

ORIGINAL ARTICLE

The gut anaerobe *Faecalibacterium prausnitzii* uses an extracellular electron shuttle to grow at oxic–anoxic interphases

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***Faecalibacterium prausnitzii* is one of the most abundant bacteria in the human gut ecosystem and it is an important supplier of butyrate to the colonic epithelium. Low numbers of faecalibacteria have been associated with inflammatory bowel disease. Despite being extremely oxygen sensitive, *F. prausnitzii* is found adherent to the gut mucosa where oxygen diffuses from epithelial cells. This paradox is now explained on the basis of gas tube experiments, flavin-dependent reduction of 5,5'-dithiobis-2-nitrobenzoate and microbial fuel cell experiments. The results show that *F. prausnitzii* employs an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen. Both compounds are present in the healthy human gut. Our observations may have important implications for the treatment of patients with Crohn's disease, for example, with flavin- or antioxidant rich diets, and they provide a novel key insight in host–microbe interactions at the gut barrier.**

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Introduction

Microbial communities in the large intestine have vital roles in the regulation of gut health and pathogenesis of disease. Dietary fibers such as resistant starch, plant cell wall materials and oligosaccharides that escape digestion by host enzymes reach the colon and serve as substrates for microbial fermentation (Saulnier *et al.*, 2009). These plant fibers have a major impact on the gut microbial ecosystem as they serve as growth substrates for gut bacteria and are fermented to short chain fatty acids (SCFAs) (Mahowald *et al.*, 2009). Moreover, they are rich sources of polyphenols that can act as redox mediators (Manach *et al.*, 2004). Due to intense microbial metabolic activity the oxygen tension in the gut lumen is considerably lower when compared to that of the gut mucosa (Due *et al.*, 2003), which possesses a sufficiently high oxygen tension to

inhibit growth of the strict anaerobes that are favored in the lumen (Hill, 1995). The major non-gaseous end products of fermentation formed in the colon are SCFAs, including acetate, propionate and butyrate (Topping and Clifton, 2001), that are important modulators of gut health. In particular, butyrate is the preferred energy source for the colonic mucosa and has a role in the protection against colitis and colorectal cancers (Hamer *et al.*, 2009).

Recently, *Faecalibacterium prausnitzii*, an acetate-consuming and butyrate-producing gut microbe has attracted great interest because of its potentially important role in promoting gut health (Sokol *et al.*, 2008; Louis and Flint, 2009). The most likely pathway utilized by this organism to produce various fatty acids including butyrate from the main carbon source glucose is shown in Figure 1. *F. prausnitzii* is one of the most abundant human colonic bacteria with numbers ranging from 5–20% of the total microbiota in stools of healthy individuals (Tap *et al.*, 2009; Walker *et al.*, 2011). Moreover, low counts of *F. prausnitzii* have been associated with several pathological disorders, such as Crohn's disease (Sokol *et al.*, 2009; Willing *et al.*, 2009). Habitually, faecalibacteria are found within the fecal mass but they are also present in the

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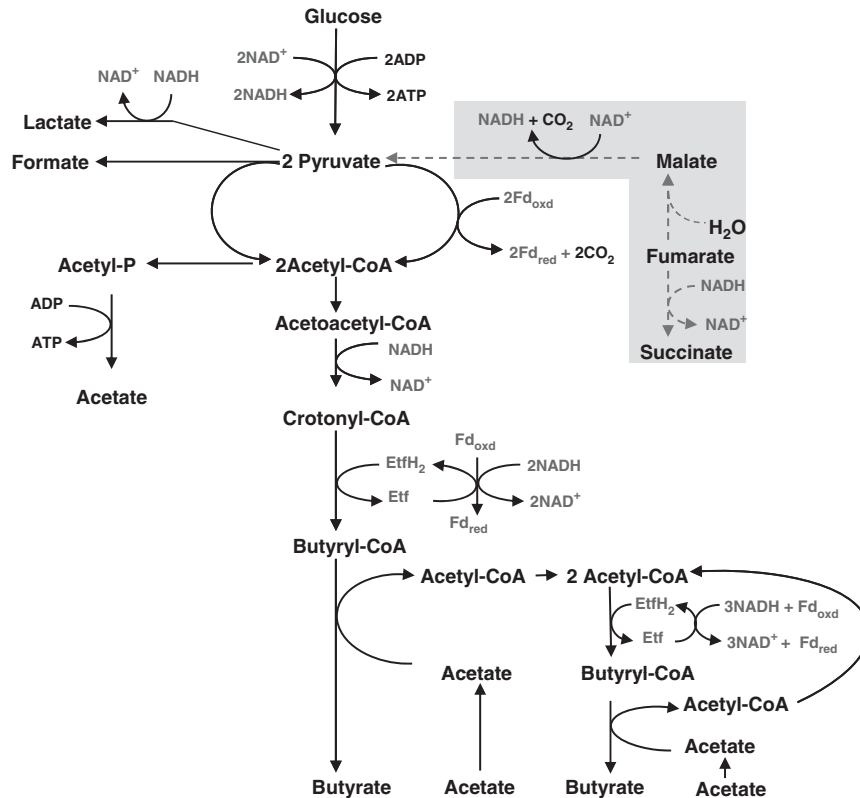


Figure 1 Presumed pathways for the production of butyrate and other short-chain fatty acids from the glucose utilized by *F. prausnitzii* strains. The diagram also depicts the net acetate consumption and cycling of general intracellular electron carriers (blue). Adapted from diagrams described by Herrmann *et al.* (2008) and by Seedorf *et al.* (2008). An alternative pathway for the use of fumarate as terminal electron acceptor and carbon source is indicated in the grey box with red dotted arrows. The colour reproduction of this figure is available at the *ISME Journal* online.

adjacent mucosa where there is oxygen influx from the gut epithelium (Swidsinski *et al.*, 2008; Van den Abbeele *et al.*, 2011). To date, *F. prausnitzii* has been considered a strict anaerobe that loses its viability within 2 min after exposure to ambient air (Duncan *et al.*, 2002). It was thus unclear how such a strict anaerobe could exist in regions of the colon with relatively high oxygen tensions. Our present studies were aimed at unraveling the factors that enable the survival of *F. prausnitzii* in oxygenated niches within the human colon.

Materials and methods

Bacterial strains and culturing conditions

F. prausnitzii strain A2-165 (DSM 17677) was described previously (Duncan *et al.*, 2002) and *F. prausnitzii* strain HTF-F was isolated from human feces and identified by 16S rRNA sequencing. *Roseburia inulinivorans* A2-194 (DSM16841) (Duncan *et al.*, 2006), *Bacteroides fragilis* ATCC 25285, *Escherichia coli* ATCC 25922 and *Bifidobacterium animalis* ATCC 25527 were used as control strains. All strains were maintained at 37 °C on yeast extract, casitone, fatty acid and glucose medium (YCFAG) in an anaerobic tent (Duncan *et al.*, 2002; Lopez-Siles *et al.*, 2012). For microbial fuel cell

experiments, the bacterial cells were grown in 50 ml of YCFAG broth to an optical density at 600 nm (A_{600}) of ~0.8. Cells were harvested by centrifugation, washed in 50 mM potassium phosphate buffer and resuspended in 0.5 ml of the same buffer. The resuspended cells were used to inoculate the anode chamber of a microbial fuel cell. All buffers and media were deoxygenated by flushing with oxygen-free nitrogen for 30 min.

Gas tube experiments

To test for growth at different oxygen concentrations, 50 µl of a bacterial overnight culture was mixed with 7 ml of molten YCFAG agar medium (~38 °C) and poured into 250 mm × 18 mm glass tubes that were sealed at both ends with gas-tight butyl rubber plugs. Sterile air was blown in at one end, while the other end contained N₂ (81%), H₂ (11%) and CO₂ (8%). The presence or absence of a growth rim was observed after 16–20 h at 37 °C. The basal medium used to test conditions for growth rim formation was YCFAG medium without hemin, resazurin, cysteine, yeast extract, acetate and other SCFAs. To determine the effect of glutathione on growth rim formation, 0.14% glutathione was added to riboflavin-containing basal medium instead of cysteine. All tested chemicals in the different

experiments were added separately at the same concentration as in YCFAG medium. Riboflavin was added in the concentration of $0.75 \mu\text{g ml}^{-1}$. SCFA production profiles of *F. prausnitzii* strains were assessed by growing the cultures in YCFAG10 medium in which the glucose concentration was reduced to 10 mM.

Oxygen consumption assays

Oxygen consumption assays were conducted in 35 ml crimp cap vials provided with butyl stoppers containing 5 ml of YCFAG medium. Medium was prepared and added in a strictly anaerobic oxygen-free nitrogen environment and 15 ml of sterile air was injected after inoculation. The residual oxygen in the gas phase was measured by gas chromatography after 48 h incubation, while the spent broth was used for SCFA analysis. In these vials oxygen acts as terminal electron acceptor. Vials having nitrogen in the gas phase served as control for fatty acid production under anaerobic conditions. Additionally, sodium fumarate (55 mM) was used as terminal electron acceptor in YCFAG10 medium and, after 24 h of incubation, the residual broth was used to determine the fatty acid production profile.

Microbial fuel cell experiments

A two-chambered microbial fuel cell with working volumes of 65 ml in the cathode and anode chambers was constructed as described by Park and Zeikus (2000). A CMI-7000S cation exchange membrane (Membranes International INC., Ringwood, NJ, USA) was used as a septum between the two chambers. A 0.5-mm platinum wire was used as cathode with 100 mM potassium-phosphate buffer as catholyte. A plain graphite plate (5 cm \times 1.5 cm \times 0.2 cm) was used as anode. Furthermore, the anode chamber contained 50 mM potassium-phosphate buffer as anolyte, 0.1 M glucose and an Ag/AgCl reference electrode. The whole system was maintained at 37 °C, and the anode chamber was continuously flushed with nitrogen gas. A voltage of -100 mV versus the Ag/AgCl reference electrode was applied on the anode, and the generated current was monitored at 30 s intervals using a potentiostat (model # 600C, CH Instruments, Austin, TX, USA). After energizing the bacterial cells with glucose for 10 min, $200 \mu\text{M}$ riboflavin was injected into the anode chamber.

Flavin-mediated DTNB reduction assay and sugar estimation

To test for 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB, Sigma Aldrich, St Louis, MO, USA) reduction, 1 ml aliquots of overnight cultures of *F. prausnitzii* A2-165 or HTF-F in YCFAG medium were harvested by centrifugation and washed twice with anaerobic potassium phosphate buffer solution (50 mM).

The cells were then added to a reaction mixture containing 50 mM potassium phosphate buffer, $60 \mu\text{M}$ CaCl_2 , $36 \mu\text{M}$ MgCl_2 , 1.3 mM DTNB, 15 mM NaCl and 3.3 mM sodium acetate. The reaction mixture was incubated anaerobically for 30 min at 37 °C with or without riboflavin ($0.75 \mu\text{g ml}^{-1}$), flavin adenine dinucleotide ($0.75 \mu\text{g ml}^{-1}$) or yeast extract (0.2%), and the development of yellow color was monitored as an increased absorbance at 420 nm (A_{420}) using UV-Visible spectroscopy. The results were expressed as fold increase in A_{420} relative to the absorbance without added flavins or yeast extract. The glucose consumption was measured using the 3, 5-dinitrosalicylic acid (DNSA) method (Miller, 1959).

Chromatography

A HPLC Ion Chromatography system (Metrohm AG, Herisau, Switzerland) with a conductivity detector was used for measuring fatty acid concentrations including lactate, formate, butyrate, propionate and acetate. A 6.1005.200 Metrosep Organic Acids column (Metrohm AG) with $5 \mu\text{m}$ particle size and dimensions of 7.8 mm \times 250 mm was used for fatty acid separation. The mobile phase was composed of 5 mM HNO_3 and acetonitrile with a v/v ratio of 98:2, respectively, and a flow rate of 0.6 ml min^{-1} was applied. Fatty acid estimation in samples having sodium fumarate as terminal electron acceptor was conducted on a HPLC system (Waters Chromatography B.V., Etten-Leur, The Netherlands) with a refractive index detector. Separation was achieved using a Varian Metacarb 67H, 300 mm \times 7.8 mm column (MetaChem Technologies Inc., Torrance, CA, USA) at room temperature in isocratic mode at a flow rate of 0.6 ml min^{-1} . A guard column of the same phase was placed in front of the analytical column. The eluent was 0.5 M sulfuric acid (AR grade, Sigma Aldrich). Oxygen consumption was analyzed by gas chromatography using a Molecular Sieve column 5A 80/100 mesh (Agilent Technologies, Amstelveen, The Netherlands).

Results

Growth of *F. prausnitzii* in the presence of oxygen

Previous tests indicated extreme oxygen sensitivity of *F. prausnitzii* cells on agar surfaces to air containing about 20% oxygen (Duncan *et al.*, 2002). In the present studies, the influence of different gas phases on growth of *F. prausnitzii* was tested under conditions that create an oxygen gradient (Figure 2a). Surprisingly, *F. prausnitzii* was found to form a growth rim at the oxygenated end (Figure 2b). In contrast, another obligate anaerobic *Firmicute* that also produces butyrate in the human colon, *R. inulinivorans* A2-194, only grew diffusely throughout the anoxic zone (Figure 2c). The rim formation by *F. prausnitzii* was due to enhanced growth at the junction of the oxidized (pink) and reduced (colorless) zones revealed by resazurin, which was used as

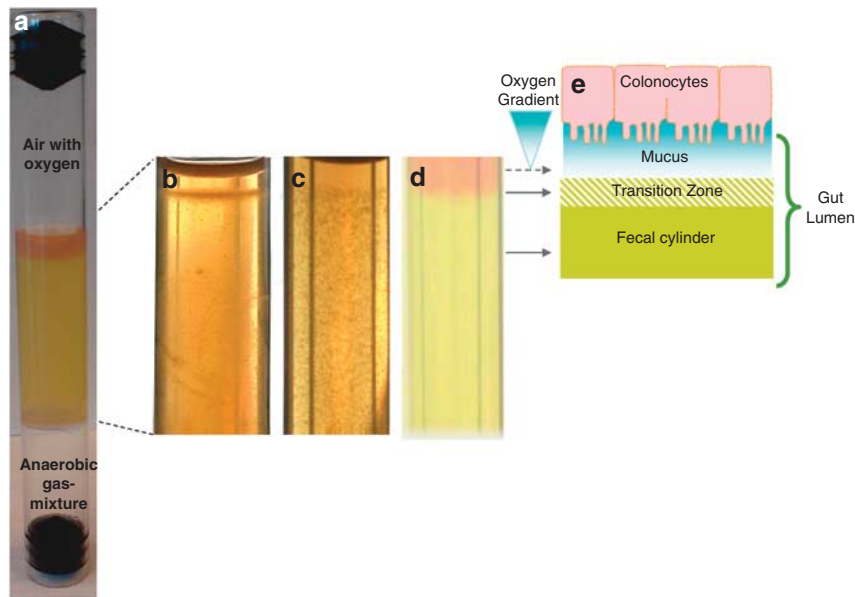


Figure 2 Growth of anaerobic gut bacteria after 16 h in agar medium with and without an oxygenated gas phase. (a) Gas tube experiments are based on glass tubes with two gas phases at the opposite ends of an agar medium plug. The gas phase at the top is composed of ~5 ml oxygen-containing air and the gas phase at the bottom is an anaerobic gas-mixture of ~81% N₂, ~11% H₂ and ~8% CO₂. (b) *F. prausnitzii* A2-165 showing enhanced growth in a rim at the oxygenated end and moderate growth in small colonies throughout the rest of the agar plug. (c) *R. inulinivorans* A2-194 showing growth in colonies throughout the agar plug except at the oxygenated zone. (d) Un-inoculated medium showing a color change of resazurin due to oxygen diffusion. (e) Schematic interpretation of the gas tube oxygen gradient on the human gut lumen and mucosal layer (Van den Abbeele *et al.*, 2011). *F. prausnitzii* can be found in the transition zone or adherent to gut-mucosa (Swidsinski *et al.*, 2008).

a redox indicator (Figure 2d). The oxygenated zone was deeper ($1.5 \text{ cm} \pm 0.2 \text{ cm}$) in uninoculated gas tubes as compared with the inoculated tubes ($0.5 \text{ cm} \pm 0.2 \text{ cm}$). This suggested that oxygen was being consumed by *F. prausnitzii* cells growing in the rim. Eight different isolates of *F. prausnitzii* were tested and all showed the ability to form the growth rim in the oxygenated gas tube zone.

Growth rim formation by F. prausnitzii requires an extracellular flavin-thiol shuttle and a terminal electron acceptor

To understand the mechanism of growth rim formation, basal medium was supplemented with different components. Of the normal components of YCFAG medium (Lopez-Siles *et al.*, 2012), cysteine and yeast extract were both required for growth rim formation by *F. prausnitzii*, but yeast extract could be replaced by riboflavin. However, cysteine alone or in combination with either hemin, resazurin or acetate did not result in the formation of a growth rim. Riboflavin, present in yeast extract was recently reported to be exploited by the fermentative bacterium *Lactococcus lactis*, as an exogenous redox mediator for extracellular electron transfer (Masuda *et al.*, 2010). To confirm the presumed role of flavins and oxidized disulfides in an extracellular electron shuttle, a 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB) assay was performed (Figure 3a). Indeed, *F. prausnitzii* A2-165 cells from the mid log phase ($\text{OD}_{660} 0.48 \pm 0.1$)

were able to use yeast extract or riboflavin as a redox mediator for extracellular electron transfer to DTNB. Reduction of flavins was accompanied by electron transfer to the disulfide bond in DTNB resulting in the yellow-colored compound 2-nitro-5-thiobenzoate (NTB⁻). As shown for strain A2-165, addition of riboflavins, flavin adenine dinucleotide or yeast extract significantly increased the relative folds of absorbance at 420 nm by 9 ± 0.2 , 7 ± 0.2 and 6.5 ± 0.4 , respectively, as compared to samples without added flavins. This implies that flavins are a prerequisite for shuttling electrons. The flavin-thiol shuttling is a feature of living cells, as cells killed with chloroform did not reduce DTNB. We therefore propose that flavins and thiols act as redox mediators in the human gut and that electrons are finally transferred to oxygen (Figure 3b). Accordingly, *F. prausnitzii* rim formation in basal medium with riboflavin was also facilitated by glutathione, which is a primary thiol-containing antioxidant in the gut (Loguercio, 2003; Burrin and Stoll, 2007). To measure the consumption of oxygen, cells were grown in a vial containing yeast extract, casitone, SCFAs including acetate and glucose (YCFAG) broth with oxygen in the gas phase. Indeed, under these conditions a considerable amount of oxygen was consumed per mmol of glucose utilized (Figures 4a and b). Interestingly, our growth experiments showed that faecalibacteria are also capable of efficiently using fumarate as an alternative electron acceptor if no oxygen is present (Figures 1, 4a and b).

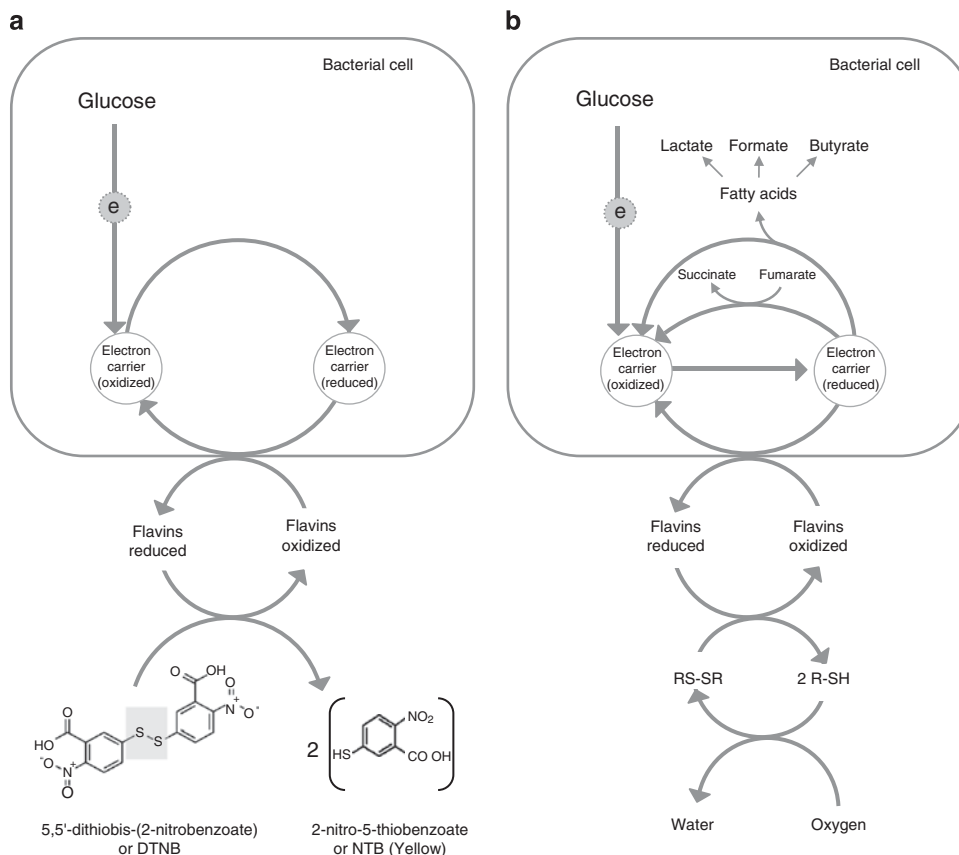


Figure 3 The faecalibacterial extracellular flavin-thiol electron shuttle. **(a)** Bacterial cell, flavin–thiol electron shuttle experiment. In this experiment the bacterial cells (schematically represented) reduce riboflavin with the help of intracellular electron carriers (see Figure 1). The electrons are subsequently transferred from riboflavin to the disulfide bond in DTNB, resulting in the yellow-colored compound NTB⁻. No yellow color is formed when the cells are incubated with DTNB in the absence of riboflavins. **(b)** Model for extracellular electron transfer to oxygen via a flavin–thiol redox shuttle. In the absence of terminal electron acceptor, reducing equivalents are used to produce SCFAs such as lactate, formate or butyrate. If fumarate is available during anaerobic growth, a membrane-associated fumarate reductase transfers electrons to fumarate, resulting in the production of succinate.

Riboflavin is essential in extracellular electron shuttling

To validate the proposed role of riboflavin in extracellular electron shuttling, microbial fuel cell experiments were performed with resting cells of *F. prausnitzii*. As shown in Figure 5 for the *F. prausnitzii* strain A2–165, no current was generated when the cells were energized with glucose. However, when riboflavin was added to the pre-energized cells in the anode chamber, a rapidly increasing current was observed that reached a plateau level of about 6 mA within 5–6 min after the addition of riboflavin (Figure 5). Importantly, no current was observed when riboflavin was added to anode chambers without cells of *F. prausnitzii*, anode chambers with glucose-starved or chloroform-treated cells of *F. prausnitzii*, or anode chambers that were inoculated with cells of *B. animalis*, *B. fragilis*, *E. coli* or *R. inulinivorans*. These findings show unambiguously that riboflavin is required for the extracellular shuttling of electrons by *F. prausnitzii* cells that metabolize glucose aerobically.

The influence of extracellular electron shuttling on SCFA production

Butyrate is generally regarded as the major fermentative end product of *F. prausnitzii* under anaerobic conditions (Figure 1) (Louis and Flint, 2009; Benus *et al.*, 2010). In view of the importance of butyrate for gut health, we investigated whether butyrate production might be altered under the newly identified growth conditions for *F. prausnitzii*. To this end, the molar yields of butyrate per mole of glucose for two metabolically different strains of *F. prausnitzii* were compared (Lopez-Siles *et al.*, 2012) under anaerobic conditions in the presence or absence of fumarate, and under aerobic conditions (carbon balances and molar yields are presented in Supplementary Table 1). Although strains A2–165 and HTF-F are closely related phylogenetically, they differ in growth rates, as the strain A2–165 grows faster than strain HTF-F. These strains also differ in detail in product formation as strain A2–165 produces four-times more formate when grown on anaerobic YCFAG medium when compared with strain HTF-F. For strain A2–165, a decreased

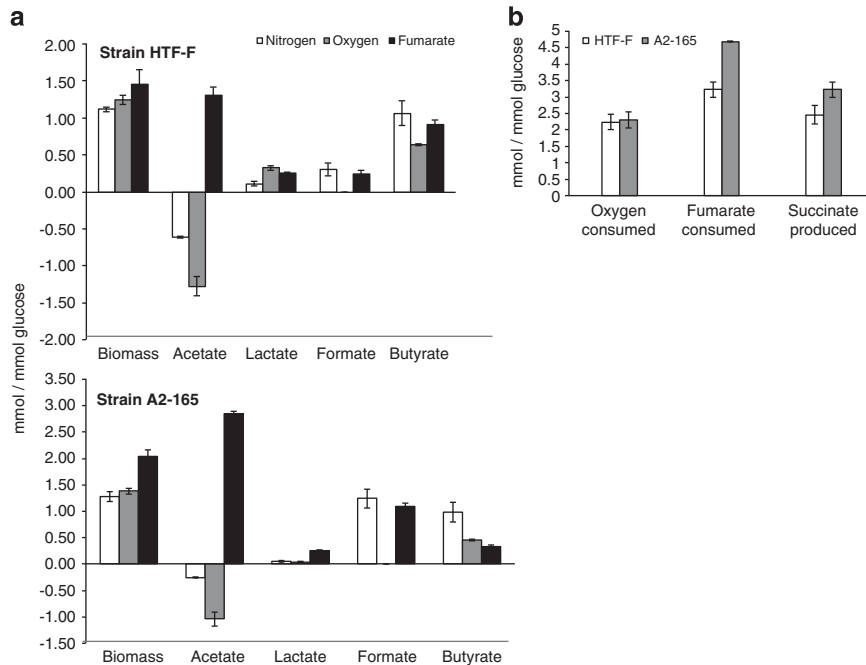


Figure 4 (a) Biomass and organic acids produced or acetate consumed (mmoles) per mmole of glucose by two different strains of *F. prausnitzii*. The strains were cultivated in YCFAG medium with a nitrogen or oxygen gas phase. Alternatively, fumarate was added to the medium of the strains cultivated with a nitrogen head. Note that both oxygen and fumarate can serve as terminal electron acceptors. (b) Oxygen or fumarate consumption per mmol of glucose, and succinate production by two isolates of *F. prausnitzii*. The data presented here is the mean of three independent replicates.

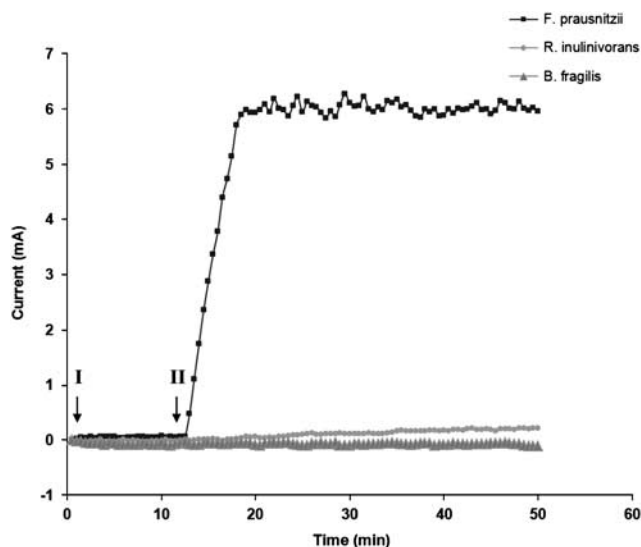


Figure 5 *F. prausnitzii* requires riboflavin for electron transfer to the anode of a microbial fuel cell. The diagram shows current production profiles for the human gut bacteria *F. prausnitzii*, *B. fragilis* and *R. inulinivorans*. (I) Bacterial cells were added to the anode chamber containing potassium phosphate buffer (50 mM) and glucose (0.1 M). A voltage of -100 mV versus Ag/AgCl reference electrode was applied and the current was measured at 30 s intervals. (II) After 10 min of incubation at 37°C , $200\ \mu\text{M}$ of riboflavin was injected as an extracellular redox mediator into the anode chamber. Under these conditions only *F. prausnitzii* generates a measurable current wave.

butyrate production was observed under oxygenated growth conditions or anoxic conditions with fumarate present (Figure 4a). This must relate to an

electron transfer shift from fermentation, that is, SCFA production, to ‘aerobic respiration’ via the flavin–cysteine redox shuttle or to the anaerobic reduction of fumarate by transferring electrons from NADH to succinate while regenerating NAD^+ (Figure 1). In contrast, strain HTF-F did not show a significant decrease in butyrate production when fumarate was used as terminal electron acceptor. This behavior might be due to physiological differences between the tested strains (Lopez-Siles *et al.*, 2012). In the presence of fumarate both strains produced malate and acetate, indicating that fumarate can also serve as carbon source. The elevated acetate formation in strain A2–165 may be explained by decreased acetate cycling (Louis and Flint, 2009). Thus, faecalibacteria metabolize glucose to acetate, CO_2 and electrons, which are disposed of via SCFA formation. Alternatively, the electrons are either transferred to oxygen via extracellular redox mediators or to fumarate, which results in succinate production. Incubations with ^{13}C acetate in the presence of oxygen did not lead to the enrichment of ^{13}C in CO_2 (data not shown), which implies that there was no operational TCA cycle under the conditions tested.

Discussion

In the present studies, we have shown that cells of *F. prausnitzii* can grow in the presence of oxygen, provided that their growth medium contains flavins and cysteine or glutathione. Based on our findings,

we propose that in the presence of oxygen the free thiols of cysteine or glutathione are oxidized to disulfide bonds that subsequently act as electron acceptors, whereas the flavins act as redox mediators for faecalibacterial cells as shown in Figures 3a and b. The rapid shuttling of flavins through the cell envelope is a rather specialized phenomenon, as *Pseudomonas* species and *Escherichia coli* produce riboflavin but do not utilize this compound for extracellular electron transfer (von Canstein *et al.*, 2008). Importantly, our findings give a rationale for the growth and survival of *F. prausnitzii* at the oxygenated fecal-mucosal interface of the human gut (Swidsinski *et al.*, 2008).

Consumed dairy products, plant foliage, fruits and fibers are the main sources of flavin in the gut, whereas thiols can also be obtained by various dietary sources such as egg yolk, dairy products and grains (Dangour, 2010). Additionally, the gut mucosa harbors considerable amounts of thiols that are secreted by colonocytes and act as antioxidants (Keshavarzian, 1992; Loguercio, 2003). Several studies have demonstrated an oxidative surge in colitis that is associated with decreased concentrations of thiols (Aw, 2003; Seril *et al.*, 2003). Oral supplementation of these compounds together with flavins is therefore likely to be useful in controlling the progression of colitis (Loguercio, 2003; Dryden *et al.*, 2005; Kim *et al.*, 2009). This also indicates that such compounds in the gut lumen promote 'gut health' (Kau *et al.*, 2011) and that they may be utilized by anaerobic bacteria in niches, where an oxygen gradient exists such as the gut mucosa.

Extracellular electron transfer by *F. prausnitzii* provides a very plausible mechanistic explanation for the survival of certain anaerobes in oxygenated environments where appropriate redox mediators are available. It remains to be established whether other human colonic anaerobes possess such a capability for extracellular electron transfer, although this was not evident for the butyrate producer *R. inulinivorans* (Figure 2c), and other gut bacteria like *B. animalis*, *B. fragilis* or *E. coli*. Furthermore, our results may provide new insights into clinical conditions such as Crohn's disease. This disease is characterized by aberrant mucosa, thiol depletion and oxidative stress leading to an altered microbiota, for instance reduction in *F. prausnitzii* numbers, which will most likely impact on the pathogenesis (Keshavarzian, 1992; Sokol *et al.*, 2008; Kim *et al.*, 2009). Taken together, the present findings highlight the potential for the flavin and antioxidant status of the diet to have a major influence on beneficial bacteria and their distribution in the human gut and hence on gut health.

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