

A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria

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Bacterial butyryl-CoA CoA-transferase activity plays a key role in butyrate formation in the human colon, but the enzyme and corresponding gene responsible for this activity have not previously been identified. A novel CoA-transferase gene is described from the colonic bacterium *Roseburia* sp. A2-183, with similarity to acetyl-CoA hydrolase as well as 4-hydroxybutyrate CoA-transferase sequences. The gene product, overexpressed in an *Escherichia coli* lysate, showed activity with butyryl-CoA and to a lesser degree propionyl-CoA in the presence of acetate. Butyrate, propionate, isobutyrate and valerate competed with acetate as the co-substrate. Despite the sequence similarity to 4-hydroxybutyrate CoA-transferases, 4-hydroxybutyrate did not compete with acetate as the co-substrate. Thus the CoA-transferase preferentially uses butyryl-CoA as substrate. Similar genes were identified in other butyrate-producing human gut bacteria from clostridial clusters IV and XIVa, while other candidate CoA-transferases for butyrate formation could not be detected in *Roseburia* sp. A2-183. This suggests strongly that the newly identified group of CoA-transferases described here plays a key role in butyrate formation in the human colon.

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INTRODUCTION

The microbial community inhabiting the human large intestine produces a range of metabolic products (Macfarlane & Gibson, 1997). The fermentation acid butyrate is of special interest, as it has been shown to serve as the preferred energy source for the gut wall and also influences cell differentiation and apoptosis in the colon, and this seems to aid in protection against colon cancer and inflammatory bowel disease (Pryde *et al.*, 2002; Wächtershäuser & Stein, 2000). A number of butyrate-producing bacteria belonging to the low-G+C-content Gram-positives have been isolated from the human colon (Barcenilla *et al.*, 2000; Louis *et al.*, 2004). Recent advances in molecular techniques have revealed that they are among the predominant bacterial groups within the human large intestine (Blaut *et al.*, 2002).

Abbreviation: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are: *Roseburia* sp. A2-183 CoA-transferase, AY796317; *Eu. hallii* L2-7 CoA-transferase, DQ072258; *F. prausnitzii* A2-165 CoA-transferase, DQ072259; *A. caccae* L1-92 CoA-transferase I, DQ151450; *A. caccae* L1-92 4-hydroxybutyrate dehydrogenase and CoA-transferase II, DQ151451.

The biosynthetic pathway for butyrate formation, including the respective genes, has been described for clostridia that are mainly found outside the gut environment, such as the solventogenic bacterium *Clostridium acetobutylicum*. Here, a central pathway leads from acetyl-CoA to butyryl-CoA (Bennett & Rudolph, 1995; Boynton *et al.*, 1996), and a CoA-transferase that acts on acetoacetyl-CoA with either acetate or butyrate as second substrate is involved in the switch from acetogenic to solventogenic fermentation (Wiesenborn *et al.*, 1989). Butyryl-CoA is converted to butyrate in a two-step reaction with the intermediate formation of butyryl-phosphate (Walter *et al.*, 1993). Until recently, it was generally believed that these two enzymes were also responsible for butyrate formation in gut bacteria (Macfarlane & Gibson, 1997; Miller & Wolin, 1979). However, studies on isolates from the rumen and the human large intestine have indicated that, instead, a CoA-transferase is utilized by some of these bacteria for the formation of butyrate (Diez-Gonzalez *et al.*, 1999; Asanuma *et al.*, 2003; Duncan *et al.*, 2002). We have shown recently that this reaction is the only available route for butyrate synthesis in the majority of human gut isolates (Louis *et al.*, 2004). However, the characteristics of this enzyme have never been reported, and the corresponding gene remains elusive to date.

Several CoA-transferases have been characterized in a range of bacteria (Barker *et al.*, 1978; Buckel *et al.*, 1981; Scherf & Buckel, 1991; Schweiger & Buckel, 1984; Sramek & Frerman, 1975; Tung & Wood, 1975; Wiesenborn *et al.*, 1989). These enzymes tend to have a relatively broad substrate specificity, and butyrate and acetate, or their respective CoA-esters, are often among the substrates utilized *in vitro*. The corresponding genes for some of the enzymes have been identified (Fischer *et al.*, 1993; Gerhardt *et al.*, 2000; Selmer *et al.*, 2002). A comparison of the gene sequences as well as the subunit structure of CoA-transferases shows a surprising diversity, despite the shared reaction mechanism of these enzymes and their overlapping substrate specificities. Here, we describe the identification of a butyryl-CoA CoA-transferase gene from the human colon bacterium *Roseburia* sp. A2-183 (Barcenilla *et al.*, 2000) and the biochemical characterization of the corresponding enzyme.

METHODS

Bacterial strains and culture conditions. Human faecal bacteria used in this study were isolated and grown as described previously (Louis *et al.*, 2004). Accession numbers of the National Collection of Industrial and Marine Bacteria (NCIMB) are as follows: *Roseburia* sp. A2-183, NCIMB 14029; *R. intestinalis* L1-82, NCIMB 13810; *Roseburia* sp. A2-194, NCIMB 14030; *Roseburia* sp. M72/1, NCIMB 14031; *Anaerostipes caccae* L1-92, NCIMB 13811. *C. acetobutylicum* DSM 792 and *Clostridium propionicum* DSM 1682 were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ). *Escherichia coli* XL-1 Blue MRF was purchased from Stratagene. To study growth on alternative carbon sources, YCFA medium (Duncan *et al.*, 2002) containing either 10 g γ -aminobutyrate l^{-1} or 100 mM succinate and 300 mM ethanol as sole carbon source was inoculated with cultures grown in YCFA medium containing 2 g l^{-1} glucose, cellobiose and soluble starch, and the optical density was followed on a Novaspec II photometer (Pharmacia Biotech) at 650 nm. Growth experiments were performed in triplicate. *E. coli* XL-1 Blue MRF was grown on LB agar plates (10 g tryptone l^{-1} , 5 g yeast extract l^{-1} , 5 g NaCl l^{-1} , 15 g agar l^{-1}), containing 100 μ g ampicillin ml^{-1} , after transformation of recombinant plasmids.

DNA cloning, sequencing and sequence analysis. A random genomic library of *Roseburia* sp. A2-183 was generated with a pSMART-LCAmp blunt cloning kit (Lucigen). Inserts were amplified with SL1up (TGAAGGTGAGCCAGTGAGTTG) and SR2down (CTTCTGCTATGGAGGTCAGG) primers and sequencing was performed with nested primers SL1mod (TTACGCTGGAGTC-TGAGGCT) and SR2 (GGTCAGGTATGATTTAAATGGTCA) on a Beckman capillary sequencer. Sequences were assembled using the Phred, Phrap and Consed programs (Ewing & Green, 1998; Ewing *et al.*, 1998; Gordon, 2004) and putative genes were identified by BLAST search against database entries in GenBank (Altschul *et al.*, 1990). Sequence alignments were performed using CLUSTALW through the BCM Search launcher (Smith *et al.*, 1996). Phylogenetic analysis was performed using the UK Human Genome Mapping Project Computing Services at www.hgmp.mrc.ac.uk (this service is no longer available online). Amino acid sequences were aligned with CLUSTALW through the interface MAGI, and a phylogenetic tree was constructed with PROTDIST through the interface PIE (distance matrix program neighbour, distance model Kimura, 100 times bootstrapping).

CoA-transferase genes were cloned according to standard protocols (Ausubel *et al.*, 1994) into restriction sites *NcoI* and *SmaI* of vector

pIVEX2.3d (Roche) after amplification from bacterial cells. Primers and restriction sites (underlined) used were: pIVEXCoATF (GACTGACCATGGATTTTCGTGAAGAATAC, *NcoI*) and pIVEXCoATR3 (GACTGACCCGGGGCGGTTGCTTCTCTCCAG, *SmaI*) for *Roseburia* sp. A2-183; L192CoATIoeF (GATTACACATGTCATTTAAGGAAG-AATACC, *BspLU11I*) and L192CoATIoe3R (ATCGATCCCGGGTTT-GTTGGATCTTCTCCAGATTG, *SmaI*) for *A. caccae* CoA-transferase I; L192CoATIoeF (GAGATATCATGAGAGATAAGAAGGACAAGC, *RcaI*) and L192CoATIoe3aR (ATCGATAGTACTTTCGTCATCAC-TCCAGGCTTC, *ScaI*) for *A. caccae* CoA-transferase II. The sequence of the recombinant genes was confirmed by sequencing the inserts on a Beckman capillary sequencer with T7 vector primers and internal primers.

Degenerate PCR and genome walking. The following protein sequences related to CoA-transferase sequences were selected for alignments to determine conserved regions suitable for degenerate primer design. *Roseburia* sp. A2-183 CoA-transferase (AY796317): *Desulfitobacterium hafniense* (ZP_00098805, ZP_00099788), *Clostridium kluyveri* (P38942), *Clostridium tetani* (NP_781174), *Archaeoglobus fulgidus* (NP_069974) and *Yersinia pestis* (NP_405485). *C. acetobutylicum* CtfB (P23673): *Clostridium beijerinckii* (AF157306_3), *Streptococcus pyogenes* (NP_268527, NP_269686), *Streptomyces coelicolor* (T35020), *Streptomyces* sp. (T47110), *E. coli* (NP_416726) and *Haemophilus influenzae* (NP_438932). *C. propionicum* propionate CoA-transferase (CAB77207): *Clostridium perfringens* (NP_561012), *C. tetani* (NP_781170, NP_781374), *Bradyrhizobium japonicum* (NP_767528), *Listeria innocua* (NP_471607) and *Fusobacterium nucleatum* (NP_603711). Degenerate primers were designed by visual inspection and contained a non-degenerate clamp region at the 5' end, based on the sequence from *Roseburia* sp. A2-183 [AY796317, primers CoATDF1 (AAGGATCTCGGIRTICAYWSIGARATG) and CoATDR2 (GAGGTCGTCICKRAIITYIGGRTGNGC)], *C. acetobutylicum* [P23673, primers CTFBfor1 (GTAACACTTIGGIR-TIGGIYTNCCNAC) and CTFBrev4 (AACAGTAACACTCIAYR-TGICCNCCNC)] or *C. perfringens* [NP_561012, primers PCTfor1 (GTAGGATTARRIACITWYRTIGAYCC) and PCTrev2 (TCCACCA-CCATCRTARSARTCRAAYTG)]. Amplification with whole bacterial cells, as described previously (Louis *et al.*, 2004), was performed with a ramped annealing approach (Skantar & Carta, 2000). The following conditions were used: initial denaturation (2 min at 94 °C), then 35 cycles of denaturation (30 s at 94 °C), annealing (20 s at 55 °C, 5 s at 50 °C, 5 s at 45 °C, 5 s at 40 °C), elongation (1 min at 72 °C), and a final extension (10 min at 72 °C). Degenerate PCR products were cloned into pGEM-T Easy (Promega) and sequenced as described previously (Louis *et al.*, 2004).

Genome walking to obtain full-length ORFs from degenerate PCR products was performed by inverse PCR (Ochman *et al.*, 1988). Briefly, genomic DNA was digested with various restriction enzymes and religated. The ligation mixes were amplified with primers reading outward from known sequences to obtain genomic regions flanking the CoA-transferase gene, and the full-length genes were amplified and sequenced in both directions.

Protein overexpression and purification. Recombinant proteins were overexpressed with an RTS-500 kit (Roche) according to the manufacturer's instructions. Overexpressed proteins were dialysed in a Slide-A-Lyser cassette (Pierce) with a 10 kDa cut-off membrane against 500 ml of 200 mM potassium phosphate buffer (pH 7.2) for 2 h, and then overnight. The enzymes were purified with a Ni-NTA Spin kit (Qiagen) and eluted with 50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl, 250 mM imidazole and 100 mM EDTA.

The endogenous CoA-transferase from *Roseburia* A2-183 was partially purified from cells grown at 37 °C under anaerobic conditions on the synthetic medium YCFAGSC (Duncan *et al.*, 2002). Bacterial cells

were collected by centrifugation (6000 g, 15 min, 4 °C), and cell pellets were resuspended in potassium phosphate buffer (50 mM, pH 7) and disrupted by sonication (MSE Soniprep, setting 150 for 5 min). The cell lysate was passed over a weak anion exchange column (DEAE Sepharose Fast Flow, Amersham BioSciences) that was equilibrated with phosphate buffer at a flow rate of 0.5 ml min⁻¹. Elution of proteins with a linear gradient of ammonium sulphate (0–120 mM) in phosphate buffer was monitored with a UV spectrophotometer (LKB Bromma 2238 uvicord II). Fractions containing enzyme activity as determined below were pooled.

CoA-transferase assays. CoA-transferase activity was determined using the citrate synthase assay (Scherf & Buckel, 1991) at 410 nm wavelength, 25 °C and pH 7, and adapted for microtitre plates, with butyryl-CoA (100 µM) and acetate (50 mM) as substrates, unless stated otherwise. For the determination of the pH optimum of the enzyme, the potassium phosphate buffer was replaced by 100 mM Bis-Tris propane. Protein concentrations were determined with a bicinchoninic acid kit (Pierce). All data are the mean and standard deviation of at least three replicates.

Proteomic techniques. One-dimensional gel electrophoresis was performed according to standard techniques (Ausubel *et al.*, 1994). Two-dimensional gel electrophoresis and trypsinization of excised spots was performed as described previously (Rincon *et al.*, 2004), using a Bio-Rad Ready Strip IPG pH gradient 4–7 for the first dimension. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) was performed with an Applied Biosystems Voyager DE PRO MALDI-TOF in reflectance mode calibrated with a peptide standard from LaserBio Labs. Theoretical peptide fingerprint profiles were determined using the Peptide Mass tool on the ExPASy server (Wilkins *et al.*, 1997), and database searches (confidence value >60) were performed with Mascot (Perkins *et al.*, 1999) (tolerance limit 0.2 Da). Separation of peptides was achieved on a nano LC system (LC Packings) with a C18 PepMap 100 nanocolumn using a water/acetonitrile gradient (5–50% acetonitrile over 30 min) at a 0.3 µl min⁻¹ flow rate. Mass spectrometry was performed using a Q-Trap (Applied Biosystems/MDS Sciex) triple quadrupole mass spectrometer fitted with a nanospray ion source, where Q3 was operated as a linear ion trap.

RESULTS

Identification and overexpression of a putative CoA-transferase gene from *Roseburia* sp. A2-183 and partial purification of the endogenous enzyme

A novel CoA-transferase gene was identified from the partial genome sequence of *Roseburia* sp. A2-183 through a BLASTX search (R. I. Aminov and others, unpublished data). The translated sequence shared 55% amino acid identity with a presumptive acetyl-CoA hydrolase sequence from *D. hafniense* and 43% with the 4-hydroxybutyrate CoA-transferase sequence from *C. tetani*. The ORF was cloned and overexpressed, and the product purified with a C-terminal histidine-tag. A single band of approximately 50 kDa was detected on an SDS-polyacrylamide gel after purification and was confirmed as being the overexpressed enzyme by MALDI-TOF analysis (not shown).

In parallel, an endogenous CoA-transferase that is able to use butyryl-CoA and acetate was partially purified from *Roseburia* sp. A2-183. A 13-fold purification was obtained

by passing a crude cell extract over a DEAE Sepharose anion exchange column. The enzyme activity eluted in a single peak. The partially purified enzyme preparation was subjected to two-dimensional gel electrophoresis, and all major spots (24) were analysed by mass spectrometry after tryptic digest (not shown). Analysis of peptide fingerprints revealed no matches to sequences in Mascot, which was not unexpected, as the genome of this strain and its close relatives has not been published. However, a comparison of the peptide fingerprints to the theoretical fingerprint for the recombinant putative CoA-transferase sequence identified in this strain revealed five spots of a mass of approximately 50 kDa and pI values of between approximately 5.5 (spot 1) and 5.9 (spot 5) with matching peptide masses (Fig. 1). To confirm these results, electrospray ionization mass spectrometry was employed to obtain amino acid sequence information. The complete sequence of one peptide and partial sequence of two other peptides could be confirmed for all five spots. For the stronger spots, 2–4, between 12 and 22% of the total amino acid sequence could be identified with this approach (Fig. 1). These results provide strong evidence that the partially purified endogenous enzyme corresponds to the cloned and overexpressed putative CoA-transferase gene product from *Roseburia* sp. A2-183.

Substrate specificity and kinetics of the CoA-transferase from *Roseburia* sp. A2-183

The partially purified native CoA-transferase from *Roseburia* sp. A2-183 had a broad pH optimum with greater than 90% maximal activity between pH 7 and 8 (not shown). The substrate specificity was determined for the recombinant CoA-transferase as well as the partially purified native enzyme from *Roseburia* sp. A2-183. Competition experiments were performed to assess the substrate specificity of the acid substrate, as the assay employed is specific for the detection of acetyl-CoA. A strong apparent inhibition was observed with butyrate and propionate, followed by isobutyrate and valerate (in decreasing order of inhibition), but not with 4-hydroxybutyrate, the preferred substrate of the most closely related CoA-transferase sequence (Fig. 2). Despite a comparable level of inhibition observed for butyrate and propionate, a comparison of the respective CoA-ester substrates revealed a higher enzyme activity with butyryl-CoA for both enzyme preparations. The observed enzyme activities in the presence of propionyl-CoA compared to butyryl-CoA were $73.4 \pm 1.6\%$ ($P < 0.001$) and $60.8 \pm 7.6\%$ ($P < 0.01$) for the endogenous partially purified CoA-transferase and the recombinant purified enzyme, respectively. None of the other CoA-ester substrates tested, caproyl-, crotonyl- and acetoacetyl-CoA, served as substrates for either enzyme preparation (enzyme activities <2% of values with butyryl-CoA).

The pure recombinant CoA-transferase from *Roseburia* sp. A2-183 was characterized kinetically. Double reciprocal plots of initial velocity versus substrate concentration

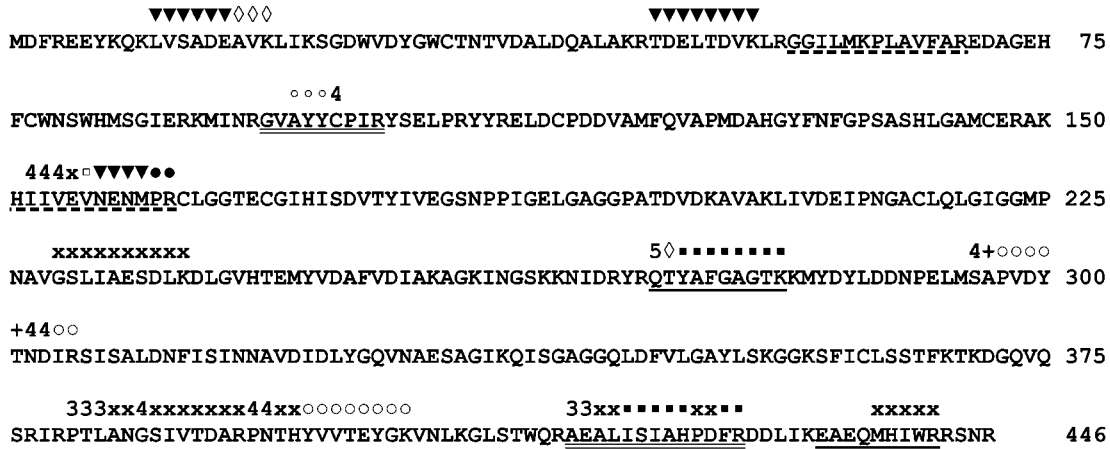


Fig. 1. Proteomic analysis of the endogenous partially purified CoA-transferase preparation from *Roseburia* sp. A2-183 and comparison with the deduced protein sequence of a candidate CoA-transferase gene from the same strain (AY796317). Lines below the sequence indicate matching theoretical peptide masses of the A2-183 sequence to peptide fingerprints of five protein spots from a two-dimensional separation of the partially purified enzyme: double line, spots 1–5; single line, spots 1–4; dashed line, spots 2–4. Symbols above the sequence indicate matching amino acid sequences from electrospray ionization mass spectrometry measurements to the five protein spots of the endogenous enzyme: ▼, spots 1–5; ●, spots 1–4; ■, spots 2–5; ○, spots 2–4; ◇, spots 3–5; °, spots 1, 2 and 4; □, spots 1, 3 and 4; +, spots 2 and 4; x, spots 3 and 4. Numbers correspond to the respective spot only.

resulted in a family of parallel lines at moderate substrate concentrations for both substrates, indicating a ping-pong bi-bi mechanism (Fig. 3). The pattern observed at high substrate concentrations of acetate is indicative of competitive substrate inhibition of a ping-pong mechanism (Cleland, 1970). K_m values for butyryl-CoA and acetate, as determined from replots of the intercepts versus the reciprocal of the concentration of the fixed substrate, were 0.098 and

6.4 mM, respectively. V_{max} values determined from these plots were both $112 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. K_m and V_{max} values for propionyl-CoA (determined at 25, 50, 100, 150 and 200 μM propionyl-CoA in the presence of 50 mM acetate) were 0.099 mM and $51 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively. Therefore the enzyme displays a similar affinity for both CoA-ester substrates, but displays a higher velocity with butyryl-CoA than with propionyl-CoA.

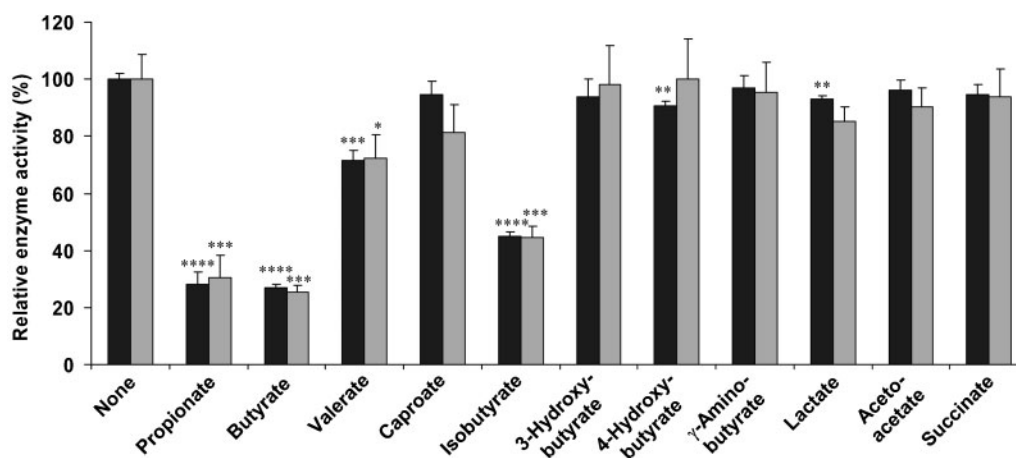


Fig. 2. Substrate specificity of *Roseburia* sp. A2-183 CoA-transferase for the carboxylic acid substrate. Acetate competed with a second acid in equimolar amounts (7.5 mM each) in the presence of butyryl-CoA (100 μM). Black bars, endogenous partially purified enzyme; grey bars, recombinant purified enzyme. The specific activities for values set at 100% are: $2.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for the endogenous enzyme and $38.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for the recombinant enzyme. Significance levels of difference between competing substrate and acetate only (labelled 'none'): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (mean and standard deviation of three independent repeats).

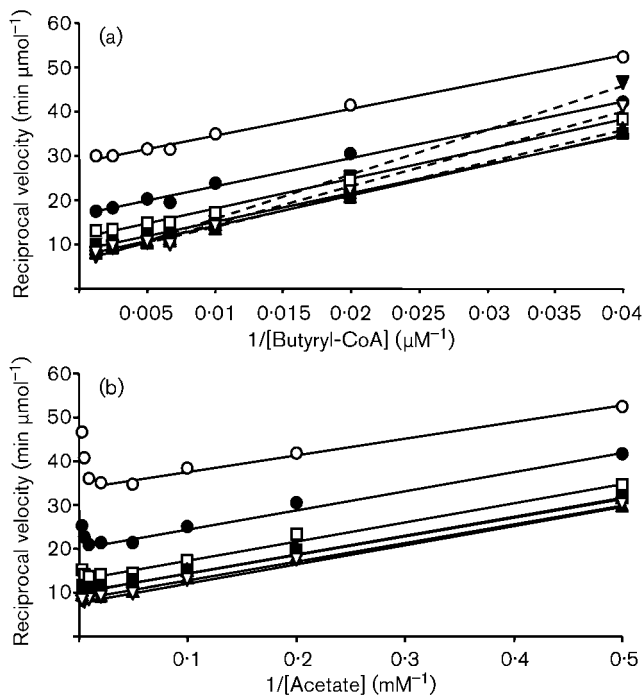


Fig. 3. Kinetics of recombinant CoA-transferase from *Roseburia* sp. A2-183. Double-reciprocal plots of initial velocity. (a) The butyryl-CoA concentration was varied with the following fixed concentrations of acetate (mM): \circ , 2; \bullet , 5; \square , 10; \blacksquare , 20; \triangle , 50; \blacktriangle , 100 (dashed line); ∇ , 200 (dashed line); \blacktriangledown , 300 (dashed line). (b) The acetate concentration was varied with the following fixed concentrations of butyryl-CoA (μM): \circ , 25; \bullet , 50; \square , 100; \blacksquare , 150; \triangle , 200; \blacktriangle , 400; ∇ , 800.

Presence of the novel CoA-transferase gene in other human gut bacteria

Degenerate primers were designed against conserved regions of the CoA-transferase gene found in *Roseburia* sp. A2-183. PCR products of the expected size were found in other butyrate-producing bacteria from the human gut [*Roseburia* sp. A2-194, M72/1, *Roseburia intestinalis* L1-82, *Eubacterium rectale* A1-86, *Butyrivibrio fibrisolvens* 16/4, *Anaerostipes caccae* L1-92, *Eubacterium hallii* L2-7 (all clostridial cluster XIVa) and *Faecalibacterium prausnitzii* A2-165 (clostridial cluster IV)], (data not shown). Full-length gene sequences were obtained by genome walking for *Eu. hallii* L2-7 and *F. prausnitzii* A2-165, and found to encode products that show, respectively, 77% and 73% amino acid identity with the CoA-transferase from *Roseburia* sp. A2-183. In strain *A. caccae* L1-92, two genes were identified, designated CoA-transferase I (74% identity to the CoA-transferase from *Roseburia* sp. A2-183) and CoA-transferase II (38% identity). A phylogenetic tree constructed from full-length CoA-transferase genes revealed that the CoA-transferase II gene was more closely related to the 4-hydroxybutyrate CoA-transferase from various clostridia, whereas the other CoA-transferase genes identified in human gut anaerobes clustered together closely (Fig. 4).

4-Hydroxybutyrate CoA-transferases are involved in the fermentation of succinate and ethanol by *C. kluyveri* and of γ -aminobutyrate by *Clostridium aminobutyricum* (Gerhardt *et al.*, 2000; Scherf & Buckel, 1991; Söhling & Gottschalk, 1996). We examined growth of *Roseburia* sp. A2-183, *Eu. hallii* L2-7, *A. caccae* L1-92 and *F. prausnitzii* A2-165 in the presence of these substrates. The increase in OD_{650} was below 0.1 for all strains and growth substrates tested, with the exception of *A. caccae* L1-92 on γ -aminobutyrate, which showed an increase of 0.169 ± 0.002 OD_{650} units over 46 h. The next gene immediately upstream of the CoA-transferase II in *A. caccae* L1-92 exhibited 50% identity to the 4-hydroxybutyrate dehydrogenase of *C. kluyveri* (4HBD_CLOKL), which catalyses the reaction directly upstream of the CoA-transferase in both *C. aminobutyricum* and *C. kluyveri*. Both CoA-transferase genes from *A. caccae* L1-92 were cloned and overexpressed and their substrate specificity was examined. The CoA-transferase I showed very similar substrate specificities to those of the enzyme from *Roseburia* sp. A2-183 (not shown). The second enzyme, however, displayed only very low enzyme activity [less than $1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (not shown)], so that the substrate specificity could not be determined.

Screening for other CoA-transferase genes by degenerate PCR

Two other types of CoA-transferase from clostridia that have been described elsewhere might also be able to convert butyryl-CoA and acetate to butyrate and acetyl-CoA. An enzyme from *C. acetobutylicum* utilizes acetoacetyl-CoA together with either acetate or butyrate as acid substrate (Wiesenborn *et al.*, 1989), while a propionate CoA-transferase from *C. propionicum* shows strong inhibition by

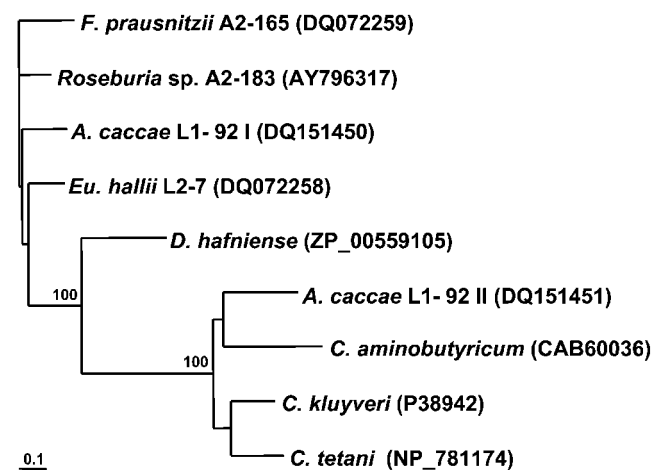


Fig. 4. Phylogenetic tree of CoA-transferase and related genes based on amino acid positions 3–439 of the *Roseburia* sp. A2-183 sequence. Accession numbers for the sequences are given in parentheses. Bootstrap values greater than 95 (per 100 replications) are shown at branch points. The scale bar represents genetic distance (10 substitutions per 100 nucleotides).

butyrate as second acid substrate *in vitro* (Schweiger & Buckel, 1984). Two pairs of degenerate PCR primers were designed here against conserved regions within the coding regions of these two CoA-transferases. The primer pairs designed to recognize the *C. propionicum* and *C. acetobutylicum* CoA-transferase genes gave products of the expected size with *C. propionicum* DSM 1682 and *C. acetobutylicum* DSM 792, respectively. Weaker bands of the expected size were also obtained with *E. coli* XL-1 Blue MRF, which possesses homologues of both genes. Neither pair, however, gave a product of the correct size with *Roseburia* sp. A2-183 (not shown), and there was no indication therefore that homologues of these two CoA-transferase genes are present in this bacterium.

DISCUSSION

Here we describe a novel type of CoA-transferase from the human gut bacterium *Roseburia* sp. A2-183 that belongs to the family I CoA-transferases (Heider, 2001), based on its ping-pong reaction mechanism as well as its sequence relationship with other CoA-transferases. The deduced protein sequence showed the highest similarity to a presumptive acetyl-CoA hydrolase sequence from *D. hafniense*. Acetyl-CoA hydrolases and CoA-transferases are related at the sequence level, and a single amino acid substitution has been shown to convert a CoA-transferase to a CoA-hydrolase (Mack & Buckel, 1997). 4-Hydroxybutyrate CoA-transferases also exhibited sequence similarity with the new sequence. However, the enzyme from *Roseburia* sp. A2-183 showed marked differences to the 4-hydroxybutyrate CoA-transferase from *C. aminobutyricum*, which has been examined enzymologically (Scherf & Buckel, 1991). A broad pH optimum close to neutral pH was found for the enzyme from *Roseburia* sp. A2-183, in accordance with that reported for other CoA-transferases (Barker *et al.*, 1978; Tung & Wood, 1975; Wiesenborn *et al.*, 1989). The 4-hydroxybutyrate CoA-transferase from *C. aminobutyricum*, on the other hand, exhibits a pH optimum at around 9.5, with only half the activity at pH 7 (Scherf & Buckel, 1991). Another characteristic that the novel CoA-transferase has in common with other CoA-transferases is its broad substrate specificity. The preferred substrates were butyrate and propionate; however, the enzyme showed no strong inhibition in the presence of 4-hydroxybutyrate, the preferred substrate of the 4-hydroxybutyrate CoA-transferase of *C. aminobutyricum* (Scherf & Buckel, 1991). These results strongly suggest that the enzyme identified from *Roseburia* sp. A2-183 represents a novel type of CoA-transferase distinct from the most closely related 4-hydroxybutyrate CoA-transferases. This is in accordance with a phylogenetic analysis of CoA-transferase sequences identified in other human gut bacteria. Sequences closely related to the *Roseburia* sp. A2-183 sequence formed a tight cluster, whereas a second sequence found in *A. caccae* L1-92 formed a distinct cluster with 4-hydroxybutyrate CoA-transferases. This second enzyme might indeed be involved in the conversion of 4-hydroxybutyrate, as *A. caccae* L1-92 shows some growth

on one of the substrates of the corresponding fermentation pathway, γ -aminobutyrate (Gerhardt *et al.*, 2000), and the adjacent gene upstream shows similarity to another enzyme of that pathway. Attempts to analyse the substrate specificity of this enzyme after overexpression in *E. coli* and purification failed due to the fact that only very low enzyme activity could be recovered. It has been reported elsewhere that the overexpression of the corresponding gene from *C. kluyveri* yields only low enzyme activity in *E. coli* (Valentin *et al.*, 2000).

In conclusion, this work establishes for the first time the substrate specificity and primary sequence of a CoA-transferase from *Roseburia* sp. A2-183 that preferentially uses butyryl-CoA as substrate. The corresponding gene could be identified in several butyrate-producing human gut bacteria, while genes for other CoA-transferases that are known to utilize butyryl-CoA seemed to be absent, based on degenerate PCR experiments. This gene is therefore a strong candidate for the CoA-transferase involved in butyrate formation in human gut bacteria. The assignment of this enzyme as a butyryl-CoA CoA-transferase is supported by the fact that *Roseburia* sp. A2-183 produces high amounts of butyrate but no propionate *in vitro* (Barcenilla *et al.*, 2000). The identification of this gene and its product will help in understanding the regulation of butyrate formation within the human gut.

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