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The *Pseudomonas aeruginosa acsA* gene, encoding an acetyl-CoA synthetase, is essential for growth on ethanol

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***Pseudomonas aeruginosa* ATCC 17933 uses a pyrroloquinoline quinone-dependent ethanol oxidation system. Two mutants of *P. aeruginosa*, unable to grow on ethanol and showing no acetyl-CoA synthetase (ACS) activity under standard test conditions, were complemented by cosmid pTB3018. Subcloning led to the isolation of a gene which encodes a protein with high similarity to acetyl-CoA synthetases. Interruption of the putative *acsA* gene by a kanamycin-resistance cassette resulted in a mutant also unable to grow on ethanol and with very low residual acetyl-CoA-forming activity. Complementation by the wild-type allele of the *acsA* gene restored growth and led to the expression of ACS activity in excess of that of wild-type cells. In wild-type *P. aeruginosa*, ACS activity was induced upon growth on ethanol, 2,3-butanediol, malonate and acetate. The wild-type and mutants defective in ACS activity showed an active acetate kinase (ACK) under the growth conditions used; however, phosphotransacetylase (PTA) could not be detected. The data indicate that *P. aeruginosa* requires active *acsA* gene product for growth on ethanol.**

Keywords: ethanol oxidation, acetate metabolism, acetate kinase, phosphotransacetylase, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa ATCC 17933 grows aerobically with ethanol as its sole source of carbon and energy. Recently, we identified an *exaABC* gene cluster, encoding components of the ethanol oxidizing system. The three genes encode an unusual quinoprotein ethanol dehydrogenase (QEDH, *exaA*), an NAD^+-dependent acetaldehyde dehydrogenase (*exaC*) and a cytochrome c_{550} (*exaB*) (Fig. 1) (Diehl *et al.*, 1998; Reichmann & Görisch, 1993; Schobert & Görisch, 1999).

Some pseudomonads use an NAD^+- and CoA-dependent acylating acetaldehyde dehydrogenase, EC 1.2.1.10, for activation of acetate (Eaton, 1996; Powlowski *et al.*, 1993). Other organisms oxidize acetaldehyde to acetate by a CoA-independent dehydrogenase. The acetate in turn can be converted to acetyl-CoA by two different routes (Fig. 1). Most organisms synthesize acetyl-CoA using either the acetyl-CoA synthetase (ACS) reaction or the acetate kinase (ACK)/phosphotransacetylase (PTA) sequence. For instance, *Pseudomonas* PAO (Champine & Goodwin, 1991) and *Methanosarcina thermophila* (Aceti & Ferry, 1988) use ACK (EC 2.7.2.1) and PTA (EC 2.3.1.8) to form acetyl-CoA. In contrast, bacteria like *Pseudomonas* AM1 (Taylor & Anthony, 1976), *Methanobacterium thermoautotrophicum* (Oberlies *et al.*, 1980), *Methanothrix soehngenii* (Jetten *et al.*, 1989; Eggen *et al.*, 1991) and *Alcaligenes eutrophus* (Steinbüchel *et al.*, 1987; Priefert & Steinbüchel, 1992) use ACS (EC 6.2.1.1). Some bacteria, such as *Escherichia coli* (Brown *et al.*, 1977; Kumari *et al.*, 1995; Chang *et al.*, 1999) and *Bacillus subtilis* (Grundy *et al.*, 1993), possess both routes for interconverting acetate and acetyl-CoA.

Recently, we isolated mutants of *P. aeruginosa* unable to grow on ethanol. Mutants MS1 and MS8 have a defect in a structural or regulatory gene of either the electron transport chain specific for the quinoprotein ethanol oxidation system or the metabolism of acetaldehyde and acetate (Schobert & Görisch, 1999). The present communication describes the identification of the gene *acsA*,

Abbreviations: ACK, acetate kinase; ACS, acetyl-CoA synthetase; PQQ, pyrroloquinoline quinone; PTA, phosphotransacetylase; QEDH, quinoprotein ethanol dehydrogenase.

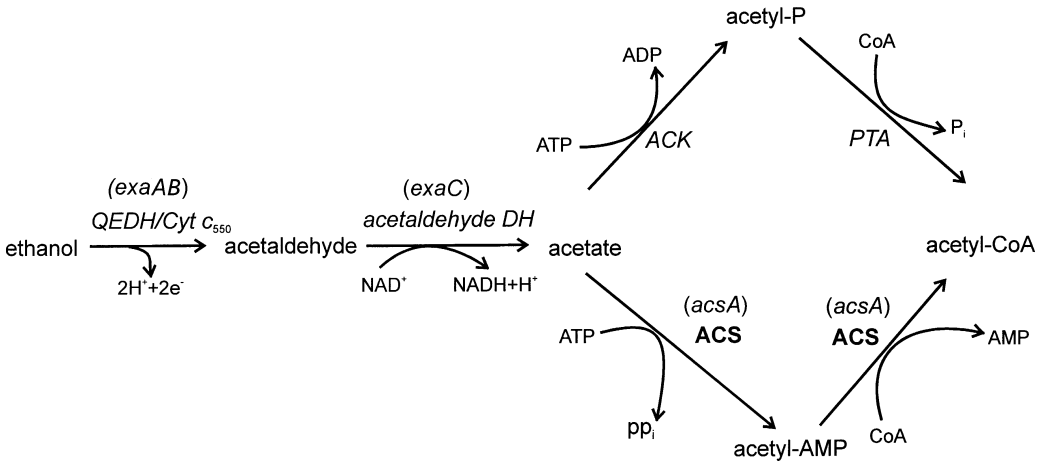

Fig. 1. Genes and gene products of the *exa* gene cluster in *P. aeruginosa* and the enzymes studied.

which encodes ACS, essential in *P. aeruginosa* ATCC 17933 for growth on ethanol.

METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids are listed in Table 1. *P. aeruginosa* mutants MS1 and MS8 contained an active QEDH and secreted pyrroloquinoline quinone (PQQ) in the culture supernatants, but were unable to grow on ethanol. *E. coli* was grown at 37 °C in LB medium. *P. aeruginosa* ATCC 17933 and mutants were cultivated at 37 °C in LB or in minimal media (Rupp & Görisch, 1988) containing different carbon sources: 86 mM (0·5 %, v/v) ethanol; 40 mM succinate; 25 mM glucose; 20 mM acetate; 22 mM 2,3-butanediol; 7 mM malonate. When appropriate, 50 μg kanamycin ml^{-1}, 20 μg tetracycline ml^{-1} or 100 μg carbenicillin ml^{-1} was added.

Recombinant DNA work and genetic techniques. Standard protocols were followed for plasmid isolation, restriction enzyme analyses, ligation, transformation (by electroporation), gel electrophoresis and other DNA manipulations (Sambrook *et al.*, 1989). For DNA sequencing, a sequenase kit and ^{35}S-labelled ATP (Amersham) were used.

Triparental matings between *E. coli* JM109 and *P. aeruginosa* mutants were carried out by mixing aliquots of overnight cultures on LB agar. *E. coli* HB101 carrying pRK2013 was used as helper strain. After 6 h at 37 °C, cells were resuspended and spread on both succinate and selective ethanol minimal medium.

For PCR amplification of the *acsA* gene, the 4·8 kb fragment of pTB4102 was used as template. *Pfu* DNA polymerase (Stratagene) was used according to the instructions of the manufacturer. As primers the oligonucleotide 5′-AGT GGA TCC GTT GAT CTC GCT GTG G-3′ complementary to a sequence 205 bp upstream of the start codon and the oligonucleotide 5′-AGT GGA TCC GAA GTG TTA CCG CGC C-3′ 109 bp downstream of the stop codon TGA of ORF2 were used.

Construction of an *acsA*::Km^r mutant. A pUC19 derivative, pTB4108, containing a 5·8 kb fragment from pTB4107 with the kanamycin-resistance gene of transposon Tn5 in the *acsA* gene, was used to transform *P. aeruginosa* by electroporation (Smith & Iglewski, 1989). Potential site-directed double-crossover mutants with a Km^r phenotype were selected for loss of ampicillin resistance.

Induction of enzyme activity. Strains were grown in minimal medium with succinate as carbon source. At an OD_{620} of 0·8, cells were collected by centrifugation, washed twice and resuspended in twice the amount of minimal medium with 0·5 % (v/v) ethanol to induce the ethanol oxidation system. After incubation for 5 h at 37 °C, cells were harvested by centrifugation.

Preparation of cell-free extracts. Bacteria were grown to late-exponential phase, harvested, and washed twice with 20 mM potassium phosphate buffer (pH 7·0). The wet cell paste (1–2 g) was resuspended in 10 ml 10 mM Tris/HCl buffer (pH 7·9). After cell disruption by sonication, the cell homogenate was centrifuged for 30 min at 6000 ***g***. The supernatant contained 1·5–4 mg protein ml^{-1} and was stored at −80 °C as cell-free extract. Under these conditions, ACS and ACK activities were stable for several days. For the detection of low enzyme activity, cell-free extracts with about 10 mg protein ml^{-1} were prepared.

Enzyme assays. Enzyme activities were determined with cell-free extracts. For standard tests, 0·5–1·3 mg protein was used in a total test volume of 1 ml. For the determination of low enzyme activity, about 6 mg protein was used per test. ACS activity was assayed in 0·1 M Tris/HCl buffer, pH 8·5, by monitoring the formation of acetyl-CoA from acetate, CoA and ATP (Jones & Lipman, 1955; Berg, 1962). ACK activity was determined by measuring the formation of acetyl-P from acetate and ATP (Aceti & Ferry, 1988; Eggen *et al.*, 1991). Acetyl-CoA and acetyl-P were determined as the trivalent iron complex of acetylhydroxamate at 540 nm. Enzyme activities below 0·025 U per assay mixture cannot be detected. One unit (U) is defined as 1 μmol product formed in 20 min. Using the same cell extract, standard deviations of the enzymic test were calculated as ±9 % for ACS and ±3 % for ACK activity. PTA activity was determined with acetyl phosphate and CoA as substrates. Formation of acetyl-CoA was followed by the absorption increase of the thioester linkage at 233 nm (Thompson & Chen, 1990). Protein concentrations were determined using the method of Groves *et al.* (1968).

Internet tools. The following internet services were used: BLAST for DNA or protein database searches (Altschul *et al.*,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Reference
Strains		
P. aeruginosa		
ATCC 17933	Wild-type	Cetin *et al.* (1965)
MS1, MS8	ATCC 17933 derivative, mutant class I	Schobert & Görisch (1999)
UK1	ATCC 17933 derivative, *acsA*::Km^r	This study
E. coli		
JM109	F′*traD36 lac*^{q} Δ(*lacZ*) M15 *proA*$^+$*B*$^+$/*recA1 endA1 gryA96 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)
DH5α	*supE44*Δ*lacU*169(*ϕ*80 *lacZ*Δ M15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Hanahan (1983)
Plasmids		
pLAFR3	Tc^r; broad-host-range cosmid	Staskawicz *et al.* (1987)
pRK2013	Km^r; helper plasmid for triparental mating	Figurski & Helinski (1979)
pSUP1021	Tc^r Cm^r Km^r; suicide vector containing Tn5	Simon *et al.* (1986)
pUC19	Ap^r; cloning and expression vector	Yanisch-Perron *et al.* (1985)
pUCP20T	Ap^r; broad-host-range plasmid	Schweizer *et al.* (1996)
pTB3018	Cosmid complementing mutants MS1 and MS8; Tc^r; ∼ 24 kb genomic DNA partially digested with *Sau*3AI from *P. aeruginosa* cloned in *Bam*HI site of pLAFR3	Schobert & Görisch (1999)
pTB3131	Ap^r Km^r; 1·0 kb PCR product with promoter and Km^r gene of Tn5 cloned between *Eco*RI–*Bam*HI sites of pUC19	This study
pTB4100	Ap^r; 6·9 kb *Hin*dIII fragment from pTB3018 cloned between *Hin*dIII sites of pUCP20T	This study
pTB4101	Ap^r; 2·8 kb fragment from pTB4100 created by digestion with *Sma*I and religation	This study
pTB4102	Ap^r; 4·8 kb *Hin*dIII–*Eco*RV fragment from pTB4100 cloned between *Hin*dIII–*Sma*I sites of pUCP20T	This study
pTB4103	Ap^r; 2·1 kb *Hin*dIII–*Eco*RV fragment from pTB4100 cloned between *Hin*dIII–*Sma*I sites of pUCP20T	This study
pTB4104	Ap^r; 2·8 kb fragment from pTB4100 created by digestion with *Sal*I–*Eco*RI, filling with Klenow fragment, and religation	This study
pTB4105	Ap^r; 2·3 kb PCR product with complete *acsA* gene cloned between *Bam*HI sites of pUCP20T (*acsA* orientation as P*lac* of pUCP20T)	This study
pTB4106	Ap^r; 2·3 kb PCR product with complete *acsA* gene cloned between *Bam*HI sites of pUCP20T (*acsA* opposite orientation to P*lac* of pUCP20T)	This study
pTB4107	Ap^r Km^r; 1·0 kb *Sma*I–*Sma*I fragment from pTB3131 containing Km^r gene of Tn5 cloned in the *Sma*I site of pTB4102	This study
pTB4108	Ap^r Km^r; 5·8 kb *Hin*dIII–*Eco*RI fragment from pTB4107 cloned between *Hin*dIII–*Eco*RI sites of pUC19	This study

* Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

1997), PROSITE for searching protein sequence motifs (Bairoch *et al.*, 1997), and *Pseudomonas* genome project version from 2.2.2000 (http://pseudomonas.bit.uq.edu.au/) for obtaining the DNA sequence of the *acsA* gene.

RESULTS AND DISCUSSION

Utilization of carbon sources

Mutants MS1 and MS8 grown in LB broth were inoculated into minimal media containing various carbon sources. In contrast to wild-type cells, ethanol, 2,3-butanediol and malonate did not support growth, whilst succinate and glucose supported growth of both wild-type and mutant cells. Acetate supported growth of both mutants; however, the lag phase and the generation time were increased and the final optical density reached was lower when compared to wild-type (Table 2).

Activity of acetate-activating enzymes

Wild-type *P. aeruginosa* was grown on various carbon sources, while mutants were grown on succinate, glucose or acetate. Cells were collected at late-exponential phase and disrupted. The supernatant after

Table 2. Growth of *P. aeruginosa* mutants on various substrates

Growth is recorded as final OD_{620} when reaching stationary phase. +++, $OD_{620} = 0{\cdot}9$–$1{\cdot}2$; ++, $OD_{620} = 0{\cdot}4$–$0{\cdot}5$; +, $OD_{620} = 0{\cdot}2$–$0{\cdot}3$; −, no growth; (+), as + but indicates a prolonged lag phase compared to the wild-type; ND, not determined. Substrate concentrations as indicated in Methods.

Substrate	Wild-type	Mutant					
		MS1	MS8	UK1	Complemented by:		
					pTB4102		pTB4105
					MS1	MS8	UK1
Ethanol	+++	−	−	−	+++	+++	+++
2,3-Butanediol	+++	−	−	ND	+++	+++	ND
Malonate	+	−	−	ND	+	+	ND
Succinate	+++	+++	+++	+++	ND	ND	ND
Glucose	+++	+++	+++	ND	ND	ND	ND
Acetate	++	(+)	(+)	(+)	++	++	ND

Table 3. Enzyme activities in cell-free extracts of wild-type and mutant strains of *P. aeruginosa* grown on or induced by various carbon sources

Strain	Carbon source		Specific activity [U (mg protein)$^{-1}$]*	
			ACS	ACK
Wild-type	Growth:	Ethanol	0·18†	0·53†
		2,3-Butanediol	0·22	0·53
		Malonate	0·16	0·59
		Succinate	ND	0·56
		Glucose	ND	0·47
		Acetate	0·18	0·26
	Induction:	Ethanol	0·28	0·60
MS1	Growth:	Succinate	ND	0·52
		Glucose	ND	0·40
		Acetate	ND	0·48
	Induction:	Ethanol	ND	0·56
MS1(pTB4106)	Growth:	Ethanol	1·05†	0·42†
UK1	Growth:	Succinate	ND	0·47
		Acetate	0·018‡	0·26‡
	Induction:	Ethanol	ND	0·48
UK1(pTB4105)	Growth:	Ethanol	0·56	0·48

ND, Not detected.

* All values are means of two independent experiments, except where indicated.

† These values are means calculated from three or four independently grown cell batches. Standard deviations of ±13% for ACS and ±27% for ACK were calculated.

‡ These activity measurements were performed with a high protein concentration of 6 mg ml^{-1}.

removal of cell debris and the supernatant after removal of membrane fragments by ultracentrifugation at 100000 ***g*** showed identical activities for ACS and ACK, indicating that the enzymes determined were soluble ones. The acyl-CoA synthesizing activity found with the wild-type after growth on ethanol was almost the same when propionate was used as substrate instead of acetate. However, with butyrate and caproate less than 15% of activity compared to acetate was found (data not shown).

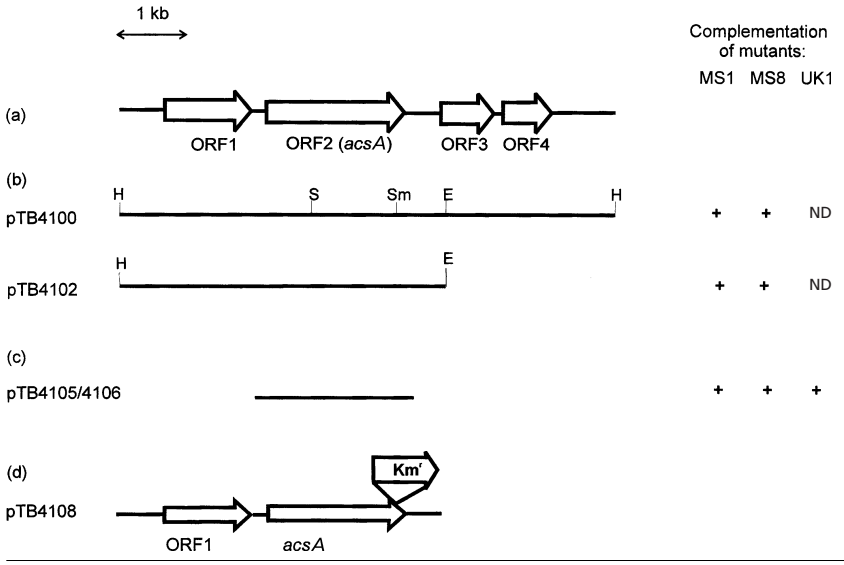

Fig. 2. Physical and restriction map of cloned genomic DNA fragments. *acsA*, acetyl-CoA synthetase gene; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; E, *Eco*RV; Km^r, kanamycin resistance; ND, not determined. (a) Map of the 6·9 kb *Hin*dIII fragment from pTB3018 containing four ORFs. (b) Fragments used to complement mutants. (c) PCR product of ORF2 (*acsA*). (d) Insert of pTB4108 with ORF1 and gene *acsA* interrupted by a Km^r cassette.

Wild-type *P. aeruginosa* expressed both ACS and ACK activity, but no PTA was found under any growth condition used (data not shown). In control experiments with cell-free extracts prepared from *E. coli* K-12 grown on glucose, PTA activity was easily demonstrated. ACS activity was induced on malonate, 2,3-butanediol, ethanol and acetate, while no activity could be detected on succinate and glucose (Table 3). In contrast, ACK activity is almost independent of the carbon source used.

Like wild-type cells, the mutants exhibited no detectable ACS activity after growth on succinate or glucose. In contrast to the wild-type, mutants also exhibited no detectable ACS activity after growth on acetate under standard test conditions. Mutant activities of ACK are similar to the specific activities of wild-type cells under similar conditions (Table 3).

The activity of ACS was also determined in the wild-type and mutant MS1 after induction on ethanol. Whilst wild-type cells displayed measurable ACS activity, the mutant did not. The ACK activity was the same (Table 3), but no PTA activity was found.

Subcloning and characterization of the *acsA* gene

Cosmid pTB3018 from a cosmid gene library of *P. aeruginosa* restored growth on ethanol to mutants MS1 and MS8. Plasmid pTB4100, carrying a 6·9 kb fragment derived from cosmid pTB3018, also complemented both mutants. Recently, we showed by sequencing a 6·7 kb fragment carrying the *exaABC* gene cluster that the DNA sequences of *P. aeruginosa* ATCC 17933 and *P. aeruginosa* PAO1 differ only slightly by about 2% (Schobert & Görisch, 1999; Diehl *et al.*, 1998). Therefore, we used the published PAO1 sequence to evaluate the relevant ATCC 17933 sequence. We sequenced 250 bp from the 5′- and 3′-end of the 6·9 kb insert of pTB4100. By comparison to the genome database of *P. aeruginosa* PAO1, we identified four ORFs. Further subclones were isolated and tested for complementation by triparental mating. Only pTB4102, with an intact ORF2, complemented both mutants MS1 and MS8 (Fig. 2). Clones carrying only the intact ORF2 were obtained by PCR. In pTB4105, ORF2 is oriented linear with the *lac* promoter of the vector pUCP20T, whilst in pTB4106, it is integrated in an antilinear orientation. Complementation with both plasmids restored growth on ethanol to mutants MS1 and MS8, indicating that the *P. aeruginosa* promoter of ORF2 is present in the 205 bp region in front of the start codon. We confirmed that the complementation occurred *in trans* and was not a result of a homologous recombination. Plasmid DNA was prepared from complemented mutant strains and used to transform *E. coli* JM109. The resulting transformants were used again in triparental matings to complement successfully the original mutants MS1 and MS8.

ORF2 shows high similarity to microbial ACS. The corresponding gene in the genome database of *P. aeruginosa* PAO1 is designated *acsA*. A putative Shine–Dalgarno sequence, GAGG, was found 8 bp upstream of the *acsA* gene start codon. A putative rho-independent transcription terminator was found 22 bp downstream of the translation stop codon TGA. The inverted repeat with a 10 bp stem was followed by a thymidine-rich sequence. The *acsA* gene is 1956 bp long and encodes a polypeptide of 652 amino acids. The deduced amino acid sequence revealed no N-terminal signal peptide, indicating that the resulting polypeptide likely is a cytosolic protein. The amino acid sequence shows a high similarity of 82% to ACS from *E. coli* (Kumari *et al.*, 1995). Similarities between 68% and 55% were found with the respective enzymes of *Alcaligenes eutrophus* (Priefert & Steinbüchel, 1992), *Saccharomyces cerevisiae* (van den Berg & Steensma, 1995), *Neurospora crassa* (Connerton *et al.*, 1990), *Methanothrix soehngenii* (Eggen *et al.*, 1991), *Pseudomonas putida* (Eaton, 1996) and *Bacillus subtilis* (Grundy *et al.*, 1993).

Proteins of the ACS family (Wang *et al.*, 1999) share two conserved amino acid sequence regions and both, motif

I and motif II, are found in the polypeptide sequence encoded by the *acsA* gene. Motif I represents the AMP-binding site of proteins that catalyse the reaction of ATP and carboxylic acids to acyl adenylates and the transfer of the acyl residue. The function of the conserved motif II is unknown.

Inactivation of the *acsA* gene

To demonstrate that the gene product of the *acsA* gene is essential for growth on ethanol, the gene was inactivated by site-directed mutagenesis using pTB4108 (Fig. 2d). A Km^r mutant, UK1, was obtained as described in Methods. The mutant was unable to grow on ethanol, and PCR with genomic DNA confirmed the presence of the kanamycin-resistance cassette in the *acsA* gene (*acsA*::Km^r) (data not shown). The Km^r gene is transcribed in the same orientation as the *acsA* gene. The *acsA*::Km^r allele in mutant UK1 does not express a dominant negative variant of ACS, since complementation with pTB4105, carrying only the *acsA* gene, restored wild-type growth on ethanol and led to a threefold higher expression of ACS activity compared to wild-type cells (Table 2 and Table 3). Like mutants MS1 and MS8, mutant UK1 showed poor growth on acetate (Table 2).

Expression of the *acsA* gene in complemented mutants MS1, MS8 and UK1

The *acsA* gene in *P. aeruginosa* indeed encodes an enzyme with ACS activity as demonstrated by the data shown in Table 3. Wild-type extracts showed ACS activity after induction by ethanol. In contrast, extracts of mutants MS1 and UK1 showed no detectable ACS activity under standard test conditions.

Mutants transformed by triparental mating with plasmid pTB4105 or pTB4106 were grown on ethanol. Cell-free extracts of the complemented mutants showed a three- to five-times higher specific activity of ACS compared to wild-type extracts (Table 3). The *acsA* gene encodes a polypeptide of 72 kDa. With cell-free extracts of induced wild-type cells, a 70 kDa polypeptide band is readily detected by SDS-PAGE, and cell-free extracts from complemented mutants showed an increased intensity of this 70 kDa band (data not shown).

Since mutant UK1, like mutants MS1 and MS8, grows on acetate, albeit poorly, we tried to detect low levels of ACS activity. Cell extract with a high protein concentration was prepared from UK1 cells grown on acetate, and up to 6 mg protein (ml assay mixture)$^{-1}$ was used. Under these conditions acetyl-CoA formation with a specific activity of about 10% of wild-type level was detected (Table 3). Apparently this low activity is sufficient to support poor growth of UK1 on acetate. The acetyl-CoA-forming enzyme in mutant UK1, however, showed a different substrate specificity to that observed with ACS in wild-type cells. While ACS in wild-type extracts activated acetate and propionate equally well, the acetyl-CoA-forming activity in UK1 extracts showed about twice the activity with propionate as substrate compared to acetate. With caproate, both, mutant and wild-type extracts, showed only about 15% of the activity compared to acetate (data not shown).

Concluding remarks

In the present paper, we demonstrate that the putative *acsA* gene of *P. aeruginosa* encodes an ACS activity. This enzyme, which so far has not been described in *P. aeruginosa*, is essential for growth on ethanol, 2,3-butanediol and malonate. In contrast, mutants with a defect in the *acsA* gene, however, can still grow on acetate, albeit poorly. By comparison with *P. aeruginosa* PAO1, where the presence of a second putative *acs* gene, *acsB*, was inferred from sequence similarities, a second *acs* gene may also be present in *P. aeruginosa* ATCC 17933. In cell extracts of mutant UK1, where the *acsA* gene is interrupted by the insertion of a kanamycin-resistance cassette, a residual low acetyl-CoA-forming activity was found, which shows a different substrate specificity compared to the wild-type *acsA* gene product. Whether this activity is caused by an acetyl-CoA-forming enzyme encoded by a putative *acsB* gene or whether the product of the *acsA*::Km^r allele shows a residual low activity with an accidentally modified substrate specificity is presently under investigation in our laboratory.

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REFERENCES

Aceti, D. J. & Ferry, J. G. (1988). Purification and characterization of acetate kinase from acetate-grown *Methanosarcina thermophila*. Evidence for regulation of synthesis. *J Biol Chem* **263**, 15444–15448.

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.

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

van den Berg, M. A. & Steensma, H. Y. (1995). ACS2, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. *Eur J Biochem* **231**, 704–713.

Berg, P. (1962). Assay and preparation of yeast aceto-CoA-kinase. *Methods Enzymol* **5**, 461–467.

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.

Brown, T. D. K., Jones-Mortimer, M. C. & Kornberg, H. L. (1977). The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. *J Gen Microbiol* **102**, 327–336.

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

Champine, J. E. & Goodwin, S. (1991). Acetate catabolism in the dissimilatory iron-reducing isolate GS-15. *J Bacteriol* **173**, 2704–2706.

Chang, D.-E., Shin, S., Rhee, J.-S. & Pan, J.-G. (1999). Acetate metabolism in a *pta* mutant of *Escherichia coli* W3110: importance of maintaining acetyl coenzyme A flux for growth and survival. *J Bacteriol* **181**, 6656–6663.

Connerton, I. F., Fincham, J. R. S., Sandeman, R. A. & Hynes, M. J. (1990). Comparison and cross-species expression of the acetyl-CoA synthetase genes of the ascomycete fungi, *Aspergillus nidulans* and *Neurospora crassa*. *Mol Microbiol* **4**, 451–460.

Diehl, A., von 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.

Eaton, R. W. (1996). P-Cumate catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA carrying the cmt operon. *J Bacteriol* **178**, 1351–1362.

Eggen, R. I. L., Geerling, A. C. M., Boshoven, A. B. P. & de Vos, W. M. (1991). Cloning, sequence analysis, and functional expression of the acetyl coenzyme A synthetase gene from *Methanothrix soehngenii* in *Escherichia coli*. *J Bacteriol* **173**, 6383–6389.

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.

Groves, W. E., Davis, F. C. & Sells, B. H. (1968). Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. *Anal Biochem* **22**, 195–210.

Grundy, F. J., Waters, D. A., Takova, T. Y. & Henkin, T. M. (1993). Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. *Mol Microbiol* **10**, 259–271.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Jetten, M. S. M., Stams, A. J. M. & Zehnder, A. J. B. (1989). Isolation and characterization of acetyl-coenzyme A synthetase from *Methanothrix soehngenii*. *J Bacteriol* **171**, 5430–5435.

Jones, M. E. & Lipmann, F. (1955). Aceto-CoA-kinase. *Methods Enzymol* **1**, 585–590.

Kumari, S., Tishel, R., Eisenbach, M. & Wolfe, A. J. (1995). Cloning, characterization, and functional expression of acs, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J Bacteriol* **177**, 2878–2886.

Oberlies, G., Fuchs, G. & Thauer, R. K. (1980). Acetate thiokinase and the assimilation of acetate in *Methanobacterium thermoautotrophicum*. *Arch Microbiol* **128**, 248–252.

Powlowski, J., Sahlman, L. & Shingler, V. (1993). Purification and properties of the physically associated meta-cleavage pathway enzymes 4-hydroxy-2-ketovalerate aldolase and aldehyde dehydrogenase (acylating) from *Pseudomonas* sp. strain CF600. *J Bacteriol* **175**, 377–385.

Priefert, H. & Steinbüchel, A. (1992). Identification and molecular characterization of the acetyl coenzyme A synthetase gene (*acoE*) of *Alcaligenes eutrophus*. *J Bacteriol* **174**, 6590–6599.

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

Rupp, M. & Görisch, H. (1988). Purification, crystallization 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.

Schobert, M. & Görisch, H. (1999). 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. *Microbiology* **145**, 471–481.

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.

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

Steinbüchel, A., Fründ, C., Jendrossek, D. & Schlegel, H. G. (1987). Isolation of mutants of *Alcaligenes eutrophus* unable to derepress the fermentative alcohol dehydrogenase. *Arch Microbiol* **148**, 178–186.

Taylor, I. J. & Anthony, C. (1976). Acetyl-CoA production and utilization during growth of the facultative methylotroph *Pseudomonas* AM1 on ethanol, malonate and 3-hydroxybutyrate. *J Gen Microbiol* **95**, 134–143.

Thompson, D. K. & Chen, J. S. (1990). Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from *Clostridium beijerinckii*. *Appl Environ Microbiol* **56**, 607–613.

Wang, S., Nakashima, S., Numata, O., Fujiu, K. & Nozawa, Y. (1999). Molecular cloning and cell-cycle-dependent expression of the acetyl-CoA synthetase gene in *Tetrahymena* cells. *Biochem J* **343**, 479–485.

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

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