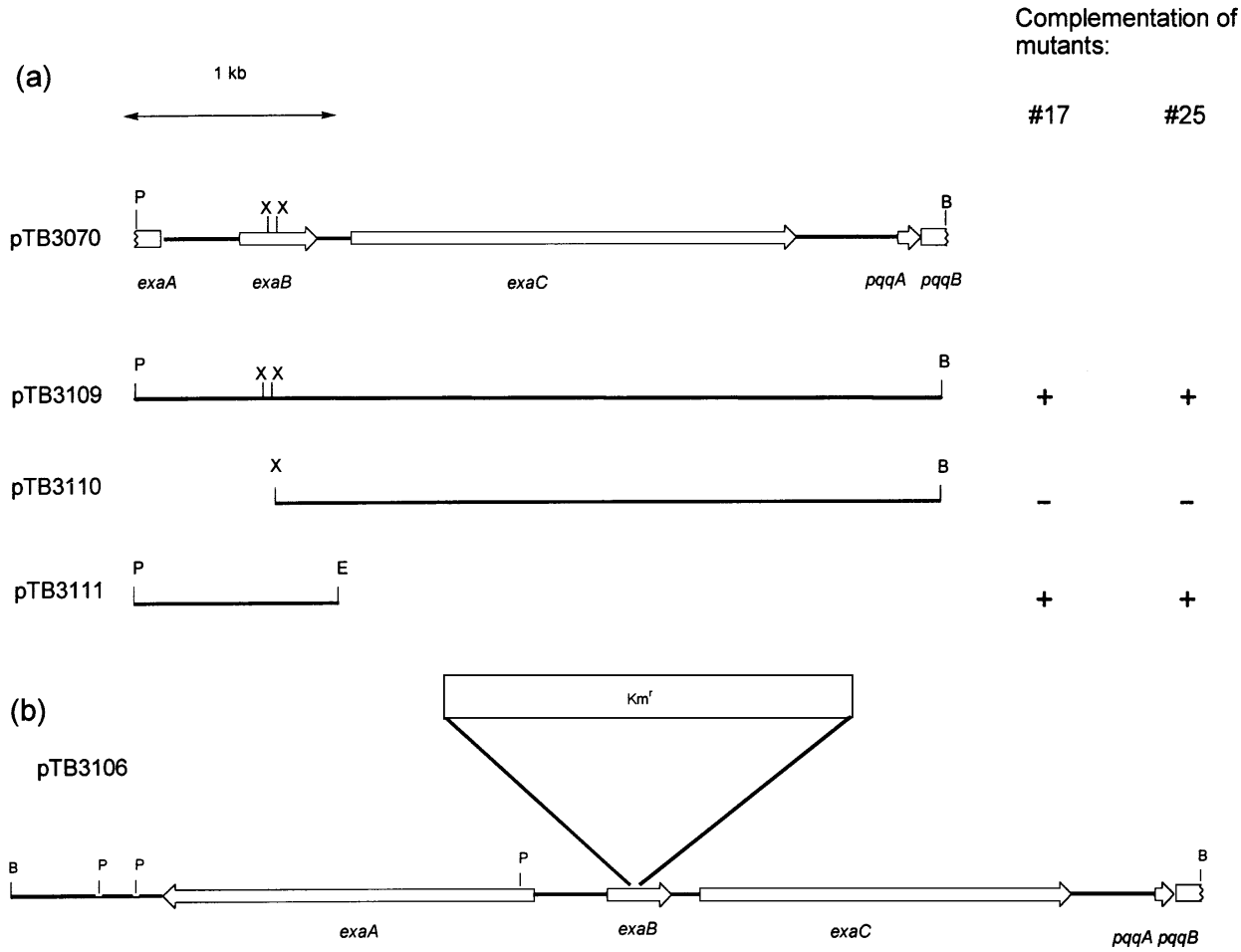

Fig. 1. Physical and restriction map of the cloned genomic DNA fragments. *exaA*, QEDH gene; *exaB*, cytochrome c_{550} gene; *exaC*, acetaldehyde dehydrogenase gene; *pqqA*, encoding putative precursor peptide for PQQ biosynthesis; *pqqB*, encoding a protein assumed to be involved in PQQ transport; P, *Pst*I; B, *Bam*HI; X, *Xho*I; E, *Eco*RI. (a) Map of the sequenced 3·2 kb genomic DNA insert of pTB3070 and of the fragments used to complement mutants MS17 and MS25. The insert of pTB3111 was obtained by digesting pTB3070 with exonuclease III. The *Eco*RI 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 Tn*5* in plasmid pTB3106. The position of *exaA* and the positions of the restriction sites downstream of *exaA* were determined by Diehl *et al.* (1998).

Hybridization and subcloning of the cytochrome c_{550} gene

The N-terminal amino acid sequence of cytochrome c_{550} from *P. aeruginosa* was determined as HGDVT PQAVD TKGLE PLGKE WRDTN PYRKP YAK. This sequence is identical with the one reported by Schrover *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 *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 *Bam*HI–*Pst*I 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 *et al.*, 1998). This gene was named *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_{550}, indicating that this gene, *exaB*, encodes cytochrome c_{550}.

The third ORF encodes a putative product of 506 amino acid residues, which shows a high similarity with bacterial NAD^+-dependent aldehyde dehydrogenases.

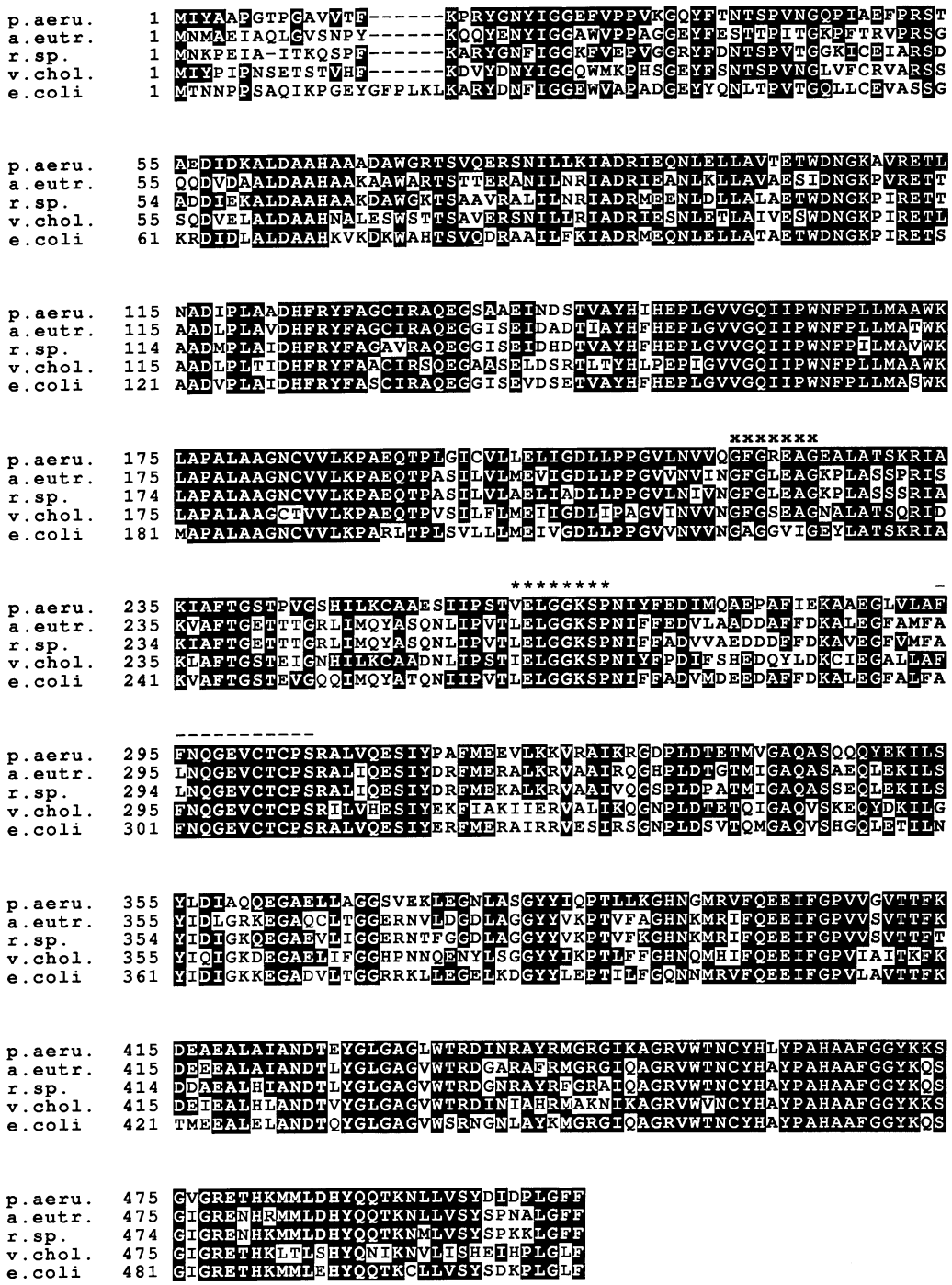

Fig. 2. Alignment of the deduced amino acid sequence of *P. aeruginosa* acetaldehyde dehydrogenase (p.aeru.) with other microbial aldehyde dehydrogenases. a.eutr, acetaldehyde dehydrogenase of *A. eutrophus* (Priefert *et al.*, 1992); r.sp., chloroacetaldehyde dehydrogenase of *Rhodococcus* sp. strain NI86/21 (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* (Priefert *et al.*, 1992), *Rhodococcus* sp. strain NI86/21 (Nagy *et al.*, 1995), *Vibrio 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

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                 .......................
cytc550   -24 MNKNNALRGLLVLAGLSLSSLALAHGDVTPQAVDTKGLEPLGKEWRDTNP

                                        xxxxx
cytc550    27 YRKPYAKHDLAVEIGAS-AYNQNCARCHGLEAKSGGIAPDLRLLETGAEG
cytc6       2          DLA--LGAQ-VFNGNCAACH-MGGRNS-VMPEKTLDKAALEQ
qh-edh    632          DEARQKDGYFMYQTFCSACHGDNAISGGVLPDLRWSGRPRGR

                                    *
cytc550    76 --DEWFKERVINGAVRDGAVYMPKMADFISQEGLWAIRSYLESVHVDE--
cytc6      39 YLDGGFKVESLIYQVENGKGAMPAWADRLSEEEIQAVAEYVFKQATDAAW
qh-edh    674 --ESFYK---LVGRGALTAYGMDRFDTSMTPEQIEDIRNFI---------
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Fig. 3. Alignment of the deduced amino acid sequence of *P. aeruginosa* cytochrome c_{550} (cytc550) with cytochrome c_6 from *C. reinhardtii* (cytc6) and the haem domain of the quinohaemoprotein ethanol dehydrogenase from *G. suboxydans* (qh-edh). Residues identical with the sequence of cytc550 are shown in black boxes. The signal peptide of cytochrome c_{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 *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_3-G-[QE]-X-C-[LIVMGSTANC]-[AGCN]-X-[GSTADNE-KR]. 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_3G (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, *exaC*, encodes an aldehyde dehydrogenase. A typical intrinsic terminator structure with a downstream stretch of 8 thymidine nucleotides is found 113 bp downstream of *exaC*.

The fourth ORF encodes a peptide of 23 amino acids, which shows high similarity to the *pqqA* gene products of several Gram-negative organisms. It shares 72% identity and 99% similarity with the respective peptide from *Pseudomonas fluorescens* (Schnider *et al.*, 1995). The *pqqA* 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 & Lidstrom (1998) showed that inactivation of this gene does not prevent PQQ biosynthesis in *M. extorquens*.

A fifth ORF downstream of *pqqA* is truncated and the stretch of 52 amino acid residues of the partial sequence shows 75% identity and 84% similarity with the *pqqB* gene product of *P. fluorescens* (Schnider *et al.*, 1995). The *pqqB* 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 *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 *P. aeruginosa* ATCC 17933 was found in the genome database of *P. aeruginosa* PAO1 with only minor sequence differences.

Sequence analysis of the cytochrome c_{550} gene

The amino acid sequence derived from *exaB* identifies the gene product as the *P. aeruginosa* preprotein of the periplasmic cytochrome c_{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_{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_{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_6 of the green alga *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 *Gluconobacter suboxydans* (Kondo & Horinouchi, 1997), *Acetobacter pasteurianus* (Takemura *et al.*, 1993) and *Acetobacter aceti* (Inoue *et al.*, 1989) (Fig. 3).

Expression of cytochrome c_{550} and aldehyde dehydrogenase in *E. coli*

To check the biological activity of the *exaB* gene product, we examined its expression in *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_{550} in *E. coli* JM109 containing pTB3071 was only observed under oxygen-

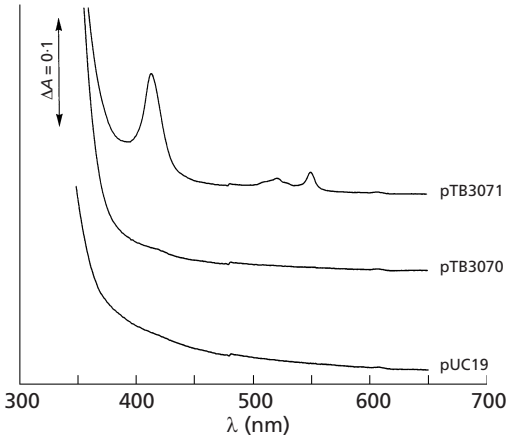

Fig. 4. Absorption spectra of the periplasmic fractions from different *E. coli* constructs. Spectra are offset relative to each other to improve clarity. *E. coli* JM109 carrying plasmid pTB3070, pTB3071 or pUC19 was grown semianaerobically and periplasmic fractions (40 μg protein ml^{-1}) were reduced with dithionite.

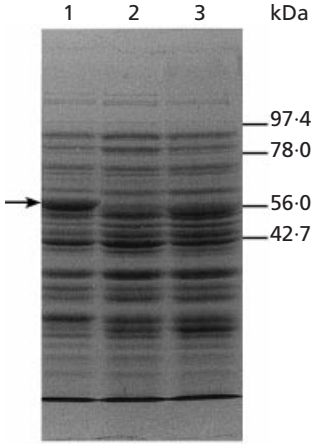

Fig. 5. Expression of acetaldehyde dehydrogenase in *E. coli* JM109. Strains were grown aerobically and pelleted cells were boiled for 3 min in treatment buffer (Sambrook *et al.*, 1989). Proteins were separated by SDS-PAGE and stained with Coomassie blue. Lanes: 1, *E. coli* JM109/pTB3071; 2, *E. coli* JM109/pTB3070; 3, *E. coli* JM109/pUC19. A bold arrow indicates the position of acetaldehyde dehydrogenase.

limiting conditions as described by Ubbink *et al.* (1992). The specific concentration in the periplasmic fraction was 1·9 nmol cytochrome c_{550} (mg protein)$^{-1}$. In control experiments with *E. coli* JM109 carrying pTB3070 or pUC19, no cytochrome c_{550} was detected (Fig. 4). The reduced-minus-oxidized difference spectrum of the heterologously expressed cytochrome was shown to be identical with that of cytochrome c_{550} 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 c_{550} (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 c_{550} 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 c_{550} 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 *Pst*I–*Bam*HI 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 c_{550} 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 c_{550} 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 c_{550} gene complemented the mutant and enhanced the growth rate on ethanol to wild-type level.

Inactivation of the cytochrome c_{550} gene

To investigate if cytochrome c_{550} is essential for growth on ethanol, the *exaB* gene was inactivated by site-directed mutagenesis using pTB3106 (Fig. 1b). A Km^r Cb^s 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 c_{550} 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

doubling times and reached a similar maximum OD_{620} 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 c_{550} 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 c_{550}, 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 c_{550} gene *exaB*.

The deduced amino acid sequence of the identified cytochrome c_{550} gene shows all characteristics of *c*-type cytochromes and encodes a signal peptide for transport to the periplasm. Interruption of the cytochrome c_{550} gene in *P. aeruginosa* impaired growth on ethanol completely and showed unambiguously that cytochrome c_{550} is an essential component of the ethanol oxidation system in this organism. The alignment of the amino acid sequences of cytochrome c_{550} and cytochrome c_6 from the alga *Chlamydomonas reinhardtii* (Hill *et al.*, 1991) revealed two conserved residues towards the C-terminus: M95 and P96. Both residues are conserved in cytochromes c_6 of algae, where methionine serves as the sixth ligand of the haem iron (Moore & Pettigrew, 1990). We suggest that in cytochrome c_{550} M95 also serves as the sixth ligand.

QEDH and cytochrome c_{550} from *P. aeruginosa* are proteins with functions equivalent to QMDH and cytochrome c_L 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 c_L from *Methylobacterium extorquens* (Nunn & Anthony, 1988). By contrast, sequence alignments of cytochrome c_{550} 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 c_{550} 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 c_{550} 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 & Vandecasteele, 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 c_{550} 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 c_{550} gene are co-transcribed 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 c_{550} gene is demonstrated by the successful complementation *in trans* of the *P. aeruginosa* mutant MS25, using pTB3109 and pTB3111.

The gene encoding cytochrome c_{550} 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 *e*thanol o*x*idation 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 c_{550} 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 *mxaFJGI* operon encoding QMDH and cytochrome c_L in methylotrophic bacteria. Furthermore, in methylotrophs the *pqq* operon does not follow the *mxaFJGI* 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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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Anderson D. J., Morris C. J., Nunn D. N., Anthony C. & Lidstrom, M. E. (1990). Nucleotide sequence of the *Methylobacterium extorquens* AM1 *moxF* and *moxJ* genes involved in methanol oxidation. *Gene* **90**, 173–176.

Anthony, C. (1992). The structure of bacterial quinoprotein dehydrogenases. *Int J Biochem* **24**, 29–39.

Arfman, N., Watling, E. M., Clement, W., van Oosterwijk, R. J., de Vries, G. E., Harder, W., Attwood, M. M. & Dijkhuizen, L. (1989). Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. *Arch Microbiol* **152**, 280–288.

Bairoch, A., Bucher, P. & Hofmann, K. (1997). The PROSITE database, its status in 1997. *Nucleic Acids Res* **25**, 217–221.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **14**, 459–472.

Cetin, E. T., Töreci, K. & Ang, Ö. (1965). Encapsulated *Pseudomonas aeruginosa* (*Pseudomonas mucosus*) strains. *J Bacteriol* **89**, 1432–1433.

Diehl, A., v. Wintzingerode, F. & Görisch, H. (1998). Quinoprotein ethanol dehydrogenase of *Pseudomonas aeruginosa* is a homodimer: sequence of the gene and deduced structural properties of the enzyme. *Eur J Biochem* **257**, 409–419.

Duine, J. A. (1995). Electron transfer from bacterial dehydrogenases. In *From Neural Networks and Biochemical Engineering to Bioelectronics*, pp. 87–94. Edited by C. Nicolini. New York: Plenum.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA***76**, 1648–1652.

Francis, R. T., Jr & Becker, R. R. (1984). Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. *Anal Biochem* **136**, 509–514.

Geiger, O. & Görisch, H. (1987). Enzymatic determination of pyrroloquinoline quinone using crude membranes from *Escherichia coli*. *Anal Biochem* **164**, 418–423.

Goodwin, P. M. & Anthony, C. (1995). The biosynthesis of periplasmic electron transport proteins in methylotrophic bacteria. *Microbiology* **141**, 1051–1064.

Goosen, N., Huinen, R. G. & van de Putte, P. (1992). A 24-amino-acid polypeptide is essential for the biosynthesis of the coenzyme pyrrolo-quinoline-quinone. *J Bacteriol* **174**, 1426–1427.

Görisch, H. & Rupp, M. (1989). Quinoprotein ethanol dehydrogenase from *Pseudomonas*. *Antonie Leeuwenhoek* **56**, 35–45.

Guerrillot, L. & Vandecasteele, J. P. (1977). Purification and characterization of two aldehyde dehydrogenases from *Pseudomonas aeruginosa*. *Eur J Biochem* **81**, 185–192.

Harms, N., de Vries, G. E., Maurer, K., Hoogendijk, J. & Stouthamer, A. H. (1987). Isolation and nucleotide sequence of the methanol dehydrogenase structural gene from *Paracoccus denitrificans*. *J Bacteriol* **169**, 3969–3975.

Hidalgo, E., Chen, Y. M., Lin, E. C. & Aguilar, J. (1991). Molecular cloning and DNA sequencing of the *Escherichia coli* K-12 *ald* gene encoding aldehyde dehydrogenase. *J Bacteriol* **173**, 6118–6123.

Hill, K. L., Li, H. H., Singer, J. & Merchant, S. (1991). Isolation and structural characterization of the *Chlamydomonas reinhardtii* gene for cytochrome c_6. Analysis of the kinetics and metal specificity of its copper-responsive expression. *J Biol Chem* **266**, 15060–15067.

Inoue, T., Sunagawa, M., Mori, A., Imai, C., Fukuda, M., Takagi, M. & Yano, K. (1989). Cloning and sequencing of the gene encoding the 72-kilodalton dehydrogenase subunit of alcohol dehydrogenase from *Acetobacter aceti*. *J Bacteriol* **171**, 3115–3122.

Kondo, K. & Horinouchi, S. (1997). Characterization of the genes encoding the three-component membrane-bound alcohol dehydrogenase from *Gluconobacter suboxydans* and their expression in *Acetobacter pasteurianus*. *Appl Environ Microbiol* **63**, 1131–1138.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lidstrom, M. E., Anthony, C., Biville, F., Gasser, F., Goodwin, P., Hanson, R. S. & Harms, N. (1994). New unified nomenclature for genes involved in the oxidation of methanol in gram-negative bacteria. *FEMS Microbiol Lett* **117**, 103–106.

de Lorenzo, V. & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn*10*-derived minitransposons. *Methods Enzymol* **235**, 386–405.

Matsushita, K., Shinagawa, E., Adachi, O. & Ameyama, M. (1982). *o*-Type cytochrome oxidase in the membrane of aerobically grown *Pseudomonas aeruginosa*. *FEBS Lett* **139**, 255–258.

Meulenberg, J. J., Sellink, E., Riegman, N. H. & Postma, P. W. (1992). Nucleotide sequence and structure of the *Klebsiella pneumoniae pqq* operon. *Mol Gen Genet* **232**, 284–294.

Miller, J. M. (1992). *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moore, G. R. & Pettigrew, G. W. (1990). *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*. Berlin & Heidelberg: Springer.

Mutzel, A. & Görisch, H. (1991). Quinoprotein ethanol dehydrogenase: preparation of the apo-form and reconstitution with pyrroloquinolin quinone and Ca^{2+} or Sr^{2+} ions. *Agric Biol Chem* **55**, 1721–1726.

Nagy, I., Schoofs, G., Compernolle, F., Proost, P., Vanderleyden, J. & de Mot, R. (1995). Degradation of the thiocarbamate herbicide EPTC (*S*-ethyl dipropylcarbamothioate) and biosafening by *Rhodococcus* sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. *J Bacteriol* **177**, 676–687.

Nunn, D. N. & Anthony, C. (1988). The nucleotide sequence and deduced amino acid sequence of the cytochrome c_L gene of *Methylobacterium extorquens* AM1, a novel class of *c*-type cytochrome. *Biochem J* **256**, 673–676.

Nunn, D. N. & Lidstrom, M. E. (1986). Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium* sp. strain AM1. *J Bacteriol* **166**, 591–597.

Parsot, C. & Mekalanos, J. J. (1991). Expression of the *Vibrio cholerae* gene encoding aldehyde dehydrogenase is under control of ToxR, the cholera toxin transcriptional activator. *J Bacteriol* **173**, 2842–2851.

Priefert, H., Krüger, N., Jendrossek, D., Schmidt, B. & Steinbüchel, A. (1992). Identification and molecular characterization of the

gene coding for acetaldehyde dehydrogenase II (*acoD*) of *Alcaligenes eutrophus*. *J Bacteriol* **174**, 899–907.

Reichmann, P. & Görisch, H. (1993). Cytochrome c_{550} from *Pseudomonas aeruginosa*. *Biochem J* **289**, 173–178.

Rupp, M. & Görisch, H. (1988). Purification, crystallisation and characterization of quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa*. *Biol Chem Hoppe-Seyler* **369**, 431–439.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schnider, U., Keel, C., Voisard, C., Defago, G. & Haas, D. (1995). Tn*5*-directed cloning of *pqq* genes from *Pseudomonas fluorescens* CHA0: mutational inactivation of the genes results in overproduction of the antibiotic pyoluteorin. *Appl Environ Microbiol* **61**, 3856–3864.

Schrover, J. M., Frank, J., van Wielink, J. E. & Duine, J. A. (1993). Quaternary structure of quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* and its reoxidation with a novel cytochrome *c* from this organism. *Biochem J* **290**, 123–127.

Schweizer, H. P., Klassen, T. R. & Hoang, T. (1996). Improved methods for gene analysis in *Pseudomonas*. In *Molecular Biology of Pseudomonads*, pp. 229–237. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.

Simon, R., O'Connell, M., Labes, M. & Pühler, A. (1986). Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol* **118**, 640–659.

Smith, A. W. & Iglewski, B. H. (1989). Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res* **17**, 10509.

van Spanning, R. J., Wansell, C. W., De Boer, T., Hazelaar, M. J., Anazawa, H., Harms, N., Oltmann, L. F. & Stouthamer, A. H. (1991). Isolation and characterization of the *moxJ*, *moxG*, *moxI*, and *moxR* genes of *Paracoccus denitrificans*: inactivation of *moxJ*, *moxG*, and *moxR* and the resultant effect on methylotrophic growth. *J Bacteriol* **173**, 6948–6961.

Springer, A. L., Chou, H. H., Fan, W. H., Lee, E. & Lidstrom, M. E. (1995). Methanol oxidation mutants in *Methylobacterium extorquens* AM1: identification of new genetic complementation groups. *Microbiology* **141**, 2985–2993.

Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. (1987). Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* **169**, 5789–5794.

Stoorvogel, J., Kraayveld, D. E., van Sluis, C. A., Jongejan, J. A., de Vries, S. & Duine, J. A. (1996). Characterization of the gene encoding quinohaemoprotein ethanol dehydrogenase of *Comamonas testosteroni*. *Eur J Biochem* **235**, 690–698.

Takemura, H., Kondo, K., Horinouchi, S. & Beppu, T. (1993). Induction by ethanol of alcohol dehydrogenase activity in *Acetobacter pasteurianus*. *J Bacteriol* **175**, 6857–6866.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

von Tigerstrom, R. G. & Razzel, W. E. (1966). Aldehyde dehydrogenase. Purification and properties of the enzyme from *P. aeruginosa*. *J Biol Chem* **243**, 2691–2702.

Toyama, H. & Lidstrom, M. E. (1998). *pqqA* is not required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AM1. *Microbiology* **144**, 183–191.

Ubbink, M., van Beeumen, J. & Canters, G. W. (1992). Cytochrome c_{550} from *Thiobacillus versutus*: cloning, expression in *Escherichia coli*, and purification of the heterologous holoprotein. *J Bacteriol* **174**, 3707–3714.

Velterop, J. S., Sellink, E., Meulenberg, J. J., David, S., Bulder, I. & Postma, P. W. (1995). Synthesis of pyrroloquinoline quinone *in vivo* and *in vitro* and detection of an intermediate in the biosynthetic pathway. *J Bacteriol* **177**, 5088–5098.

Vijgenboom, E., Busch, J. E. & Canters, G. W. (1997). *In vivo* studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that *azu* expression is under control of *rpoS* and ANR. *Microbiology* **143**, 2853–2863.

West, S. E. & Iglewski, B. H. (1988). Codon usage in *Pseudomonas aeruginosa*. *Nucleic Acids Res* **16**, 9323–9335.

Wilson, K. (1994). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, vol. I, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel. New York: Wiley.

Xu, J. & Johnson, R. C. (1995). *aldB*, an RpoS-dependent gene in *Escherichia coli* encoding an aldehyde dehydrogenase that is repressed by Fis and activated by Crp. *J Bacteriol* **177**, 3166–3175.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

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