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# Genomic and functional analysis of *Romboutsia ilealis* CRIB<sup>T</sup> reveals adaptation to the small intestine

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**Background.** The microbiota in the small intestine relies on their capacity to rapidly import and ferment available carbohydrates to survive in a complex and highly competitive ecosystem. Understanding how these communities function requires elucidating the role of its key players, the interactions among them and with their environment/host.

**Methods.** The genome of the gut bacterium *Romboutsia ilealis* CRIB<sup>T</sup> was sequenced with multiple technologies (Illumina paired end, mate pair and PacBio). The transcriptome was sequenced (Illumina HiSeq) while growing on three different carbohydrate sources and short chain fatty acids were measured via HPLC.

**Results.** Hence, we present the complete genome of *Romboutsia ilealis* CRIB<sup>T</sup>, a natural inhabitant and key player of the small intestine of rats. *R. ilealis* CRIB<sup>T</sup> possesses a circular chromosome of 2,581,778 bp and a plasmid of 6,145 bp, carrying 2,351 and eight predicted protein coding sequences, respectively. Analysis of the genome revealed limited capacity to synthesize amino acids and vitamins, whereas multiple and partially redundant pathways for the utilization of different relatively simple carbohydrates are present. Transcriptome analysis allowed pinpointing the key components in the degradation of glucose, L-fucose and fructo-oligosaccharides.

**Discussion.** This revealed that *R. ilealis* CRIB<sup>T</sup> is adapted to a nutrient-rich environment where carbohydrates, amino acids and vitamins are abundantly available and uncovered potential mechanisms for competition with mucus-degrading microbes.

1 **Genomic and functional analysis of *Romboutsia ilealis* CRIB<sup>T</sup> reveals adaptation to the**  
2 **small intestine**

3

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29

31 **Abstract**

32 **Background.** The microbiota in the small intestine relies on their capacity to rapidly import and  
33 ferment available carbohydrates to survive in a complex and highly competitive ecosystem.  
34 Understanding how these communities function requires elucidating the role of its key players,  
35 the interactions among them and with their environment/host.

36 **Methods.** The genome of the gut bacterium *Romboutsia ilealis* CRIB<sup>T</sup> was sequenced with  
37 multiple technologies (Illumina paired end, mate pair and PacBio). The transcriptome was  
38 sequenced (Illumina HiSeq) while growing on three different carbohydrate sources and short  
39 chain fatty acids were measured via HPLC.

40 **Results.** Hence, we present the complete genome of *Romboutsia ilealis* CRIB<sup>T</sup>, a natural  
41 inhabitant and key player of the small intestine of rats. *R. ilealis* CRIB<sup>T</sup> possesses a circular  
42 chromosome of 2,581,778 bp and a plasmid of 6,145 bp, carrying 2,351 and eight predicted  
43 protein coding sequences, respectively. Analysis of the genome revealed limited capacity to  
44 synthesize amino acids and vitamins, whereas multiple and partially redundant pathways for the  
45 utilization of different relatively simple carbohydrates are present. Transcriptome analysis  
46 allowed pinpointing the key components in the degradation of glucose, L-fucose and fructo-  
47 oligosaccharides.

48 **Discussion.** This revealed that *R. ilealis* CRIB<sup>T</sup> is adapted to a nutrient-rich environment where  
49 carbohydrates, amino acids and vitamins are abundantly available and uncovered potential  
50 mechanisms for competition with mucus-degrading microbes.

51

52 **Introduction**

53 Intestinal microbes live in a complex and dynamic ecosystem, and to survive in this highly  
54 competitive environment they have developed close (symbiotic) associations with a diverse array  
55 of other intestinal microbes and with their host. This has led to a complex network of host-  
56 microbe and microbe-microbe interactions in which the intestinal microbes and the host co-  
57 metabolise many substrates (Backhed et al. 2005; Scott et al. 2013). In addition to competition  
58 for readily available carbohydrates in the diet, intestinal microbes are able to extract energy from  
59 dietary polysaccharides that are indigestible by the host (Flint et al. 2012). Furthermore,  
60 intestinal microbes can utilize host-derived secretions (e.g. mucus) as substrates for metabolic  
61 processes (Ouwerkerk et al. 2013). In turn, the metabolic activities of the intestinal microbes  
62 result in the production of a wide array of compounds of which some are important nutrients for  
63 the host. For example, short chain fatty acids (SCFA), the main end-products of bacterial  
64 fermentation in the gut, can be readily absorbed by the host and further metabolized as energy  
65 sources (Elia & Cummings 2007) (Lange et al. 2015). All together, the metabolic activity of the  
66 intestinal microbiota has a major impact on health of the host, and recent studies have indicated  
67 an important role for microbial activity in (human) diseases such as inflammatory bowel disease,  
68 irritable bowel syndrome and obesity (Gerritsen et al. 2011a; Quigley 2013).

69 The wide array of microbial genes present in the intestinal tract in addition to the host's own  
70 genome provides insight into the complex network of possible host-microbe and microbe-  
71 microbe interactions. To this end, it has been estimated that together the human intestinal  
72 microbes contribute ~150 times more genes than the human genome (Qin et al. 2010). To be able  
73 to maintain themselves in an ecosystem such as the intestinal tract, microbes have adapted or

74 even specialized in foraging certain niche-specific substrates. However, we have only limited  
75 understanding of the spatial and temporal heterogeneity in community composition and activity  
76 in the different niches along the length of the intestinal tract. To unravel the functional  
77 contribution of specific intestinal microbes to host physiology and pathology, we have to  
78 understand their metabolic capabilities at a higher resolution. It is still difficult, however, to  
79 associate a functionality in an ecosystem to specific sets of genes and in turn to individual  
80 microbial species, and vice versa. To this end, the combination of genome mining and functional  
81 analyses with single microbes or simple and defined communities can provide an overall insight  
82 in the genetic and functional potential of specific members of the intestinal microbial community  
83 (Heinken et al. 2013; Li et al. 2008; Xu et al. 2003).

84 Little is known about the intestinal microbes adapted to the small intestine (Booijink et al. 2007;  
85 van den Bogert et al. 2013b; Zhang et al. 2014). The small intestine is a nutrient-rich  
86 environment, and previous studies have shown that the microbial communities in the (human)  
87 small intestine are driven by the rapid uptake and conversion of simple carbohydrates (Zoetendal  
88 et al. 2012) (Leimena et al. 2013). Community composition and activity in the small intestine is  
89 largely determined by host digestive fluids such as gastric acid, bile and pancreatic secretions.  
90 In-depth genomic analysis on *Streptococcus* isolates of small intestinal origin has shown that  
91 these microbes are adapted to a highly dynamic environment (Van den Bogert et al. 2013a). Here  
92 we present the completely sequenced and annotated genome of *Romboutsia ilealis* CRIB<sup>T</sup>, a  
93 Gram-positive bacterium that was recently isolated from the small intestinal tract of rats  
94 (Gerritsen et al. 2014). It was found to be a natural inhabitant of the rat small intestine,  
95 specifically of the ileum, and a correlation with improved health status of the rats was observed  
96 in an experimental model of acute pancreatitis (Gerritsen et al. 2011b).

97 Here we describe a model driven analysis of the small intestinal inhabitant *R. ilealis* CRIB<sup>T</sup> and  
98 provide an overview of the metabolic capabilities and nutritional potential of this organism.  
99 These examinations revealed that *R. ilealis* CRIB<sup>T</sup> is able to degrade a wide range of relatively  
100 simple carbohydrates. In addition, we were able to pinpoint potential mechanisms that enable  
101 this organism to survive in the competitive small intestinal environment. These mechanisms  
102 include a bile salt hydrolase and an urease, which enhance the organisms ability to handle  
103 intestinal conditions, and potential mechanisms for competition for fucose with mucus-degrading  
104 microbes.

105

106

## 107 **Materials and methods**

108

### 109 **Genome sequencing, assembly and annotation**

110 *R. ilealis* CRIB<sup>T</sup> (DSM 25109) was routinely cultured in CRIB medium at 37 °C as previously  
111 described (Gerritsen et al. 2014). Genomic DNA extraction was performed as previously  
112 described (Van den Bogert et al. 2013a). Genome sequencing was done using 454 Titanium  
113 pyrosequencing technology (Roche 454 GS FLX), as well as Illumina (Genome Analyzer II and  
114 HiSeq2000) and PacBio sequencing (PacBio RS). Mate-pair data was generated by BaseClear  
115 (Leiden, the Netherlands). All other data was generated by GATC Biotech (Konstanz, Germany).  
116 The genome was assembled in a hybrid approach with multiple assemblers and scaffolds.  
117 Genome annotation was carried out with an in-house pipeline. Prodigal v2.5 was used for  
118 prediction of protein coding DNA sequences (CDS) (Hyatt et al. 2010), InterProScan 5RC7 for  
119 protein annotation (Hunter et al. 2012), tRNAscan-SE v1.3.1 for prediction of tRNAs (Lowe &

120 Eddy 1997) and RNAMmer v1.2 for the prediction of rRNAs (Lagesen et al. 2007). Additional  
121 protein function predictions were derived via BLAST identifications against the UniRef50  
122 (Suzek et al. 2007) and Swissprot (UniProt-Consortium 2014) databases (download August  
123 2013). Afterwards the annotation was further enhanced by adding EC numbers via PRIAM  
124 version March 06, 2013 (Claudel-Renard et al. 2003). Non-coding RNAs were identified using  
125 rfam\_scan.pl v1.04, on release 11.0 of the RFAM database (Burge et al. 2013). CRISPRs were  
126 annotated using CRISPR Recognition Tool v1.1 (Bland et al. 2007). See the Supplemental  
127 Methods in Text S1 for details on the genomic DNA extraction, genome sequencing, assembly,  
128 annotation, and metabolic modelling.

129

### 130 **Whole-genome transcriptome analysis**

131 *R. ilealis* CRIB<sup>T</sup> was grown in a basal bicarbonate-buffered medium (Stams et al. 1993)  
132 supplemented with 16 g/L yeast extract (BD, Breda, The Netherlands) and an amino acids  
133 solution as used for the growth of *C. difficile* (Karasawa et al. 1995). In addition the medium  
134 was supplemented with either 0.5 % (w/v) D-glucose (Fisher Scientific Inc., Waltham, MA  
135 USA), L-fucose (Sigma-Aldrich, St. Louis, MO, USA) or fructo-oligosaccharide (FOS) P06 (DP  
136 2-4; Winclove Probiotics, Amsterdam, The Netherlands). The final pH of the medium was 7.0.  
137 For each condition triplicate cultures were set up.  
138 For RNA-seq analysis, the cells were harvested in mid-exponential phase ( $OD_{600nm} = 0.25-0.55$ ,  
139 ~8-10h incubation) (Table S1).

140 Total RNA was purified using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany).  
141 Depletion of rRNA was performed using the Rib-Zero<sup>TM</sup> Kit for bacteria (Epicentre  
142 Biotechnologies, Madison, WI, USA). Library construction for whole-transcriptome sequencing



143 (RNA-seq) was done by use of the ScriptSeq™ v2 RNA-seq Library Preparation Kit in  
144 combination with ScriptSeq™ Index PCR primers (Epicentre Biotechnologies).

145 The barcoded cDNA libraries were pooled and sent to GATC Biotech (Konstanz, Germany)  
146 where 150 bp sequencing was performed on one single lane using the Illumina HiSeq2500  
147 platform in combination with the TruSeq Rapid SBS (200 cycles) and TruSeq Rapid SR Cluster  
148 Kits (Illumina Inc., San Diego, CA, USA). Reads were mapped to the genome with Bowtie2  
149 v2.0.6 (Langmead & Salzberg 2012) using default settings. Details on the RNA-seq raw data  
150 analysis can be found in Table S2 and Supplemental Methods in Text S1.

151 Gene expression abundance estimates and differential expression analysis was performed using  
152 Cuffdiff v2.1.1 (Trapnell et al. 2013) with default settings. Differentially expressed genes were  
153 determined by pairwise comparison of a given condition to the other three conditions for a total  
154 of six pairwise comparisons. Genes were considered significantly differentially expressed when  
155 they showed a  $\geq 1.5 \log_2(\text{fold change})$  in any of the conditions with a false discovery rate (FDR)-  
156 corrected P value (q value)  $\leq 0.05$  (Tables S3-S6). Principal component analysis was performed  
157 with Canoco 5.0 (ter Braak & Smilauer 2012) on log-transformed gene transcript abundances  
158 using Hellinger standardization. Gene expression heatmaps were generated based on gene  
159 transcript abundances using R v3.1.0 and R-packages svDialogs and gplots.

160 See the Supplemental Methods in Text S1 for details on the carbohydrate growth experiment and  
161 whole genome transcriptome analysis.

162

### 163 **Nucleotide sequence accession number**

164 All related data has been deposited at the European Nucleotide Archive. The raw reads for the  
165 genome of *R. ilealis* CRIB<sup>T</sup> can be accessed via the accession numbers ERR366773,

166 ERX397233, ERX397242 and ERX339449. The assembly can be accessed under LN555523-  
167 LN555524. The RNAseq data has been deposited under the numbers ERS533849- ERS533861.

168

## 169 **Results**

### 170 **Genome analysis**

#### 171 *Global genome features*

172 *R. ilealis* CRIB<sup>T</sup> contains a single, circular chromosome of 2,581,778 bp and a plasmid of 6,145  
173 bp (Table 1 and Fig. 1). The chromosome contains 2,351 predicted protein CDS, of which 321  
174 were annotated as hypothetical and for 91 only a domain of unknown function could be assigned.  
175 The plasmid carries eight predicted protein CDS, of which none was recognized for having a  
176 metabolic or replicative function. Furthermore, it appears to be a non-mobilizable plasmid, given  
177 that it lacks any known mobilization-associated genes. The overall G+C content of the genome is  
178 27.9 %, which is in good agreement with a G+C content of 28.1 mol% previously determined for  
179 *R. ilealis* CRIB<sup>T</sup> by HPLC methods (Gerritsen et al. 2014).

180

181 INSERT TABLE 1

182

183 INSERT FIGURE 1

184

185 With a total of 14 copies of the 16S ribosomal RNA (rRNA) gene, *R. ilealis* CRIB<sup>T</sup> is among the  
186 species with the highest number of 16S rRNA gene copies reported up to this date (Lee et al.  
187 2009). However, it should be noted, that not all 16S rRNA copies in *R. ilealis* CRIB<sup>T</sup> are  
188 embedded in the conserved 16S-23S-5S rRNA operon structure. This has been reported for other

189 genomes containing multiple rRNA operons as the result of duplications (Bensaadi-Merchermek  
190 et al. 1995; Schwartz et al. 1992).

191 A COG category could only be assigned to 1,647 of the predicted proteins (70 %) including 372  
192 proteins (16 %) assigned to the categories R (general function prediction only) and S (function  
193 unknown) (Fig. S1). This low coverage in function prediction is most likely due to the fact that  
194 the COG categorization has not been updated since 2003. With InterProScan a predicted function  
195 could be assigned to 82 % of the predicted proteins. Based on the InterPro and PRIAM  
196 classifications (Claudel-Renard et al. 2003), an enzymatic function could be predicted for more  
197 than 500 proteins.

198

### 199 ***General metabolic pathways***

200 Analysis of the CDS predicted from the *R. ilealis* CRIB<sup>T</sup> genome revealed the presence of a  
201 complete set of enzymes for the glycolytic pathway. In line with the anaerobic lifestyle of the  
202 organism, enzymes for the oxidative phase of the pentose phosphate pathway could not be  
203 detected. Additionally, the genes that encode enzymes involved in the tricarboxylic acid cycle  
204 were lacking. Subsequently a metabolic model was constructed with Pathway tools v18.0. A flux  
205 balance analysis with the model was performed, suggesting that *R. ilealis* CRIB<sup>T</sup> is a mixed acid  
206 fermenter as was previously reported (Gerritsen et al. 2014). Predicted end products of  
207 fermentation are a mixture of acetate, formate, lactate and ethanol, with the possibility of gas  
208 formation (CO<sub>2</sub> and H<sub>2</sub>). In addition to ethanol, which can be produced during mixed acid  
209 fermentation, 1,2-propanediol was predicted to be formed via the L-fucose degradation pathway.  
210 The fermentation end products formate, acetate and lactate are predicted to be produced from  
211 pyruvate. No other solvents were predicted to be produced by the metabolic model, however,

212 low amounts of propionate production (up to 3 mM) were observed repeatedly during *in vitro*  
213 growth (Gerritsen et al. 2014). None of the three established pathways for propionate production  
214 in the intestinal tract, i.e. the succinate, acrylate or the propanediol pathway (Reichardt et al.  
215 2014), could be identified at the genetic level, but it might be possible that propionate is  
216 produced together with formate from threonine fermentation by the activity of acetate kinase in  
217 the final step instead of a dedicated propionate kinase, as these enzymes have very similar  
218 catabolic mechanisms and carboxylic acid binding sites (Ingram-Smith et al. 2005). No other  
219 probable underground reactions (known minor side reactions besides the main reaction catalysed  
220 by enzymes) (D'Ari & Casadesus 1998) (Notebaart et al. 2014) for the production of propionate  
221 were detected while investigating the BRENDA database (Chang et al. 2015).

222 The analysis of the genome and the prediction by the model indicated that fermentation is  
223 probably the main process for energy conservation in *R. ilealis*. However, the presence of a  
224 sulfite reductase gene cluster (CRIB\_1284-CRIB\_1286) of the dissimilatory *asrC*-type (Dhillon  
225 et al. 2005) points at possible anaerobic respiration. Similar siroheme-dependent sulfite  
226 reductases are found in many close-relatives of *R. ilealis* such as *Intestinibacter bartlettii*,  
227 *Clostridium sordellii* and *C. difficile* (Czyzewski & Wang 2012). Sulfite reduction by *R. ilealis*  
228 CRIB<sup>T</sup>, and close relatives, has been previously demonstrated *in vitro* (Gerritsen et al. 2014), and  
229 increased growth yield and metabolite production was observed in the presence of sulfite for *R.*  
230 *ilealis* CRIB<sup>T</sup> (data not shown). In the intestinal tract sulfite is derived from food sources that  
231 contain sulfite as preservative, and it has been shown that neutrophils release sulfite as part of the  
232 host defence against microbes (Mitsuhashi et al. 1998).

233

234 ***Metabolism of growth factors and cofactors***

235 Many genes encoding enzymes required for amino acid biosynthesis appeared to be absent in *R.*  
236 *ilealis* CRIB<sup>T</sup>. Complete pathways are present for the biosynthesis of aspartate, asparagine,  
237 glutamate, glutamine and cysteine, using carbon skeletons available from central metabolites or  
238 via conversion of other amino acids.

239 The absence of genes to produce branched-chain amino acids (leucine, isoleucine and valine)  
240 was also reflected in the absence of branched chain fatty acids in the cell membrane of *R. ilealis*,  
241 which is characteristic for the genus *Romboutsia* (Gerritsen et al. 2014). From these observations  
242 it can be concluded that *R. ilealis* depends on a number of exogenous amino acids, peptides  
243 and/or proteins to fuel protein synthesis. The dependency on an exogenous source of amino acids  
244 is reflected by the identification of multiple amino acid transporters, including an  
245 arginine/ornithine antiporter, multiple serine/threonine exchangers, a transporter for branched  
246 amino acids, and several amino acid symporters and permeases without a predicted specificity.  
247 Furthermore, numerous genes have been annotated as protease or peptidase, including several  
248 with a signal peptide.

249 *R. ilealis* CRIB<sup>T</sup> appears to contain all genes for *de novo* purine and pyrimidine synthesis, as  
250 well as for the production of the coenzymes NAD and FAD via salvage pathways from niacin  
251 and riboflavin, respectively. While some organic cofactors can be produced by *R. ilealis* CRIB,  
252 including siroheme, it mainly relies on salvage pathways (e.g. for lipoic acid) or exogenous  
253 sources for the supply of precursors mainly in the form of vitamins (e.g. thiamin, riboflavin,  
254 niacin, pantothenate, pyridoxine, biotin, vitamin B12).

255

256 ***Carbohydrate transport and metabolism***

257 As previously reported *R. ilealis* CRIB<sup>T</sup> is able to utilize a wide variety of carbohydrates  
258 (Gerritsen et al. 2014). Previously good growth of *R. ilealis* on L-fucose, glucose, raffinose and  
259 sucrose was described in addition to moderate growth on D-arabinose and D-galactose and weak  
260 growth on D-fructose, inulin, lactose, maltose and melibiose. Growth on L-fucose, fructose,  
261 galactose, glucose, lactose, maltose, melibiose, raffinose and sucrose was predicted from the  
262 genome-scale metabolic model as well. Besides the central glycolytic genes, which are present in  
263 a single genomic area, genes encoding the specific carbohydrate degradation enzymes are  
264 distributed throughout the genome in gene clusters together with their respective transporters and  
265 transcriptional regulator. No separate pathway for the use of D-arabinose could be predicted,  
266 however, the L-fucose degradation pathway is likely also used for D-arabinose utilization  
267 (LeBlanc & Mortlock 1971). An arabinose transporter, similar to the maltose and sucrose  
268 transporters, could be identified. In addition, a gene cluster involved in the degradation of the  
269 host-derived carbohydrate sialic acid could be predicted (CRIB\_613-CRIB\_619) (Almagro-  
270 Moreno & Boyd 2009). The structure of this gene cluster is similar to the one identified in *C.*  
271 *difficile* (Ng et al. 2013). The ability to degrade the predominantly host-derived carbohydrates L-  
272 fucose and sialic acid suggest a role in the utilization of mucin, an abundant host-derived  
273 glycoprotein in the intestinal tract (Derrien et al. 2010; Ouwkerk et al. 2013). However, no  
274 growth on mucin was observed (data not shown), which is in line with the lack of a predicted  
275 extracellular fucosidase and/or sialidase.

276

277 ***Additional genes of ecological interest***

278 A gene cluster encoding a urease, consisting of three subunits (*ureABC*), and a number of urease  
279 accessory genes were identified (CRIB\_1381-CRIB\_1388). The gene cluster identified in *R.*  
280 *ilealis* CRIB is very similar to the urease gene cluster in the genome of *C. sordellii* (Fig. S3), a  
281 species in which the urease activity is used to phenotypically distinguish *C. sordellii* strains from  
282 *C. bifermentans* strains (Roggentin et al. 1985). Furthermore, a possible ammonium transporter  
283 (CRIB\_1389) was identified in the genome of *R. ilealis* CRIB<sup>T</sup> next to the urease gene cluster.  
284 Ureases are nickel-containing metalloenzymes that catalyse the hydrolysis of urea to ammonia  
285 and carbon dioxide, and thereby these enzymes allow microbes to use urea as nitrogen source by  
286 assimilation via glutamate. They are ubiquitous proteins occurring in diverse organisms (Mobley  
287 et al. 1995). In the intestinal environment, where urea is abundantly present (Fuller & Reeds  
288 1998), some bacteria use ureases to survive the acidic conditions in the upper part of the  
289 intestinal tract as urea hydrolysis leads to a local increase in pH (Rutherford 2014).

290 Another function of ecological interest is the predicted choloylglycine hydrolase. Proteins within  
291 the choloylglycine hydrolase family are bile salt hydrolases (BSHs), also known as conjugated  
292 bile acid hydrolases (CBAHs), that are widespread among intestinal microbes (Ridlon et al.  
293 2006). They are involved in the hydrolysis of the amide linkage in conjugated bile salts,  
294 releasing primary bile acids. There is a large heterogeneity among BSHs, for example with  
295 respect to their substrate specificity. The BSH of *R. ilealis* CIRB<sup>T</sup> was found to be the most  
296 similar to the one found in *C. butyricum*. Although the physiological advantages of BSHs for the  
297 microbes are not completely understood, it has been hypothesized that they constitute a  
298 mechanism to detoxify bile salts and thereby enhance bacterial colonization (Czyzewski & Wang  
299 2012).

300

301 **Metabolite and transcriptome analysis**302 *Metabolite and transcriptome analysis of R. ilealis CRIB<sup>T</sup> during growth on different*  
303 *carbohydrates*

304 To study key pathways predicted to be involved in carbohydrate utilization and their regulation  
305 in more detail, a whole-genome transcriptome analysis was performed, focussing on four  
306 experimental conditions. Firstly, growth on glucose, a preferred substrate for many microbes  
307 present in the intestinal tract, was studied. Secondly the growth on fructans, oligo-  
308 /polysaccharides present in many food items, was examined. Previously weak growth on inulin, a  
309 polysaccharide consisting of long chains of  $\beta 1 \rightarrow 2$  linked fructose units, was observed (Gerritsen  
310 et al. 2014). For this study a shorter fructan (FOS P06, DP2-4) was chosen, because growth on  
311 shorter fructans is likely more relevant for microbes living in the small intestine (Zoetendal et al.  
312 2012). Thirdly, growth on L-fucose was examined, as growth on this substrate was found to be  
313 unique for *R. ilealis* CRIB<sup>T</sup> compared to other related microbes. Finally, *R. ilealis* CRIB<sup>T</sup> was  
314 also grown in the basal medium in the absence of an additional carbon source for comparison  
315 (control condition).

316 Based on measurements of optical density and pH during growth (growth characteristics of  
317 individual cultures can be found in Table S1), sampling was done in mid-exponential phase (~8-  
318 10 h incubation; used for transcriptome analysis) and in stationary phase (24 h incubation), and  
319 sugar utilization and fermentation products were measured with HPLC (Table 2). The fact that in  
320 neither of the experimental conditions the supplied carbohydrates were depleted and metabolites  
321 were still produced during the sampling points at ~8-10 h and 24 h, confirmed that samples  
322 obtained for transcriptome analysis at ~8-10 h were taken during exponential growth. In the FOS



323 cultures an accumulation of extra-cellular fructose was observed. As predicted from the  
324 metabolic model, growth on glucose resulted in the production of formate, acetate and lactate  
325 (Table 2).

326

327 INSERT TABLE 2

328

329 Growth on FOS was slightly less than on glucose, however, after 24 h of growth the same  
330 fermentation products were observed in similar amounts (Table 2). Growth on L-fucose showed  
331 production of 1,2-propanediol instead of lactate. The fact that 1,2-propanediol was observed in  
332 one of the control cultures could be explained by the fact that an L-fucose grown culture was  
333 used as inoculum for this culture, leading to carry-over of minor amounts of metabolites.

334 For the genome-wide transcriptome analysis of triplicate cultures grown in the four different  
335 conditions (i.e. a total of 12 cultures), a total of 159,250,634 150bp-reads were generated by  
336 RNA-seq (overview in Table S2). Principal component analysis of the transcriptomes of the  
337 individual cultures showed that the cultures clustered by condition (Fig. 2).

338 INSERT FIGURE 2

339

340 ***Differential expression of genes involved in carbohydrate degradation and fermentation in R.***  
341 ***ilealis CRIB<sup>T</sup>***

342 To identify differentially regulated genes, pairwise comparisons were done with cuffdiff  
343 (Trapnell et al. 2013) using a cut off of  $\geq 1.5 \log_2$  (fold-change) and q-value  $\leq 0.05$ . Figure 3  
344 shows a heat map of all differentially regulated genes, exact numbers can be found in Tables S3-  
345 6.

346

347 INSERT FIGURE 3

348

349 INSERT FIGURE 4

350

351 The gene cluster involved in glycolysis (CRIB\_186-CRIB\_191) was most abundantly expressed  
352 in the conditions that support the highest growth rates determined by the highest cell density  
353 reached in the time period that was measured (glucose, followed by FOS; Fig. 3). This was also  
354 reflected in the fact that expression of genes involved in replication such as ribosomal proteins,  
355 proteins involved in cell wall biosynthesis and general cell division processes were most strongly  
356 expressed during growth in the presence of glucose and to a lesser extent FOS. Other genes  
357 involved in the central sugar metabolic pathways (e.g. CRIB\_1849, CRIB\_140, CRIB\_2223, and  
358 CRIB\_105) were upregulated in these conditions, albeit not significantly differentially regulated.  
359 This suggests that these are less tightly regulated at the transcriptional level, probably because  
360 they are also involved in other processes than sugar degradation (Commichau et al. 2009). The  
361 metabolic model suggests that this is indeed the case, as some of the enzymes produce  
362 intermediates which can be consumed by fatty acid biosynthesis and amino acid biosynthesis  
363 processes.

364 Altogether, the transcriptome of *R. ilealis* CRIB<sup>T</sup> grown on FOS was very similar to its  
365 transcriptome when grown on glucose (Fig. 2), with only 18 genes significantly upregulated  
366 during growth in the presence of FOS compared to glucose (Table S4). Apparent was the  
367 upregulation of the gene clusters involved in the transport and degradation of the respective  
368 sugars or their derivatives (Fig. 3). In the presence of glucose the glucose-specific PTS system

369 (CIRB\_2017-CRIB\_2018) was significantly upregulated, together with its associated  
370 transcriptional regulator (CRIB\_2019). In turn, in the presence of FOS two clusters predicted to  
371 be involved in sucrose degradation (CRIB\_148-CRIB\_152 and CRIB\_1458-1461) were  
372 significantly upregulated. The third gene cluster predicted to be involved in sucrose degradation  
373 (CRIB\_1399-1400) was not significantly regulated during growth on FOS, however, it should be  
374 noted that these genes are located in a cluster functionally annotated to melibiose metabolism  
375 and are most likely regulated by the transcriptional regulator in this cluster. In addition to the two  
376 sucrose degradation clusters, a transport cluster of unknown function (CRIB\_1506-CRIB\_1509)  
377 was upregulated during growth on FOS, albeit only significant compared to growth on glucose.  
378 During growth in the presence of L-fucose, the gene cluster predicted to be involved in L-fucose  
379 degradation (CRIB\_1294-CRIB\_1298) was significantly upregulated, including the gene  
380 encoding the corresponding transcriptional regulator (CRIB\_1299). An overview of the main  
381 carbohydrate degradation pathways regulated in the different conditions is given in Figure 4.  
382 During growth on glucose, L-lactate dehydrogenase (CRIB\_684) was significantly upregulated,  
383 albeit not significant compared to growth on FOS. This enzyme catalyses the reduction of  
384 pyruvate resulting in the production of L-lactate and the reoxidation of the NADH formed during  
385 glycolysis. Only at time point 24 h lactate was observed (Table 2). This suggests that at time  
386 point ~8-10 h the cells were starting to regenerate NAD by upregulating this gene. In the  
387 presence of L-fucose, NAD<sup>+</sup> regeneration is achieved via the reduction of lactaldehyde to 1,2-  
388 propanediol by lactaldehyde reductase (CRIB\_1300), which was upregulated in the presence of  
389 L-fucose together with the L-fucose degradation gene cluster. In the spent medium of L-fucose  
390 grown cells, 1,2-propanediol was already seen at time point ~8-10 h whereas no lactate  
391 production was observed. Another way to regenerate NAD<sup>+</sup> is to reduce pyruvate to ethanol (Fig.

392 4). In the presence of both glucose and FOS an upregulation was seen of the gene encoding the  
393 bifunctional aldehyde/alcohol dehydrogenase (CRIB\_2231), which converts acetyl-CoA to  
394 ethanol. However, in none of the samples ethanol was measured by HPLC analysis.

395

#### 396 ***Expression and regulation of other environmentally relevant processes in R. ilealis CRIB<sup>T</sup>***

397 Remarkable was the significant upregulation of a gene cluster related to iron transport  
398 (CRIB\_892-CRIB-898) during growth on glucose and FOS compared to growth on L-fucose.  
399 The significance of this gene cluster for carbohydrate utilization is not known, however, several  
400 enzymes could be identified in the genome of *R. ilealis* CRIB<sup>T</sup> that use different forms of iron as  
401 cofactor, for example the hydrogenases involved in hydrogen metabolism (Calusinska et al.  
402 2010), several ferredoxins, and the L-threonine dehydratase (CRIB-426) that was significantly  
403 upregulated during growth on L-fucose. As multiple transporters involved in the transport of iron  
404 compounds were predicted, it might also be that the uptake of iron provides a competitive  
405 advantage to other microbes that are dependent on iron for respiration and other metabolic  
406 processes (Kortman et al. 2014).

407 During growth on FOS, a small gene cluster (CRIB\_601-CRIB\_603) that includes a gene  
408 encoding an alternative sigma factor was significantly upregulated. Interestingly, this was also  
409 apparent in the control culture that was inoculated with FOS-preconditioned cells. This suggests  
410 that in the presence of FOS (or its derivatives sucrose or fructose) transcription is also regulated  
411 by RNA polymerase promoter recognition.

412 In the intestinal environment *R. ilealis* CRIB will encounter a wide array of carbohydrates that  
413 are either continually or transiently present. Prioritization of carbohydrate utilization is partly  
414 achieved at a transcriptional level by the selective expression of genes. The primary mechanism

415 by which bacteria regulate the utilization of non-preferred carbohydrates in the presence of  
416 preferred carbon sources is known as carbon catabolite repression (CCR), a hierarchical system  
417 for coordinating sugar metabolism (Deutscher 2008). The fact that, compared to glucose and  
418 FOS, L-fucose is utilized by a pathway that does not directly involve fructose-1,6-bisphosphate,  
419 a key metabolite in the regulation of CCR of Gram-positive bacteria, made it possible to study  
420 CCR by either glucose or FOS. The transcriptome analysis suggests that some genes and operons  
421 in *R. ilealis* CRIB<sup>T</sup> were indeed subject to CCR in response to the presence of glucose. For  
422 example, two gene clusters predicted to be involved in hexuronate metabolism (CRIB\_649-  
423 CRIB\_652 and CRIB\_2244-CRIB\_2249), pathways that make the use of D-glucuronate and D-  
424 galacturonates as sole carbon source possible, were significantly upregulated during growth in  
425 the presence of L-fucose compared to growth on glucose (Table S5). In addition, the gene cluster  
426 predicted to be involved in sialic acid utilization (CRIB\_613-CRIB\_616) was downregulated in  
427 the presence of glucose as well. Furthermore, when comparing the expression of the gene cluster  
428 involved in L-fucose degradation during growth on glucose relative to the growth in the absence  
429 of a carbon source (control condition), also this gene cluster appeared to be under CCR in the  
430 presence of glucose (Table S5). These results suggest that in *R. ilealis* CRIB<sup>T</sup> multiple gene  
431 clusters that are involved in the use of alternative carbon sources are subject to CCR. The urease  
432 encoding gene cluster (CRIB\_1381-CRIB\_1388) was significantly upregulated when grown in  
433 the absence of an additional carbon source. The fact that this was significant compared to growth  
434 on glucose suggests possible CCR of the urease gene cluster, however, upregulation of this gene  
435 cluster in the absence of an exogenous carbon source might also be a possible mechanism.

436

437 **Discussion**

438 Gerritsen *et al.* (2011b) have shown by 16S rRNA gene sequence-based analysis that *R. ilealis*  
439 CRIB<sup>T</sup> is a dominant member of the small intestine microbiota in rats, especially in the ileum.  
440 The genomic and transcriptional analysis of *R. ilealis* CRIB<sup>T</sup> reported here provides new insights  
441 into the genetic and functional potential of this inhabitant of the small intestine. Genomic  
442 analysis revealed the presence of metabolic pathways for the utilization of a wide array of  
443 ‘simple’ carbohydrates in addition to a multitude of carbohydrate uptake systems that included a  
444 series of PTS systems, carbohydrate specific ABC transporters, permeases and symporters.  
445 Considering the habitat of *R. ilealis* CRIB<sup>T</sup>, we chose to focus on key pathways involved in the  
446 utilization of specific diet- and host-derived carbon sources by whole-genome transcriptome  
447 analysis.

448 In the intestinal tract, the diet-derived carbohydrates that the host is unable to digest are  
449 important sources of energy for many microbes. Here we examined the growth of *R. ilealis*  
450 CRIB<sup>T</sup> on FOS, a relatively simple oligosaccharide that is indigestible by the host. The  
451 transcriptome of *R. ilealis* CRIB<sup>T</sup> grown on FOS was very similar to its transcriptome when  
452 grown on glucose, a monosaccharide used by the majority of microbes present in the intestinal  
453 tract. This is not surprising considering that glucose is in addition to fructose one of the two  
454 subunits present in FOS. Differential gene expression analysis demonstrated the apparent FOS-  
455 induced upregulation of two separate gene clusters that were predicted to be involved in sucrose  
456 degradation. Remarkable was the accumulation of fructose in the culture supernatant during  
457 growth of *R. ilealis* CRIB<sup>T</sup> on FOS. A simple explanation could be the release of fructose or  
458 beta-fructofuranosidase activity after cell lysis. The fact that *R. ilealis* only grows weakly on D-

459 fructose (Gerritsen et al. 2014), and no transporter specific for fructose could be identified as in  
460 close relatives that are able to grow on D-fructose, could explain fructose accumulation. This  
461 might indicate a possible symbiotic relationship with other microbes in the close environment,  
462 which are most likely able to utilize the excess fructose. Another explanation could be  
463 extracellular degradation of FOS, followed by import of sucrose and/or glucose into the cell.  
464 Fructan degradation by extracellular enzymes is described for other (intestinal) microbes (*van*  
465 *Hijum et al. 2006*). However, no extracellular fructansucrase or glucansucrase could be  
466 predicted. Furthermore, no new candidates for this activity could be identified via the differential  
467 gene expression analysis described here. One possible candidate could be the predicted beta-  
468 fructofuranosidase present in the PTS system-containing sucrose degradation gene cluster. Next  
469 to the beta-fructofuranosidase-encoding gene, a gene was found to which no function could be  
470 assigned, but that was predicted to have a transmembrane region and a domain which could be  
471 involved in transport. Given that both loci overlap by a few nucleotides, and that the overlap is  
472 within a homopolymer region, it is possible that both loci form one protein due to ribosomal  
473 slippage on the homopolymer (Sharma et al. 2014). This could possibly lead to an external  
474 membrane-bound enzymatically active protein, which would explain the accumulation of  
475 fructose. Future studies with mutant strains might shed more light on the specific contribution of  
476 the two predicted sucrose degradation gene clusters to the degradation of FOS, or even longer  
477 fructans (inulin), in *R. ilealis* CRIB<sup>T</sup>.

478 Unlike other members of the family *Peptostreptococcaceae*, *R. ilealis* CRIB<sup>T</sup> is able to grow on  
479 L-fucose, a predominantly host-derived carbon source (Gerritsen et al. 2014). The whole-genome  
480 transcriptome analysis confirmed the presence of a functional L-fucose degradation pathway,  
481 similar to the pathways previously identified in other intestinal inhabitants such as *E. coli*

482 (Baldoma & Aguilar 1988), *Bacteroides thetaiotaomicron* (Hooper et al. 1999) and *Roseburia*  
483 *inulinivorans* (Scott et al. 2006). By gene sequence homology a similar pathway was found in  
484 *Clostridium perfringens* and the more closely related *C. sordellii* (Fig. S2). L-fucose is a  
485 common sugar present within the intestinal environment, as it is a monosaccharide that is an  
486 abundant component of many N- and O-linked glycans and glycolipids produced by mammalian  
487 cells, including the fucosylated glycans that are found at the terminal positions of mucin  
488 glycoproteins (Becker & Lowe 2003). Fucosylated mucin glycoproteins are especially found in  
489 the (human) ileum (Robbe et al. 2004; Robbe et al. 2003). For both intestinal commensals and  
490 pathogens the ability to utilize L-fucose has been demonstrated to provide a competitive  
491 advantage in the intestinal environment (Hooper et al. 1999; Stahl et al. 2011). In *R. ilealis* all  
492 enzymes for L-fucose degradation are present in one cluster, however, no fucosidase-encoding  
493 gene could be identified, which means that *R. ilealis* is not able to release L-fucose units from  
494 fucosylated glycans (e.g. mucin) by itself. Hence, in the intestinal environment *R. ilealis* is  
495 dependent on free L-fucose monosaccharides released by other microbes. Furthermore, a gene  
496 cluster involved in degradation of sialic acid (Almagro-Moreno & Boyd 2009; Vimr 2013; Vimr  
497 et al. 2004) was predicted from the genome, but no extracellular sialidase could be identified  
498 similar to *C. difficile* (Ng et al. 2013). This suggests that also for sialic acid, a common residue  
499 found in mucin glycoproteins, *R. ilealis* CRIB<sup>T</sup> seems to be dependent on the activity of other  
500 microbes. Recently it was demonstrated that the host is able to regulate fucosylation of its  
501 intestinal epithelial cells in response to pathogen-induced stress (Pickard et al. 2014). This  
502 suggests that the ability of microbes to use fucose as an energy source may contribute to the  
503 protection of the host against infections by endogenous pathogens.



504 Interesting was the identification of a urease gene cluster in *R. ilealis* CRIB<sup>T</sup>, which appeared to  
505 be induced in carbon source limiting circumstances. Urea in the intestinal tract is derived from  
506 the breakdown of amino acids. *Helicobacter pylori* is a well-known example where urease  
507 activity contributes to the survival of the bacterium in the acidic environment of the stomach  
508 (Marshall et al. 1990). For some of the urease-positive bacteria, this enzyme has been shown to  
509 act as a virulence factor as it is responsible for urea hydrolysis that leads to increased pH and  
510 ammonia toxicity (Rutherford 2014). However, for commensal intestinal bacteria ureases can  
511 probably function as colonization factors as well, as they contribute in general to acid resistance  
512 and thereby play a role in gastrointestinal survival (Marshall et al. 1990). Urea is released into all  
513 parts of the intestinal tract via diffusion from the blood, but it has been reported that pancreatic  
514 excretions and bile are a main route of entry (Bergner et al. 1986). We have not been able to  
515 demonstrate urease activity in *R. ilealis* CRIB<sup>T</sup> during growth in the presence of urea (data not  
516 shown). However, different mechanisms for the expression of urease have been identified in  
517 other microbes: constitutive, inducible by urea, or controlled by nitrogen source availability  
518 (Moblely et al. 1995). For *C. perfringens* for example, the urease activity, which is plasmid  
519 borne, was shown to be only expressed in nitrogen-limiting conditions (Dupuy et al. 1997). The  
520 increased urease gene expression by *R. ilealis* CRIB<sup>T</sup> observed in the control condition, in the  
521 absence of an additional carbohydrate, suggests an alternative mechanism for regulation of  
522 urease gene expression.

## 523 **Conclusions**

524 We are just starting to elucidate the composition and function of the microbial communities in  
525 the mammalian small intestine. Recently we have reported the isolation and characterization of  
526 *R. ilealis* CRIB<sup>T</sup> from the small intestine of a rat (Gerritsen et al. 2014). In rats this species was

527 identified to be a dominant member of the ileal microbiota (Gerritsen et al. 2011b). Here we  
528 applied a holistic systems biology approach, involving several fields of wet and dry biology, to  
529 study *R. ilealis* CRIB<sup>T</sup>, a natural and abundant inhabitant of the small intestinal tract of rats. Its  
530 ability to use host sugars that are liberated by other microbes suggests that *R. ilealis* CRIB<sup>T</sup> is in  
531 competition with mucus-degrading microbes, like *B. thetaiotaomicron* or *Akkermansia*  
532 *muciniphila*. In conclusion, *R. ilealis* is a species that is able to utilize an array of carbohydrates  
533 using different and partially redundant pathways. In contrast, it has only limited ability to *de*  
534 *novo* synthesize amino acids and vitamins, and hence the organism shows an adaption to a  
535 nutrient-rich environment in which carbohydrates and exogenous sources of amino acids and  
536 vitamins are abundantly available. A deeper investigation of key players in the intestinal tract  
537 like *R. ilealis* and others will lead to a better understanding of how the microbial communities in  
538 us function as a whole. The more we understand how each organism works, and how they  
539 interact, the better we get an insight into these environments and can predict how nutrition will  
540 influence our health and well-being.

541

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546

547

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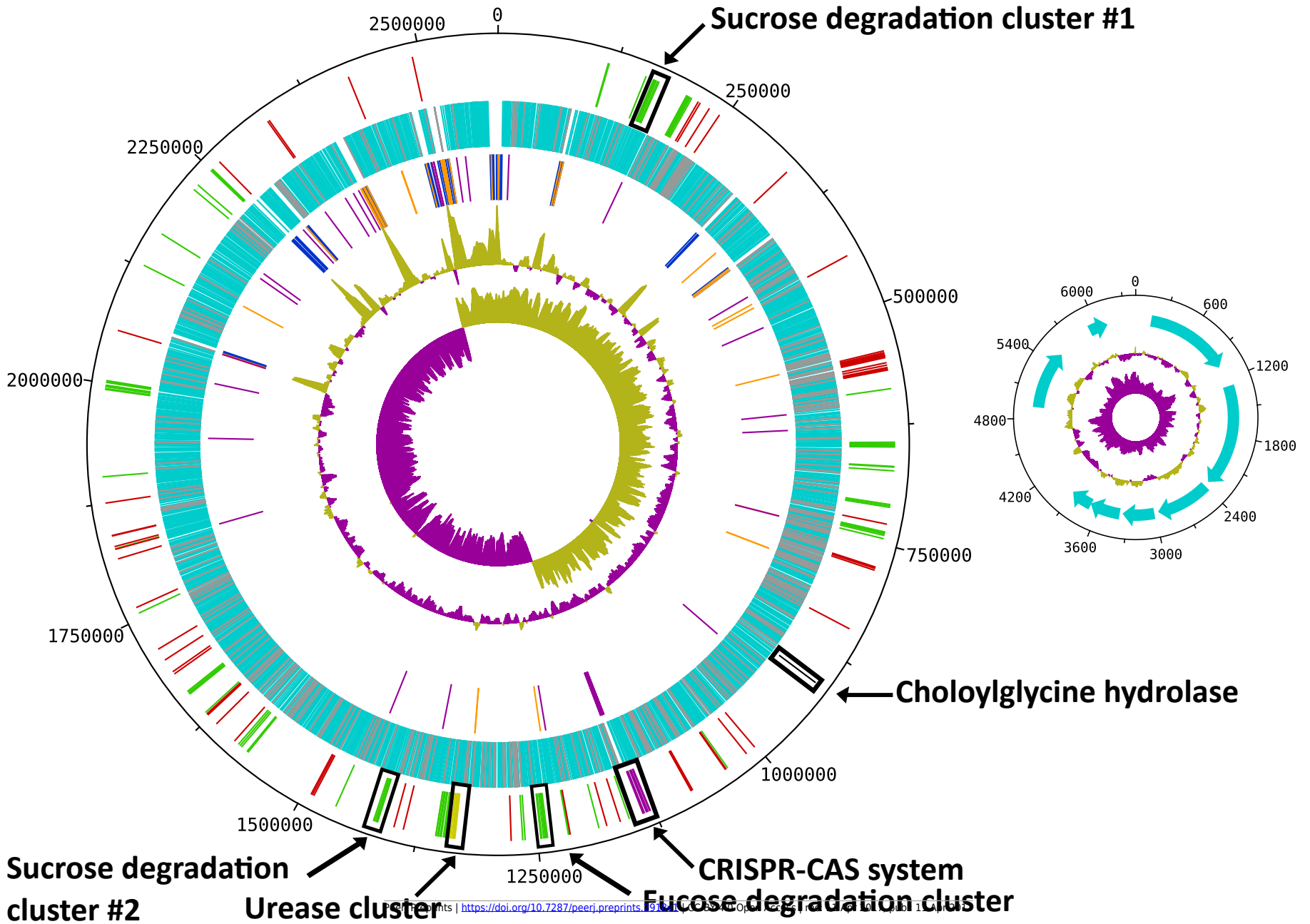
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- 735

736

**Figure 1**(on next page)

Circular map of the *R. ilealis* CRIB<sup>T</sup> genome

Both chromosome and non-mobilizable plasmid are shown. For the chromosome tracks from inside to outside are as follows: 1, GC skew; 2, G+C content; 3, RNAs [rRNAs (blue), tRNAs (orange) and ncRNAs (purple)]; 4, all predicted protein CDS [with predicted function (light-blue), hypothetical proteins and proteins to which only a domain of unknown function could be assigned (grey)]; 5, genes or gene clusters of interest [(mobile genetic elements (red), Cas proteins (pink), urease gene cluster (yellow), choloylglycine hydrolase (black), gene clusters involved in carbohydrate utilization (green)]. For the plasmid tracks from inside to outside are as follows: 1, GC skew; 2, G+C content; 3, all predicted CDS.

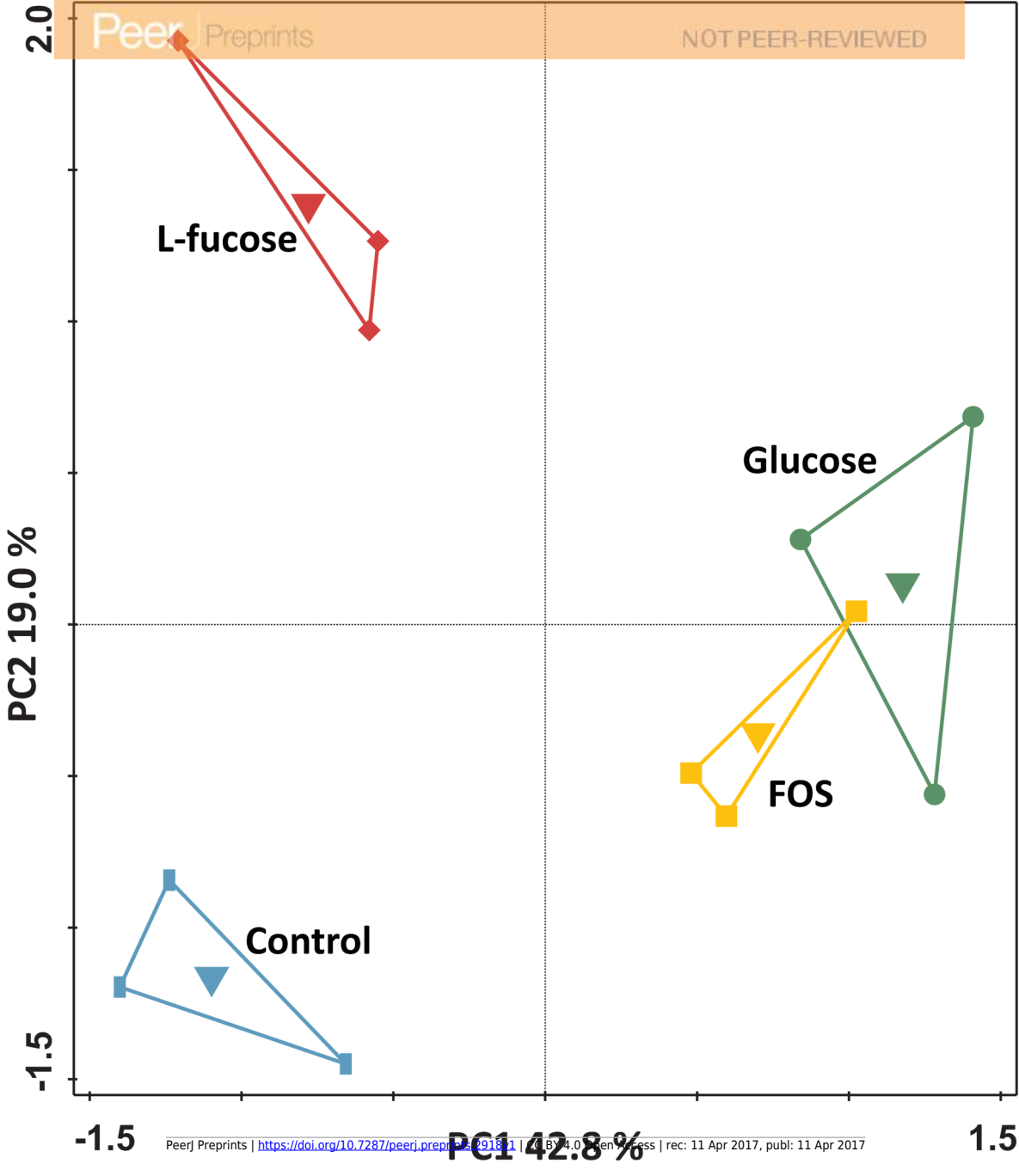




**Figure 2** (on next page)

Principal component analysis of the transcriptomes of *R. ilealis* CRIB<sup>T</sup> grown on different carbohydrates (glucose, FOS and L-fucose) or in the absence of an additional carbon source (control).

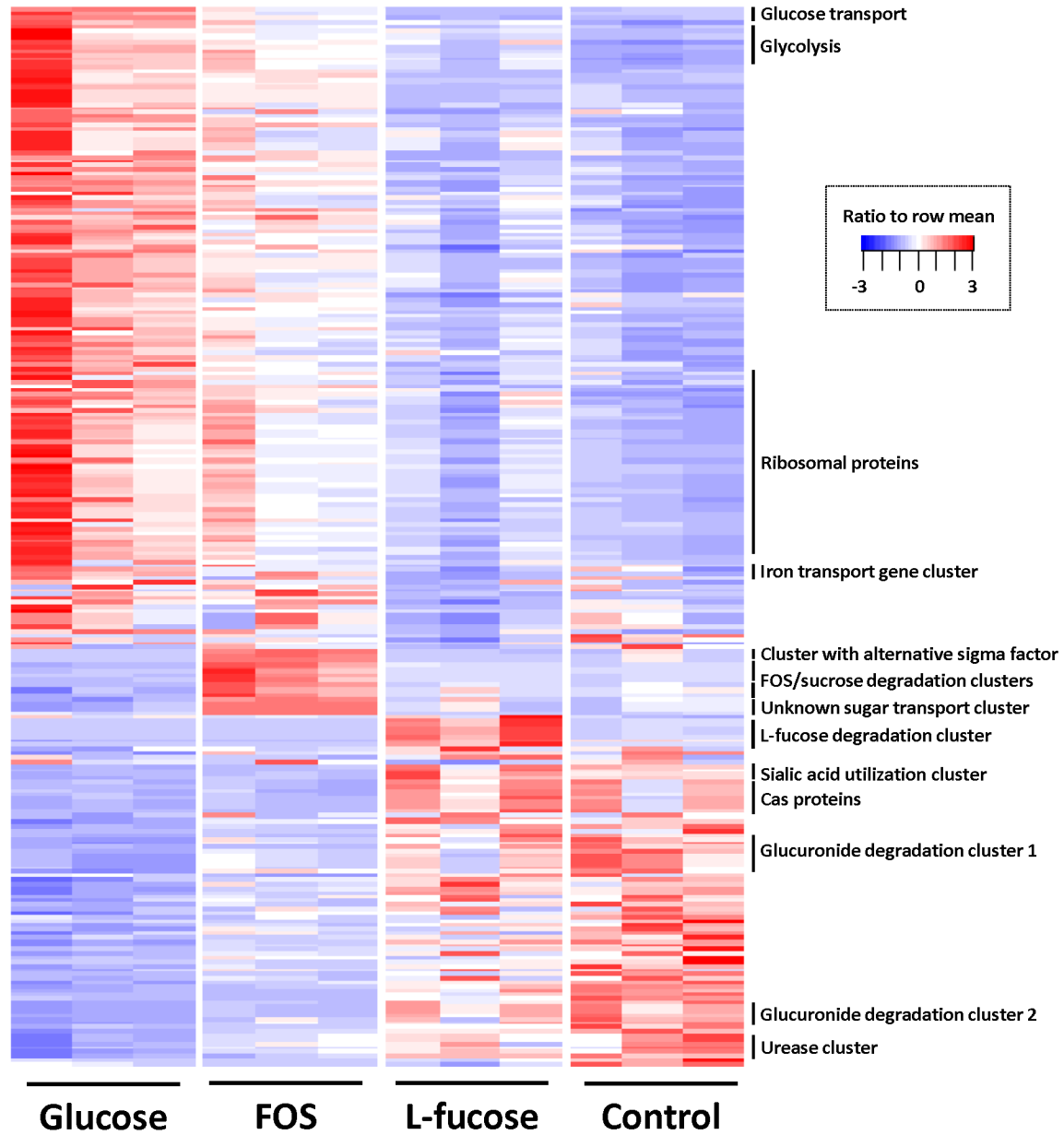
First and second ordination axes are plotted, explaining 42.8 % and 19.0 % of the variability in the data set, respectively. Individual transcriptomes are symbol-coded by experimental condition: glucose (circles), FOS (squares), L-fucose (diamonds) and control (rectangles). The experimental conditions were used as supplementary variables as well and could explain 62.9 % of the variation.



**Figure 3**(on next page)

Heatmap of genes differentially expressed in at least one of the four conditions ( $\geq 1.5$   $\log_2(\text{fold change})$  and  $q$  value  $\leq 0.05$ ).

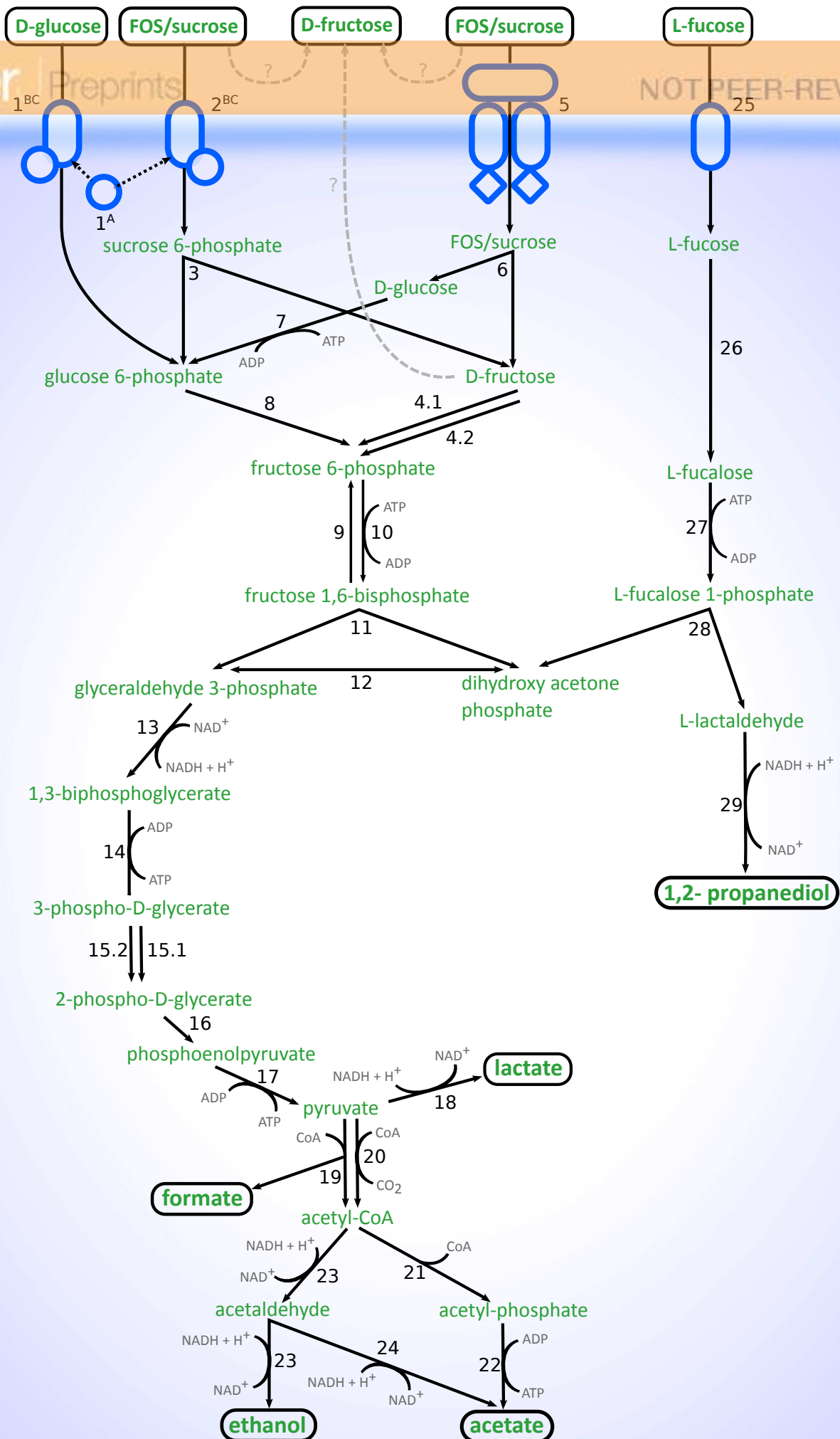
Colour coding by ratio to row mean. Key gene clusters are indicated.



**Figure 4**(on next page)

Schematic overview of the pathways involved in degradation of glucose, FOS and L-fucose in *R. ilealis* CRIB<sup>T</sup>

1<sup>A</sup>; PTS system glucose-specific EIIA component (CRIB\_2018); 1<sup>BC</sup>, PTS system glucose-specific EIIBC component (CRIB\_2017); 2<sup>BC</sup>, PTS system sucrose-specific EIIBC component (CRIB\_1461); 3,  $\beta$ -fructofuranosidase with RDD family protein (CRIB\_1459 and CRIB\_1460); 4, fructokinase (CRIB\_152 and CRIB\_1458); 5; ABC-type transporter (CRIB\_148-CRIB\_150); 6,  $\beta$ -fructofuranosidase (CRIB\_151); 7, glucokinase (CRIB\_1849); 8, glucose 6-phosphate isomerase (CRIB\_140); 9, fructose 1,6-bisphosphatase (CRIB\_45 and CRIB\_2020); 10, 6-phosphofructokinase ; (CRIB\_104); 11, fructose-bisphosphate aldolase (CRIB\_2223); 12, triosephosphate isomerase (CRIB\_189); 13, glyceraldehyde-3-phosphate dehydrogenase (CRIB\_187); 14, phosphoglycerate kinase; 15, phosphoglycerate mutase (CRIB\_1223) and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (CRIB\_190); 16, enolase (CRIB\_191); 17, pyruvate kinase (CRIB\_105); 18, L-lactate dehydrogenase (CRIB\_684); 19, formate acetyltransferase (CRIB\_2141); 20, pyruvate-flavodoxin oxidoreductase (CRIB\_2021); 21, phosphate acetyltransferase (CRIB\_2171); 22, acetate kinase (CRIB\_1927); 23, bifunctional aldehyde-alcohol dehydrogenase (CRIB\_2231); 24, fatty aldehyde dehydrogenase (CRIB\_2231); 25, L-fucose permease (CRIB\_1294); 26, L-fucose isomerase (CRIB\_1298); 27, L-fuculokinase (CRIB\_1297); 28, L-fuculose phosphate aldolase (CRIB\_1297); 29, lactaldehyde reductase (CRIB\_1300).



**Table 1** (on next page)

General features of the *R. ilealis* CRIB<sup>T</sup> genome.

1

	Chromosome	Plasmid
<b>Size (bp)</b>	2,581,778	6,145
<b>G+C content (%)</b>	27.9	29.3
<b>Protein CDS</b>	2,351	8
<b>Pseudogenes</b>	12	0
<b>Coding density</b>	1.10	1.02
<b>Average gene size (bp)</b>	899	531
<b>rRNA genes</b>		
<b>16S rRNA genes</b>	14	0
<b>23S rRNA genes</b>	14*	0
<b>5S rRNA genes</b>	14	0
<b>tRNAs</b>	109	0
<b>ncRNAs</b>	28	0
<b>CRISPR repeats</b>	1*71	0

2 \* An additional 23S rRNA gene is expected in one of the gaps.



**Table 2** (on next page)

Fermentation end products of *R. ilealis* CRIB<sup>T</sup> produced during growth on different carbohydrates (glucose, FOS or L-fucose) or in basal medium in the absence of a carbon source (control condition)

Samples were obtained during mid-exponential phase (~8-10 h incubation; used for transcriptome analysis) and in stationary phase (24 h incubation). For the control cultures, fermentation products are shown for the individual cultures separating the carbohydrates used for preconditioning of the inoculum. For the three other conditions, values represent means of triplicate cultures with standard deviations. N.D., not detected.

1

	Formate		Acetate		Propionate		Lactate		1,2-propanediol	
	8-10 h	24 h	8-10 h	24 h	8-10 h	24 h	8-10 h	24 h	8-10 h	24 h
Control: basal medium	3.2	7.7	2.0	6.2	2.0	2.2	N.D.	N.D.	N.D.	N.D.
(glucose inoc.)	4.5	9.2	2.4	7.4	2.4	2.9	N.D.	N.D.	N.D.	N.D.
(FOS inoc.)	4.8	10.8	2.3	9.8	2.3	3.0	N.D.	N.D.	1.0	1.0
(L-fucose inoc.)										
Basal medium + glucose (5 % w/v)	4.4±1.2	28.2±4.3	1.0±0.9	16.3±2.2	1.0±0.9	1.3±0.1	N.D.	3.0±0.7	N.D.	N.D.
Basal medium + FOS (5 % w/v)	4.7±0.6	27.3±2.5	1.4±0.0	17.7±1.4	1.4±0.0	1.6±0.1	N.D.	2.5±0.3	N.D.	N.D.
Basal medium + L-fucose (5 % w/v)	6.7±0.1	19.5±3.6	2.8±0.1	16.3±2.9	2.8±0.1	2.8±0.4	N.D.	N.D.	1.3±0.1	7.7±1.4

2