

# Butyrate production in phylogenetically diverse *Firmicutes* isolated from the chicken caecum

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## Summary

Sixteen butyrate-producing bacteria were isolated from the caecal content of chickens and analysed phylogenetically. They did not represent a coherent phylogenetic group, but were allied to four different lineages in the *Firmicutes* phylum. Fourteen strains appeared to represent novel species, based on a level of  $\leq 98.5\%$  16S rRNA gene sequence similarity towards their nearest validly named neighbours. The highest butyrate concentrations were produced by the strains belonging to clostridial clusters IV and XIVa, clusters which are predominant in the chicken caecal microbiota. In only one of the 16 strains tested, the butyrate kinase operon could be amplified, while the butyryl-CoA : acetate CoA-transferase gene was detected in eight strains belonging to clostridial clusters IV, XIVa and XIVb. None of the clostridial cluster XVI isolates carried this gene based on degenerate PCR analyses. However, another CoA-transferase gene more similar to propionate CoA-transferase was detected in the majority of the clostridial cluster XVI isolates. Since this gene is located directly downstream of the remaining butyrate pathway genes in several human cluster XVI bacteria, it may be involved in butyrate formation in these bacteria. The

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present study indicates that butyrate producers related to cluster XVI may play a more important role in the chicken gut than in the human gut.

## Introduction

In the chicken gastrointestinal tract, the main sites of bacterial activity are the crop and the caeca and, to a lesser extent, the small intestine. *Lactobacillus* spp. dominate the crop (Barnes *et al.*, 1972; Watkins and Kratzer, 1983) and small intestinal tract (Lu *et al.*, 2003), while the caecal microbiota are dominated by species of the *Clostridiales* order, followed by *Lactobacillales* and *Bacteroidales* (Dumonceaux *et al.*, 2006). A molecular, culture-independent study from Zhu and colleagues (2002) on the caecal microbiota of chickens identified 243 different sequences, representing 50 phylogenetic groups or sub-groups of bacteria, with the majority of the caecal sequences being near or above 95% identical to their closest relatives in the database. Only about 10% of these sequences corresponded with validly named species, indicating that the knowledge of the intestinal microbiota of poultry is incomplete (Apajalahti *et al.*, 2004; Bjerrum *et al.*, 2006).

The activity and composition of the caecal microbiota is largely influenced by diet-derived substrates that resist small intestinal digestion. Fermentation of these substrates leads to formation of metabolites such as short-chain fatty acids (SCFAs) of which the concentration and relative proportion is affected by the type and quantity of the available substrates (Wolin *et al.*, 1999). The quantitatively most important SCFAs are acetic, propionic and butyric acid. Butyrate in particular is known to serve as the direct energy source for the colonic epithelium (Roediger, 1980) and possesses anti-inflammatory properties resulting from inhibition of the transcription factor NF $\kappa$ B activity (Place *et al.*, 2005). In addition, butyric acid is capable to reinforce the colonic defence barrier by increasing the production of mucins and host antimicrobial peptides (Barcelo *et al.*, 2000; Schaubert *et al.*, 2003). Butyric acid also promotes the body weight of broilers and has an inhibitory activity against *Salmonella* and *Clostridium perfringens* (Leeson *et al.*, 2005; Van Immerseel *et al.*, 2005; Hu and Guo, 2007; Timbermont *et al.*, 2010). Little is known about the endogenous butyrate-producing capacity in the lower intestinal tract of chickens, most likely

**Table 1.** Number of isolates within a butyrate-production range per sampled chicken and number of isolates consuming at least 2 mM acetate.

Chicken (suffix)	Butyrate production (mM)					Acetate consumption ( $\geq 2$ mM) among all isolates	
	0–2.0	2.1–5.0	5.1–10	10.1–15	> 15	Butyrate producers	Non-butyrate producers
14-week-old layer (a)	37	16	8	4	0	12/28	0/37
4-week-old layer (b)	50	2	0	1	5	7/8	9/50
4-week-old broiler (c)	28	3	4	3	0	1/10	0/28
4-week-old broiler (d)	23	5	5	3	1	4/14	0/23
4-week-old broiler (e)	53	2	2	1	4	4/9	0/53

because only limited information is available on the identity and diversity of butyrate-producing bacteria in the chicken gut microbiota. Therefore the objective of the present study was to isolate butyrate-producing bacteria colonizing the caecum of chickens and to determine their pathway for butyrate production.

## Results

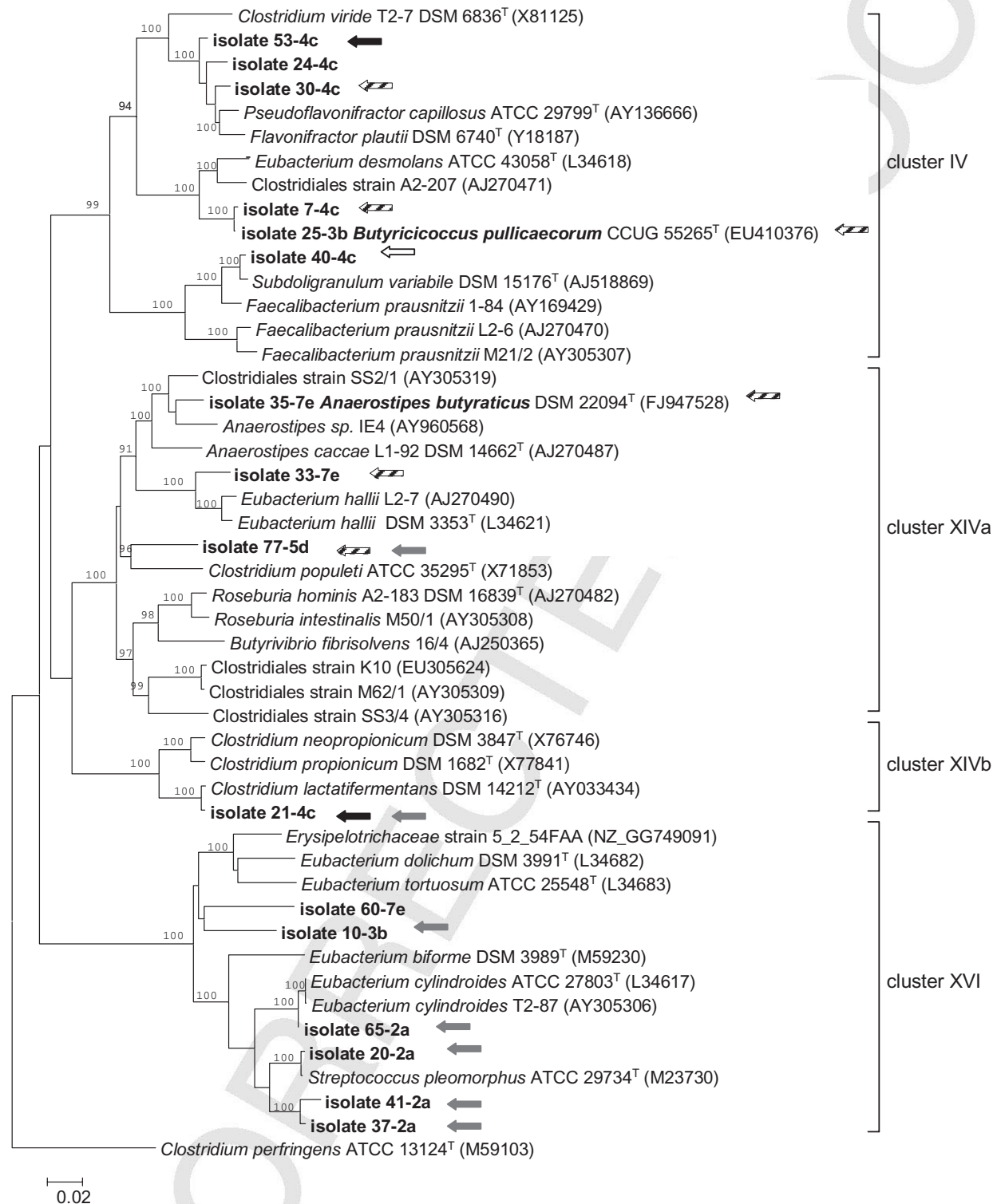
### Isolation of butyrate-producing bacteria

Dilutions of the caecal content were plated on M2GSC agar, since this medium was shown successful for the isolation of different types of butyrate producers from the human gut (Barcenilla *et al.*, 2000). Sixty-five, 58, 38, 37 and 62 colonies from a 14- and a 4-week-old layer pullet and from 34 week-old broilers, respectively, were randomly picked, grown overnight in M2GSC broth and screened for fatty acid production. In accordance with the study of Barcenilla and colleagues (2000), the cut-off value was set at 2 mM butyrate for consideration as a butyrate producer. Twenty-six per cent of all tested isolates produced more than 2 mM butyrate, with the proportion of butyrate-producing isolates varying between 14% and 43% for the five sampled chickens. The highest number of high-concentration butyrate producers (> 15 mM) was isolated from the chickens, in which the total number of butyrate producers was the lowest (b and e) (Table 1). At least 2 mM of the acetate present in the M2GSC medium was consumed by 41% of all butyrate producers. All isolates from the 14-week-old layer and the three 4-week-old broiler chickens that consumed acetate proved to be butyrate producers, in contrast with the isolates from the 4-week-old layer where only 44% of the acetate consumers were butyrate producers (Table 1).

### Identification of the butyrate producers

Randomly amplified polymorphic DNA (RAPD) and repetitive-extragenic-palindromic (REP) PCR fingerprints were determined for the 45 out of the 68 butyrate-producing isolates that could be recultured. Numerical

analysis of both types of fingerprints yielded comparable results (data not shown) and 16 isolates with very distinct RAPD and REP-PCR fingerprints were selected for further identification through 16S rRNA gene sequence analysis. Approximately 1300 bp of the 16S rRNA genes were determined, compared with all sequence data in public databases using the BLAST algorithm (Altschul *et al.*, 1990) and used for the construction of a phylogenetic tree (Fig. 1). The 16 isolates were distributed among four clostridial clusters, cluster IV, XIVa, XIVb and XVI (Collins *et al.*, 1994), within the *Firmicutes* phylum. When considering only validly named bacteria, three cluster IV isolates (53-4c, 24-4c and 30-4c) were most closely related to *Flavonifractor plautii* (formerly *Clostridium orbiscindens*) (Carlier *et al.*, 2010) (97.7% and 95.7% 16S rRNA sequence similarity) and *Pseudoflavonifractor capillosus* (formerly *Bacteroides capillosus*) (Carlier *et al.*, 2010) (97.5% 16S rRNA sequence similarity) respectively; one cluster IV isolate (40-4c) was most closely related (97.8% 16S rRNA sequence similarity) to the butyrate-producing organism *Subdoligranulum variabile*; and two isolates (7-4c and 25-3b) were most closely related (91.5% and 92.7% 16S rRNA sequence similarity) to *Eubacterium desmolans*. From this cluster, isolate 25-3b was recently classified into a novel species within a novel genus, *Butyricoccus pullicaecorum* (Eeckhaut *et al.*, 2008). Three isolates (35-7e, 33-7e and 77-5d) were most closely related to members of the clostridial cluster XIVa, i.e. *Anaerostipes caccae* (94.5% 16S rRNA similarity), *Eubacterium hallii* (95.2% 16S rRNA similarity) and *Clostridium populeti* (92.5% 16S rRNA similarity) respectively. In this cluster, the highest butyrate concentration was produced by isolate 35-7e which was recently further characterized and classified into the novel species *Anaerostipes butyraticus* (Eeckhaut *et al.*, 2010). Isolate 21-4c was most closely related (99.1% 16S rRNA sequence identity) to *Clostridium lactifermentans*, a lactate-fermenting bacterium of the clostridial cluster XIVb (van der Wielen *et al.*, 2002). All isolates within cluster XIVa and XIVb showed the ability to utilize the lactic acid present in the medium (Table 2). Three of the cluster XVI isolates (20-2a, 41-2a and 37-2a) were most closely



1 **Fig. 1.** Phylogenetic tree showing the relationship between the different butyrate-producing chicken isolates based on 16S rRNA gene  
 2 sequences. The tree was constructed by use of the neighbour-joining method. The 16S rRNA gene sequence of *Clostridium perfringens*  
 3 (ATCC 13124) was used as an outgroup to root the tree. Accession numbers are given in brackets. The numbers shown at the nodes of the  
 4 tree indicate bootstrap values out of 100 bootstraps resamplings (values under 90% are not shown). The strains isolated in this study are  
 5 shown in boldface and are labelled with an arrow when possessing a sequence related to the butyryl-CoA:acetate CoA transferase gene  
 6 (black arrow: CoATDF1, CoATDR2 primers; striped arrow: CoATDF1, CoATDR2 and BCoATscrF, BCoATscrR primers), the propionate  
 7 CoA-transferase gene (grey arrow) or the butyrate kinase gene (white arrow). Scale bar: 0.02 substitutions per nucleotide position.



**Table 2.** Acidic fermentation products and relationships of the butyrate-producing strains.

Strain	Cluster	Fermentation acids (mM) <sup>a,b</sup>					Highest 16S rRNA gene sequence similarity	
		Butyric acid	Acetic acid	Propionic acid	Lactic acid	Formic acid	Type strain of validly named species (% 16S rRNA sequence similarity)	Accession number
53-4c	IV	10.8 ± 1.4	-4.6 ± 0.7	-0.3 ± 0.6	5.9 ± 0.8	0.1 ± 0.1	<i>Pseudoflavonifractor capillosus</i> (97.7)	AY136666
24-4c		6.7 ± 1.2	2.9 ± 0.1	-0.8 ± 1.8	3.7 ± 0.01	1.3 ± 0.02	<i>Pseudoflavonifractor capillosus</i> (95.7)	AY136666
30-4c		11.8 ± 0.7	0.9 ± 0.3	0.6 ± 0.1	3.6 ± 0.1	0.1 ± 0.1	<i>Flavonifractor plautii</i> (97.5)	Y18187
7-4c		11.6 ± 0.9	-0.7 ± 0.2	-0.6 ± 1.1	-0.4 ± 0.3	0.5 ± 0.2	<i>Butyricoccus pullicaecorum</i> (97.9)	EU410376
25-3b		15.3 ± 0.2	-4.9 ± 0.5	-0.6 ± 1.5	-0.6 ± 0.3	1.7 ± 0.3	<i>Butyricoccus pullicaecorum</i> (100)	EU410376
40-4c	XIVa	6.8 ± 0.7	2.7 ± 0.3	0.4 ± 0.2	1.8 ± 0.02	16 ± 0.2	<i>Subdoligranulum variabile</i> (98.2)	AJ518869
35-7e		21.7 ± 0.6	-5.2 ± 0.2	-0.4 ± 0.3	-4.3 ± 0.6	1.7 ± 0.2	<i>Anaerostipes butyraticus</i> (100)	FJ947528
33-7e		15.1 ± 0.7	-1.3 ± 0.2	-4.1 ± 0.4	-8.1 ± 0.1	0	<i>Eubacterium hallii</i> (95.2)	L34621
77-5d		9.5 ± 0.8	4.8 ± 1.2	-1.9 ± 1.6	-2.1 ± 0.9	0.3 ± 0.2	<i>Clostridium populeti</i> (92.5)	X71853
21-4c		XIVb	5.7 ± 0.8	8.1 ± 1.3	10.5 ± 2.1	-3.3 ± 0.8	0.2 ± 0.1	<i>Clostridium lactatifermentans</i> (99.1)
60-7e	XVI	3.1 ± 0.8	0.2 ± 0.01	-1.4 ± 0.04	6.7 ± 0.5	4.7 ± 0.4	<i>Eubacterium tortuosum</i> (90.4)	L34683
10-3b		2.5 ± 0.9	0	-1.1 ± 0.3	4.9 ± 0.01	5.6 ± 0.2	<i>Eubacterium tortuosum</i> (90.6)	L34683
65-2a		3.6 ± 0.3	-0.2 ± 0.06	-0.8 ± 0.1	8.3 ± 0.2	2.9 ± 0.07	<i>Eubacterium cylindroides</i> (98.7)	L34617
20-2a		2.9 ± 0.8	-0.4 ± 0.5	-1.0 ± 0.3	8.5 ± 0.4	3.6 ± 0.2	<i>Streptococcus pleomorphus</i> (98.5)	M23730
41-2a		3.9 ± 0.6	-0.9 ± 0.2	-1.8 ± 1.7	7.1 ± 0.1	2.9 ± 0.1	<i>Streptococcus pleomorphus</i> (95.5)	M23730
37-2a		2.9 ± 0.9	-0.8 ± 0.4	-0.8 ± 0.3	8.1 ± 0.2	2.2 ± 0.2	<i>Streptococcus pleomorphus</i> (95.2)	M23730

a. Concentrations are averages of results of three replicates ± standard deviations and were calculated by subtracting the values at  $t_0$  from these at  $t_{24}$ .

b. Strains were grown overnight in M2GSC medium supplemented with 8 mM DL-lactate.

related (98.5%, 95.5% and 95.2% 16S rRNA sequence similarity) to the generically misnamed (Täpp *et al.*, 2003) *Streptococcus pleomorphus*; one cluster XVI isolate (65-2a) was most closely related to *Eubacterium cylindroides* (96.5% 16S rRNA sequence identity), and two further isolates (60-7e and 10-3b) were remotely (90.4% and 90.6% 16S rRNA sequence identity) related to *Eubacterium tortuosum* (Fig. 1, Table 2). Given their distinct RAPD and REP-PCR fingerprints (data not shown) and phylogenetic positions (Fig. 1), these 16 isolates are considered to represent 16 distinct strains in the remainder of the text.

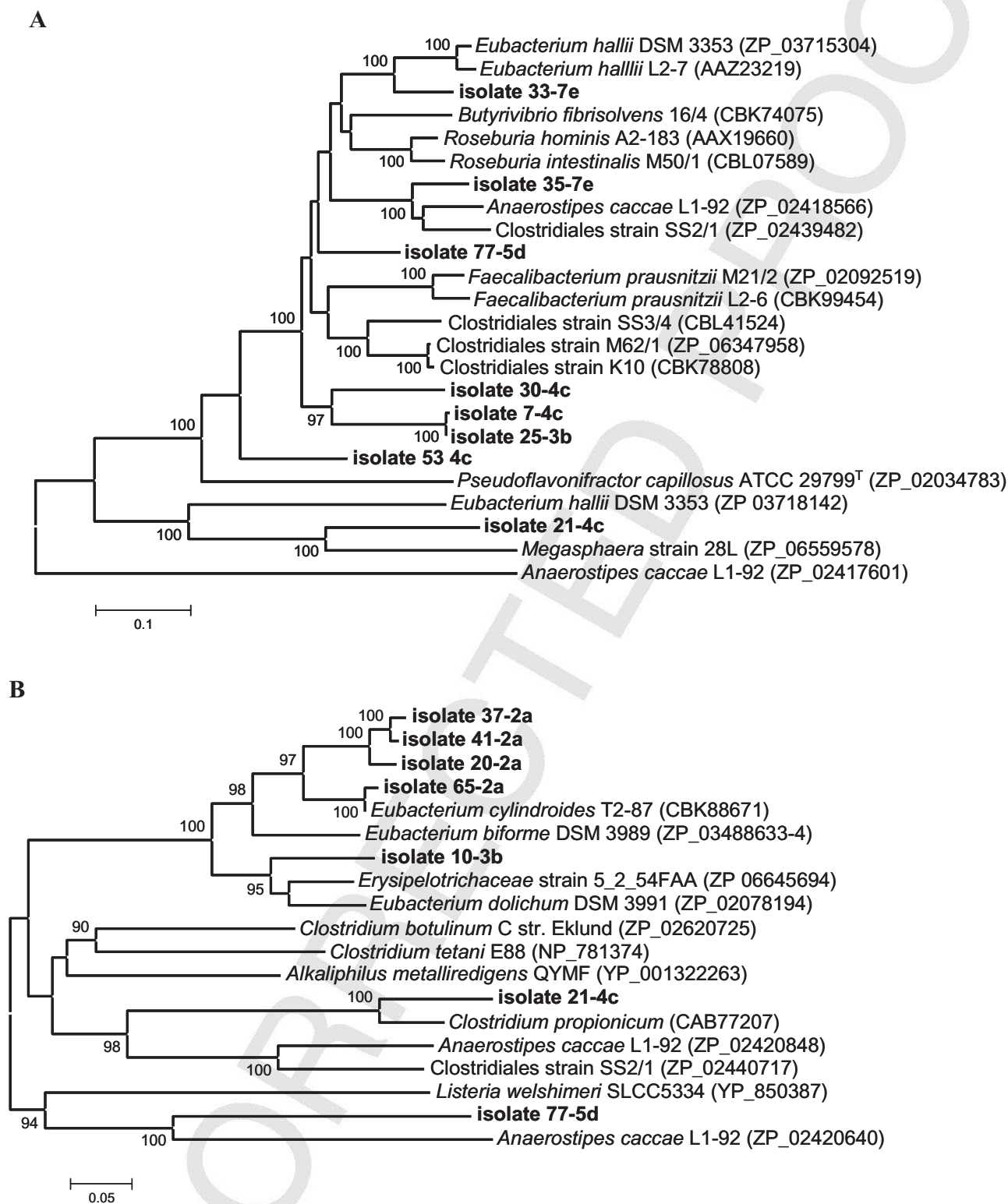
#### Detection of genes encoding enzymes involved in butyrate production

All 16 chicken-derived butyrate-producing strains were screened for the presence of the butyrate kinase operon and/or the butyryl-CoA : acetate CoA-transferase gene which carry out the final step of butyrate synthesis.

In strain 40-4c an amplicon of the expected size (about 771 bp) was obtained after PCR using PTBfor1 and BUKrev2 primers targeted against the butyrate kinase operon. The sequence of the amplicon was 98.6% similar to the butyrate kinase gene of the human faecal bacterium *S. variabile* (DSM 15176), its closest phylogenetic neighbour (Fig. 1, white arrow). The butyrate kinase operon could not be amplified in any of the other strains with the primer set used.

Butyryl-CoA : acetate CoA-transferase gene amplicons of the expected size (about 582 bp) using primer pair CoATDF1 and CoATDR2 were found in clostridial cluster

IV, XIVa and XIVb strains 53-4c, 30-4c, 7-4c, 25-3b, 35-7e, 33-7e, 77-5d and 21-4c. In these isolates (Fig. 1, striped arrows), except for 21-4c and 53-4c (Fig. 1, black arrows), an amplicon (about 530 bp) was also obtained using the BCoATscrF and BCoATscrR primers. Cluster IV strain 24-4c, nor any of the cluster XVI isolates yielded a PCR product with the two primer pairs targeting the butyryl-CoA : acetate CoA-transferase gene. All the isolated cluster XVI strains, except 60-7e, but also isolate 77-5d and 21-4c from cluster XIVa and XIVb (Fig. 1, grey arrows), showed an amplicon of the expected size (about 702 bp) after PCR using degenerate primers designed against propionate CoA-transferases (PCT primers, Table S1). All CoA-transferase gene amplicons were sequenced and deduced amino acid sequences subjected to database searches using the BLASTP algorithm. Two phylogenetic trees were constructed based on the gene sequences related to butyryl-CoA : acetate CoA-transferase (Fig. 2A) and propionate CoA-transferases (Fig. 2B). For most strains, the phylogeny of the CoA-transferase gene sequences agreed well with the 16S rRNA gene-based phylogeny (strains 33-7e, 35-7e, 77-5d, 30-4c, 7-4c, 25-3b, Fig. 2A, and isolates 37-2a, 41-2a, 20-2a, 65-2a, 10-3b, Fig. 2B). However, the CoA-transferase gene and 16S rRNA gene-based phylogenies of strains 53-4c and 21-4c were discordant, two strains (77-5d and 21-4c) carried more than one CoA-transferase gene and neither a CoA-transferase gene nor the butyrate kinase operon could be amplified in strains 24-4c and 60-7e. Inspection of draft genome sequences from human isolates belonging to cluster XVI revealed that the CoA



**Fig. 2.** Phylogenetic tree of deduced protein sequences related to butyryl-CoA:acetate CoA-transferase (A) and propionate CoA-transferase (B) respectively. The strains isolated in this study are shown in boldface, while the remaining strains are reference sequences. Accession numbers are given in brackets. The numbers shown at the nodes of the tree indicate bootstrap values out of 100 bootstraps resamplings (values under 90% are not shown). Scale bar: 0.1 substitutions per nucleotide position.

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**Fig. 3.** Arrangement of butyrate pathway genes in human cluster XVI strains. THL, thiolase; BCD, butyryl-CoA dehydrogenase; ETF $\beta$ , electron transfer protein  $\beta$ ; ETF $\alpha$ , electron transfer protein  $\alpha$ ; BHBD,  $\beta$ -hydroxybutyryl-CoA dehydrogenase; CRO, crotonase; CoAT, CoA-transferase. Corresponding genes from draft genome sequences: *E. cylindroides* T2-87 (CBK88667–88671; part of BHBD sequence and CRO sequence are missing due to a stretch on N connecting contigs); *E. dolichum* DSM 3991 (EUBDOL\_02008–14); *E. biforme* DSM 3989 [EUBIFOR\_01208–16; two THL genes are present in tandem (EUBIFOR\_01208–9), the putative CoA-transferase (EUBIFOR\_01215–16) contains a frameshift].

transferase gene (Fig. 2B) was located directly downstream of the other butyrate pathway genes (Fig. 3), indicating that it may be linked to butyrate metabolism in these bacteria.

### Discussion

In several molecular-based studies on broiler gut microbiota using complete caecal as well as mucosa-associated caecal 16S rRNA clone libraries, the largest set of retrieved sequences represented *Clostridium* cluster XIVa and *Clostridium* cluster IV bacteria (Gong *et al.*, 2002; Lan *et al.*, 2002; Zhu *et al.*, 2002; Dumonceaux *et al.*, 2006). Although in the human gut these two phylogenetic lineages contain many butyrate-producing bacteria (Louis and Flint, 2009), so far from the chicken caecum, only a few *Faecalibacterium prausnitzii*-like strains have been cultured and found to produce butyrate (Bjerrum *et al.*, 2006). These butyrate producers were isolated during a study in which the overall microbial community composition in the different intestinal segments was analysed. In the present study we only looked for butyrate producers and found that 69 out of 260 isolates from two layer type and three broiler chickens produced at least 2 mM butyrate. So 26.5% were butyrate producers which is comparable with the 30.9% Barcenilla and colleagues (2000) obtained in the human study. The clonal relatedness of 45 of these isolates (13 isolates could not be recultured after storage) was estimated using RAPD and REP-PCR fingerprinting; 16 strains with unique RAPD and REP-PCR fingerprints were selected for further identification using 16S rRNA gene sequence analysis which revealed that they belonged to the clostridial clusters IV, XIVa, XIVb and XVI. Although 16S rRNA gene sequences of *F. prausnitzii*- and *S. variabile*-related bacteria within cluster IV are abundant in the lumen as well as in the mucus of the chicken caecum (Gong *et al.*, 2002; Bjerrum *et al.*, 2006), only one *S. variabile*-related strain (97% 16S rRNA sequence similarity) was isolated in the present study. Although strains belonging to *Roseburia* are important butyrate producers in the human colon, none was found in the present study.

It remains to be established whether they are also an important component of the chicken microbiota. The 16S rRNA gene sequence similarity of the 16 butyrate-producing strains towards the type strains of their nearest validly named neighbours varied from 90.4% to 99.1%. As organisms sharing less than 98.5% of their 16S rRNA sequence typically correspond with distinct species (Stackebrandt and Ebers, 2006) these 16 strains most probably represent 14 novel bacterial species.

In the caeca of chickens, lactate is detected during the first 15 days of live, thereafter only low lactate concentrations are found, presumably as a result of metabolic cross-feeding from lactate-producing bacteria such as lactobacilli and bifidobacteria to lactate-utilizing bacteria (van der Wielen *et al.*, 2000). So far only *E. hallii*, *A. caccae* and *Clostridiales* strain SS2/1 within clostridial cluster XIVa and *Megasphaera elsdenii* within clostridial cluster IX are known to use lactate as precursor for butyrate synthesis (Soto-Cruz *et al.*, 2001; Duncan *et al.*, 2004). In the present study, all three clostridial cluster XIVa strains consumed some of the DL-lactate which was added to the M2GSC medium.

For the production of intracellular butyrate, two distinct pathways are described in clostridia (Gottschalk, 1986). In the human gut, only a few bacterial species, including isolates related to *Clostridium nexile* and *Coprococcus* spp., use the butyrate kinase pathway (Louis *et al.*, 2004). Those strains are members of clostridial cluster XIVa, while the strain (40-4c) carrying the butyrate kinase operon isolated in this study belongs to cluster IV. The majority of the cultured human butyrate-producing strains are found to carry the butyryl-CoA : acetate CoA-transferase gene (Louis *et al.*, 2004; 2010; Charrier *et al.*, 2006) encoding the enzyme which consumes acetate during the process of butyrate formation. The butyryl-CoA : acetate CoA-transferase gene was detected in all cluster XIVa and in the majority of the cluster IV strains examined here. Its phylogeny matched the 16S rRNA gene-based phylogeny for most isolates, except for isolate 53-4c. The CoA-transferase gene of strain 21-4c from cluster XIVb also differed markedly from the other CoA-transferase sequences and clustered more closely with a second class of CoA-transferase genes. For both isolates, only one of the degenerate primer sets (CoATDF1 and CoATDR2) led to an amplicon, while the primers amplifying a narrow range of CoA-transferases, BcoATscrF and BcoATscrR, did not result in an amplicon. Thus these genes may encode CoA-transferases with a different substrate specificity and it remains to be established whether they are involved in butyrate formation. The butyryl-CoA : acetate CoA-transferase gene present in cluster IV, XIVa and XIVb bacteria could not be found in cluster XVI strains by degenerate PCR using primer pairs CoATDF1, CoATDR2 and BCoATscrF, BCoATscrR.



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1 Instead, a CoA-transferase gene more closely related to  
 2 propionate CoA-transferases was found in all but one of  
 3 the cluster XVI isolates. Analysis of draft genome  
 4 sequences from cluster XVI butyrate producers revealed  
 5 that this gene is present directly downstream of the  
 6 central pathway genes for butyrate formation, leading  
 7 from acetyl-CoA to butyryl-CoA. Based on degenerate  
 8 PCR experiments against several central pathway genes,  
 9 the arrangement in *E. cylindroides* strains had previously  
 10 been assumed to be similar to cluster I strains (Louis and  
 11 Flint, 2009). However, the evidence from draft genomes  
 12 shows that cluster XVI strains isolated from human faeces  
 13 show a different arrangement (Fig. 3) which is also in line  
 14 with the previous degenerate PCR results. Thus, *E. cylin-*  
 15 *droides* in fact carries the same gene arrangement as  
 16 *Eubacterium dolichum* rather than the one present in  
 17 cluster I strains. Many CoA-transferases that have been  
 18 examined biochemically have a broad substrate specific-  
 19 ity *in vitro*. The butyryl-CoA : acetate CoA-transferase  
 20 found in many human gut butyrate producers exhibited a  
 21 similar affinity for propionyl-CoA as for butyryl-CoA (Char-  
 22 rier *et al.*, 2006), while the propionate CoA-transferase  
 23 from *Clostridium propionicum* also converts butyrate  
 24 (Schweiger and Buckel, 1984). It is therefore hypoth-  
 25 esized that a CoA-transferase related to the gene from *C.*  
 26 *propionicum* is responsible for butyrate formation in  
 27 cluster XVI bacteria, especially as its gene lies directly  
 28 downstream of the butyrate central pathway genes in the  
 29 cluster XVI butyrate-producing bacteria that genome  
 30 sequence is available for (Fig. 3). Clostridial cluster XIVb  
 31 isolate 21-4c is the only isolate found here that produces  
 32 high amounts of propionate (Table 2) and it carries a  
 33 CoA-transferase closely related to the one from *C. propi-*  
 34 *onicum*, which may be responsible for butyrate production  
 35 in this strain.

36 To our knowledge, this is the first study investigating the  
 37 diversity and phylogenetic relationship of culturable  
 38 butyrate-producing bacteria from chicken caeca. It would  
 39 be useful to confirm the importance of the strains found  
 40 here on a bigger number of animals using PCR  
 41 approaches for the detection of the functional genes on  
 42 the caecal content and clone libraries to see if the same  
 43 sequences can be retrieved. Although more extensive  
 44 surveys are likely to reveal additional phylogenetic groups  
 45 of butyrate-producing bacteria, the present study indi-  
 46 cates that butyrate producers related to cluster XVI may  
 47 play a more important role in the chicken gut than in the  
 48 human colon.

**Experimental procedures***Chickens*

52 Caecal samples from five chickens were taken. The first two  
 53 samples were taken from a 14-week-old Isa Brown layer type

pullet and a 4-week-old White Leghorn layer type pullet. For  
 the third, fourth and fifth sampling, the caeca of 4-week-old  
 broiler (Ross) chickens, purchased from different commercial  
 farms, were used.

*Caecal sampling*

Immediately after euthanasia, the caeca were transferred into  
 an anaerobic (84% N<sub>2</sub>, 8% CO<sub>2</sub> and 8% H<sub>2</sub>) workstation  
 (Ruskin Technology, Bridgend, UK). One gram of the caecal  
 content was homogenized in 9 ml of anaerobic M2GSC  
 medium pH 6 containing per 100 ml, 30 ml of rumen fluid, 1 g  
 of casitone, 0.25 g of yeast extract, 0.2 g of glucose, 0.2 g  
 of cellobiose, 0.2 g of soluble starch, 0.045 g of K<sub>2</sub>HPO<sub>4</sub>,  
 0.045 g of KH<sub>2</sub>PO<sub>4</sub>, 0.09 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.09 g of NaCl,  
 0.009 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.009 g of CaCl<sub>2</sub>, 0.1 mg of resazurin,  
 0.4 g of NaHCO<sub>3</sub> and 0.1 g of cysteine hydrochloride  
 (Barcenilla *et al.*, 2000). From this suspension, a 10-fold  
 serial dilution was made and from each dilution, 0.3 ml was  
 spread onto agar plates containing M2GSC medium with  
 1.5% agar. Plates were incubated at 41–42°C for 48 h. Single  
 colonies were randomly picked from dilutions 10<sup>-7</sup> or 10<sup>-8</sup> and  
 grown overnight in 10 ml of M2GSC broth.

*SCFA and lactic acid quantification*

Quantitative determination of the acidic fermentation prod-  
 ucts (butyric acid, acetic acid, propionic acid, lactic acid and  
 formic acid) was performed using a validated HPLC-UV  
 method after 24 h of growth in M2GSC broth or in M2GSC  
 broth supplemented with 8 mM DL-lactic acid. After centrifuga-  
 tion of the overnight cultures, the supernatant was acidified  
 using concentrated hydrochloric acid and extracted with  
 diethylether for 20 min. After centrifugation, the organic layer  
 was transferred to another extraction tube and extracted  
 again for 20 min with sodium hydroxide. After centrifugation  
 the aqueous phase was transferred to an autosampler vial  
 and concentrated hydrochloric acid was added. An aliquot  
 was injected on the HPLC-UV instrument. The HPLC instru-  
 ment consisted of a P1000XR type quaternary gradient  
 pump, an AS3000 type autosampler, a UV1000 type ultraviolet  
 detector and a SN4000 type system controller, all from  
 ThermoFisher Scientific (Breda, the Netherlands). Chromato-  
 graphic separation was achieved using a PLRP-S column  
 (250 × 4.6 mm i.d., Varian, Middelburg, the Netherlands). A  
 gradient elution was performed using sodium dihydrogen  
 phosphate in HPLC grade water and HPLC grade acetonitrile  
 as mobile phase A and B respectively. The detector was set  
 at a wavelength of 210 nm. For data processing, the Chrom-  
 quest software (ThermoFisher Scientific) was used. Quanti-  
 fication was performed using linear calibration curves  
 (calibration range: 0.5–50.0 mM for each compound). Limits  
 of quantification (LOQ) were set at 0.5 mM for formic acid,  
 lactic acid, acetic acid and propionic acid and at 1.0 mM for  
 butyric acid. Limits of detection (LOD) were: 0.19 mM for  
 formic acid, 0.18 mM for lactic acid, 0.19 mM for acetic acid,  
 0.14 mM for propionic acid and 0.33 mM for butyric acid.

*Identification of the butyrate-producing isolates*

RAPD and REP-PCR were used to investigate the genetic  
 diversity and clonality of the isolates. Genomic DNA was

extracted using alkaline lysis as previously described (Scheirlinck *et al.*, 2007). The DNA was used for REP-PCR with the (GTG)<sub>5</sub> primer and RAPD typing using the 10-nucleotide primer 272 (Table S1) (Versalovic *et al.*, 1994; Coenye *et al.*, 2002). The resulting RAPD and REP-PCR fingerprints were analysed using the BioNumerics V4.61 software package (Applied Maths, Sint-Martens-Latem, Belgium) as described before (Scheirlinck *et al.*, 2007). The similarities among the banding patterns were calculated using Pearson's correlation coefficient (expressed as a percentage-of-similarity value) and a dendrogram was constructed based on the UPGMA algorithm (Pearson and Lipman, 1988).

The extracted DNA was further used as target for amplification of 16S rRNA genes using the 'universal' eubacterial primers fD1 and rD1 (corresponding to positions 8 and 1541 in the *Escherichia coli* numbering system) (Table S1) (Weisburg *et al.*, 1991). The purified amplicons were sequenced using the BigDye Terminator sequencing kit with primers pD,  $\gamma^*$ , 3 and O\* on an ABI PRISM Genetic Analyser (Table S1) (Coenye *et al.*, 1999). The sequences obtained were compared with entries in the EMBL and GenBank database using the BLAST program (Altschul *et al.*, 1990). The most similar database sequences were aligned with the isolate sequences using the CLUSTALW program (Chenna *et al.*, 2003). To outline the phylogenetic relationship, a neighbour-joining tree (Saitou and Nei, 1987) was constructed via the PHYLIP package (Felsenstein, 1989), using DNADIST for distance analysis (Kimura, 1980) and bootstrap values of 100 replicates.

#### Amplification and phylogenetic analysis of the genes involved in butyrate formation

The route for butyrate synthesis in the phylogenetically diverse butyrate-producing strains was determined using degenerate primers PTBfor2 and BUKrev1 for amplification of part of the operon encoding phosphotransbutyrylase and butyrate kinase, via a ramped annealing approach as described previously (Louis *et al.*, 2004). Two degenerate primer pairs were used to amplify part of the butyryl-CoA : acetyl CoA-transferase gene. Primer set CoATDF1, CoATDR2 (Table S1) was designed using a broad range of CoA-transferase-related sequences and had a non-degenerate clamp region at the 5' end, based on the sequence from *Roseburia* sp. A2-183 (Charrier *et al.*, 2006). Conserved regions in butyryl-CoA : acetyl CoA-transferases but not in 4-hydroxybutyrate CoA transferase and acetyl-CoA hydrolase were used for the design of the primers BCoATscrF and BCoATscrR (Table S1) (Louis and Flint, 2007). Degenerate primers PCTfor1 and PCTrev2 (Table S1) were designed against conserved regions of CoA-transferase genes related to a propionate CoA-transferase from *C. propionicum* (AJ276553) (Charrier *et al.*, 2006). PCR products of the expected size were purified and sequenced using the BigDye Terminator sequencing kit. Contigs were generated with Lasergene 6 (DNASTAR) and deduced protein sequences were compared with entries in the GenBank database using BLASTP (Altschul *et al.*, 1990). The deduced protein sequences and reference sequences were aligned with CLUSTALW (Chenna *et al.*, 2003) and manually inspected. Phylogenetic trees were constructed with MEGA4 (Tamura

*et al.*, 2007) using the neighbour-joining method, 100 times bootstrap, pairwise gap deletion and Poisson correction. Butyrate pathway gene arrangements of clostridial cluster XVI strains *E. dolichum*, *E. bifforme* and *E. cylindroides* were examined in draft genome sequences available in the GenBank database by performing BLASTP analyses with the corresponding genes from human isolate L2-50 (Louis *et al.*, 2007; accession number of corresponding DNA sequence DQ987697) and the propionate CoA-transferase gene from *C. propionicum* (Selmer *et al.*, 2002; Accession No. CAB77207). All hits were between 45% and 79% identical.

#### Nucleotide sequence accession numbers

The sequences of the 16S rRNA genes and the CoA-transferase genes used in the construction of the phylogenetic trees are together with the sequence of the butyrate kinase gene deposited under GenBank Accession No. HQ452851–HQ452864, HQ452835–HQ452849 and HQ452850 respectively.

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**Table S1.** Sequences of the used primers. 58

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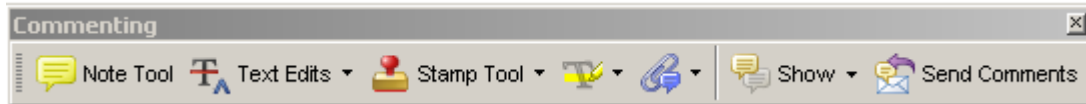


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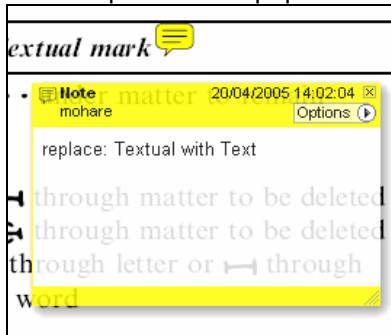
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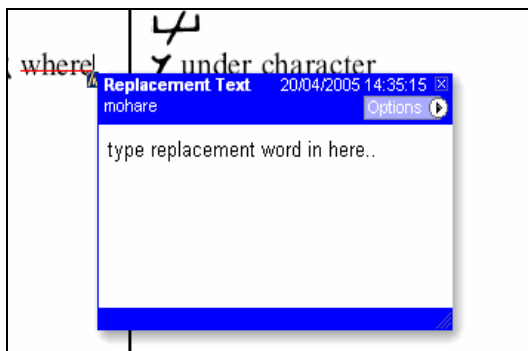


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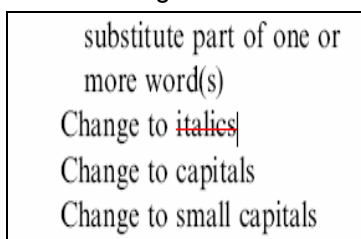


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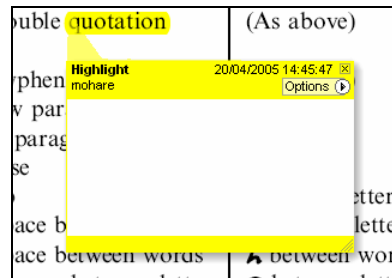


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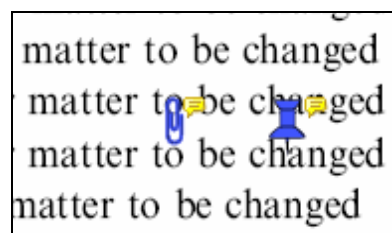


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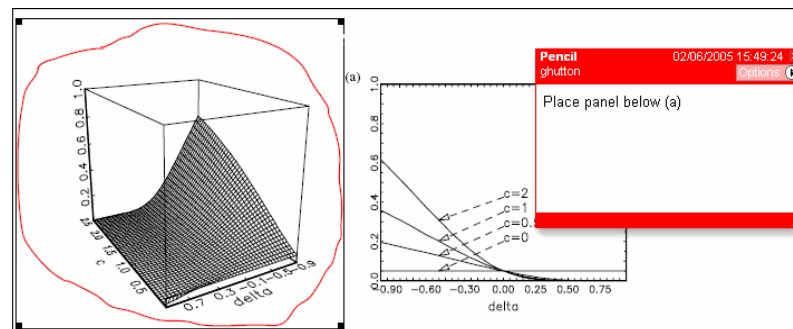


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