Bacterial Responses to a Simulated Colon Tumor Microenvironment*†

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One of the few bacteria that have been consistently linked to colorectal cancer (CRC) is the opportunistic pathogen Streptococcus galleyticus. Infections with this bacterium are generally regarded as an indicator for colonic malignancy, while the carriage rate of this bacterium in the healthy large intestine is relatively low. We speculated that the physiological changes accompanying the development of CRC might favor the colonization of this bacterium. To investigate whether colon tumor cells can support the survival of S. galleyticus, this bacterium was grown in spent medium of malignant colonocytes to simulate the altered metabolic conditions in the CRC microenvironment. These in vitro simulations indicated that S. galleyticus had a significant growth advantage in these spent media, which was not observed for other intestinal bacteria. Under these conditions, bacterial responses were profiled by proteome analysis and metabolic shifts were analyzed by 1H-NMR-spectroscopy. In silico pathway analysis of the differentially expressed proteins and metabolite analysis indicated that this advantage resulted from the increased utilization of glucose, glucose derivatives, and alanine. Together, these data suggest that tumor cell metabolites facilitate the survival of S. galleyticus, favoring its local outgrowth and providing a possible explanation for the specific association of S. galleyticus with colonic malignancy. Molecular & Cellular Proteomics 11: 10.1074/mcp. M112.019315, 851–862, 2012.

The human intestine is the habitat for several hundred different bacterial species with an increasing bacterial concentration and variability toward the distal colon (1). The resident gut microbiota is essential for human health by making dietary nutrients available to the host and preventing the invasion of pathogens by competitive colonization and nutrient competition (2, 3). Strikingly, the part of the intestine with the highest bacterial colonization, the colon, is also most affected by cancer, with 146,970 annual cases in the United States of America (4). In a healthy colonic environment, the host has several defense mechanisms to shield itself from bacterial infection, such as the viscous mucus layer overlaying the epithelium. However, the progression of CRC1 is accompanied by changes in the integrity of the colon, including reduced mucus production (5) and increased epithelial permeability (6). These physiological changes can drive the intestinal ecosystem, which is relatively stable during adult life, into dysbiosis (7, 8). As a consequence, the host may become more susceptible to opportunistic bacterial infections (9–11).

One of the few bacteria that have been consistently linked to CRC is the opportunistic pathogen Streptococcus galleyticus (previously known as Streptococcus bovis biotype I). In CRC patients the fecal carriage rate of this bacterium is increased from 10% to about 50% (12), which suggests that this disease facilitates the colonic survival of S. galleyticus. Importantly, ~60% of patients that present with S. galleyticus endocarditis have concomitant CRC (both adenomas and carcinomas) (13, 14), which largely exceeds the rates reported in the general population (~25%) (15). These patients had no gastro-intestinal signs or clinical symptoms of malignancy and CRC was only detected because this bacterial infection guided the physician to perform a colonoscopy.

Several mechanisms for this apparent association between S. galleyticus and CRC can be envisaged. Recently, we postulated a model in which the collagen binding ability of S. galleyticus contributes to the specific colonization of malignant colonic sites (16). However, the altered microenvironment of the tumor may also provide conditions that favor survival and outgrowth of S. galleyticus in this newly formed intestinal niche. For example, Hirayama et al. have shown that glucose-1-phosphate and fructose-1-phosphate levels as well

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† The abbreviations used are: BHI, brain heart infusion broth; CRC, colorectal cancer; DMEM, Dulbecco’s modified Eagle’s medium; FC, fold change; FCS, fetal calf serum; FDR, false discovery rate; F6P, fructose 6-phosphate; G13P, glycerate-1,3-phosphate; G6P, glucose-6-phosphate; MRS, Man, Rogosa, and Sharpe broth; OD, optical density; PCA, principal component analyses; 3PG, 3-phosphate glyceric acid.
as amino acid concentrations were significantly higher in tumor tissue than in normal tissue (17). To investigate if this altered nutritional status of the CRC microenvironment could facilitate the foraging of *S. galloyticus*, we simulated the influence of colon tumor cell metabolites on *S. galloyticus* growth by incubating this bacterium in spent medium of malignant cells. Subsequently, the bacterial responses were profiled by two-dimensional proteome analysis, and metabolic shifts in the culture medium were assessed by 1H-NMR spectroscopy. In *silico* pathway analysis and further in *vitro* simulations showed that, unlike other intestinal bacteria, *S. galloyticus* had a growth advantage under these conditions, which could mainly be attributed to increased glycolysis. These results provide the first molecular support that tumor metabolites may facilitate the local outgrowth of tumor-foraging bacteria, such as *S. galloyticus*.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Bacterial Strains—**Colorectal adenocarcinoma cell lines HT-29, SW480, HCT116, and Caco-2 (www.atcc.org) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza) supplemented with 10% Fetal Calf Serum (further indicated as standard medium) at 37 °C/5% CO₂. These culturing conditions were used unless stated otherwise. The following bacterial strains were used; *Enterococcus faecalis* 19433 (www.atcc.org), *Lactobacillus plantarum* WCSF1 (18) and *Escherichia coli* Nissile 1917 (obtained from Julian Marchesi).

*S. galloyticus* subsp. *galloyticus* UCN54 (further designated as *S. galloyticus*) and the genetically resembling non-pathogenic strain *S. galloyticus* subsp. *macedonicus* CIP105685T (further designated as *S. macedonicus*) from the Pasteur collection (19, 20), *Escherichia coli* NCTB5, *Salmoneilla typhimurium* NTB6 (16), *Staphylococcus lugdunensis* NTB8, *Enterobacter cloacae* NTB9, and *Klebsiella pneumonia* NTB10 from the Radboud strain collection. All strains were grown on Columbia blood agar or in Brain Heart Infusion broth (Difco) supplemented with 1% glucose at 37 °C/5% CO₂. *L. plantarum* was grown in de Man, Rogosa, and Sharpe (MRS)-broth and *E. coli* and *E. coli* Nissile were grown in Brain Heart Infusion at 200 rpm at 37 °C/5% CO₂.

**Bacterial Growth Experiments—**To determine bacterial growth characteristics in the presence of tumor cell metabolites, CRC cell lines were first cultured for 3 days in standard DMEM growth medium. Next, the culture supernatants were collected (designated as spent medium) and bacterial growth in this spent medium was compared with the growth in standard medium (control condition). Every 20 min optical density was measured by determining the OD₆₀₀ in a microplate reader (Ascent).

To determine bacterial growth rates in simulation experiments, standard DMEM medium was supplemented with the amino acids L-alanine (2 mM) or phenylalanine (2 mM). Besides these amino acids, this medium was supplemented with fructose 6-phosphate (F6P) or 3-phosphate glyceric acid (3PG), both at a concentration of 2 mM. Combinations of F6P (1 mM) and 3PG (1 mM) with the amino acids L-alanine (2 mM) and phenylalanine (2 mM) were made to assess the combined effect of these substances on *S. galloyticus* and *E. coli* growth. Media were inoculated with ~1 × 10⁶ *S. galloyticus* or *E. coli* and OD₆₀₀ was measured every 20 min. Bacterial growth rates were determined at the linear part of the bacterial growth curves and plotted using Graphpad 4.0. Slope differences were determined in Graphpad 4.0 using linear regression models and considered significant if p < 0.05.

**Protein Sample Collection—**For 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis, *S. galloyticus* was grown to mid-log phase (OD₆₀₀ of 0.4) in spent medium of Caco-2 or HCT116 cells. Alternatively, *S. galloyticus* was grown in spent medium of Caco-2 cells in the presence of viable Caco-2 cells. As control condition *S. galloyticus* was grown to mid-log phase in standard medium (OD₆₀₀ of 0.2). All incubations were performed in triplicate. At the appropriate OD₆₀₀, bacteria were collected (4000 g, 10 min) and washed 2 times with phosphate-buffered saline (PBS; 8000 g, 3 min). Next, bacteria were suspended in lysis solution (2 mM MgCl₂) containing 3 mg/ml lysozyme (Biochemica) and 100 μg/ml mutanolysin (Sigma Aldrich) and incubated at 37 °C for 60 min. After incubation, 1% SDS was added together with a protease inhibitor mixture (Roche). Samples were sonicated 3 times on ice at 30% with 0.3 s intervals for 20 s on a DIGITAL Sonifier® UNITS Models S-250D (Branson, Danbury, CT). After sonication, benzonase (Novagen, Madison, WI) treatment (0.625 UI/μl) for 30 min on ice was performed to degrade remaining DNA. Insoluble material was removed by spinning the samples at 4 °C for 30 min at 14000 g. The supernatants were solubilized 1:1 in solubilization solution (7 mM Urea, 2 mM Thiourea, 10% CHAPS, 50 mM dithioretioleol (DTT) and 0.2% carrier ampholytes (pH 3–10)) and stored at –80 °C until further analysis. Protein concentration in the samples was measured with the Bradford protein assay (BioRad) according to the manufacturers’ instruction.

2D-PAGE—After thawing, 350 μg protein per sample was diluted in rehydration solution (final concentration: 5 mM Tris-HCl pH 8.8, 7 mM Urea, 3% SDS, 20% glycerol, 2% DTT) for 30 min and subsequently with equilibration solution 2 (0.375 mM Tris-HCl (pH 8.8), 6 mM urea, 2% SDS, 20% glycerol, 4% iodoacetamide) for 10 min. The second dimension was run on Protein II Ready gels (12% Tris-HCl; Bio-Rad) in xTris-Glycerine electrophoresis buffer (Bio-Rad) at 24 mA/gel during 5–6 h using a continuous cooling system. Next, gels were fixed for 1 h in 10% acetic acid and 50% methanol in ddH₂O. Proteins were visualized with Blue silver staining (0.12% Coommassie G-250, 10% (NH₄)₂SO₄, 10% HSP04, 20% methanol). After protein staining, gels were scanned on an Odyssey gel system at 700 nm (focus offset 0.5 mm and scan intensity 4.0).

**Delta-2D Analysis—**High-resolution TIFF files of the protein gels were loaded into Delta-2D (Decodon) and warped following the group warping strategy as outlined in the Delta-2D manual. Because of imperfect IEF, 1 gel from the condition in which *S. galloyticus* was grown in spent medium from Caco-2 cells in the presence of viable Caco-2 cells was excluded from analysis. After warping, a fusion image was generated for spot detection using one of the “control” gels as master image. Spots were automatically detected by the software and visually evaluated to remove “noise” spots. Spot intensities were generated using overall normalization. For a first evaluation of the samples, scatter plots were generated. Furthermore, sample and protein expression clustering were evaluated by principal component analyses (PCA) and hierarchical clustering using complete linkage with Euclidian distance. Statistical analysis was performed using ANOVA for overall comparison of the groups and student’s t test (based on permutations) was used for individual comparisons of the experimental groups to the control group. Expression differences were considered significant at a p value below 0.01. The False Discovery Rate (FDR) was set at 10 proteins or less. Spots were selected for spot identification based on statistical tests, a fold-change of at least 2 and a normalized expression value of at least 0.05 (Fig. 1).
Global Metabolic Pathway Analysis—To obtain a global picture of the affected metabolic pathways of *S. gallolyticus* in all three spent media conditions, significantly changed proteins that were identified in the 2D gel analysis (120 proteins, Fig. 1) were mapped to the KEGG global map of metabolic pathways (21) via orthologous groups (COGs, see supplemental Table S4) using the Signature web server (22). These pathways were visualized on the global metabolic map using iPath (23), where the color represents the average fold-change in expression of all proteins mapped to a specific pathway. A more detailed outline of the affected cellular functions was obtained by using the annotations provided by the RAST server (24), which assigns each function to a subsystem (see supplemental Table S4). Both annotations in KEGG and RAST were used to obtain a general overview of the affected metabolic pathways.

**Fig. 1. Flow-diagram of protein and pathway analysis.** A total of 665 proteins was identified with 2D-PAGE analysis. Based on statistical analysis with ANOVA and t-tests a total 87 differentially expressed protein spots (*p* < 0.01) and an additional 89 protein spots with a fold change of more than 2 (*p* < 0.05) were selected for identification. By in-gel tryptic digestion, vMALDI-MS/MS peptide sequencing and subsequent SEQUEST protein database searches, a total of 120 protein spots could be identified resulting in a total of 78 unique identified proteins. The expression values of these 78 unique proteins were subjected to pathway analysis; average fold changes were used when a protein was found in multiple spots. A total of 71 proteins could be mapped to orthologous groups and of these proteins 32 mapped to the KEGG database, whereas 60 of the 78 proteins could be directly mapped to the RAST database in an alternative pathway annotation strategy.

**Pathway analysis**

- 32 proteins mapping to KEGG
- 60 proteins mapping to RAST

**Identification**

- 176 proteins selected for identification
- 120 protein spots identified with vMALDI-MS/MS
  - contains 78 unique proteins

**2D-analysis**

- 665 proteins in 2D-PAGE analysis
- 87 proteins selected based on ANOVA or t-test
  - *p* < 0.01 - expression > 0.05
- 89 proteins selected based on FC
  - *p* < 0.05 - FC > 2 - expression > 0.05

**vMaldi-MS/MS Protein Identification**—Preparative 2D-PAA gels were prepared as described above. Protein spots were excised with a clean scalpel in a laminar flow hood to reduce keratin pollution and stored in a microtiter plate. Spots were dehydrated with acetonitrile and stored in -80°C until further analysis. Next, gel-pieces were thawed and subjected to in-gel trypsin digesting as described previously (25). After digestion, peptides were extracted by sonication in a water bath for 15–20 s, concentrated using a centrifugal evaporator, and diluted 1:1 with 2% trifluoroacetic acid (TFA). Next, samples were spotted on a stainless steel MALDI plate. Sample analysis was performed on a linear ion trap fitted with an intermediate pressure matrix-assisted laser-desorption ionization source (vMALDI-LTQ; Thermo Fisher Scientific) (26). In total, 5 full MS runs were analyzed, each resulting in a selection of the 10 highest peaks that were further analyzed by collision-induced dissociation fragmentation analysis. Generated RAW-data files, were analyzed with SEQUEST (Ver. 28 Rev.12) and identifications were considered significant with a peptide probability > 1e-002, and a protein probability > 1e-003. The following modifications were allowed in the search: carbamidomethylation of cysteines (fixed), oxidation of methionine (variable) and deamination of asparagine and glutamine (variable). See supplemental Table S1 for further details on vMALDI-MS/MS protein identification, data analysis settings and validation criteria.

**1H-NMR-spectroscopy**—Biological duplicates of media and spent media samples of Caco-2 and HCT116 with or without *S. gallolyticus* were deproteinized using Sartorius Centrisart I filters (cut-off 10 kDa; Sartorius AG, Goettingen, Germany). Before use, the filter was washed twice by centrifugation of water to remove glycerol. A small volume (20 μl) of 20.2 mM trimethylsilyl-2,2,3,3-tetra-deuteropropionic acid (TSP, sodium salt; Aldrich) in D2O was added.
to 700 μl of the ultrafiltrate, providing a chemical shift reference (0.00 ppm) and a deuterium lock signal. Finally, 650 μl of the sample was placed in a 5 mm NMR tube (Wilmad Royal Imperial; Wilmad LabGlass, USA).

Single pulse 1H-NMR spectra (500 MHz) were obtained on a Bruker DMX-500 spectrometer as described by Engelke et al. (27). Phase and baseline were corrected manually.

All spectra were scaled to TSP and metabolite signals were fitted semi-automatically with a Lorentzian line shape. Metabolite concentrations in the spent media were calculated relative to the known concentration in the standard medium and correspondingly expressed as mM or mg/ml. The concentration of lactate in the standard medium was calculated by comparing the area of lactate with the area of the valine doublet at 1.04 ppm.

RESULTS

Growth Advantage of S. gallolyticus in Spent Medium of Colorectal Cancer Cells—To investigate the influence of CRC metabolites on the growth of S. gallolyticus and other intestinal bacterial strains, bacteria were inoculated in spent medium from Caco-2 cells. As indicated in Fig. 2, the bacterial growth rate of S. gallolyticus and S. macedonicus in this medium was significantly increased compared with the growth rate in control medium with a factor 3.1 and 13.9 respectively (p < 0.001). Conversely, the growth rates of L. plantarum, E. coli, S. lugdunensis (p < 0.01), and S. typhimurium (not...
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pathways were enriched in To investigate which metabolic Medium from Tumor Cells— further experiments focused on S. gallolyticus. Proteome Profiling of S. gallolyticus Cells Grown in Spent Medium from Tumor Cells—To investigate which metabolic pathways were enriched in S. gallolyticus while growing in spent media, the changes in the S. gallolyticus proteome were explored by 2D-PAGE analysis in the two media with the most pronounced effects, namely HCT116 and Caco-2 (see supplemental Fig. S2 for original 2D-gels). To this purpose, S. gallolyticus responses on incubation in spent medium from HCT116 cells, and in spent medium from Caco-2 cells in the absence or presence of viable Caco-2 cells, were compared with the control condition in which S. gallolyticus was grown in standard medium. The condition with viable Caco-2 cells was included to allow cross-talk within this hybrid co-culture system. A total of 665 proteins were detected with 2D-proteome analysis (supplemental Fig. S3). Global analysis of these proteomes with both hierarchical clustering (Fig. 3A) and principal component analysis (PCA; Fig. 3B) showed that the proteomes of the replicate conditions cluster together. This indicates that the biological replicates yielded highly similar and reproducible expression profiles, but that distinct protein spots were induced under the different experimental conditions. The expression profiles of S. gallolyticus grown in spent medium of Caco-2 cells were similar to the condition in which also viable Caco-2 cells were present, which implies that components that were already present in the spent medium dominated the altered expression of S. gallolyticus proteins. The protein expression profile of S. gallolyticus grown in spent medium of HCT116 cells showed a somewhat different pattern than the two Caco-2 conditions (Fig. 3A and 3B).

Overall comparison with ANOVA and the individual comparisons with students t-tests of the experimental conditions with the control resulted in a total of 87 differentially expressed protein spots (p < 0.01). An additional 89 proteins were selected for identification based on a fold change of more than 2 (p < 0.05) (Fig. 1).

Identification of Differentially Expressed Proteins—As indicated above, a total of 176 protein spots from the master gel were selected for in-gel tryptic digestion and peptide sequencing by vMALDI-MS/MS. Subsequent SEQUEST searches resulted in the identification of 120 protein spots (68%) (see supplemental Table S2 for annotated profiles and supplemental Table S3 for peptide hits per identified spot with probability scores). These 120 identified protein spots consisted of 78 unique proteins (Fig. 1, Table I). Thus, as depicted in Table I, several proteins were detected in more than one protein spot. This is most likely because of post-translational protein processing such as glycosylation, phosphorylation, and/or proteolytical processing. Since it is difficult to make a functional distinction between the observed isoforms, we have chosen to present an average fold change per identified protein (Table I). Functional classification of these proteins shows that most proteins have a function in metabolism, especially in carbohydrate metabolism, amino acid metabolism, and metabolism of nucleotides (Table I). Other identified proteins are involved in protein synthesis, cell wall synthesis and cellular processes. A total of 14 proteins could not be functionally annotated. Overall, the protein expression differences of S. gallolyticus when this bacterium was grown in HCT116 and Caco-2 spent media compared with the control condition pointed in the same direction. Only 6 of the 78 unique S. gallolyticus proteins showed opposite expression patterns in HCT116 medium compared with Caco-2 medium (indicated with an asterisk in Table I). Thus, whereas hierarchical clustering and PCA of all 665 proteins suggested different expression patterns when S. gallolyticus was grown in Caco-2 or HCT116 medium, investigation of only the 176 S. gallolyticus proteins with significantly altered expression levels showed similar protein expression changes. This suggests that the consistent growth advantage of S. gallolyticus in both media conditions is most likely mediated by the same metabolic factors.

Pathway Analysis Reveals Up-regulation of Glycolysis—To visualize whether significantly altered proteins of S. gallolyticus in both HCT116 and Caco-2 media belonged to common pathways, the average fold changes were subjected to pathway analysis (Fig. 1, supplemental Table S4). Pathway analysis with both KEGG (supplemental Fig. S4) and RAST annotation tools indicated similar changes in bacterial metabolism when grown in Caco-2 or HCT116 medium (Table II), although the overall changes were more pronounced in spent medium from Caco-2 cells. Most of the identified proteins belonged to the glycolysis (up-regulated) and purine metabolism pathways (down-regulated) (Table 2A and 2B) and showed similar outcomes when either one of the annotation tools was used. The average fold changes in the glycerolipid, glycolysis, and fructose utilization pathways suggest that increased utilization of glucose and glucose derivatives is a central event in S. gallolyticus cells grown in spent medium from both Caco-2 and HCT116 cells. A closer look, combining RAST and KEGG annotation, reveals that the glycolysis pathway was up-regulated between the enzymes glucose-6-phosphate (G6P) and glyceraldehyde-3-phosphate (3PG) in both Caco-2 (Fig. 4A) and HCT116 (Fig. 4B) medium. However, conversion of 3PG to glyceraldehyde-3-phosphate (G13P) was increased in Caco-2 medium and decreased in HCT116 medium, whereas the conversion of G13P to glyceraldehyde-3-phosphate was increased in HCT116 medium alone. Taken together, these findings suggest that S. gallolyticus preferentially forages on glucose and its break-down products, and
that these secondary glucose metabolites of tumor cells provide *S. gallolyticus* with improved growth characteristics. It should be noted that the observed changes in metabolic pathways are not just the simple result of the increased growth rate of *S. gallolyticus* but of a different employment of its preferred metabolic pathways, as shown by the fact that several pathways, such as purine metabolism, have also been down-regulated under the spent medium conditions (Table II).

**Metabolome Analysis Confirms Up-regulation of Glycolysis**—To further investigate which tumor cell metabolites in spent medium could be responsible for the growth advantage of *S. gallolyticus*, the metabolites in standard medium (control) and spent medium from Caco-2 and HCT116 cells were analyzed by ¹H-NMR-spectroscopy before and after incubation with *S. gallolyticus* (Fig. 5). Duplicate analyses were highly reproducible and indicated that both Caco-2 and HCT116 cells had a high glucose metabolism as indicated by i) the reduction of glucose (box in Fig. 5A–5C) and pyruvate, and ii) an increase in lactate levels in spent medium compared with standard medium (Fig. 5A–5C; Table III). In addition, *S. gallolyticus* had an increased ability to metabolize glucose in both Caco-2 and HCT116 spent medium (Fig. 5D–5F), which was shown by a reduction in glucose level of 31% in both spent media compared with 7.5% in standard medium (Table III). That notion that glycolysis in *S. gallolyticus* was up-regulated when grown in these tumor cell media was confirmed by the increased production of pyruvate under these conditions (0% in standard medium, versus 14.7% in Caco-2 and 34.6% in HCT116; Fig. 5K–5L). It must be noted, however, that intermediate glucose metabolites, such as G6P, fructose-6-phosphate (F6P) and 3PG could not be discriminated by ¹H-NMR spectroscopy. Therefore, the increased utilization of glucose in spent media could as well be attributed to these glucose metabolites as indicated by pathway analysis of the *S. gallolyticus* proteins with significantly altered expression levels (Fig. 4). Furthermore, alanine was found to have increased levels in spent medium of both Caco-2 and HCT116 cells, whereas the alanine levels decreased after growth of *S. gallolyticus* by 30% in these media (Fig. 5G–5L, Table III), suggesting that alanine might also be a growth factor for *S. gallolyticus*. The levels of acetoin (Fig. 5J–5L), a bacterial metabolite of pyruvate metabolism, nicely reflect the increase of the bacterial glycolysis pathway in the spent media, and could therefore be used as a surrogate marker for increased glucose and glucose derivate consumption under these conditions. Taken together, pathway and ¹H-NMR-anal-

**Fig. 3.** Hierarchical clustering and principal component analysis. Protein expression levels of *S. gallolyticus* grown in standard medium (A), spent medium of Caco-2 cells (B) or HCT116 cells (C), or in spent medium of Caco-2 cells in the presence of viable Caco-2 cells (D) were visualized using hierarchical clustering analysis (A) and principal component analysis (B); numbers refer to the biological replicas of each experimental condition. Both these analyses showed that the replicate experiments of each condition clustered together. Furthermore, the two independent Caco-2 conditions, with and without viable cells, (B and D) had similar expression patterns.
### TABLE I

Identified S. gallolyticus proteins with significant changes in expression. *Proteins that have opposite expression in Caco-2 versus HCT116 medium.* v-Caco-2 = S. gallolyticus grown in medium of viable Caco-2 cells.

| spot nr | functional category | GALLO-code | protein | Caco-2 | HCT116 | v-Caco-2 |
|---------|---------------------|------------|---------|--------|--------|----------|
| 1, 2    | Detoxification      | GALLO_0604 | NADP⁺-H⁺ oxidoreductase | 1.05 | 0.98 | 1.08 |
| 3       | Detoxification      | GALLO_0211 | NADH oxidoreductase | 1.45 | 0.92 | 1.00 |
| 4, 67   | Metabolism of amino acids and related molecules | GALLO_1129 | glutamate dehydrogenase | 1.50 | 1.30 | 1.00 |
| 7       | Metabolism of amino acids and related molecules | GALLO_0830 | ATP-dependent amidohydrolase | 1.59 | 1.25 | 1.00 |
| 64      | Membrane bioenergetics | GALLO_1555 | phosphoenolpyruvate carboxykinase | 1.03 | 0.97 | 1.00 |
| 65      | Membrane bioenergetics | GALLO_0829 | pyruvate-ferredoxin oxidoreductase | 1.03 | 1.03 | 1.00 |
| 66      | Membrane bioenergetics | GALLO_0791 | ATP synthase alpha chain | 1.03 | 1.00 | 1.00 |

**Intermediary metabolism**

| spot nr | functional category | GALLO-code | protein | Caco-2 | HCT116 | v-Caco-2 |
|---------|---------------------|------------|---------|--------|--------|----------|
| 10, 16, 18 | Metabolism of carbohydrates and related molecules | GALLO_0028 | fructose-1,6-bisphosphatase | 1.39 | 1.50 | 1.00 |
| 19      | Metabolism of carbohydrates and related molecules | GALLO_1129 | glutamate dehydrogenase | 1.50 | 1.30 | 1.00 |
| 26      | Metabolism of carbohydrates and related molecules | GALLO_0830 | ATP-dependent amidohydrolase | 1.59 | 1.25 | 1.00 |
| 27, 76  | Metabolism of carbohydrates and related molecules | GALLO_1555 | phosphoenolpyruvate carboxykinase | 1.03 | 0.97 | 1.00 |
| 28, 58, 88 | Metabolism of carbohydrates and related molecules | GALLO_0829 | pyruvate-ferredoxin oxidoreductase | 1.03 | 1.03 | 1.00 |
| 29      | Metabolism of carbohydrates and related molecules | GALLO_0791 | ATP synthase alpha chain | 1.03 | 1.00 | 1.00 |

**Pathways**

| spot nr | functional category | GALLO-code | protein | Caco-2 | HCT116 | v-Caco-2 |
|---------|---------------------|------------|---------|--------|--------|----------|
| 32, 36, 96 | Functional categories: 1.1 Cell Wall; 1.2 Transport/binding proteins and lipoproteins; 1.4 Membrane bioenergetics; 1.7 Cell division; 2.1 Metabolism of carbohydrates and related molecules; 2.2 Metabolism of amino acids and related molecules; 2.3 Metabolism of nucleotides and nucleic acids; 2.4 Metabolism of lipids; 3.5 RNA synthesis; 3.7 Protein synthesis; 3.9 protein folding; 4.1 Adaptation to atypical functions; 4.2 detoxification; 4.3 antibiotic production; 5.2 From other organisms; 5.3 from Streptococcus. | GALLO_1555 | phosphoenolpyruvate carboxykinase | 1.03 | 0.97 | 1.00 |

### Other functions

| spot nr | functional category | GALLO-code | protein | Caco-2 | HCT116 | v-Caco-2 |
|---------|---------------------|------------|---------|--------|--------|----------|
| 100     | Functional categories: 1.1 Cell Wall; 1.2 Transport/binding proteins and lipoproteins; 1.4 Membrane bioenergetics; 1.7 Cell division; 2.1 Metabolism of carbohydrates and related molecules; 2.2 Metabolism of amino acids and related molecules; 2.3 Metabolism of nucleotides and nucleic acids; 2.4 Metabolism of lipids; 3.5 RNA synthesis; 3.7 Protein synthesis; 3.9 protein folding; 4.1 Adaptation to atypical functions; 4.2 detoxification; 4.3 antibiotic production; 5.2 From other organisms; 5.3 from Streptococcus. | GALLO_0604 | hypothetical protein | 0.09 | 0.16 | 0.50 |

**Similar to unknown proteins**

| spot nr | functional category | GALLO-code | protein | Caco-2 | HCT116 | v-Caco-2 |
|---------|---------------------|------------|---------|--------|--------|----------|
| 52, 53, 63, 113, 120 | Functional categories: 1.1 Cell Wall; 1.2 Transport/binding proteins and lipoproteins; 1.4 Membrane bioenergetics; 1.7 Cell division; 2.1 Metabolism of carbohydrates and related molecules; 2.2 Metabolism of amino acids and related molecules; 2.3 Metabolism of nucleotides and nucleic acids; 2.4 Metabolism of lipids; 3.5 RNA synthesis; 3.7 Protein synthesis; 3.9 protein folding; 4.1 Adaptation to atypical functions; 4.2 detoxification; 4.3 antibiotic production; 5.2 From other organisms; 5.3 from Streptococcus. | GALLO_1879 | hypothetical protein | 1.00 | 0.41 | 1.00 |
| 55      | Functional categories: 1.1 Cell Wall; 1.2 Transport/binding proteins and lipoproteins; 1.4 Membrane bioenergetics; 1.7 Cell division; 2.1 Metabolism of carbohydrates and related molecules; 2.2 Metabolism of amino acids and related molecules; 2.3 Metabolism of nucleotides and nucleic acids; 2.4 Metabolism of lipids; 3.5 RNA synthesis; 3.7 Protein synthesis; 3.9 protein folding; 4.1 Adaptation to atypical functions; 4.2 detoxification; 4.3 antibiotic production; 5.2 From other organisms; 5.3 from Streptococcus. | GALLO_0604 | hypothetical protein | 0.09 | 0.16 | 0.50 |

### Fold changes of the specified condition compared to the control condition with a p value <0.05. Fold changes of proteins that have been identified in more than 1 protein spot are presented as an average fold change. NOTE: proteins can be detected in more than one spot due to protein phosphorylation, glycosylation and/or proteolytically processing. Since all of these isoforms may contribute to protein function, we have chosen to present an average fold-change for each unique protein.
**Streptococcus gallolyticus** Forages on Tumor Cell Metabolites

### TABLE II

| KEGG pathway                              | # Proteins (up/down) | Average FC |
|------------------------------------------|----------------------|------------|
|                                           | Caco-2 | HCT116 | Caco-2 | HCT116 |
| Alanine, aspartate and glutamate metabolism | 2 (2/0) | 2 (2/0) | 2.31 | 1.71 |
| Glycerolipid metabolism                   | 3 (2/1) | 3 (2/1) | 4.14 | 0.38 |
| Glycolysis/Gluconeogenesis                | 15 (8/7) | 15 (9/6) | 0.72 | 0.56 |
| Purine metabolism                         | 12 (2/10) | 12 (3/9) | −1.73 | −1.26 |
| Pyrimidine metabolism                     | 5 (5/2) | 5 (5/2) | 0.32 | −0.24 |
| Starch and sucrose metabolism             | 1 (0/1) | 1 (0/1) | −1.16 | −1.27 |
| Valine, leucine and isoleucine biosynthesis | 4 (4/0) | 4 (3/1) | 2.04 | 0.77 |
| Vitamine B6 metabolism                    | 7 (3/4) | 7 (3/4) | 0.7 | −0.46 |
| Bile acid biosynthesis                    | 4 (3/1) | 4 (3/1) | 0.68 | 0.53 |
| Butyrate metabolism                       | 5 (4/1) | 5 (4/1) | 0.87 | 1.09 |
| RAST annotation                           |         |         |       |       |
| Fatty acid biosynthesis                   | 2 (2/0) | 2 (2/0) | 1.90 | 1.53 |
| Fructose utilization                      | 2 (2/0) | 2 (2/0) | 3.88 | 2.29 |
| Vitamin B6 biosynthesis                   | 8 (4/4) | 8 (4/4) | 0.84 | 0.38 |
| Bacterial cell division                   | 6 (3/3) | 6 (2/4) | −0.95 | −0.10 |
| Amino acid biosynthesis                   | 4 (3/1) | 4 (3/1) | 1.12 | 1.31 |
| Purine biosynthesis                       | 10 (0/10) | 10 (1/9) | −2.74 | −1.91 |
| Pyrimidine synthesis                      | 4 (2/2) | 4 (3/1) | 1.48 | 1.66 |
| Glycolysis/Gluconeogenesis                | 19 (11/8) | 19 (13/6) | 0.76 | 0.70 |

**Discussion**

The association between *S. gallolyticus* and malignancy of the intestinal tract has long been recognized, however, the underlying mechanisms still need to be resolved. Here we show for the first time that CRC cell metabolites can provide this bacterium with a specific growth advantage. Proteome and metabolome analyses point toward the increased utilization of glucose and its metabolites together with the amino acid alanine by *S. gallolyticus*, suggesting that these CRC metabolites are of particular importance for the observed growth advantage. It can be envisaged that this phenomenon may be one of the molecular mechanisms that supports the high incidence of *S. gallolyticus* infections in CRC patients (16, 28). Below, we will speculate how such a mechanism could have its place in the complex ecosystem of the gut.

The colonic lumen is a highly competitive environment, in which a balanced microbial composition is maintained at a rather stable level. The current state-of-the-literature suggests that *S. gallolyticus* is a bad colonizer of the healthy colon, whereas it has recently been shown that about 47% of the distal small intestinal (ileum) microbiota consists of *Streptococci*. In the ileum, where high levels of carbohydrates and dietary nutrients are available (29, 30), carbohydrate fermentation by *Streptococci* leads to the formation of lactate. Adaptation to this carbohydrate-rich environment is reflected by the enrichment for genes encoding functions in substrate transport and carbohydrate metabolism in the *S. gallolyticus* genome (19). In line with this observation, *S. bovis* strains were shown to be among the most efficient glucose fermenting bacteria in an experimental human *in vitro* gut fermentation model (TIM-2; 31).

It seems plausible that *S. gallolyticus* cells that are shed from the ileum and enter the colonic lumen are gradually outcompeted by the colonic microbiota and disappear below the detection level. This is reflected by the relative low fecal carriage of about 10%, and colonic mucosal colonization of 2% in the healthy human population (12, 32). However, the...
CRC microenvironment has some interesting similarities with the nutritional status of the ileum. Recent metabolome studies have shown that CRC tissues are associated with increased levels of lactate, glucose derivates, amino acids, lipids, and fatty acids (17, 33–35). Our current in vitro metabolome analysis of CRC cell supernatants were in-line with these in vivo data and strongly suggest that the metabolic changes in the surrounding of tumor cells result in increased glucose metabolism and shedding of amino acids, which might attract glucose fermenting bacteria, such as S. gallolyticus that are not normally present in the colon. This could explain the fact that both fecal carriage and colonic mucosal colonization of S. gallolyticus in CRC patients was found to be strongly increased, to respectively 50% and 48% (12, 32). Recent deep pyrosequencing studies of 16S rRNA amplicons derived from CRC tissue biopsies and adjacent non-malignant mucosa showed that colonic malignancies did indeed strongly affect the local composition of mucosa-associated microbiota, and resulted in an increased abundance of Fusobacteria, Streptococcaceae, and Coriobacteria (8, 36–38).

Although the evidence for increased glycolysis and amino acid metabolism appears to be strong, it cannot be excluded
that we have missed certain metabolic features with our proteomics-based pathway analysis. First of all, not all protein spots could be identified with vMALDI-MS/MS (68% identified) and secondly not all identified proteins could be functionally annotated using KEGG (41%) or RAST (77%). Despite these limitations, both annotation systems showed similar pathway alterations and both proteome and metabolome analysis pointed in the direction of increased glucose and glucose metabolite utilization in spent media of cancer cells. Unfortunately, the direct measurement of these glucose metabolites with NMR-spectroscopy was not possible and therefore it remains to be determined why *S. gallolyticus* is not able to use glucose as a source in standard medium, while it is more efficiently used in spent media of cancer cells or when F6P and 3PG are added to standard medium. Future studies should be aimed at fractionation of spent media from CRC cells to identify the factors that increase the growth rate of *S. gallolyticus*. Such a biochemical approach is needed to further increase our insight in the competitive growth advantage of certain bacteria in a CRC microenvironment.

**TABLE III**

Metabolic profiles of standard and spent medium of Caco-2 and HCT116 cells. *nm, **mg/L*

|                          | Valine* | Alanine* | Tyrosine* | Phenyl-alanine* | Glucose** | Pyruvate** | Lactate* | Acetoina |
|--------------------------|---------|----------|-----------|----------------|-----------|------------|----------|---------|
| **Without bacteria**     |         |          |           |                |           |            |          |         |
| Standard mediumb         | 1.04    | 1.48     | 6.9       | 7.34           | 5.24      | 2.38       | 1.33     | 1.38    |
| Caco-2 spent medium      | 0.8     | 0.1      | 0.4       | 0.4            | 4.5       | 110        | 1.61     | 0       |
| HCT116 spent medium      | 0.73    | 0.2      | 0.4       | 0.4            | 3.4       | 29.5       | 8.4      | 0       |
| **After growth of S. gallolyticus** |         |          |           |                |           |            |          |         |
| Standard medium          | 0.78    | 0.09     | 0.4       | 0.4            | 4.2       | 110        | 4.97     | 4       |
| Caco-2 spent medium      | 0.66    | 0.17     | 0.37      | 0.37           | 2.5       | 45.6       | 22.06    | 20      |
| HCT116 spent medium      | 0.4     | 0.09     | 0.27      | 0.27           | 0         | 54         | 44.5     | 22      |

*a The production of acetoin is expressed as an arbitrary unit compared to internal control TSP (see Materials and Methods).

b Standard medium concentrations were used as reference values for calculation of concentrations and percentages.
**Streptococcus gallolyticus** Forages on Tumor Cell Metabolites

![Diagram](image)

**Fig. 6. Effect of F6P, 3PG, alanine and phenylalanine on the growth of S. gallolyticus and E. coli.** A. The growth rates of *S. gallolyticus* and *E. coli* in standard medium (control, black), were compared with those in standard medium supplemented with 2 mM fructose-6-phosphate (F6P, dark-gray) and 2 mM glycerate-3-phosphate (3PG, light-gray). The effect of alanine (dark-gray) or phenylalanine (light-gray) supplementation on the growth rates of *E. coli* and *S. gallolyticus* in standard medium with or without 1 mM F6P or 3PG is shown in panels **B** and **C**, respectively. Calculation of the growth rates was based on 3 independent experiments. Bars represent the ratio between the growth rate in supplemented medium to that in standard medium. Evaluation with linear regression analysis was used to define if the growth rates were significantly different from each other. Significant differences (*p < 0.05*) are indicated with an asterisk (*).

They can selectively favor the growth of *S. gallolyticus*. The full impact of such tumor-associated metabolic changes on *S. gallolyticus* within a colonic microbiota should be further investigated by *in vitro* gut systems or *in vivo* models that include the ecological complexity of the gastro-intestinal tract. Ultimately, this knowledge may provide leads for novel microbiome-related tools for early detection of CRC, which is one of the major issues in human health care. Furthermore, understanding the cross-talk between CRC tumors and the intestinal microbiota may provide leads toward novel therapies or strategies to detect this disease at an early stage.

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