Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside

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Abstract

The human intestinal microbiota is essential for the conversion of the dietary lignan secoisolariciresinol diglucoside (SDG) via secoisolariciresinol (SECO) to the enterolignans enterodiol (ED) and enterolactone (EL). However, knowledge of the species that catalyse the underlying reactions is scant. Therefore, we focused our attention on the identification of intestinal bacteria involved in the conversion of SDG. Strains of *Bacteroides distasonis*, *Bacteroides fragilis*, *Bacteroides ovatus* and *Clostridium cocleatum*, as well as the newly isolated strain *Clostridium* sp. SDG-Mt85-3Db, deglycosylated SDG. Demethylation of SECO was catalysed by strains of *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, *Eubacterium limosum* and *Peptostreptococcus productus*. Dehydroxylation of SECO was catalysed by strains of *Clostridium scindens* and *Eggerthella lenta*. Finally, the newly isolated strain ED-Mt61/PYG-s6 catalysed the dehydrogenation of ED to EL. The results indicate that the activation of SDG involves phylogenetically diverse bacteria, most of which are members of the dominant human intestinal microbiota.

Introduction

The human intestinal tract harbours complex bacterial communities, which constantly interact with host cells and dietary factors. Owing to their metabolic potential, these communities play a key role in energy balance and in the metabolism of undigested food components, with presumed consequences for health (Backhed *et al.*, 2005). Lignans, for example, are phyto-oestrogens that are metabolised by intestinal bacteria (Cassidy *et al.*, 2000). Secoisolariciresinol diglucoside (SDG) is one of the most abundant dietary lignans. It is found in a variety of food items, with particularly high concentrations in flaxseed (Mazur, 1998). SDG is of interest because of its possible implications for the prevention of breast and colon cancer (Chen *et al.*, 2003), atherosclerosis (Prasad, 1999) and diabetes (Prasad, 2001). The underlying mechanisms include antioxidative and enzyme-inhibiting properties (Wang *et al.*, 1994; Kitts *et al.*, 1999), as well as oestrogen-dependent activities (Schottner *et al.*, 1998; Mueller *et al.*, 2004). The enterolignans enterodiol (ED) and enterolactone (EL) are produced from SDG by intestinal bacteria and show enhanced biological activities (Fig. 1) (Borriello *et al.*, 1985; Prasad, 2000; Kilkkinen *et al.*, 2002; Bowey *et al.*, 2003; Jacobs *et al.*, 2005). Hence, the intestinal microbiota is essential for SDG activation. The bacterial transformation of SDG includes deglycosylation, demethylation, dehydroxylation and dehydrogenation (Wang *et al.*, 2000). The conversion of secoisolariciresinol (SECO), the aglycone form of SDG, results from the interaction between dominant and subdominant anaerobic bacterial communities (Clavel *et al.*, 2005). Two isolated faecal bacterial strains, *Peptostreptococcus productus* SECO-Mt75m3 and *Eggerthella lenta* SECO-Mt75m2, catalyse the demethylation and dehydroxylation of SECO, respectively. Although their occurrence was related to the proportion of EL-producing communities in faeces, the diversity of SECO-metabolizing bacteria is certainly not restricted to these two strains.

Because an understanding of the mechanisms underlying the bacterial production of enterolignan is a prerequisite to studying the health effects of dietary lignans, we aimed at describing SDG-converting communities at the species level. Specifically, we intended to identify intestinal anaerobic bacterial strains that catalyse one of the four reactions underlying the conversion of SDG to EL. This was achieved on the one hand by the isolation of strains from human faeces, and on the other hand by the screening of phylogenetically or functionally related strains obtained from bacterial culture collections.

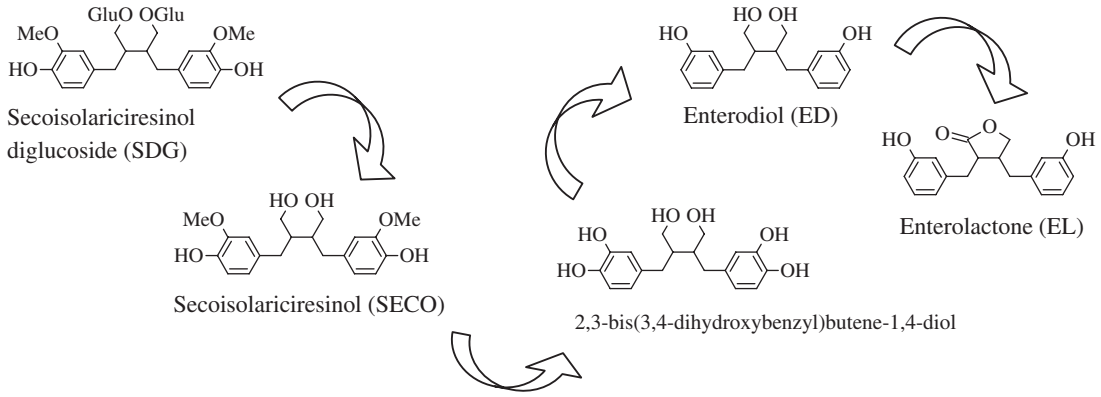

Fig. 1. Chemical structure of secoisolariciresinol diglucoside and its bacterial metabolites.

Materials and methods

Chemicals

Secoisolariciresinol diglucoside was isolated from flaxseed (Degenhardt *et al.*, 2002). SECO was purchased from Sigma-Aldrich (Taufkirchen, Germany). ED and EL were purchased from the VTT Technical Research Centre of Finland (Espoo, Finland). These compounds were racemic mixtures. Stock solutions of SECO (100 mM), ED and EL (50 mM) were prepared in MeOH. Stock solutions of SDG (100 mM) were prepared in H_2O.

Culture conditions for strains obtained from bacterial culture collections

Strictly anaerobic culture techniques were used (Attebery & Finegold, 1969; Breznak & Costilow, 1994). Freeze-dried cultures of *Butyribacterium methylotrophicum* DSM 3468^T, *Clostridium amygdalinum* DSM 12857^T, *Clostridium cocleatum* DSM 1551^T, *Clostridium ramosum* DSM 1402^T, *Clostridium saccharolyticum* DSM 2544^T, *Clostridium scindens* DSM 5676^T, *Eggerthella lenta* DSM 2243^T, *Enterobacter cloacae* DSM 30054^T and *Eubacterium callanderi* DSM 3662^T were revived as recommended by the supplier (DSMZ, Braunschweig, Germany). Cryostocks or grown cultures of *Acetobacterium woodii* DSM 1030^T, *Bacteroides distasonis* DSM 20701^T, *Bacteroides fragilis* DIfE-05, *Clostridium barkeri* DSM 1223^T, *Clostridium coccoides* DSM 935^T, *Clostridium spiroforme* DSM 1552^T, *Eubacterium limosum* DSM 20543^T, *Peptostreptococcus productus* DSM 2950^T, *P. productus* DSM 3507, *Ruminococcus hansenii* DSM 20583^T, *Ruminococcus obeum* ATCC 29174^T and *Ruminococcus schinkii* DSM 10518^T were obtained from the collection of the German Institute of Human Nutrition Potsdam-Rehbrücke or the French National Institute of Agricultural Research. The strains were grown in Brain Heart Infusion (Merck, Darmstadt, Germany) supplemented with 5 g L^{-1} yeast extract and 5 mg L^{-1} haemin (YHBHI). To ensure purity, they were streaked two times on YHBHI-agar. Purity was examined by comparison of colony morphology and cell morphology after Gram-staining. Gram-stains were confirmed by means of the KOH-test (Gregerson, 1978). Strains were incubated on YHBHI-agar under aerobic conditions to check for the presence of aerobic contaminants.

Culture media for isolation and conversion experiments

Media were prepared using strictly anaerobic techniques. The media Mt-6 and Mt-75, as well as Salt 1 solution, Salt 2 solution, and trace element solution 2, have been described previously (Clavel *et al.*, 2005).

Medium Mt-3 contained per litre: 4 g $NaHCO_3$, 0.5 g sodium acetate · $3H_2O$, 0.5 g sodium formate, 0.5 g cysteine · HCl · H_2O, 0.3 g yeast extract, 1 mg resazurin, 100 mL 10-fold-concentrated basal solution (154.9 mM Na_2HPO_4, 100 mM Na_2HPO_4, 57.4 mM NH_4Cl, 24.1 mM K_2HPO_4, 11.8 mM KH_2PO_4 and 8.3 mM $MgSO_4$) (Diekert, 1992), 20 mL trace element solution 1 (Diekert, 1992), 10 mL rumen fluid and 1 mL vitamin solution (Diekert, 1992). SDG was added to a final concentration of 500 μM. The pH was adjusted to 7.5, the medium was gassed with 80% N_2 plus 20% CO_2 (volume in volume, v/v) and autoclaved at 121 °C for 15 min.

Medium Mt-61 was modified after Mt-6. Concentrations were adjusted as follows: rumen fluid, 3.5% (v/v); yeast extract, 500 mg L^{-1}; sodium acetate and sodium formate, 800 mg L^{-1} each; haemin, 0.25 mg L^{-1}. Medium Mt-61 was not supplemented with glucose and fructose.

Medium Mt-85 contained per litre: 500 mg cysteine · HCl · H_2O, 250 mg sodium formate, 250 mg $Na_2S \cdot 9H_2O$, 50 mg yeast extract, 1 mg resazurin, 1 mL rumen fluid, 1 mL vitamin solution (Diekert, 1992), 100 mL Salt 1 solution, 2 mL Salt 2 solution, and 0.1 mL trace element solution 2.

SDG was added to a final concentration of 500 μM. The pH was adjusted to 7.6 and the medium was gassed and autoclaved as described above.

Isolation of SDG-deglycosylating and ED-dehydrogenating bacteria

All steps were carried out using strictly anaerobic techniques. Incubations were performed at 37 °C. Faeces were obtained from a healthy male adult with dominant enterolignan-producing bacterial communities, as determined by most probable number enumerations (Clavel *et al.*, 2005). Faecal dilutions were prepared as described previously (Clavel *et al.*, 2005). The purity of isolates was ensured as described for strains from culture collections.

The isolation of SDG-deglycosylating bacteria was performed in an anaerobic chamber (MACS variable atmosphere workstation, Don Whitley Scientific, Shipley, UK). Faecal dilutions were spread-plated onto Mt-85 supplemented with 14 g L^{-1} agar. Plates were incubated for 103 h. Twenty-two colonies from the 10^{-3}, 10^{-4} and 10^{-5} faecal dilutions were picked and cultured in peptone–yeast–glucose broth (medium no. 104, DSMZ, Braunschweig, Germany). The resulting cultures were subsequently used in conversion experiments to test their ability to convert SDG.

For isolation of ED-dehydrogenating bacteria, handling of samples was performed in an anaerobic tent (Coy Laboratory Products, Grass Lake, MI). Mt-6 and Mt-61 media were supplemented with 15 μL sterile-filtered stock solution of ED. A 10-fold faecal dilution (50 μL) was incubated for 24 h in 1.5 mL Mt-6 broth. A volume of 50 μL of the faecal culture was transferred once in 1.5 mL Mt-6 and then twice in 1.5 mL Mt-61, every time after 24 h of growth. The conversion of ED was monitored after each transfer by liquid chromatography. Serial dilutions of the final ED-converting enrichment (10^{-1}–10^{-6}) were spread-plated twice onto Mt-61 supplemented with 14 g L^{-1} agar. In total, 39 single colonies were picked after 62 and 86 h of growth. None of the selected colonies converted ED. However, not all bacteria that grew on the plates formed distinct colonies. Some grew as fine smears. Dehydrogenation of ED was observed after incubation of such smears, which were streaked onto Columbia- (5% sheep blood, Biomérieux, Marcy l'Etoile, France), PYG- and Wilkins–Chalgren-agar (Oxoid, Hampshire, UK) to support better growth. After 95 h of growth, bacteria were picked from the edge of 32 colonies and tested for ED dehydrogenation.

Conversion experiments with pure cultures and isolated strains

To test for the deglycosylation of SDG, 50 μL overnight liquid cultures were incubated in 1.5 mL Mt-3 broth. To test for the demethylation of SECO or the dehydrogenation of ED or SECO, 50 μL overnight liquid cultures or bacteria from isolated colonies were incubated in 1.5 mL Mt-6 broth containing 1 mM SECO or 500 μM ED. To test for dehydroxylation of SECO, 20 μL overnight cultures of each of *P. productus* SECO-Mt75m3 and the dehydroxylating candidate strain were co-incubated in 1 mL Mt-75 broth containing 1 mM SECO. Incubations were performed at 37 °C. Controls consisted of bacteria in media without substrate and media containing substrate without bacteria. Samples were collected after approximately 24 and 48 h of growth and kept at −20 °C until liquid chromatography analysis.

High-performance liquid chromatography (HPLC)

Samples were centrifuged (13 000 ***g***, 3 min) and the supernatants were further analysed. Separation was carried out with a RP-18 column (Lichrocart® Lichrospher®100, 250 × 4 mm, 5 μm, Merck, Darmstadt, Germany) maintained at 37 °C and protected with a guard RP-18 column (4 × 4 mm, 5 μm). The gradient elution was modified after Nurmi *et al.* (2003). Eluents were: A, 80% 50 mM sodium actetate (adjusted to pH 5 with 100% acetic acid) plus 20% MeOH (v/v); B, 40% 50 mM sodium acetate plus 40% MeOH and 20% acetonitrile (v/v/v). The gradient was 20%–80% B within 10 min, 80%–100% B within 14 min, 100% B for 5 min, and back to 20% B within 1 min. The system was equilibrated with 20% B for 10 min at the end of each run. The flow rate was 0.3 mL min^{-1} and the injection volume was 20 μL. Lignans were detected at 285 nm using a UV diode array detector. The Chromeleon software version 6.40 (Dionex, Idstein, Germany) was used for data acquisition and analysis. The retention times of standard lignans were: SDG, 17.2 min; SECO, 22.2 min; ED, 25.2 min; EL, 28.5 min. Metabolites were identified by comparison with the retention times and spectra of standards. The molecular mass of peaks, for which no standards were available, was determined by electrospray ionization mass spectrometry (ESI-MS).

ESI-MS experiments

A triple quadrupole mass spectrometer fitted with a Z-spray API electrospray source (Quattro II, Micromass, Manchester, UK) was used. Metabolites were separated by HPLC as described above. Downstream of the column, the flow was split (6 : 1) so that a continuous flow of 0.05 mL min^{-1} was directed into the spectrometer. MS analyses were carried out in negative ionization mode. The temperature of the ion source was 120 °C. The cone and capillary voltages were 25 V and 3.0 kV, respectively. The desolvation temperature was 380 °C, and the desolvation gas (N_2) was maintained at 400 L h^{-1}. Data were analysed using the Mass Lynx 3.5 software (Micromass).

Identification of SDG-converting isolates and maintenance of bacterial strains

Pure isolates were identified by 16S rRNA gene sequence analyses as described previously (Clavel *et al.*, 2005). PCR products were also sent to AGOWA (Berlin, Germany) for sequencing with primers 27f (5′ AGAGTTTGATCCTGGCTCAG) and 1492r (5′ TACCTTGTTACG ACT T) (Kageyama *et al.*, 1999). Similarities were calculated following the unambiguous alignment of consensus 16S rRNA gene sequences. Scanning electron micrographs of the isolates were obtained as described previously (Grund *et al.*, 1995). Cryostocks of isolated cultures were maintained in Microbank tubes (MAST Diagnostica, Reinfeld, Germany) according to the manufacturer's instructions and stored at −80 °C. For all strains, working stocks were maintained in YHBHI broth and subcultured every two to three weeks. Purity controls consisted of microscopic observations of Gram-stained bacterial cells.

Results and discussion

Identification of SDG-deglycosylating bacteria

Four SDG-deglycosylating strains were isolated from human faeces. The 16S rRNA gene sequence of a Gram-positive helically coiled rod (1416 nucleic acids) showed 99.4% similarity with *Clostridium* sp. 14774 (GenBank accession no. AJ315981) and 96.7% similarity with *Clostridium cocleatum* DSM 1551^T (Y18188). Figure 2 shows the conversion of SDG to SECO by the isolate, referred to as *Clostridium* sp. SDG-Mt85-3Db (DQ100445). In addition, the 16S rRNA gene sequence of one Gram-negative rod-shaped isolate (1355 nucleic acids) showed 99.7% similarity with *Bacteroides ovatus* DSM 1896^T (X83952). The organism was named *B. ovatus* SDG-Mt85-3Cy (DQ100446). Finally, the 16S rRNA gene sequences of two other Gram-negative rod-shaped isolates (1426 nucleic acids) showed 98.5% similarity with *Bacteroides fragilis* DSM 2151^T (X83935). The organisms were named *B. fragilis* SDG-Mt85-4C (DQ100447) and SDG-Mt85-5B (DQ100448). Four additional strains from culture collections deglycosylated SDG: *Bacteroides distasonis* DSM 20701^T, *B. fragilis* DIfE-05, *C. cocleatum* DSM 1551^T and *Clostridium ramosum* DSM 1402^T. Thus, five out of eight SDG-deglycosylating strains belong to *Bacteroides* species, which have been extensively studied for their ability to metabolise sugars, indigestible polysaccharides and glycosylated compounds (Bokkenheuser *et al.*, 1987; Backhed *et al.*, 2005). Since the genus *Bacteroides* is one of the most prevailing and prevalent bacterial genera in the human intestinal tract, it is reasonable to assume that the identified *Bacteroides* species play a major role in SDG deglycosylation (Rigottier-Gois *et al.*, 2003; Eckburg *et al.*, 2005).

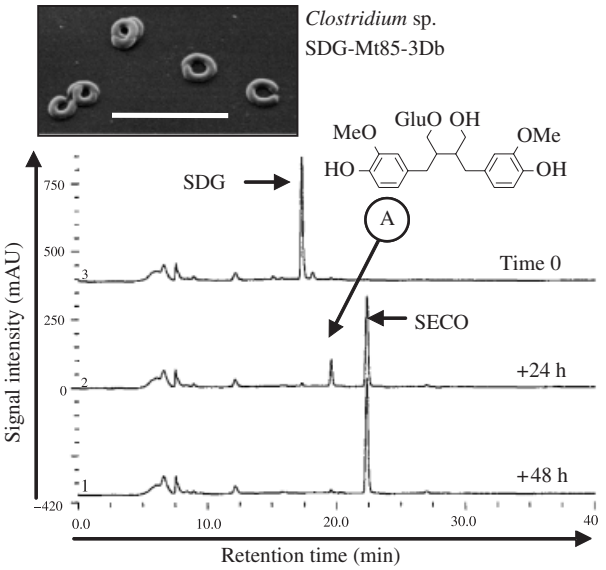

Fig. 2. Deglycosylation of secoisolariciresinol diglucoside by the newly isolated strain *Clostridium* sp. SDG-Mt85-3Db grown in Mt-3 broth. Samples were collected at the times indicated on the chromatograms and supernatants were analysed by high-performance liquid chromatography. The deglycosylation of secoisolariciresinol diglucoside led to the formation of secoisolariciresinol, via compound A (19.6 min). The molecular mass of A was 524 g mol^{-1}, as determined by mass spectrometry. It corresponds to secoisolariciresinol diglucoside with one glucose molecule removed. The bar in the photograph represents 5 μm.

Identification of SECO-demethylating bacteria

Five strains from culture collections demethylated SECO: *Bacteroides methylotrophicum* DSM 3468^T, *Eubacterium callanderi* DSM 3662^T, *Eubacterium limosum* DSM 20543^T, *Peptostreptococcus productus* DSM 2950^T and *P. productus* DSM 3507. Figure 3 shows the demethylation of SECO by *P. productus* DSM 2950^T. The previously isolated strain *P. productus* SECO-Mt75m3 was not able to demethylate SDG (data not shown). All the SECO-demethylating strains belong to the functional group of acetogens, which produce acetate at the expense of H_2 plus CO_2. Some acetogens also utilize methyl groups from various compounds for acetate formation (Frazer, 1994; Hur & Rafii, 2000). Two non-methane-excreting individuals were previously shown to harbour 7.2×10^7 and 3.1×10^8 acetogens g^{-1} wet faeces (Dore *et al.*, 1995). Besides, the occurrences of *P. productus* and *E. limosum* reported in the literature using PCR or fluorescent *in situ* hybridization are in the range of approximately 10^8 CFU g^{-1} as revealed previously by most probable number enumerations of ED-producing bacteria (Wang *et al.*, 1996; Kageyama & Benno, 2001; Clavel *et al.*, 2005). Since three strains of *P. productus* demethylated SECO, the activity appears to be conserved within this species. In contrast, a number of functional or phylogenetic relatives of *E. limosum* and *P. productus* did not demethylate SECO.

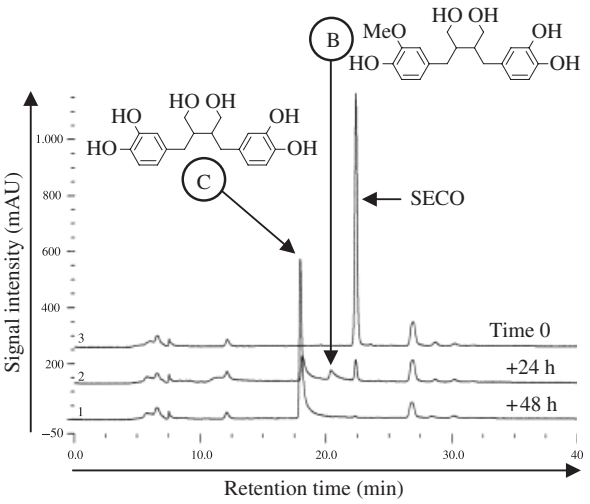

Fig. 3. Demethylation of secoisolariciresinol by *Peptostreptococcus productus* DSM 2950^T grown in Mt-6 broth. The demethylation of secoisolariciresinol led to the formation of compound C (18.0 min), via compound B (20.2 min). The molecular mass of B was 348 g mol^{-1}, as determined by mass spectrometry. It corresponds to secoisolariciresinol with one methyl group removed. Compound C was identified as the 2,3-bis(3,4-dihydroxybenzyl)butene-1,4-diol by comparison with the retention time and the spectrum of the standard. It lacks the two methoxy groups of secoisolariciresinol.

Identification of SECO-dehydroxylating bacteria

When co-incubated with *P. productus* SECO-Mt75m3, two strains from culture collections catalysed the dehydroxylation of SECO: *Clostridium scindens* DSM 5676^T and *E. lenta* DSM 2243^T. Figure 4 illustrates the dehydroxylation of SECO by *Eggerthella lenta* DSM 2243^T. The previously isolated strain *Eg. lenta* SECO-Mt75m2 incubated alone in Mt-6 did not show any activity towards SECO (data not shown). Early molecular studies showed that *C. scindens* and *Eg. lenta*, which both belong to the functional group of biliary steroid-metabolising bacteria, are common members of the human intestinal microbiota (Bokkenheuser *et al.*, 1979; Doerner *et al.*, 1997; Schwiertz *et al.*, 2000; Kitahara *et al.*, 2001). Besides, as in the case of SECO demethylation by *P. productus*, the dehydroxylation activity was observed for several strains of *Eg. lenta*.

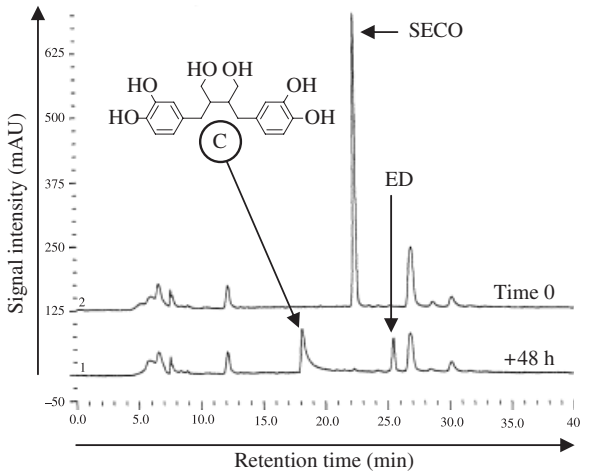

Fig. 4. Formation of enterodiol from secoisolariciresinol by a co-culture of *Peptostreptococcus productus* SECO-Mt75m3 and *Eggerthella lenta* DSM 2243^T grown in Mt-75 broth.

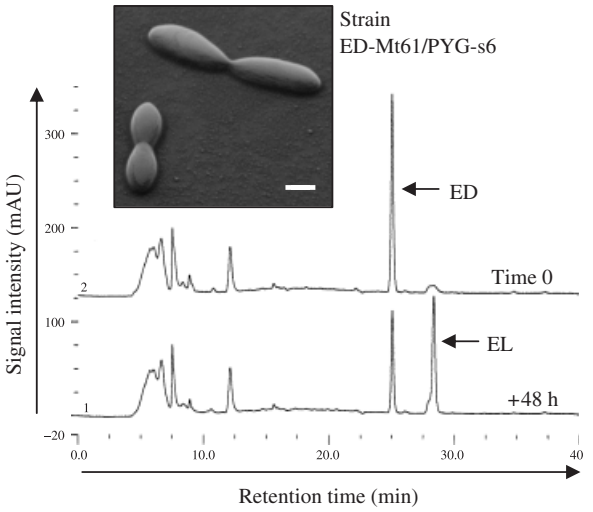

Fig. 5. Dehydrogenation of enterodiol by the newly isolated strain ED-Mt61/PYG-s6 grown in Mt-6 broth. The bar in the photograph represents 1 μm.

Identification of ED-dehydrogenating bacteria

One Gram-positive rod-shaped strain capable of dehydrogenating ED was isolated from faeces (Fig. 5). Its 16S rRNA gene sequence (1437 nucleic acids) showed 93.6% similarity with *Clostridium amygdalinum* DSM 12857^T (AY353957) and 93.4% similarity with *Clostridium saccharolyticum* DSM 2544^T (Y18185). The organism is referred to as strain ED-Mt61/PYG-s6 (DQ100449). It was not able to dehydrogenate SECO (data not shown). The most probable number enumerations of approximately 3×10^5 CFU g^{-1} dried faeces for EL-producing bacteria suggest that the isolate belongs to subdominant communities (Clavel *et al.*, 2005).

Overview of the identified SDG-activating bacteria

Figure 6 illustrates the phylogenetic relationships of the SDG-converting anaerobic bacteria identified in this study. Sixteen strains from the human intestinal tract have been shown to contribute to the conversion of SDG. Two other SECO-demethylating organisms, *Butyribacterium methylotrophicum* and *E. callanderi*, are not known yet as members of the intestinal microbiota. Thus, the production of EL requires the interaction of phylogenetically and functionally

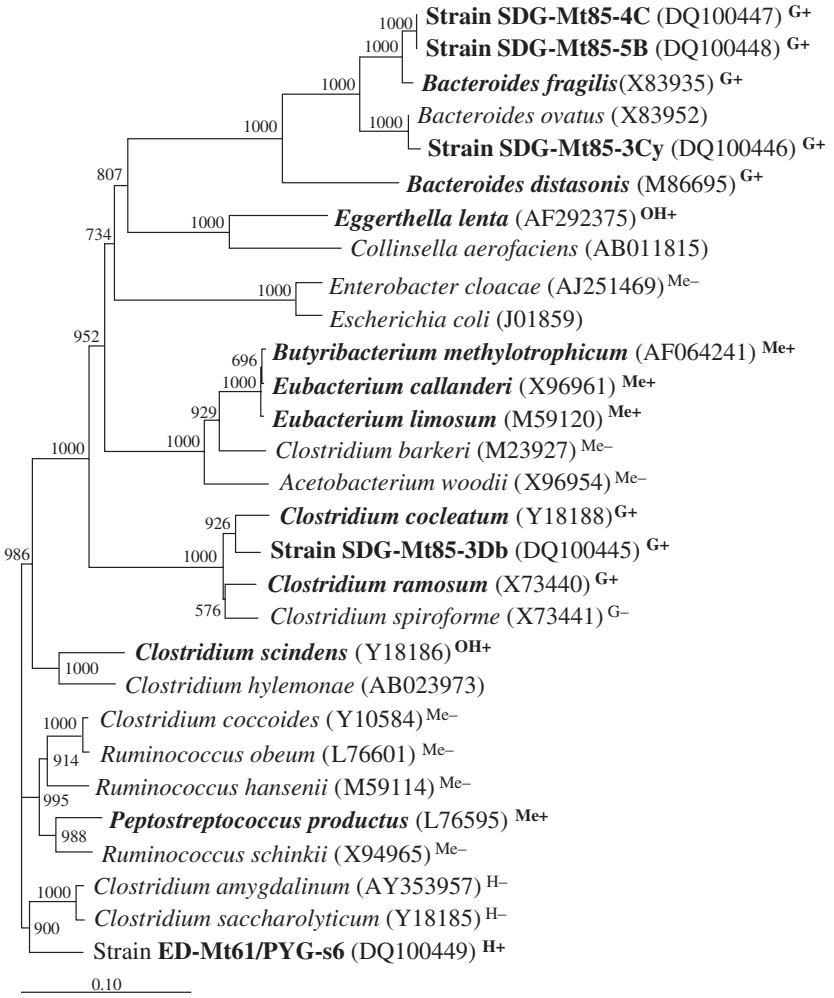

Fig. 6. Phylogenetic tree of secoisolariciresinol-diglucoside-converting bacteria. Strains were tested for their ability to catalyse one of the reactions of secoisolariciresinol diglucoside conversion, as indicated by superscript letters as follows: ^{G}for deglycosylation, ^{Me}for demethylation, ^{OH}for dehydroxylation, ^{H}for dehydrogenation. Bacteria capable of converting the corresponding substrate appear in bold letters with an additional superscript '+'. The non-converting strains tested are marked with a superscript '−'. The GenBank accession numbers of the sequences used to construct the tree are indicated in brackets. Sequences were aligned using the Vector NTI Suite 9.0.0, and the tree was constructed with Clustal X 1.8 using bootstrap values calculated from 1000 trees.

distantly related species, most of which are members of the dominant intestinal microbiota. Variations in their proportions within intestinal communities of different human subjects may explain the large interindividual differences observed previously with regard to the occurrence of SECO-converting bacteria and enterolignan production (Clavel *et al.*, 2005). With 11 species falling into six different genera, SDG-converting intestinal bacteria exhibit a relatively high diversity. Among the isolated organisms, *Clostridium* sp. SDG-Mt85-3Db and strain ED-Mt61/PYG-s6, which deglycosylated SDG and dehydrogenated ED, respectively, may be one new species and one new genus, on the basis of their 16S rRNA gene sequences (Stackebrandt & Goebel, 1994). Further biochemical, enzymatic and molecular experiments

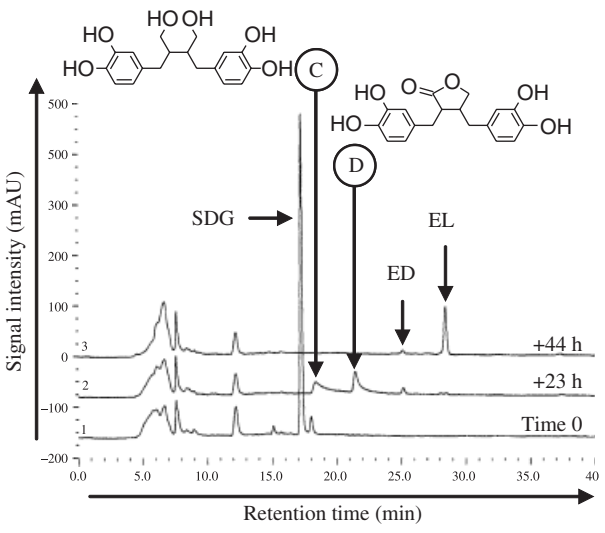

Fig. 7. Formation of enterolactone from secoisolariciresinol diglucoside by a co-culture of *Clostridium* sp. SDG-Mt85-3Db, *Peptostreptococcus productus* SECO-Mt75m3, *Eggerthella lenta* SECO-Mt75m2 and strain ED-Mt61/PYG-s6 grown in Mt-6 broth. The molecular mass of D was 330 g mol^{-1}, as determined by mass spectrometry. It corresponds to matairesinol, the lactone form of secoisolariciresinol, with its two methoxy groups removed.

will be performed for a complete description of these organisms. The fact that several species catalyse the same reaction towards SDG and that the conversion potential seems to be wide-spread among different strains of one species may explain the high prevalence of enterolignan production in humans (Clavel *et al*., 2005).

The incubation of SDG with four of the identified organisms, one for each of the four reactions of SDG conversion, led to the formation of EL (Fig. 7). Figure 7 also illustrates the possible identification of a new intermediate in the formation of EL from SDG. Thus, the newly isolated strain ED-Mt61/PYG-s6 might not only catalyse the dehydrogenation of ED, but also the dehydrogenation of SECO lacking its two methoxy groups.

Conclusion

Eleven species previously unknown for their role in the anaerobic conversion of SDG have been identified. They may be of use for the biotechnological production of EL, the commercial availability of which is restricted. The study was deliberately limited to a qualitative description of the bacterial conversion of SDG. Furthermore, our results do not exclude the possibility that other bacteria may contribute to the conversion of SDG. However, the identified bacteria are now useable as models to characterize the mechanisms of EL production in more detail. In parallel, their relevance could be assessed by the design and optimization of specific 16S rRNA-targetting probes or primers. Such approaches may form the basis of future animal experiments or human intervention studies to assess the influence of changes in SDG-activating microbial communities on the *in vivo* bioavailability and activity of the oestrogen-like compounds ED and EL.

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