

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

K. Bendrat, U. Müller, A.-G. Klees, W. Buckel\*

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität, Karl-von-Frisch-Straße, D-35032 Marburg, Germany

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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  $\beta$ -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 ( $\alpha$  54 kDa,  $\beta$  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<sub>2</sub>, 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' pre-

ceeded by at least four additional open reading frames, one of which, *gcdA*, encodes the carboxytransferase subunit of glutaconyl-CoA decarboxylase, the consecutive 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.

## 2. MATERIALS AND METHODS

### 2.1. Bacteria and phages

*Acidaminococcus fermentans* ATCC 25085 [5]

*Escherichia coli* DH5 $\alpha$  [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 *EcoRI* and *HindIII*.

### 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 (*HindIII*, *EcoRI*) 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- $\beta$ -D-galactoside after the culture had reached an optical density of  $\Delta E_{578} = 1$ .

\*Corresponding author. Fax: (49) (6421) 285833.

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

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 *HindIII/EcoRI* 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* DH5 $\alpha$  cells were transformed with pAGK63, and expression of HGDC was stimulated by adding up to 0.2 mM isopropyl-1-thio- $\beta$ -D-galactoside after the culture had reached an optical density of  $\Delta E_{578} = 1$ . Anaerobically prepared cell-free extracts of transformed *E.*

*coli* DH5 $\alpha$  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.

Sequencing of the cloned *hgdC* revealed an ATG start codon at position 1827 preceded 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 oxygen-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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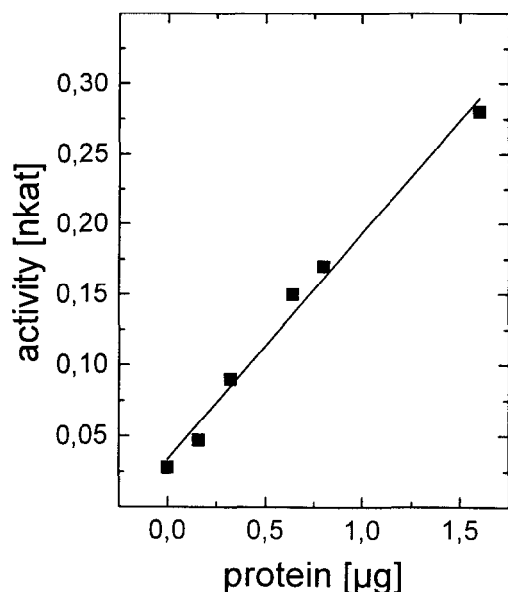


Fig. 1. Stimulation of pure HGDA/B preparation from *A. fermentans* by cell-free extracts of *E. coli* transformed with pAGK63. Each assay contained 5 mM MgCl<sub>2</sub>, 0.15 mM ATP, 5 mM dithiothreitol, 0.1 mM acetylphosphate, 0.1 mM CoASH, 1 mM NAD<sup>+</sup>, 0.14 mM Ti(III)citrate, 10 nkat glutaconyl-CoA 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.

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*gdaA*

I R G Y V E A F T E A A Y Q N P E S I C  
 5' GATCCGCGGGTATGTGGAAGCCTTCACGGAAGCTGCITATCAGAAATCCGGAAGCATCTG 3'  
 1690 1700 1710 1720 1730 1740

P F H Q M I L P R A I R E F E T F V K K  
 5' CCCGTTCCATCAGATGATTCTGCCTCGTCCATCAGAGAGTTTGAGACTTTCGTAAGAA 3'  
 1750 1760 1770 1780 1790 1800

*hgdC*

SD M S I Y T L G I D V G  
 5' ATAATCCTTACGGAGGAAATGTTTTAATGAGTATCTATACCTTGGGAATCGATGTTGGAT 3'  
 1810 1820 1830 1840 1850 1860

S T A S K K C I I L K D G K E I V A K S L  
 5' CTACTGCATCCAAGTGCATTATCCTGAAAGATGGAAAAGAAATCGTGGCGAAATCCCTGG 3'  
 1870 1880 1890 1900 1910 1920

V A V G T G T S G P A R S I S E V L E N  
 5' TAGCCGTGGGGACCGGAACCTCCGGTCCCGCACGGTCTATTTTCGGAAGTCTGGAAAATG 3'  
 1930 1940 1950 1960 1970 1980

A H M K K E D M A F T L A T G Y G R N S  
 5' CCCACATGAAAAAGAAGACATGGCCTTTACCTGGCTACCGGCTACGGACGCAATTTCGC 3'  
 1990 2000 2010 2020 2030 2040

L E G I A D K Q M S E L S C H A M G A S  
 5' TGGAAGGCATTGCCGACAAGCAGATGAGCGAAGTGAAGTGCATGCCATGGGCGCCAGCT 3'  
 2050 2060 2070 2080 2090 2100

F I W P N V H T V I D I G G Q D V K V I  
 5' TTATCTGGCCCAACGTCCATACCGTCATCGATATCGGCGGGCAGGATGTGAAGGTCACTCC 3'  
 2110 2120 2130 2140 2150 2160

H V E N G T M T N F Q M N D K C A A G T  
 5' ATGTGGAAAACGGGACCATGACCAATTTCCAGATGAATGATAAATGCGCTGCCGGGACTG 3'  
 2170 2180 2190 2200 2210 2220

G R F L D V M A N I L E V K V S D L A E  
 5' GCCGTTTCTCGGATGTTATGGCCAATATCCTGGAAGTGAAGGTTTCCGACCTGGCTGAGC 3'  
 2230 2240 2250 2260 2270 2280

L G A K S T K R V A I S S T C T V F A E  
 5' TGGGAGCCAAATCCACCAACGGGTGGCTATCAGCTCCACCTGTACTGTGTTTCAGAGAA 3'  
 2290 2300 2310 2320 2330 2340

S E V I S Q L S K G T D K I D I I A G I  
 5' GTGAAGTCATCAGCCAGCTGTCCAAAGGAACCGACAAGATCGACATCATTGCCGGGATCC 3'  
 2350 2360 2370 2380 2390 2400

H R S V A S R V I G L A N R V G I V K D  
 5' ATCGTTCTGTAGCCAGCCGGGTCTTGGTCTTGCCAATCGGGTGGGATTGTGAAGACG 3'  
 2410 2420 2430 2440 2450 2460

V V M T G G V A Q N Y G V R G A L E E G  
 5' TGGTCATGACCGGCGGTGTAGCCAGAACTATGGCGTGAGAGGAGCCCTGGAAGAAGGCC 3'  
 2470 2480 2490 2500 2510 2520

L G V E I K T S P L A Q V N G A L G A A  
 5' TTGGCGTGGAAATCAAGACGTCTCCCTGGCTCAGTACAACGGTGCCCTGGGTGCCGCTC 3'  
 2530 2540 2550 2560 2570 2580

SD  
 5' TGTATGCGTATAAAAAAGCAGCCAAATAAGCTGTATATCATGTAAAGAAGGAAGATCAT 3'  
 2590 2600 2610 2620 2630 2640

*hgdA*

M P K T V S P G V Q A L R D V V E K V Y  
 5' TATGCCAAAGACAGTAAGCCCTGGCGTTTCAGGCATTGAGAGATGATGTTGAAAAGGTTTA 3'  
 2650 2660 2670 2680 2690 2700

R E L  
 5' CAGAGAACTGCG 3'  
 2710

Fig. 2. Complete DNA and derived amino acid sequence of *hgdC*. *hgdC* (780 bp) is located between *gdaA* [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].