Isolation and properties of acetate kinase- and phosphotransacetylase-negative mutants of *Thermoanaerobacter thermohydrosulfuricus*

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***Thermoanaerobacter thermohydrosulfuricus* (*Clostridium thermohydrosulfuricum*) ferments carbohydrate substrates to acetate, ethanol and L-lactate. To block acetate production, mutants defective in acetate kinase and/or phosphotransacetylase were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitroguanidine by selection for fluoroacetate resistance and subsequent counterselection for growth on pyruvate. Upon growth on glucose, all mutants formed only minor amounts of acetate and markedly less ethanol than the wild-type. The mutants produced L-lactate as the main fermentation product, yielding up to 1.8 mol lactate (mol glucose)⁻¹ as compared to 0.7 mol lactate (mol glucose)⁻¹ obtained with the parent strain. Expression of the genes encoding acetate kinase and phosphotransacetylase was found to be negatively controlled by acetate.**

Keywords: *Thermoanaerobacter*, mutagenesis, lactate, acetate kinase, phosphotransacetylase

INTRODUCTION

Thermoanaerobic bacteria have received considerable interest in recent years due to their ability to convert a wide range of carbohydrates into commodity chemicals (Canganella & Wiegel, 1993). However, the variety of products formed via branched fermentation pathways generally limits the usefulness of thermoanaerobes for production of bulk chemicals. Thus, the economic success of industrial-scale biotechnological processes will depend on directing the fermentation towards one or two major products (Rogers & Gottschalk, 1993).

Thermoanaerobacter thermohydrosulfuricus (*Clostridium thermohydrosulfuricum*) catabolizes hexoses to pyruvate via the Embden–Meyerhof–Parnas pathway. Subsequently, the catabolism branches off into reduction of pyruvate to L-lactate (Turunen *et al.*, 1987) and the ferredoxin-dependent cleavage of pyruvate to acetyl-CoA and CO₂ (Lovitt *et al.*, 1988). Acetyl-CoA is either reduced to ethanol or converted to acetate. Phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) and acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) catalyse the conversion of acetyl-

CoA and ADP to acetate and ATP, yielding 1 mol ATP (mol acetate produced)⁻¹. In addition, these enzymes catalyse the activation of acetate to acetyl-CoA, since both enzymic reactions are readily reversible.

The reversibility of acetate formation has been exploited for the isolation of acetate-negative mutants from *Escherichia coli* (Brown *et al.*, 1977) and *Clostridium thermosaccharolyticum* (Rothstein, 1986) by selection for fluoroacetate resistance. Fluoroacetate is taken up and converted to fluoroacetyl-CoA and further to fluorocitrate, which exerts a lethal effect by irreversibly binding to aconitase (Glusker, 1971). Thus wild-type bacteria are killed in the presence of fluoroacetate, whereas mutants unable to metabolize it survive. In this paper we report the isolation and characterization of fluoroacetate-resistant *T. thermohydrosulfuricus* mutants defective in acetate kinase and/or phosphotransacetylase.

METHODS

Organism and culture conditions. *T. thermohydrosulfuricus* DSM 570 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Bacteria were grown under anaerobic conditions in a modified reinforced clostridial medium (RCM) containing (l⁻¹): 3 g yeast extract, 5 g beef extract, 5 g tryptone, 10 g Bis-Tris, 1 mg resazurin and 15 g agar, when required. The pH of the medium

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; RCM, reinforced clostridial medium.

was adjusted to pH 7.2 with HCl. Glucose or pyruvate was added to autoclaved RCM to a final concentration of 10 g l⁻¹. Liquid cultures were grown at 68 °C without agitation. Plates were incubated anaerobically in sealed plastic bags in a steam-saturated atmosphere at 60 °C. For strain maintenance, early exponential phase cultures were stored in 50 % (v/v) glycerol at -20 °C.

Analysis of fermentation products. Glucose, pyruvate, ethanol, acetate and lactate were quantified by HPLC using a Bio-Rad HPX-87H column maintained at 45 °C by a thermostat and continuously degassed 5.0 mM H₂SO₄ at a flow rate of 0.8 ml min⁻¹ as eluant. Samples were prepared by centrifugation at 14000 g for 5 min, followed by filtration through 4 mm Nalgene syringe filters (0.2 µm pore). Volumes of 20 µl were injected manually by means of a sample loop. Eluting substances were quantified with an ERMA 7512 refractive index detector and the integration of peak areas was performed by a Hewlett Packard Integrator 3396A.

Mutagenesis and selection. Mutagenesis of growing cells was carried out according to Rothstein (1986). *T. thermohydrosulfuricus* culture (50 ml) in exponential growth phase (OD₆₀₀ = 0.9) was centrifuged at 1500 g for 10 min. After resuspension of the cell pellet in 10 ml RCM containing 1 % (w/v) glucose at 60 °C, fresh *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) solution (1 mg ml⁻¹ in 0.1 M sodium citrate buffer, pH 6.0, prewarmed to 60 °C) was added to a final concentration of 0.4 mg ml⁻¹. Mutagenesis was carried out for 3 min at 60 °C and stopped by rapidly chilling the cultures in an ice-water bath, followed by centrifugation (1500 g, 10 min, 4 °C) and two washing steps in ice-cold medium. Finally the washed cells were resuspended in 10 ml medium and incubated overnight at 60 °C for phenotypic expression.

Mutagenesis of resting cells was performed according to Murray *et al.* (1983). Exponentially growing cells from a 150 ml culture were harvested (1500 g, 10 min) and washed twice in 20 ml RCM without carbohydrate source, prewarmed to 60 °C. To exhaust endogenous nutrients, the washed cells were incubated in RCM at 60 °C for 2 h and then chilled to room temperature. Mutagenesis was accomplished by adding ice-cold MNNG solution to a final concentration of 0.4 mg ml⁻¹ and incubating the mixture with gentle shaking. After 20, 40 and 60 min, 6 ml aliquots were taken, rapidly chilled in ice-water and further manipulated as described above.

Selection for fluoroacetate resistance was carried out by plating on RCM agar plates containing 1 % glucose and 120 mM fluoroacetate. After an incubation of 4 d at 60 °C, single colonies were replica-plated on RCM agar plates containing 120 mM fluoroacetate and either 1 % glucose or 1 % (w/v) pyruvate as sole carbon source. Fluoroacetate-resistant colonies which showed no growth on pyruvate were chosen for further studies.

Preparation of cell extracts. Cells from late exponential phase were harvested by centrifugation (7000 g, 30 min, 8 °C), washed twice in 50 mM Tris/HCl, pH 8.0, containing 1 mM EDTA, and resuspended in the same buffer at 0.25 g ml⁻¹. Cells were incubated with lysozyme (1 mg ml⁻¹) for 30 min at 37 °C and disrupted by three passes through an Aminco French pressure cell (16000 p.s.i., 4 °C). After removing cell debris (25000 g, 30 min, 4 °C) the clear supernatant was stored at -20 °C.

Cell extracts (20 ml, 100–200 mg protein) were fractionated by chromatography on a Pharmacia Q-Sepharose Fast Flow column (16 × 100 mm) equilibrated with 20 mM Tris/HCl buffer at pH 8.0. Elution was performed with a 500 ml linear NaCl gradient (0–600 mM) in equilibration buffer at a flow rate of 2 ml min⁻¹. Fractions (8 ml) were collected and assayed for

enzyme activity. Protein concentrations were determined by the method of Sedmak & Grossberg (1977).

Enzyme assays. Acetate kinase assay mixtures (1 ml) containing 50 mM Tris/HCl, pH 7.5, 10 mM MgSO₄, 750 mM acetate, 1.16 M NH₂OH.HCl adjusted to pH 7.5 with KOH, were preincubated for 3 min at 60 °C. The reaction was started by the addition of 30 µl ATP (333 mM in 1 M Tris base) and stopped after 5 min incubation at 60 °C by transferring 300 µl of the assay mixture into 750 µl 77 mM FeCl₃ in 1 M HCl. Acetohydroxamate was quantified at 540 nm (Clark *et al.*, 1989). One unit of activity is defined as the amount of enzyme producing 1 µmol hydroxamate min⁻¹.

Phosphotransacetylase activity was determined at 25 °C by measuring the release of coenzyme A from acetyl-CoA with 5,5'-dithiobis(2-nitrobenzoic acid). Assay mixtures (1 ml) contained 100 mM potassium phosphate buffer, pH 7.4, and 80 µM 5,5'-dithiobis(2-nitrobenzoic acid). The reaction was started by the addition of 200 µM acetyl-CoA. The OD₄₁₂ of the nitrothiobenzoate anion was measured after 2 min incubation. One unit of activity is defined as the amount of enzyme liberating 1 µmol enzyme A min⁻¹.

Lactate dehydrogenase (L-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27) activity was determined by a modification of the method of Turunen *et al.* (1987). Assay mixtures (1 ml) contained 100 mM imidazole/HCl buffer, pH 6.4, 0.5 mM D-fructose-1,6-bisphosphate and 10 mM sodium pyruvate. The reaction was started by the addition of 0.2 mM NADH/H⁺. One unit of activity is defined as the amount of enzyme oxidizing 1 µmol NADH/H⁺ min⁻¹.

RESULTS

Mutagenesis and selection

Wild-type *T. thermohydrosulfuricus* is unable to grow at fluoroacetate concentrations above 60 mM. Upon mutagenesis with MNNG, two different colony types could be distinguished on agar plates containing 120 mM fluoroacetate: very small, clear colonies with diameters below 0.5 mm and larger, opaque colonies with diameters of 1–3 mm. Similar colony morphologies on fluoroacetate plates have been noted for mutagenized *C. thermosaccharolyticum* cultures and only large colonies were found to breed true for fluoroacetate resistance (Rothstein, 1986). Mutants defective in acetate kinase and/or phosphotransacetylase should be unable to grow on pyruvate as sole carbon source, since they cannot gain ATP via the acetate kinase reaction (Murray *et al.*, 1983). Therefore, only large colonies which showed no growth on pyruvate were selected for further investigation.

Mutagenesis of growing *T. thermohydrosulfuricus* cells, according to Rothstein (1986), yielded only one mutant. In contrast, 17 mutants were obtained upon resting cell mutagenesis (Murray *et al.*, 1983) whereby the efficiency of mutagenesis increased with the exposure time of cells to the mutagen.

Growth of acetate-negative mutants and distribution of fermentation products

The mutants grew with generation times comparable to that of the parent strain (50 min) but had longer lag phases and reached lower cell densities (Fig. 1). It should be

Fig. 1. Growth of *T. thermohydrosulfuricus* wild-type and acetate-negative mutants in serum vials containing 60 ml RCM/glucose at 68 °C without agitation. □, Wild-type parent; ▲, mutant 1001 (Pta⁻); ●, mutant 1057 (Ack⁻); ■, mutant 1061 (Ack⁻ Pta⁻). Experiments were performed in triplicate.

noted that a biphasic growth curve was reported previously for *Thermoanaerobacter ethanolicus* (*C. thermohydrosulfuricum* 39E) under non-shaking incubation conditions and attributed to hydrogen inhibition (Lovitt *et al.*, 1988). Upon reaching the stationary phase, all of the mutants rapidly lost viability. Analysis of fermentation products (Table 1) indicated that all mutants produced only minor amounts of acetate. Moreover, formation of ethanol was markedly lower than in the wild-type, whereas lactate production was unimpaired and 1.5–1.8 mol lactate were produced per mol glucose used. By HPLC analysis, no other fermentation products were detectable. The incomplete substrate consumption of the mutants was due to the acidification of the medium by lactic acid production resulting in a drop of pH to below 4.0 in serum vial cultures. Complete substrate utilization was achieved in pH-controlled fermentations (data not shown).

Enzyme activities of acetate-negative mutants

Acetate kinase and/or phosphotransacetylase activities in cell extracts from late exponential phase mutant cultures were drastically reduced to 1–4 % of the values obtained with wild-type cell extracts (Table 1). Furthermore, cell extracts of the Pta⁻ mutants consistently showed increased acetate kinase activity, whereas extracts of the Ack⁻ mutants had higher levels of phosphotransacetylase activity as compared to the wild-type. Strains selected for further studies included mutants defective in acetate kinase (1057) or phosphotransacetylase activity (1001) and a mutant (1061) defective in both activities.

For further analysis, cell extracts were fractionated by anion exchange chromatography and the fractions assayed for enzyme activities (Fig. 2). In comparison to the wild-type, the activity peaks of the defective enzymes are barely detectable, whereas the intact enzymes are present in increased amounts. No increase in lactate dehydrogenase activity was observed in mutant extracts.

Effect of acetate on enzyme production

The enhanced production of acetate kinase in Pta⁻ mutants and phosphotransacetylase in Ack⁻ mutants might indicate that the expression of both genes of the acetate pathway is negatively controlled by acetate. To investigate the influence of different acetate concentrations on enzyme production, fermentations were performed with addition of 10 mM or 100 mM acetate buffer, pH 7.2, prior to inoculation. Analysis of the fermentation products of wild-type cultures indicated that *T. thermohydrosulfuricus* produced less acetate and ethanol and more lactate in media containing acetate (data not shown). When the cultures reached the stationary phase, cell extracts were prepared and assayed for acetate kinase and phosphotransacetylase activities. The data shown in Table 2 demonstrate that acetate kinase and phosphotransacetylase activities are drastically reduced in media containing acetate. In the presence of 10 mM acetate, acetate kinase activity dropped to background levels,

Table 1. Fermentation products and enzyme activities of fluoroacetate-resistant *T. thermohydrosulfuricus* mutants

Cultures (60 ml) in serum vials were grown in triplicate at 68 °C in RCM medium containing 1 % glucose. Cells were harvested in late exponential phase for assay of enzyme activity or grown to stationary phase for analysis of fermentation products. Determination of fermentation products was performed in triplicate, SD values were less than 4 % of the means shown; enzyme activities were determined in duplicate, SD values were less than 8 % of the means shown.

Strain	Glucose used (g l ⁻¹)	Fermentation products (g l ⁻¹) [mol products (mol glucose) ⁻¹]			Carbon recovery (%)	Specific activity (mU mg ⁻¹)		Phenotype
		Lactate	Acetate	Ethanol		Acetate kinase	Phosphotransacetylase	
DSM 570	9.7	3.3 [0.68]	1.3 [0.40]	2.6 [1.05]	107	2700	2480	Wild-type
Mutant 1001	3.9	3.6 [1.85]	0.07 [0.05]	0.2 [0.20]	105	3190	90	Pta ⁻
Mutant 1057	4.2	3.2 [1.52]	0.09 [0.06]	0.3 [0.28]	93	80	3690	Ack ⁻
Mutant 1061	4.0	3.2 [1.60]	0.03 [0.02]	0.2 [0.20]	91	40	20	Ack ⁻ Pta ⁻

Fig. 2. Q-Sepharose anion exchange chromatography of *T. thermohydrosulfuricus* cell extracts from wild-type (a), mutant 1057 (b) and mutant 1001 (c). ▲, Acetate kinase; ●, phosphotransacetylase; □, lactate dehydrogenase; , protein.

whereas phosphotransacetylase activity was lowered to 10–20% of the control.

DISCUSSION

Mutagenesis of *T. thermohydrosulfuricus* with MNNG and selection for fluoroacetate resistance led to the isolation of mutants defective in acetate kinase and/or phosphotransacetylase, exhibiting an acetate-negative phenotype. This is in contrast to the fluoroacetate-resistant *C. thermosaccharolyticum* mutants obtained by Rothstein (1986), all but one of which produced wild-type levels of acetate. This phenotype presumably results from mutations in genes encoding proteins for (fluoro-)acetate uptake or metabolism of (fluoro-)acetyl-CoA, i.e. citrate synthase or aconitase. Junelles *et al.* (1987) even observed a twofold increase in acetate production by fluoroacetate-resistant mutants of *Clostridium acetobutylicum* and assigned it to their inability to re-metabolize the produced acids. Counterselection for growth on pyruvate is therefore a prerequisite for the selection isolation of fluoroacetate-resistant mutants defective in acetate production.

The production of acetate kinase and phosphotransacetylase in *T. thermohydrosulfuricus* was found to be negatively controlled by acetate. This finding explains the observation that single mutants tend to overexpress the respective intact enzyme of the acetate pathway (Table 1). Repression of acetate kinase biosynthesis by acetate has previously been observed in *C. acetobutylicum* (Ballongue *et al.*, 1986). It should be noted that the acetate kinase (*ack*) and phosphotransacetylase (*pta*) genes of *E. coli*, *Salmonella typhimurium* and *Methanosarcina thermophila* are organized as operons (Kakuda *et al.*, 1994; Van Dyk & LaRossa, 1987; Latimer & Ferry, 1993). Similarly, the butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*) genes of *C. acetobutylicum* ATCC 824 and *Clostridium*

Table 2. Effect of external acetate on enzyme production

Cultures (60 ml) in serum vials were grown in triplicate at 68 °C in RCM medium containing 1% glucose and acetate as indicated. Determination of enzyme activities was performed in duplicate, SD values were less than 8% of the means shown.

Strain	Acetate concn (mM)	Specific activity (mU mg ⁻¹)	
		Acetate kinase	Phosphotransacetylase
DSM 570 (wild-type)	0	2700	2480
	10	10	570
	100	3	280
Mutant 1001 (Pta ⁻)	0	3200	86
	10	6	4
	100	5	6
Mutant 1057 (Ack ⁻)	0	80	3690
	10	0	220
	100	17	40

beijerinckii (*C. acetobutylicum* NCIMB 8052) form operons (Walter *et al.*, 1993; Oultram *et al.*, 1993). Coregulation of the *ack* and *pta* genes of *T. thermohydrosulfuricus* suggests that these genes also constitute a transcription unit. The lower sensitivity of *pta* expression to acetate repression (Table 2) intimates that *pta* can be transcribed independently from *ack*, as has been observed for the *pta* gene of *E. coli* (Kakuda *et al.*, 1994).

Inactivation of the acetate pathway of *T. thermohydrosulfuricus* not only blocks acetate formation but also has an adverse effect on ethanol production (Table 1). It should be pointed out that the reduction of acetyl-CoA to ethanol requires two molecules of NAD(P)H, whereas only a single molecule of NADH is consumed in the reduction of pyruvate to lactate. A defect in acetate production will therefore result in an accumulation of acetyl-CoA under conditions where ethanol formation is limited by the supply of NAD(P)H. An increase in the level of acetyl-CoA might slow down the ferredoxin-dependent cleavage of pyruvate to acetyl-CoA and CO₂ and thus channel the carbon flux towards lactate formation. Considering the nearly identical properties of *Ack*⁻ and *Pta*⁻ mutants, a regulatory role of acetyl phosphate seems unlikely. *T. thermohydrosulfuricus* differs in this respect from enteric bacteria, where acetyl phosphate is thought to act as a global signal indicating an imbalance between glycolysis and acetyl-CoA utilization (McCleary *et al.*, 1993). On the other hand, acetyl-CoA was previously shown to activate the NAD(P)H:ferredoxin oxidoreductase of saccharolytic clostridia, thereby increasing the amount of NAD(P)H available for ethanol production (Jungermann *et al.*, 1973). Acetyl-CoA may therefore act as a key regulatory element for carbon and electron flux in *T. thermohydrosulfuricus*.

Lactate production of acetate-negative *T. thermohydrosulfuricus* mutants is comparable to that reported for *Clostridium thermolacticum*, the only thermoanaerobe producing lactic acid as the main fermentation product (Le Ruyet *et al.*, 1985). To achieve the theoretical maximum of 2 mol lactate (mol glucose)⁻¹, it will be necessary to obtain acetate-negative mutants with an additional defect in ethanol formation. Such *T. thermohydrosulfuricus* mutants carrying out a homolactic fermentation might be of interest for the industrial production of lactic acid (Canganella & Wiegel, 1993).

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