Cloning, sequence analysis, expression and inactivation of the *Corynebacterium glutamicum* *pta-ack* operon encoding phosphotransacetylase and acetate kinase

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The *Corynebacterium glutamicum* *ack* and *pta* genes encoding the acetate-activating enzymes acetate kinase and phosphotransacetylase were isolated, subcloned on a plasmid and re-introduced into *Corynebacterium glutamicum*. Relative to the wild-type, the recombinant strains showed about tenfold higher specific activities of both enzymes. Sequence analysis of a 3657 bp DNA fragment revealed that the *ack* and *pta* genes are contiguous in the corynebacterial chromosome, with *pta* upstream and the last nucleotide of the *pta* stop codon (TAA) overlapping the first of the *ack* start codon (ATG). The predicted gene product of *pta* consists of 329 amino acids (*M*, 35 242), that of *ack* consists of 397 amino acids (*M*, 43 098) and the amino acid sequences of the two polypeptides show up to 60% (phosphotransacetylase) and 53% (acetate kinase) identity in comparison with respective enzymes from other organisms. Northern (RNA) blot hybridizations using *pta*- and *ack*-specific probes and transcriptional *cat* fusion experiments revealed that the two genes are transcribed as a 2.5 kb bicistronic mRNA and that the expression of this operon is induced when *Corynebacterium glutamicum* grows on acetate instead of glucose as a carbon source. Directed inactivation of the chromosomal *pta* and *ack* genes led to the absence of detectable phosphotransacetylase and acetate kinase activity in the respective mutants and to their inability to grow on acetate. These data indicate that no isoenzymes of acetate kinase and phosphotransacetylase are present in *Corynebacterium glutamicum* and that a functional acetate kinase/phosphotransacetylase pathway is essential for growth of this organism on acetate.

Keywords: *Corynebacterium glutamicum*, acetate metabolism, acetate kinase, phosphotransacetylase, *pta-ack* operon

INTRODUCTION

Corynebacterium glutamicum and its subspecies are widely used for large-scale amino acid production (Leuchtenberger, 1996). This organism grows aerobically on glucose and on acetate as the sole carbon sources and both can also serve as substrates for the production of glutamate, lysine and threonine (reviewed by Kinoshita & Tanaka, 1972). Growth on, and amino

acid production from, acetate as the sole carbon source requires the uptake of acetate, its activation to acetyl-CoA and the operation of the glyoxylate shunt as an anaplerotic sequence (Clark & Cronan, 1996). Key enzymes of acetate metabolism in *Corynebacterium glutamicum* are acetate kinase, phosphotransacetylase and the glyoxylate-cycle enzymes isocitrate lyase and malate synthase (Ozaki & Shiio, 1969; Shiio *et al.*, 1969). Acetate kinase activates acetate to acetyl phosphate, which is subsequently converted to acetyl-CoA by phosphotransacetylase. Isocitrate lyase catalyses the cleavage of isocitrate to succinate and glyoxylate, and malate synthase condenses glyoxylate with acetyl-CoA

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to give malate. The latter enzymes are required to bypass the decarboxylation steps of the tricarboxylic acid cycle (Kornberg, 1966). Whereas not much is known about the acetate-activating enzymes of *Corynebacterium glutamicum*, the isocitrate lyase and malate synthase of this organism have been the subjects of intensive investigation. Both enzymes were purified and biochemically characterized and it turned out that their activities are controlled by allosteric regulation by a variety of intermediates of central metabolism (Reinscheid *et al.*, 1994a, b). Isolation and analysis of the *Corynebacterium glutamicum* genes encoding isocitrate lyase and malate synthase (*aceA* and *aceB*) revealed that the genes are clustered on the chromosome and oriented in opposite directions (Reinscheid *et al.*, 1994a, b). Further studies showed that the expression of *aceA* and *aceB* is regulated at the transcriptional level, resulting in high and low specific activities of both enzymes in the presence and absence, respectively, of acetate in the growth medium (Wendisch *et al.*, 1997). Acetate kinase and phosphotransacetylase of *Corynebacterium glutamicum* also show higher specific activities upon growth in the presence of acetate (Wendisch *et al.*, 1997). However, so far the basis for the different acetate kinase and phosphotransacetylase activities has not been investigated and it remains unclear whether the observed regulation is also due to transcriptional control of the respective genes.

To understand the molecular basis for acetate activation and its regulation in *Corynebacterium glutamicum*, we initiated studies on both the acetate kinase and the phosphotransacetylase of this organism. We describe the biochemical characterization of acetate kinase and phosphotransacetylase in extracts of *Corynebacterium glutamicum*, the isolation and analysis of the respective genes *ack* and *pta*, and we show data on the expression and regulation of the two genes under different growth conditions. Finally, we present data on the construction and characterization of defined *ack* and *pta* mutants of *Corynebacterium glutamicum*.

METHODS

Bacteria, plasmids and culture conditions. The bacterial strains and plasmids, their relevant characteristics and their sources are given in Table 1. The minimal medium used for *Corynebacterium glutamicum* has been described previously (Eikmanns *et al.*, 1991b) and contained 4% (w/v) glucose or 2% (w/v) potassium acetate. M9 medium (Sambrook *et al.*, 1989) containing 0.5% (w/v) glucose or 0.4% (w/v) potassium acetate was used as a minimal medium for *Escherichia coli* and LB medium (Sambrook *et al.*, 1989) was used as the complex medium for both organisms. For the growth of *E. coli* JRG1061, 2 mM tryptophan was added. When appropriate, ampicillin (100 µg ml⁻¹) or kanamycin (50 µg ml⁻¹) was added to the medium. *Corynebacterium glutamicum* was grown aerobically at 30 °C, *E. coli* at 37 °C.

DNA preparation, transformation and conjugation. The isolation of chromosomal DNA from *Corynebacterium glutamicum*, the isolation of plasmids from *E. coli* and *Corynebacterium glutamicum* and the transformation of both organisms were performed as previously described (Eikmanns *et al.*, 1994).

The conjugation between *E. coli* S17-1 and *Corynebacterium glutamicum* was performed as described by Schäfer *et al.* (1990), and transconjugants were selected on LB agar plates containing kanamycin (25 µg ml⁻¹) and nalidixic acid (50 µg ml⁻¹).

DNA manipulations. Restriction enzymes, T4 DNA ligase, Klenow polymerase, calf intestine phosphatase, proteinase K, DNase I, RNase A, T7 RNA polymerase and RNasin were obtained from Boehringer Mannheim and used as instructed by the manufacturer. Restriction-generated fragments were separated on 0.8% agarose gels and isolated and purified by using the Qiaex II Gel Extraction Kit from Qiagen.

DNA hybridization experiments were performed as previously described (Reinscheid *et al.*, 1994a). The 1.4 kb *Cla*I fragment isolated from pJU2 was labelled with digoxigenin-dUTP and used as a probe. Labelling, hybridization, washing and detection was performed using the non-radioactive DNA Labelling and Detection Kit and the instructions from Boehringer Mannheim.

For sequencing, the 3.3 kb *Xba*I–*Bam*HI fragment from pJU2 was ligated into pUC18 and, after restriction with either *Sph*I/*Xba*I or *Bam*HI/*Sac*I, progressive unidirectional deletions of the inserted DNA were created with the Erase-a-Base System from Promega. Appropriate subclones were sequenced using the AutoRead Sequencing Kit from Pharmacia with subsequent electrophoretic analysis with an ALF DNA sequencer from Pharmacia. The nucleotide sequence containing the terminator structure was determined using pJU2, appropriate primers and the AutoRead Sequencing Kit from Pharmacia. Sequence data were compiled and analysed with the HUSAR program package from EMBL.

RNA isolation and Northern hybridization. Total RNA from *Corynebacterium glutamicum* was isolated as described by Börmann *et al.* (1992). For hybridization, aliquots of the RNA were separated on an agarose gel containing 17% (v/v) formaldehyde and transferred onto a nylon membrane (Eikmanns *et al.*, 1994). The *pta*- and *ack*-specific antisense RNA probes were prepared by ligating the 0.49 kb *Eco*RI–*Sal*I *pta* fragment into pGEM-4Z and the 0.33 kb *Hind*III–*Kpn*I *ack* fragment into pGEM-3Z, linearizing the resulting plasmids with *Hind*III and *Eco*RI, respectively, and synthesizing digoxigenin-dUTP-labelled RNAs using T7 RNA polymerase and the RNA Labelling Kit from Boehringer Mannheim. Hybridization (at 46 °C, in the presence of 50% formamide), washing and detection was performed with the 'Nucleic Acid Detection Kit' from Boehringer Mannheim.

Construction of cat fusions. The promoter probe vector pEKpCm was used for construction of a transcriptional fusion of the *pta*–*ack* promoter region to the *cat* (chloramphenicol acetyltransferase) gene. The fusion was generated by insertion of the blunt-ended 1.26 kb *Xba*I–*Eco*RI fragment (see Fig. 1) into pEKpCm restricted with *Sal*I and blunt-ended by treatment with Klenow polymerase. The orientation of the insert was determined by restriction mapping. The copy number of the pEKpCm derivative was determined by the method described by Nesvera *et al.* (1997) and found to be comparable to the original vector, thus ruling out major errors in evaluation of the promoter activity caused by different *cat* gene dosage.

Gene disruption. The chromosomal *pta* and *ack* genes were disrupted by a method described by Schwarzer & Pühler (1991). A *pta*-internal 0.5 kb *Eco*RI–*Sal*I fragment and a 0.33 kb *ack*-internal *Hind*III–*Kpn*I fragment from pJU2 were ligated into the mobilizable *E. coli* vector pEM1, which is non-replicative in *Corynebacterium glutamicum*. The resulting

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44</i> , <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i>	Hanahan (1985)
JRG1061*	<i>ack-11</i> , <i>trpA9761</i> , <i>trpR72</i> , <i>iclR7</i> , <i>gal-25</i> , λ^-	Guest (1979)
S17-1	Mobilizing donor strain	Simon <i>et al.</i> (1983)
<i>C. glutamicum</i>		
WT	Wild-type strain ATCC 13032	American Type Culture Collection
13AK	Fluoroacetate-resistant mutant of strain WT, acetate kinase negative	Wendisch <i>et al.</i> (1997)
13PTA	Fluoroacetate-resistant mutant of strain WT, phosphotransacetylase negative	Wendisch <i>et al.</i> (1997)
13AK-PTA	Fluoroacetate-resistant mutant of strain WT, acetate kinase and phosphotransacetylase negative	Wendisch <i>et al.</i> (1997)
Cosmids/plasmids		
pHC79-based gene library	<i>C. glutamicum</i> WT chromosomal DNA cloned in cosmid pHC79	Börmann <i>et al.</i> (1992)
pHC3a	recombinant pHC79 cosmid able to complement <i>E. coli</i> JRG1061	This work
pJC1	<i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, Ap ^R Km ^R	Cremer <i>et al.</i> (1988)
pJU2	pJC1 containing 4.3 kb <i>Sau3AI</i> fragment from pHC3a	This work
pUC18	Cloning vector, Ap ^R	Vieira & Messing (1982)
pEKpCm	Promoter probe vector carrying the promoterless <i>cat</i> gene, Km ^R	Eikmanns <i>et al.</i> (1991a)
pEKpCm-DR2	pEKpCm containing 1255 bp <i>XbaI</i> - <i>EcoRI</i> fragment from pJU2	This work
pEM1	Integration vector, <i>oriV oriT</i> , Km ^R	Schrumpf <i>et al.</i> (1991)
pGEM-3Z/pGEM-4Z	Transcription vector carrying the T7 and SP6 promoters, Ap ^R	Promega

* Kindly provided by Barbara Bachmann, *E. coli* Genetic Stock Center, USA.

plasmids (pEM-pta and pEM-ack) were introduced into *Corynebacterium glutamicum* by conjugation from *E. coli* S17-1. To prove the integration of pEM-pta and pEM-ack at the chromosomal *pta*-*ack* locus of the *Corynebacterium glutamicum* transconjugants IN-pta and IN-ack, we performed Southern-blot analysis with the respective mutants. *EcoRI*-restricted chromosomal DNA from these strains was hybridized to the labelled 1.4 kb *ClaI* fragment, resulting in two signals at 2.3 kb and 3.8 kb with the DNA from strain IN-pta and one signal at 5.8 kb with the DNA from strain IN-ack. These sizes were expected from integration of pEM-pta within the *pta* gene locus and of pEM-ack within the *ack* gene locus of *Corynebacterium glutamicum*.

Enzyme assays. To determine enzyme activities in cell-free extracts, *Corynebacterium glutamicum* cells were grown in minimal medium to the exponential growth phase, washed twice in 20 ml 50 mM Tris/HCl buffer, pH 7, and resuspended in 1 ml of the same buffer containing 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT (except for determination of chloramphenicol acetyltransferase activity) and 30% (w/v) glycerol. After disruption of the cells by sonication (Eikmanns *et al.*, 1991b) and subsequent centrifugation for 30 min at 13000 g and 4 °C, the supernatant was used for the assays. The protein concentration was determined by the Biuret method (Gornall *et al.*, 1949) using BSA as the standard.

Acetate kinase activity was analysed in the acetyl phosphate forming direction as described by van Dyk & LaRossa (1987) in 1 ml 70 mM Tris/HCl, pH 7.6, 50 mM MgCl₂, 3 mM ATP, 3 mM phosphoenolpyruvate, 0.3 mM NADH, 7 U pyruvate kinase, 10 U lactate dehydrogenase, and 340 mM acetate as substrate. One unit of acetate kinase activity is defined as 1 μ mol NADH consumed per min at 30 °C.

Phosphotransacetylase activity was assayed by monitoring the conversion of acetyl phosphate to acetyl-CoA according to a modified method described by Brown *et al.* (1977). In a final volume of 1 ml, the assay contained 100 mM Tris/HCl, pH 7.6, 5 mM MgCl₂, 0.5 mM cysteine, 20 mM NH₄Cl, 3 mM CoA and 20 mM acetyl phosphate. The formation of acetyl-CoA was followed photometrically at 232 nm. One unit of activity corresponds to 1 μ mol acetyl-CoA formed per min at 30 °C.

Chloramphenicol acetyltransferase activity was assayed photometrically at 412 nm as described by Shaw (1975) in 1 ml 100 mM Tris/HCl, pH 7.8, 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM acetyl-CoA and 0.25 mM chloramphenicol. One unit of activity is defined as 1 μ mol chloramphenicol acetylated per min at 37 °C.

RESULTS

Acetate kinase and phosphotransacetylase activities in *Corynebacterium glutamicum*

The specific activities of acetate kinase and phosphotransacetylase were determined in cell-free extracts of *Corynebacterium glutamicum* WT grown on minimal medium containing glucose or acetate as the carbon source (Table 2). As already shown by Wendisch *et al.* (1997), the specific activities of both enzymes were about threefold higher in acetate-grown cells than in glucose-grown cells, confirming that the two enzymes are subject to coordinated regulation by the carbon source in the growth medium.

The analysis of the acetate kinase activity in extracts of *Corynebacterium glutamicum* WT revealed apparent K_m values of 7.9 mM for acetate and 0.23 mM for ATP, which are both somewhat lower than those reported for acetate kinases from *Methanosarcina thermophila* (Aceti & Ferry, 1988) and *Clostridium acetobutylicum* (Winzer *et al.*, 1997), and are in the same range as those reported for acetate kinases from *E. coli* and *Salmonella typhimurium* (Fox & Roseman, 1986). As for the acetate kinases from other organisms (Fox & Roseman, 1986), the *Corynebacterium glutamicum* enzyme required $MgCl_2$ (2 mM), which could be substituted by $MnCl_2$ at the same concentration. The enzyme activity was inhibited by $CaCl_2$ (10 mM led to an inhibition of about 50%) but not by NaCl and LiCl (up to 20 mM). The substrate specificity of the *Corynebacterium glutamicum* acetate kinase was tested with propionate, butyrate and succinate. The enzyme was active with propionate, although the apparent K_m was higher (15 mM) and the apparent V_{max} was about 25% lower than with acetate. When butyrate or succinate were tested as substrates, the activity was below the detection level of 0.01 U (mg protein)⁻¹. The *Corynebacterium glutamicum* enzyme was not active with GTP or ITP at concentrations similar to, or greater than, those required for saturation by ATP as has been shown for the respective enzymes from *E. coli* and *S. typhimurium* (Fox & Roseman, 1986).

Characterization of the phosphotransacetylase activity in extracts of *Corynebacterium glutamicum* WT revealed apparent K_m values of 0.13 mM for acetyl phosphate, which is about tenfold lower than that of phosphotransacetylases from most other bacteria (Shimizu *et al.*, 1969; Lundie & Ferry, 1989) and 0.4 mM for CoA, which is slightly higher than those reported for

the phosphotransacetylase enzymes from other organisms (Shimizu *et al.*, 1969; Rado & Hoch, 1973; Lundie & Ferry, 1989). As described for the *Bacillus subtilis* enzyme (Rado & Hoch, 1973), the phosphotransacetylase activity in *Corynebacterium glutamicum* was unaffected by pyruvate, isocitrate, succinate and malate at concentrations up to 10 mM.

Isolation and subcloning of the *ack* and *pta* genes from *Corynebacterium glutamicum*

The *Corynebacterium glutamicum* *ack* gene, encoding acetate kinase, was isolated by heterologous complementation of *E. coli* mutant JRG1061 using a *Corynebacterium glutamicum* WT cosmid gene library based on vector pH79. Due to its acetate kinase deficiency, strain JRG1061 is not able to grow on acetate minimal medium (Guest, 1979). Pooled recombinant cosmids were transformed into *E. coli* JRG1061 and, by testing about 2000 transformants for growth on acetate minimal medium, four clones were obtained which grew on acetate as the sole carbon source. After isolation of the four cosmids (pHC3, pHC3a, pHC8 and pHC9) and retransformation into *E. coli* JRG1061, the transformants again grew on acetate minimal medium, suggesting that the cosmids carried the *ack* gene. For subcloning purposes, cosmid pHC3a was partially digested with *Sau3A*I and fragments between 2 and 6 kb were ligated into the *Bam*HI site of the *E. coli*-*Corynebacterium glutamicum* shuttle vector pJC1. The ligation mixture was transformed into *E. coli* JRG1061 and transformants were again screened for growth on acetate minimal medium. By this procedure, five clones carrying plasmids able to complement the acetate kinase deficiency of *E. coli* JRG1061 were obtained. The

Table 2. Specific activities of acetate kinase and phosphotransacetylase in cell-free extracts of *Corynebacterium glutamicum* strains

The cells were grown in minimal medium (MM) containing glucose or acetate as the carbon source. The values are means $\pm$ SD obtained from at least three independent cultivations and two determinations per experiment. NG, No growth

Strain	Acetate kinase activity*		Phosphotransacetylase activity*	
	MM + glucose	MM + acetate	MM + glucose	MM + acetate
WT	0.36 $\pm$ 0.08	0.90 $\pm$ 0.11	1.28 $\pm$ 0.08	3.14 $\pm$ 0.15
WT/pJC1	0.34 $\pm$ 0.04	1.07 $\pm$ 0.09	1.36 $\pm$ 0.08	2.99 $\pm$ 0.12
WT/pJU2	3.64 $\pm$ 0.30	8.56 $\pm$ 0.45	10.14 $\pm$ 0.89	35.76 $\pm$ 4.56
13AK	< 0.01	NG	0.90 $\pm$ 0.09	NG
13AK/pJU2	2.19 $\pm$ 0.92	5.43 $\pm$ 1.42	6.23 $\pm$ 0.42	19.12 $\pm$ 4.01
13PTA	0.24 $\pm$ 0.04	NG	< 0.01	NG
13PTA/pJU2	2.25 $\pm$ 0.40	7.54 $\pm$ 0.82	8.65 $\pm$ 1.15	31.37 $\pm$ 3.45
13AK-PTA	< 0.01	NG	< 0.01	NG
13AK-PTA/pJU2	3.66 $\pm$ 0.42	10.27 $\pm$ 0.95	7.77 $\pm$ 0.97	28.34 $\pm$ 3.05
IN-ack	< 0.01	NG	1.15 $\pm$ 0.02	NG
IN-pta	< 0.01	NG	< 0.01	NG

* U (mg protein)⁻¹

Fig. 1. Restriction map of the *Corynebacterium glutamicum* chromosomal 4.3 kb *Sau3AI* fragment in pJU2. The arrows represent the computer-predicted coding regions of the *pta* and *ack* genes. The double-headed arrows indicate the fragments used for the preparation of the Northern hybridization probes. B, *Bam*HI; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nci*I; P, *Pst*I; S, *Sal*I; S3, *Sau3AI*; St, *Stu*I; X, *Xba*I. The *Xba*I site originates from the cloning site of the vector pJC1.

restriction map of the 4.3 kb insert from one of the plasmids, pJU2, is shown in Fig. 1.

To confirm the origin of the cloned DNA fragment in pJU2, genomic DNA from *Corynebacterium glutamicum* was analysed by Southern hybridization. For this purpose, the 1.4 kb *Cl*aI fragment from pJU2 was isolated, labelled with digoxigenin-dUTP and hybridized to *Eco*RI-restricted and size-fractionated chromosomal DNA from *Corynebacterium glutamicum*. The hybridization resulted in a single signal at 2.25 kb (not shown), which was expected from the restriction map of the pJU2 insert. This result confirms that the isolated DNA fragment corresponds to a fragment within the genome of *Corynebacterium glutamicum* with no detectable structural alteration.

In a previous study, we isolated fluoroacetate-resistant *Corynebacterium glutamicum* mutants which were unable to grow on acetate minimal medium and which were defective in either acetate kinase (strain 13AK), phosphotransacetylase (strain 13PTA) or in both enzymes (strain 13AK-PTA) (Wendisch *et al.*, 1997). To confirm that pJU2 carries the *ack* gene, pJU2 was tested for its ability to complement the acetate kinase-negative strain *Corynebacterium glutamicum* 13AK. Considering the fact that in *E. coli*, *S. typhimurium* and *Methanobrevibacterium thermophilum* the *ack* gene is located in the neighbourhood of the phosphotransacetylase gene *pta*, we also tested pJU2 for its ability to complement the phosphotransacetylase deficiency of strain 13PTA and the deficiency of both enzymes in the double mutant 13AK-PTA. After transformation with pJU2, all three mutants were able to grow on acetate as the sole carbon source. These results suggested that pJU2 not only carried the *ack* gene but also the *pta* gene of *Corynebacterium glutamicum*.

To ensure the presence of the *ack* and *pta* genes on the isolated DNA fragment, pJU2 was transformed into *Corynebacterium glutamicum* WT and the specific enzyme activities of the host and of the transformant, *Corynebacterium glutamicum* WT/pJU2, were determined (Table 2). After growth on glucose minimal

medium, the recombinant strain displayed seven- to tenfold higher specific activities of acetate kinase and phosphotransacetylase than did the host strain or its derivative carrying the cloning vector pJC1. Since the specific activities of acetate kinase and phosphotransacetylase in the *Corynebacterium glutamicum* WT were significantly higher when the cells were grown on acetate instead of glucose, it was interesting to study the activities in *Corynebacterium glutamicum* WT/pJU2 also after growth on acetate. As shown in Table 2, the specific activities of acetate kinase and phosphotransacetylase in *Corynebacterium glutamicum* WT/pJU2 were still about tenfold higher compared to the host when acetate was used for growth. We also measured the specific acetate kinase and phosphotransacetylase activities in cell-free extracts of the *Corynebacterium glutamicum* strains 13AK, 13PTA and 13AK-PTA with and without pJU2. As shown in Table 2, pJU2 conferred acetate kinase and phosphotransacetylase activity to all three mutants and the activities of the plasmid-carrying strains were about three- to fourfold higher when the cells were grown on acetate instead of glucose. These results show that the cloned fragment contained functional *ack* and *pta* genes including the structures necessary for expression and regulation in *Corynebacterium glutamicum*.

Nucleotide sequence of the DNA containing the *ack* and *pta* genes and amino acid sequence comparisons

The nucleotide sequence of a 3209 bp *Sau3AI*–*Bam*HI fragment from pJU2 was determined from both strands and has been deposited at the EMBL Nucleotide Sequence Database under the accession number X89084. Computer analysis revealed two ORFs extending from bp 1009 to 1995 (ORF1) and from bp 1998 to 3188 (ORF2), the last nucleotide of the TAA stop codon of ORF1 being the first nucleotide of ORF2. Both ORFs exhibited a codon usage corresponding to that of highly expressed corynebacterial genes (Eikmanns, 1992). The amino acid sequences encoded by ORF1 and ORF2 show significant identity to phosphotransacetylases and acetate kinases, respectively, from other organisms (see below). These results indicate that ORF1 represents the *pta* gene and ORF2 represents the *ack* gene of *Corynebacterium glutamicum*. The predicted *pta* and *ack* gene products consist of 329 amino acids with an M_r of 35 242 and 397 amino acids with an M_r of 43 098, respectively. To identify a possible terminator downstream of the *ack* gene, the nucleotide sequence of the 460 bp downstream of *ack* was determined and included in the sequence deposited at the EMBL database. Centred 34 bp downstream of *ack*, at positions 3206 to 3246 in the assembled sequence, a region of dyad symmetry followed by several T residues similar to rho-independent transcription terminators (Rosenberg & Court, 1979) was found. The mRNA hairpin loop predicted from this sequence has a ΔG (25 °C) of -20.8 kcal mol $^{-1}$ (-87.4 kJ mol $^{-1}$). This result indicates transcriptional termination downstream of the *ack* gene.

An alignment of the deduced amino acid sequences of

Fig. 2. Multiple amino acid sequence alignment of phosphotransacetylases from different organisms. Identical amino acids are boxed. The aligned sequences are from *Corynebacterium glutamicum* [pta-Cgl; SWISS-PROT (SP)/swiss-prot-Trembl (SPT) accession no. SPT P77844], *Mycobacterium tuberculosis* (pta-Mtu; SP P96254), *Synechocystis* sp. (pta-Ssp; SP P73662), *H. influenzae* (pta-Hin; SP P45107), *B. subtilis* (pta-Bsu; SP P39646), *E. coli* (pta-Eco; SP P39184), *Clostridium acetobutylicum* (pta-Cac; SP P71103) and *Methanosarcina thermophila* (pta-Mth; SP P38503). The numbers adjacent to species designation refer to the amino acid residue position at the beginning of the line.

Fig. 3. Alignment of the deduced acetate kinase sequences from different organisms. Identical amino acids are boxed. The aligned sequences are from *Corynebacterium glutamicum* (ack-Cgl; SWISS-PROT accession no. [SP] P77845), *Mycobacterium tuberculosis* (ack-Mtu; SP P96255), *E. coli* (ack-Eco; SP P15046), *B. subtilis* (ack-Bsu; SP P37877), *H. influenzae* (ack-Hin; SP P44406), *Clostridium acetobutylicum* (ack-Cac; SP P71104), *Synechocystis* sp. (ack-Ssp; SP P73162), *Methanosarcina thermophila* (pta-Mth; SP P38502) and *Mycoplasma genitalium* (ack-Mge; SP P47599). The numbers adjacent to species designation refer to the amino acid residue position at the beginning of the line.

the *Corynebacterium glutamicum* *ack* and *pta* gene products with sequences of phosphotransacetylases and acetate kinases from a variety of other bacteria is shown in Figs 2 and 3, respectively. Homology analysis by the

method of Myers & Miller (1988) revealed that the *Corynebacterium glutamicum* phosphotransacetylase shows a high degree of identity to phosphotransacetylase enzymes (or the carboxy-terminal parts of the respective

Fig. 4. Northern-blot analysis of the *Corynebacterium glutamicum* (a) *pta* (phosphotransacetylase) gene and (b) *ack* (acetate kinase) gene after growth of the cells on minimal medium containing acetate (lanes 1 and 2) or glucose (lanes 3) as a carbon source. Total RNA (6 µg in lanes 1 and 3; 0.86 µg (=1/7) in lanes 2) was electrophoresed and probed with *pta*-specific (a) or *ack*-specific (b) antisense RNA probes. The positions of RNA standards (Gibco-BRL) are shown on the left.

enzymes) from *Mycobacterium tuberculosis* (60%), *Synechocystis* sp. (57%), *Haemophilus influenzae* (49%), *B. subtilis* (47%), *E. coli* (47%), *Clostridium acetobutylicum* (44%) and *Methanosarcina thermophila* (42%). The deduced acetate kinase from *Corynebacterium glutamicum* showed a relatively high degree of identity to the acetate kinases from *Mycobacterium tuberculosis* (53%), *E. coli* (46%), *B. subtilis* (45%), *H. influenzae* (45%), *Clostridium acetobutylicum* (42%), *Synechocystis* sp. (42%), *Methanosarcina thermophila* (42%) and *Mycoplasma genitalium* (33%).

Northern-blot analysis of the *pta* and *ack* genes and transcriptional fusion experiments

RNA hybridization experiments were performed to identify the *pta* and *ack* mRNA transcript(s). For this purpose, total RNA from acetate- and from glucose-grown cells of *Corynebacterium glutamicum* WT was isolated and aliquots were electrophoresed, blotted onto a nylon membrane and hybridized to *pta*- and *ack*-specific digoxigenin-labelled antisense RNA probes. Hybridization to both probes resulted in a signal at about 2.5 kb (Fig. 4), which corresponds approximately to the total size of *pta* and *ack* (1 + 1.2 kb). This result indicates that the *pta* and *ack* genes from *Corynebacterium glutamicum* form a bicistronic operon. The concentration of the *pta-ack* transcript in acetate-grown

cells of *Corynebacterium glutamicum* was estimated to be four- to sixfold higher than in glucose-grown cells. This difference is slightly higher than the differences observed in the specific activities of acetate kinase and phosphotransacetylase on medium containing either glucose or acetate. However, the results indicate that the observed regulation of the two enzymes occurs primarily at the mRNA level.

To study whether the differences in the level of the *pta-ack* transcript in acetate- and glucose-grown cells were caused by transcriptional regulation (i.e. promoter control) or by different *pta-ack* mRNA degradation rates, a transcriptional fusion between the *pta-ack* promoter region and the promoterless *cat* gene was constructed in the promoter probe vector pEKplCm. The resulting plasmid, pEKplCm-DR2 (carrying the 1.26 kb *Xba*I-*Eco*RI fragment from pJU2), was transformed into *Corynebacterium glutamicum* WT and chloramphenicol acetyltransferase activity was determined in the recombinant strains after growth on acetate and glucose minimal medium. Whereas *Corynebacterium glutamicum* carrying the host vector pEKplCm showed no chloramphenicol acetyltransferase activity [<0.01 U (mg protein) $^{-1}$] on either medium, the strain carrying pEKplCm-DR2 showed 0.077 ± 0.010 U (mg protein) $^{-1}$ during growth on glucose and two- to threefold higher activity [0.186 ± 0.003 U (mg protein) $^{-1}$] during growth on acetate as the carbon source. The specific phosphotransacetylase and acetate kinase activities in these strains were comparable to those of the WT strain, i.e. also two- to threefold higher when the cells were grown on acetate instead of glucose (data not shown). These results and the results obtained by the Northern-hybridization experiments show that expression of the *Corynebacterium glutamicum pta-ack* operon is controlled at the transcriptional level and that this regulation is responsible for the different specific activities of phosphotransacetylase and acetate kinase observed in cells grown on acetate or glucose minimal medium.

Inactivation of the chromosomal *pta* and *ack* genes in *Corynebacterium glutamicum*

To study whether *Corynebacterium glutamicum* requires the *pta* and *ack* genes for growth, the chromosomal copies of both genes were disrupted in *Corynebacterium glutamicum* WT, resulting in strains IN-*ack* (*ack* disrupted) and IN-*pta* (*pta* disrupted). The two mutant strains were tested for their phosphotransacetylase and acetate kinase activities and for growth on acetate and glucose minimal medium. As shown in Table 2, *Corynebacterium glutamicum* IN-*ack* displayed no detectable acetate kinase activity and *Corynebacterium glutamicum* IN-*pta* displayed neither phosphotransacetylase nor acetate kinase activity. The lack of both enzyme activities in the latter strain was expected due to the organization of *pta* and *ack* as an operon. Both mutants were unable to grow on acetate as the sole carbon source, whereas they showed wild-type levels of growth on glucose minimal medium. These results

strongly indicate that *Corynebacterium glutamicum* WT possesses only one acetate kinase and one phosphotransacetylase enzyme and they show that the two enzymes are essential for growth of *Corynebacterium glutamicum* on acetate.

DISCUSSION

Acetate kinase and phosphotransacetylase genes are known from a variety of different bacteria, e.g. *E. coli* (Matsuyama *et al.*, 1989, 1994; Kakuda *et al.*, 1994), *Methanosarcina thermophila* (Latimer & Ferry, 1993), *Mycobacterium tuberculosis* (Bergh & Cole, 1994), *Clostridium acetobutylicum* (Boynton *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *Mycoplasma genitalium* (Fraser *et al.*, 1995), *Synechocystis* sp. (Kaneko *et al.*, 1996) and *B. subtilis* (Kunst *et al.*, 1997). However, the four latter sequences were identified during genome-sequencing projects solely by sequence similarity, and can therefore not be considered as being well analysed at the molecular level. The present study describes the genetic characterization of acetate kinase and phosphotransacetylase from *Corynebacterium glutamicum*, physiological effects after disruption of the respective genes, and the biochemical analysis of acetate kinase and phosphotransacetylase in cell-free extracts of this organism.

Chromosomal organization of the *ack* and *pta* genes

Analysis of the *Corynebacterium glutamicum* acetate kinase and phosphotransacetylase genes revealed they are clustered on the chromosome. Clustering has also been reported for the homologous genes from *Mycobacterium tuberculosis* (Bergh & Cole, 1994), *Clostridium acetobutylicum* (Boynton *et al.*, 1996), *E. coli* (Kakuda *et al.*, 1994) and *Methanosarcina thermophila* (Latimer & Ferry, 1993) whereas the *Mycoplasma genitalium* and *B. subtilis* *ack* and *pta* genes are separated by more than 80 kb (Fraser *et al.*, 1995; Kunst *et al.*, 1997). The arrangement of *ack* and *pta* in *Corynebacterium glutamicum* is in the same relative order as in *Mycobacterium tuberculosis*, *Methanosarcina thermophila* and *Clostridium acetobutylicum*, with *pta* located in front of *ack* (Bergh & Cole, 1994; Latimer & Ferry, 1993; Boynton *et al.*, 1996). The opposite arrangement of *ack* and *pta*, i.e. *ack* located in front of *pta*, was found in *E. coli* and *S. typhimurium* (Kwan *et al.*, 1988; Kakuda *et al.*, 1994). Taken together, the data indicate a high degree variation concerning the location and the arrangement of the *ack* and *pta* genes in different organisms.

Transcriptional organization and regulation of the *ack* and *pta* genes

Northern-blot analysis of total *Corynebacterium glutamicum* RNA revealed that in *Corynebacterium glutamicum* the *pta* and *ack* genes constitute an operon as has also been shown for the homologous genes from *Methanosarcina thermophila*, *Clostridium acetobutylicum* and *E. coli* (Boynton *et al.*, 1996; Kakuda *et al.*,

1994; Singh-Wissmann & Ferry, 1995). Moreover, our Northern analysis and the results of the *cat*-fusion experiment indicate that the *pta-ack* operon in *Corynebacterium glutamicum* is subject to transcriptional regulation. Singh-Wissmann & Ferry (1995) showed that expression of the *Methanosarcina thermophila* *pta-ack* operon is regulated by the growth substrate: the steady-state levels of *pta-ack* transcript were several-fold higher in acetate-grown cells than in cells grown on other substrates such as methanol, mono-, di- or trimethylamine. Transcriptional regulation was also shown for the acetate kinase gene from *B. subtilis* (Grundy *et al.*, 1993). However, in this organism the expression of *ack* is induced in the presence of excess glucose by a mechanism involving the catabolite-control regulator protein CcpA (Grundy *et al.*, 1993). In addition, *B. subtilis* *ack* mutants show unaltered growth on acetate but impaired growth on glucose (Grundy *et al.*, 1993). Therefore, it was suggested that acetate kinase and phosphotransacetylase in *B. subtilis* are not required for the utilization of acetate but for its formation in the presence of glucose. In *E. coli* and *S. typhimurium* the *ack-pta* operon is constitutively expressed, with only a modest (less than twofold) induction upon anaerobiosis or starvation conditions and there are no indications for induction by acetate (Brown *et al.*, 1977; Kwan *et al.*, 1988; Nyström, 1994; Clark & Cronan, 1996). This is in agreement with the observations that in *E. coli* the acetate kinase/phosphotransacetylase pathway operates in both directions and thus serves not only for growth on acetate as carbon source but also for acetate excretion (Huisman *et al.*, 1996) and for energy conservation during anaerobic growth on carbohydrates (Thauer *et al.*, 1977). In the present study, the defined *pta* and *ack* mutants of *Corynebacterium glutamicum* showed no impairment of growth on glucose but were unable to grow on acetate as the sole carbon source. Furthermore, our experiments revealed transcriptional induction of the *pta-ack* operon from *Corynebacterium glutamicum* by acetate. These data indicate that the acetate kinase/phosphotransacetylase pathway in *Corynebacterium glutamicum* mainly serves for acetate utilization.

Significance of acetate kinase and phosphotransacetylase

In *E. coli*, acetate can be activated not only by acetate kinase/phosphotransacetylase but also by acetyl-CoA synthetase, which directly converts acetate to acetyl-CoA (Brown *et al.*, 1977). While in *E. coli* the former pathway is required for growth on high concentrations of acetate, the acetyl-CoA synthetase is used when acetate is present at low concentrations (Kumari *et al.*, 1995). Testing of *Corynebacterium glutamicum* crude extracts for the presence of acetyl-CoA synthetase revealed no enzymic activity (data not shown) as has previously been reported for *Corynebacterium glutamicum* subsp. *flavum* (Ozaki & Shio, 1969). Although we cannot definitely exclude that *Corynebacterium glutamicum* possesses an acetyl-CoA synthetase, our results indicate that in *Corynebacterium glutamicum*

under our conditions, acetate is primarily activated by the acetate kinase/phosphotransacetylase pathway.

It is interesting to note that *Corynebacterium glutamicum* showed distinct acetate kinase and phosphotransacetylase activities, even when the cells were grown on glucose as the sole carbon source. One reason for the presence of both enzymes in the absence of acetate might be their requirement for acetate overflow during oxygen-limited growth (Dominguez *et al.*, 1993). Another reason for the background level of acetate kinase and phosphotransacetylase in the absence of acetate might be that in *Corynebacterium glutamicum*, efficient acetate activation is required for the induction of acetate kinase and phosphotransacetylase and also of the glyoxylate cycle enzymes isocitrate lyase and malate synthase (Wendisch *et al.*, 1997).

Coordinated transcriptional regulation of acetate kinase, phosphotransacetylase, isocitrate lyase and malate synthase

When *Corynebacterium glutamicum* grows in the presence of acetate, the cells show higher specific activities of all four key enzymes of acetate metabolism (acetate kinase, phosphotransacetylase, isocitrate lyase and malate synthase) than during growth on other substrates (Reinscheid *et al.*, 1994a, b; Wendisch *et al.*, 1997). In the cases of isocitrate lyase and malate synthase, this regulation has been shown to be exerted at the level of transcription of the respective *aceA* and *aceB* genes (Wendisch *et al.*, 1997). Our findings that acetate kinase and phosphotransacetylase in *Corynebacterium glutamicum* are also transcriptionally regulated in response to the presence of acetate in the growth medium may suggest that the four key enzymes of acetate metabolism are under common transcriptional control in this organism. Therefore, it can be speculated that a transcriptional regulator acts on the promoters of *aceA*, *aceB* and the *pta-ack* operon. However, the induction of the *aceA* and *aceB* genes was much stronger (100- and 50-fold, respectively) than that of the *pta-ack* operon (threefold). This might be caused by different affinities of the postulated transcriptional regulator to the respective operator regions of *aceA*, *aceB* and the *pta-ack* operon or by differences in the promoter region of the respective genes. Close inspection of the upstream regions of the *aceA*, *aceB* and *pta* genes did not reveal a common operator motif and further studies are necessary to unravel the mechanisms and the proteins involved in the transcriptional regulation of the genes encoding the enzymes involved in acetate metabolism of *Corynebacterium glutamicum*.

Comparison of the phosphotransacetylase and acetate kinase amino acid sequences from *Corynebacterium glutamicum* with those from other organisms

Structural analysis of the deduced phosphotransacetylase amino acid sequence from *Corynebacterium glutamicum* revealed a polypeptide of 329 amino acids

with an M_r of 35242. With this size, the phosphotransacetylase of *Corynebacterium glutamicum* is comparable to the homologous enzymes from *B. subtilis*, *Clostridium acetobutylicum* and *Methanosarcina thermophila* (Kunst *et al.*, 1997; Boynton *et al.*, 1996; Latimer & Ferry, 1993), whereas the phosphotransacetylase enzymes deduced from the *pta* sequences from *Mycobacterium tuberculosis*, *Synechocystis* sp., *H. influenzae* and *E. coli* consist of about 700 residues (M_r 75000–80000) (Bergh & Cole, 1994; Kakuda *et al.*, 1994; Fleischmann *et al.*, 1995; Kaneko *et al.*, 1996). The comparison of the *Corynebacterium glutamicum* enzyme with other phosphotransacetylases (Fig. 2) revealed a high degree of identity to the 'small' phosphotransacetylases and to the carboxy-terminal 330 amino acids of the 'large' enzymes. Highly conserved sequence motifs were found around two arginine residues (R⁹² and R³⁰⁸ in the *Corynebacterium glutamicum* sequence) which have been suggested to be essential for enzymic activity or for the binding of CoA in the *Methanosarcina thermophila* enzyme (R⁸⁷ and R³¹⁰, respectively, in the *Methanosarcina thermophila* enzyme) (Rasche *et al.*, 1997).

Amino acid sequence comparison of the deduced acetate kinase from *Corynebacterium glutamicum* with the respective enzymes from other organisms revealed high levels of similarity with respect to size and primary structure (Fig. 3). Recently, the three-dimensional structure of acetate kinase from *Methanosarcina thermophila* was elucidated (Buss *et al.*, 1997) and by site-directed mutagenesis, three glutamate residues were found to be essential for catalysis (E³⁸⁴) or for substrate binding (E⁹⁷ and E³⁸⁵) (Singh-Wissmann *et al.*, 1997). The alignment of all known acetate kinases revealed a 100% conservation of E³⁸⁴ and E³⁸⁵, emphasizing the relevance of these two residues proposed by Singh-Wissmann *et al.* (1997).

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