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Identification of the gene encoding the activator of (*R*)-2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans* by gene expression in *Escherichia coli*

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(*R*)-2-Hydroxyglutaryl-CoA dehydratase (HGDA/B) from *Acidaminococcus fermentans* requires an activator protein for activity. This activator (HGDC) has not yet been purified from its natural source due to its low concentration combined with an extreme sensitivity towards oxygen. Gene expression in *Escherichia coli* identified an open reading frame (780 bp) as the gene encoding HGDC. Dehydratase activity was stimulated at least tenfold by cell-free extracts of *E. coli* cells transformed with a plasmid carrying *hgdC*. On the chromosome the *hgdC* gene is located just before *hgdA* and *hgdB*.

(*R*)-2-Hydroxyglutaryl-CoA dehydratase (EC 4.2.1.-); Gene expression; Oxygen sensitivity; DNA sequence; *Acidaminococcus fermentans*

1. INTRODUCTION

Several anaerobic bacteria such as *Acidaminococcus fermentans* and *Fusobacterium nucleatum* contain enzymes catalysing the reversible elimination of water from (*R*)-2-hydroxyglutaryl-CoA to glutaconyl-CoA in a *syn*-manner, which is a key step in the fermentation of glutamate to ammonia, carbon dioxide, acetate, butyrate and hydrogen [1]. This reaction is of considerable mechanistic interest since it involves cleavage of a C-H bond in the β-position, which is not activated by the thiolester.

The (*R*)-2-hydroxyglutaryl-CoA dehydratase system (HGD) from *A. fermentans* consists of two components, the actual dehydratase and an activator. The dehydratase component HGDA/B is an $\alpha_2\beta_2$ heterotetramer (α 54 kDa, β 42 kDa) containing riboflavin and Fe–S clusters that can be purified from cell-free extracts of *A. fermentans* [2,3]. The purified dehydratase is not active; in order to get activity the dehydratase has to be incubated with ATP, $MgCl_2$, the artificial reducing agent Ti(III)citrate and an activator protein (HGDC), which is required in substoichiometric amounts. This activator protein is extremely oxygen-sensitive and cannot yet be purified from its natural source.

The genes for the two subunits of the dehydratase, *hgdA* and *hgdB*, have been cloned and sequenced [3]. These genes are clustered in a 'glutamate operon' preceeded by at least four additional open reading frames, one of which, *gcdA*, encodes the carboxytransferase subunit of glutaconyl-CoA decarboxylase, the consuctive enzyme of the same pathway [4]. Another of these open reading frames, ORF3 (780 bp), is located between *gcdA* and *hgdA*. This paper describes cloning, sequencing and identification by expression in *E. coli* of ORF3 as the gene encoding the activator HGDC.

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Abbreviations: HGD, (*R*)-2-hydroxyglutaryl-CoA dehydratase; ORF, open reading frame; PCR, polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. *Bacteria and phages*

Acidaminococcus fermentans ATCC 25085 [5]
Escherichia coli DH5α [6]
Phage EMBL12 [7]

2.2. *Plasmids*

pUC19 [8] was used for subcloning and sequencing, pAGK63 for sequencing and expression experiments. pAGK63 was derived from pJF118HE [9] by inserting PCR amplified ORF3 after digestion with *Eco*RI and *Hin*dIII.

2.3. *Oligonucleotides*

Oligo 1: 5′-GCT TAT CAG AAT CCG GAA AGC TTC TGC CCG TTC C-3′
Oligo 2: 5′-TTC AAC TAC ATC TCT GAA TTC CTG AAC GCC AG-3′
Heterologous restriction (*Hin*dIII, *Eco*RI) sites used for cloning of the PCR fragment are underlined.

2.4. *DNA Sequencing*

Subcloned fragments were sequenced with Sequenase 2.0 (USB).

2.5. *Preparation of cell-free* E. coli *extracts*

E. coli was aerobically cultivated at 37 °C on Standard I nutrient broth (Merck, Darmstadt) containing 0.1 mg/ml ampicillin. Gene expression was stimulated by adding 0.2 mM isopropyl-1-thio-β-D-galactoside after the culture had reached an optical density of $\Delta E_{578} = 1$.

After two hours of further aerobic incubation cells were harvested by centrifugation and lysed anaerobically by pressure. Cell debris were removed by centrifugation.

2.6. *Enzyme assay*

HGD activity was measured according to Klees et al. [10] with pure HGDA/B preparations which were obtained following the protocol of Schweiger et al. [2].

3. RESULTS AND DISCUSSION

Sequencing an EMBL12 clone from a genomic library of *A. fermentans* revealed an open reading frame (ORF3, 780 bp) between *gcdA* and *hgdA/hgdB*, which we supposed to encode the activator, hereafter called HGDC [4]. To test this we amplified ORF3 by PCR from genomic *A. fermentans* DNA [4]. The oligonucleotides used for this purpose (oligo 1 and 2) contained heterologous *Hin*dIII/*Eco*RI restriction sites to facilitate cloning of the PCR fragment into the expression vector pJF118HE to create pAGK63. Sequencing of the cloned PCR fragment confirmed the sequence obtained from the EMBL12 clone.

E. coli DH5α cells were transformed with pAGK63, and expression of HGDC was stimulated by adding up to 0.2 mM isopropyl-1-thio-β-D-galactoside after the culture had reached an optical density of $\Delta E_{578} = 1$. Anaerobically prepared cell-free extracts of transformed *E. coli* DH5α stimulated the activity of pure HGDA/B preparations at least tenfold (Fig. 1), indicating that ORF3 does in fact encode the activator HGDC. Control experiments showed that extracts of *E. coli* cells transformed with the vector pJF118HE did not stimulate dehydratase activity. HGDC produced in *E. coli*, like that from *A. fermentans*, was extremely sensitive towards oxygen. Cell-free *E. coli* extracts lost 99% of this HGDC activity in the presence of oxygen within one minute. Surprisingly, active HGDC could be obtained from aerobic as well as from anaerobic cultures, if cells were harvested during the exponential growth phase. Presumably the oxygen concentration inside *E. coli* is very low during the exponential growth phase. After entering stationary phase the oxygen concentration rises, whereby HGDC becomes inactivated.

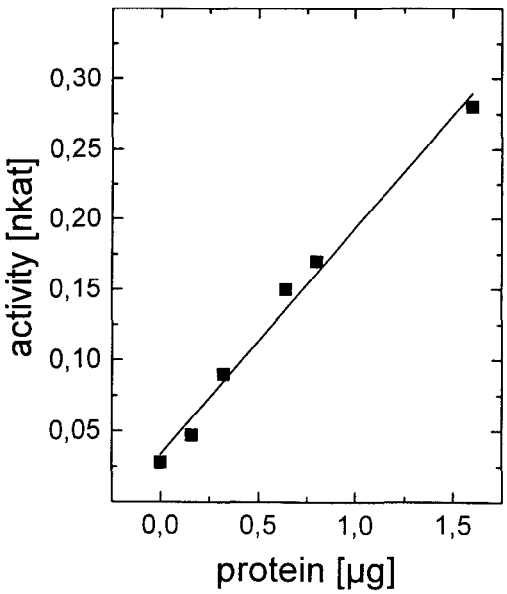

Fig. 1. Stimulation of pure HGDA/B preparation from *A. fermentans* by cell-free extracts of *E. coli* transformed with pAGK63. Each assay contained 5 mM $MgCl_2$, 0.15 mM ATP, 5 mM dithiothreitol, 0.1 mM acetylphosphate, 0.1 mM CoASH, 1 mM NAD^+, 0.14 mM Ti(III)citrate, 10 nkat glutaconyl-CoA decarboxylase and 0.2 mg auxiliary enzymes from *A. fermentans* [11]. Reactions were started by adding 1 mM (*R*)-2-hydroxyglutarate. No stimulating activity was measured in control experiments by testing either cell-free extracts exposed to air for two minutes or cell-free extracts from *E. coli* transformed with pJF118HE.

Sequencing of the cloned *hgdC* revealed an ATG start codon at position 1827 preceeded by a potential ribosome binding site highly homologous to those found for *gcdA* and *hgdA/hgdB* [3,4]. Therefore it appears likely that this ATG codon represents the translation start. Thus *hgdC* is 780 bp long and encodes the activator HGDC (27250 Da). Fig. 2 shows the complete DNA and derived amino acid sequences.

A protein data base research did not reveal any homologies of HGDC to other proteins (SWISSPROT 20). From the sequence it is not apparent why the activator is so oyxgen-labile. Since a plain protein should be much more stable, oxygen-sensitivity could be due to the presence of a prosthetic group. However, the prosthetic group, if there is one, would be derived from *E. coli* and correctly incorporated. Hopefully, the purification of HGDC from *E. coli* will turn out to be feasible.

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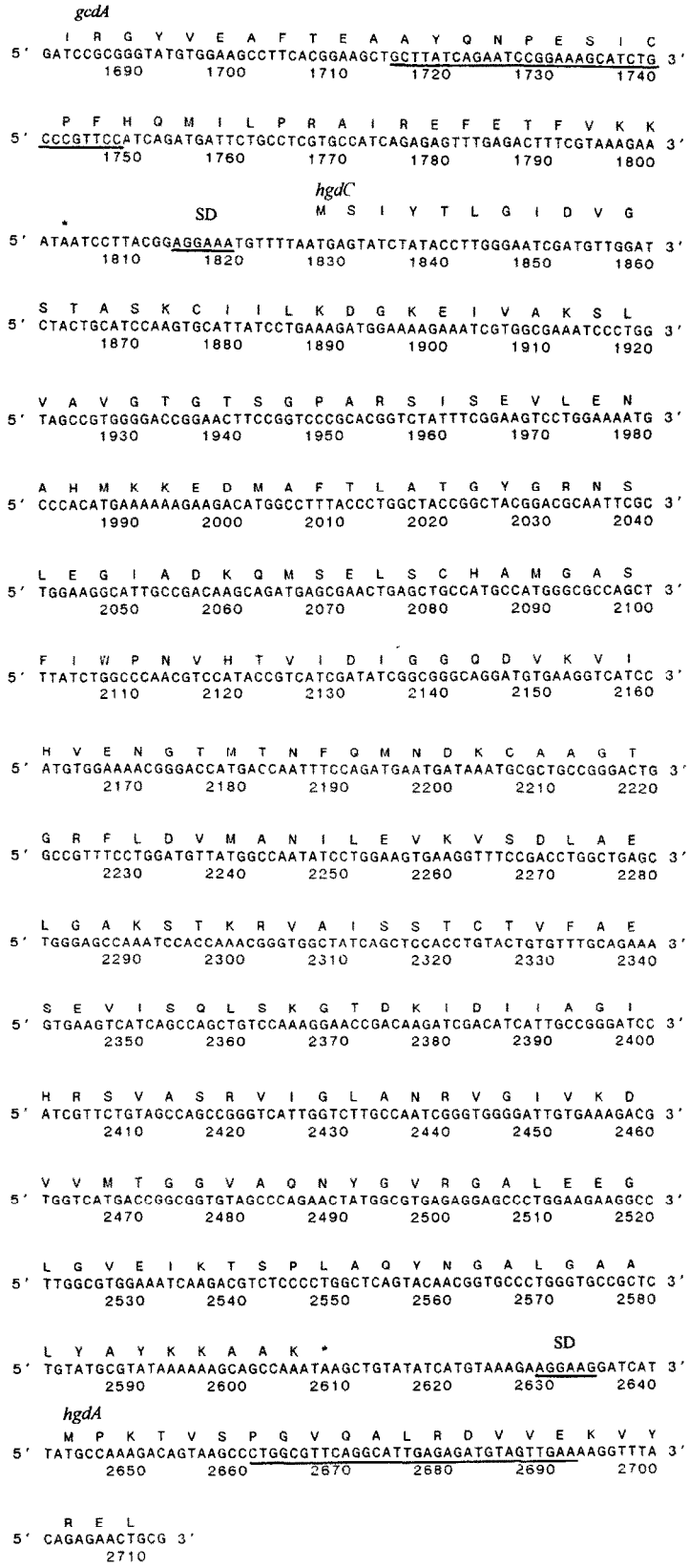

Fig. 2. Complete DNA and derived amino acid sequence of *hgdC*. *hgdC* (780 bp) is located between *gcdA* [4] and *hgdA/hgdB* [3] and codes for a protein of 27,250 Da. Putative ribosome binding sites (SD) and binding sites of the oligonucleotides used for PCR are underlined. The numbering of the nucleotides refers to Bendrat and Buckel [4].