Escherichia coli must synthesize purines and pyrimidines to establish in the mouse intestine

Running title:

Nucleotide dependent establishment of E. coli in mice

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To study the adaptation of an intestinal bacterium to its natural environment, germfree mice were associated with commensal *Escherichia coli* MG1655. Two-dimensional gel electrophoresis was used to identify proteins differentially expressed in *E. coli* MG1655 collected from either cecal contents or anaerobic *in vitro* cultures. Fourteen differentially expressed proteins (>3-fold, p<0.05) were identified, nine of which were up-regulated in cecal versus *in vitro*-grown *E. coli*. Four of these proteins were investigated further for their role in gut colonization. After deletion of the corresponding genes, the resulting *E. coli* mutants were tested for their ability to establish in the intestine of gnotobiotic mice in competition with the wild type strain. A mutant devoid of *ydfG*, which encodes a putative NADH-dependent methylglyoxal reductase, reached a 1.2 log lower cecal concentration than the wild type. Deletion of the *nanA* gene encoding N-acetyleneuraminate lyase affected the colonization and persistence of *E. coli* in the intestine of the gnotobiotic mice only slightly. A mutant devoid of 5′-phosphoribosyl 4-(N-succinocarboxamide)-5-aminimidazole synthase, a key enzyme of purine synthesis, displayed intestinal cell counts >4 logs lower than those of the wild type. Deletion of the gene encoding aspartate carbamoyltransferase, a key enzyme of pyrimidine synthesis, even resulted in the washout of the corresponding mutant from the mouse intestinal tract. These findings indicate that *E. coli* needs to synthesize purines and pyrimidines to successfully establish in the mouse intestine.
Introduction

The human gastrointestinal tract harbors a complex community of approximately $10^{14}$ microorganisms (11). Whereas the impact of intestinal bacteria on the host has been studied in detail, knowledge about the impact of host factors on gut microorganisms is still limited. Only few proteomic studies investigated the response of bacteria to the intestinal environment. The application of proteome analysis to the infant fecal ecosystem was hampered by the complexity of the microbial community: Only one of 11 determined peptide sequences could be linked to an enzyme, the bifidobacterial transaldolase (8). In contrast, several metabolic pathways involved in carbon assimilation were demonstrated to be up-regulated in *Lactococcus lactis* when investigated in mice monoassociated with this organism. YwcC, a phosphogluconolactonase probably involved in the pentose phosphate pathway, was found to be essential for the organism’s ability to colonize the mouse intestinal tract (15). *Bifidobacterium longum* was reported to express after short exposure to the gastrointestinal environment several sets of proteins including adhesion factors, and metabolic genes (18). Comparative proteome analysis of *E. coli* grown *in vitro* on LB or in the intestine of monoassociated mice indicated that *E. coli* utilizes a wider range of substrates in the mouse intestine than under *in vitro* conditions and is exposed to various forms of stress including starvation (2).

The present study aimed at the identification of proteins essential for the colonization and persistence of *E. coli* in the gastrointestinal environment. The well characterized non-pathogenic *E. coli* strain MG1655 was grown *in vitro* and its proteome compared with that of the same strain isolated from the cecum of monoassociated mice. To reduce the differences in nutrient availability between the *in vivo* and the *in vitro* situation the *in vitro* cultures were grown anaerobically on a broth prepared from the mouse chow. Selected bacterial proteins undergoing the most prominent up-regulation in the mouse cecum were identified and tested for their
possible role in colonization. For that purpose, targeted deletion mutants were tested in
competition with the wild type strain in respect of their ability to successfully establish in the
germbre free mouse intestine. The results suggest that *E. coli* needs to synthesize purines and
pyrimidines to establish and persist in the mouse intestine.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *E. coli* K-12 MG1655 (CGSC 6300) was kindly
provided by K. Schnetz, University of Cologne, Germany. *E. coli* cells were grown anaerobically
in MOPS-buffered Ringer-solution (100 mM MOPS, 8.6 g/l NaCl, 0.3 g/l KCl, 0.33 g/l CaCl$_2$,
pH 7.0; 0.25 g/l L-cysteine and 1 mg/l resazurine) supplemented with 2.4% (w/v) of standard
mouse chow (Altromin 1310) at 37°C. The bacteria were harvested by a two-step procedure:
particles of the mouse chow were removed by a low speed centrifugation (300 g, 4°C, 3 min) and
the bacterial cells were subsequently sedimented at 10000 g, 4°C for 3 min. For proteome
analysis of *in vitro* grown *E. coli*, bacterial cells were collected in the exponential and the
stationary phase. To minimize the influence of growth phase-dependent variations, the mean of
the *in vitro* protein expression in both phases was compared with the protein expression of *E. coli*
cells from the cecum.

**Mouse colonization experiments and sample preparation.** The germbre free status of the
animals was confirmed before each experiment. Twenty C3H mice (9-12 weeks old) were kept in
filter top cages under a laminar flow hood for the duration of the experiment. The mice had free
access to sterile food and autoclaved water. Each mouse was orogastrically inoculated with 1x10$^9$
*E. coli* cells. After 21 days the mice were killed and the cecum contents were collected, weighed
and diluted 1:10 (w/v) with phosphate buffered saline (PBS) containing a protease-inhibitor mix
(GE-Healthcare) at a 100-fold dilution. After homogenization of intestinal contents by agitation with a Uniprep 24 (speed 2; Uniequip, Martinsried, Germany) in the presence of glass beads (diameter 2.85–3.33 mm) the samples were centrifuged (300 g, 4°C, 3 min) to remove coarse particles originating from the feed and cell counts were determined from the supernatants by plating on LB-Lennox (9) agar.

**Isolation of bacteria from cecal contents and in vitro cultures.** The above supernatants were centrifuged (10000 g, 4°C, 3 min) and the pelleted cells were resuspended in washing buffer (10 mM Tris, pH 8; 5 mM magnesium acetate; 30 mg/l chloramphenicol; protease-inhibitor mix 1:100 diluted). The cells were subsequently isolated by Nycodenz (Axis-shield PoC; AS, Oslo, Norway) gradient centrifugation as described by Roy et al. (15), with slight modifications. Briefly, 0.5 ml of a Nycodenz solution (40% w/v) was overlaid with 0.5 ml cell suspension and centrifuged for 15 min at 186000 g and 4°C. The *E. coli* cells at the interface were recovered and washed 5 times with washing buffer at 4°C. Washed cells were stored at -80°C.

**Preparation of whole bacterial cell protein extracts.** Frozen cells were thawed, resuspended in 0.8 ml lysis buffer (8 M urea, 30 mM Tris, 4% (w/v) CHAPS, pH 8.5) and mechanically disrupted with zirconia/silica-beads (0.1 mm; Roth, Karlsruhe, Germany) in an FP120 FastPrep cell disruptor (Thermo Scientific, Waltham, MA, USA) by applying three 20 s cycles of homogenization, using a speed of 4.0 m/sec, interrupted by 5 min intervals for cooling on ice. Unbroken cells were removed by centrifugation (14.000 g, 4°C, and 20 min). Components interfering with proteomic analysis were removed by selective precipitation of proteins (2-D Clean-Up Kit™, GE Healthcare). The concentration of resuspended proteins was determined with the Bradford-Assay (BioRad, Madrid, Spain). The pH of the protein solution was adjusted to pH 8.5 with non-pH-adjusted lysis buffer (8 M urea, 30 mM Tris, 4% (w/v) CHAPS) for an optimal reaction with the CyDye in the following step.
2-Dimensional Difference-in-Gel-Electrophoresis. The protein extracts were labeled according to the manufacturer’s instructions with CyDyes (GE Healthcare) for Difference in Gel Electrophoresis (DIGE) and focused on immobilized pH gradient-strips (pH-range 4-7, 24 cm) in an Ettan IPGphor 3 (GE Healthcare, Uppsala, Sweden). Active rehydration (30 V, 10 h) was followed by isoelectric focusing of the samples for a total of 60.2 kVh at 20°C. The second dimension was run on 12.5% SDS-gels in an Ettan-Dalt II apparatus at 1W per gel for 45 min followed by 17 W per gel for 3.5 hours. The proteins were visualized with a Typhoon Trio laser scanner and image analysis was done with the DeCyder-Software V 6.5 (both GE Healthcare, Uppsala, Sweden).

In-gel protein digestion. Preparative gels were stained with ruthenium II tris (bathophenanthroline disulfonate) (14). Subsequently proteins of interest were excised automatically using an Ettan-Dalt spot picker (GE Healthcare, Uppsala, Sweden) and subjected to tryptic digestion. Gel plugs were equilibrated twice with 50 mM NH₄HCO₃ and 50% MeOH for 30 min and dehydrated with 100% acetonitrile using a 96-well plate format. Proteins were digested with 50 ng trypsin (Promega) in 25 mM NH₄HCO₃ over night at 37°C. Peptides were extracted from the gel plugs with 50% acetonitrile and 0.1% trifluoro acetic acid for 20 min. The tryptically digested peptides were dried by vacuum centrifugation.

Identification of proteins. The peptide mixtures were dissolved in 12 µl of 0.1% formic acid and analyzed by NanoLC–ESI-MS/MS (injection volume usually 0.5–5 µl). Data were acquired using a Waters nanoACQUITY UPLC system (Milford, MA, USA) and a Waters Micromass QTOF Ultima API hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Manchester, UK) fitted with a NanoLockspray™ ion source. The nanoACQUITY UPLC system consisted of a Binary and an Auxiliary Solvent Manager and a Sample Manager with cooled sample tray and it was equipped with a 180 µm × 20 mm, 5 µm Waters Symmetry
C18 trap column and a 100 µm × 100 mm, 1.7 µm Waters BEH130 C18 analytical column. Samples were loaded from Binary Solvent Manager and trapped for online desalting with 1% B for 3 min at a flow rate of 5 µl/min (solvent A: aqueous 0.1% formic acid (v/v); solvent B: 0.1% formic acid/acetonitrile (v/v)). For elution and separation of the peptides, an increasing organic solvent concentration (1% B to 25% B in 16 min, 25% B to 85% B in 1 min) was applied at a flow rate of 400 nl/min.

ESI-MS and MS/MS analysis, data processing and protein identification were performed as described previously (1, 2), except that ProteinLynxTMGlobalServer2.3 software version (http://www.waters.com) and SwissProt version 57.2 (http://www.expasy.org/sprot/) were used for processing of the MS/MS data and subsequent databank searching. In order to eliminate false positive results, a second search was performed using the randomized database. Some proteins were in addition identified by the Proteome Factory AG (www.proteomefactory.com).

**Generation of deletion mutants.** Chromosomal sequences comprising the targeted genes were replaced by a kanamycin-resistance cassette according to the technique of Datsenko and Wanner (6) using pKD13 as a template for the antibiotic resistance gene. Primers used for construction of the deletion mutants are listed in Table 1. Mutant candidates were tested for the loss of the target gene by PCR with kanamycin- (k1 and k2) and locus-specific primers. In addition mutants were sequenced for genotype confirmation and tested for their growth phenotype.

**Complementation of the pyrBI mutant.** The *pyrLBI* genes were PCR-amplified from MG1655 with primers GGAATTCTTAATTGGCCAGCACCACATTA and GCAAGCTTATAGCGCGCATCCCTGAGCA and a 1688 nt *EcoRI*-*PstI* fragment was cloned into the low-copy number plasmid pSU19 (3) giving *ppyrLBI*. The insert was verified by sequencing.
Mouse competition experiments. For each competition experiment four germfree C3H mice (9-12 weeks old) were kept in positive-pressure isolators (Metall + Plastik, Radolfzell-Stahringen, Germany) and housed individually in polycarbonate cages. The mice had free access to sterile food and autoclaved water. Each mouse was orogastrically inoculated with $5 \times 10^8$ E. coli MG 1655 and $5 \times 10^8$ of the respective E. coli mutant (purC, pyrBI, nanA or ydjKJIHG). Fecal samples were collected starting one day after the association and analyzed by plate counting for wild type and mutant cell numbers. After 21 days the mice were killed and cecum contents were collected and analyzed as described above.

Determination of nucleosides and nucleobases in luminal gut contents. The procedure is based on the method of Czarnecka et al. (5) with major modifications. The nucleosides and nucleobases were isolated from cecum contents and from in vitro samples by solid phase extraction with Strata X columns, 30 mg/ml (Phenomenex, Aschaffenburg, Germany). The columns were sequentially conditioned with 1 ml 25 mM ethanolamine pH 8.0, loaded with 0.5 ml of supernatant from resuspended and centrifuged cecum content or in vitro sample and washed with 1 ml 50% methanol. The flow-through and the 50% methanol fractions were lyophilized, resuspended in 50 µl deionized water and defatted with n-hexane (1:5; v/v). For HPLC analysis 40 µl of the water phase was used.

Nucleosides and nucleobases were separated by reversed-phase, ion pair chromatography on a C18 column (LiChrospher 100 RP-18; 5 µm; 250 by 4 mm; Merck, Darmstadt, Germany). The mobile phase was 100 mM potassium acetate, pH 4.5, and 2 mM hexane sulfonic acid (solvent A), acetonitrile (solvent B) and deionized water (solvent C) (1 to 4.2% solvent B in 6 min; 4.2 to 4.8% solvent B in 5 min; 4.8 to 7% solvent B in 3 min, 7% solvent B for 2 min, 100%...
solvent C in 5 min; 100% solvent B in 5 min, 100% solvent C in 5 min and 1% solvent B for 5 min) at a flow rate of 1 ml/min. The nucleosides and nucleobases were detected at 255 nm. Thymine, thymidine, cytosine, cytidine, uracil, uridine, guanine, guanosine, adenine and adenosine (Sigma) served as standards for calibration.

Characterization of deletion mutants. Cells were precultured aerobically overnight in MOPS-minimal medium (12) supplemented with 50 mM glucose and 100 µM adenine (purC) or 100 µM uracil (pyrBI), washed with minimal medium lacking nucleobases, and inoculated at 6.6 × 10^7 cfu/ml into MOPS-minimal medium containing 50 mM glucose and either 100 µM adenine, guanine, adenosine or guanosine for the purC mutant; or 100 µM uracil, cytosine, thymine, uridine, cytidine or thymidine for the pyrBI mutant, respectively. The cultures were incubated aerobically at 37°C for 24 h and growth was monitored by measuring the OD_{600}. Similarly, the nan mutants were tested in MOPS-minimal medium supplemented with either glucose (10 mM) or N-acetylneuraminic acid (10mM) as the carbon source.

RESULTS AND DISCUSSION

Identification of proteins involved in the adaptation of E. coli to the intestinal environment. To gain insight into the mechanisms that enable bacteria to adapt to the intestinal environment, mice monoassociated with commensal E. coli MG1655 were used as a simplified model of host-microbiota interactions. Protein expression of E. coli in the cecum of these mice was compared with that of E. coli grown anaerobically in vitro on rodent chow (2.4%; w/v). The comparative analysis of the in vivo versus in vitro conditions revealed fourteen differentially expressed proteins (≥3-fold, p<0.05) (Table 2), 9 proteins being up-regulated and 5 proteins being down-regulated in the in vivo versus the in vitro grown cells. Four of the up-regulated proteins were selected for more detailed investigations. The choice was based on the difference in
expression and their suspected biological importance. Proteins whose abundance increased maximally under in vivo conditions were the aspartate transcarbamoylase (ATCase, PyrB) and phosphoribosylaminomimidazole-succinocarboxamide synthetase (SAICAR-synthetase, PurC) (Table 2). The ATCase catalyzes the first committed step of pyrimidine nucleotide synthesis, namely the conversion of carbamoylphosphate and L-aspartate to N-carbamoyl-L-aspartate. The SAICAR-synthetase catalyzes the eighth step in the biosynthesis of IMP, the precursor of ADP and GMP: 5′-phosphoribosyl-5-aminoimidazole-4-carboxylate + L-aspartic acid + ATP → (5′-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole + ADP+P

The uncharacterized protein YdjG, a putative NADH-dependent methylglyoxal reductase, was 3.5-fold up-regulated in vivo. The corresponding gene ydjG is part of an operon which encodes additional proteins with the predicted functions of two oxidoreductases, an aldolase, a kinase and a transport protein. This led us to hypothesize that YdjG could be part of a new metabolic pathway for the degradation of a so far unidentified substrate required for colonization. Moreover, the N-acetylneuraminic acid lyase (NanA) was 3-fold up-regulated in vivo. This enzyme catalyzes the first step in the degradation of N-acetylneuraminic acid (Neu5Ac). It cleaves off a pyruvate moiety from Neu5Ac yielding N-acetylmannosamine. An increased level of NanA in the cecum suggests that E. coli utilizes Neu5Ac as an energy and carbon source. This amino-sugar serves as a terminal carbohydrate moiety in the side chain of numerous glycoconjugates present on the surface of epithelial cells and in mucus.

The observed up-regulation of PyrB, PurC, YdjG and NanA suggested that E. coli depends on these proteins to establish and persist in the mouse intestinal tract. To test this hypothesis, each of the corresponding genes was deleted and the resulting deletion mutants were subsequently tested for their ability to colonize the intestinal tract of mice in competition with the parental E. coli strain.
Deletion of the genes encoding NanA and YdjG affects intestinal colonization by *E. coli* only slightly. In *vitro*, the nanA mutant did not grow on Neu5Ac as the sole source of carbon and energy indicating that the deletion of this gene prevents the utilization of Neu5Ac (data not shown). Following the application of equal cell numbers of the wild type and the nanA mutant strain to germfree mice the two strains did not differ in their fecal cell concentrations until day 8 (9-10 log cfu/g feces), indicating that the nanA mutant colonized the mouse intestine with the same efficiency as the parental strain. However, twelve days after the association, the fecal cell number of the mutant strain was one log lower than that of the wild type strain, but this difference was only significant at some sampling times (Fig. 1). Analysis of different sections of the gastrointestinal tract revealed that the mutant strain displayed cell numbers in small intestine and cecum that were one log lower than those of the wild type (p<0.05). The difference of 1.2 log observed in the colon between the wild type and the nanA mutant was not statistically significant (Fig. 2).

NanA is required for growth of *E. coli* on Neu5Ac, the predominant sialic acid found in mammalian cells (16, 17). Neu5Ac is a frequent component of glycoproteins on the surface of intestinal cells and of acidic mucopolysaccharides secreted into the intestinal tract. *E. coli* is capable of utilizing Neu5Ac as the sole source of carbon and energy (4). Chang et al. demonstrated that *E. coli* must be able to degrade Neu5Ac to permanently establish in the mouse intestinal tract. In contrast to these findings we observed that deletion of nanA affected the ability of the *E. coli* to colonize the intestinal tract of germfree mice in competition with the wild type *E. coli* only slightly. This discrepancy could be due to the fact, that Chang et al. (4) used the streptomycin-treated mouse model in which only facultative bacteria are eliminated while strict anaerobic bacteria are still present. The latter might keep the concentration of potential alternative substrates in the gut so low that the nanA deletion mutants find no substrates that can
replace Neu5Ac. Since in our mouse model only wild type \textit{E. coli} competes with the mutant strain, it is conceivable that substrates other than Neu5Ac are still available in sufficient amounts to enable growth of the mutant.

The fecal bacterial cell counts of mice associated with equal numbers of a \textit{ydjLKIHG} deletion mutant and wild type \textit{E. coli} only differed on a few days between the wild type and the deletion mutant. On these days the cell numbers of the mutant were always lower than those of the wild type (Fig. 3). The cell numbers in the different sections of the intestine were one log lower for the mutant strain as compared to the wild type strain, but only the difference observed in the cecum was statistically significant (p<0.03) (Fig. 4).

Since the deletion of the whole operon did not affect the mutant’s ability to establish in the intestinal tract in competition with the wild type, it may be concluded that YdjG and its companion proteins are not a prerequisite to establish in the intestinal tract, but that they may improve the growth and competitiveness of \textit{E. coli} in certain sections of the digestive tract, such as the cecum, where the concentration of the wild type was somewhat higher than that of the mutant.

\textbf{SAICAR-synthetase and aspartate transcarbamoylase support the establishment and persistence of \textit{E. coli} in the mouse intestine.} The fecal cell number of the \textit{purC} mutant decreased within one week after association by four logs, while the cell number of the wild type strain remained unchanged (Fig. 5). This difference in cell numbers between wild type and mutant was also seen in the contents of small intestine, cecum and colon (Fig. 6), where the mutant strain exhibited cell counts five logs lower than those of the wild type.

An even stronger reduction of cell numbers was caused by the \textit{pyrBI} mutation, which deletes both subunits of the aspartate transcarbamoylase. While the fecal concentration of the wild type strain remained unchanged throughout the experiment (9 to 10 log cfu/g feces), the
fecal cell numbers of the pyrBI mutant decreased with time and fell below the detection limit ($10^3$ cfu g$^{-1}$ dry feces) after day 13 (p<0.002) (Fig. 7). Moreover, the mutant strain was not detectable in the intestinal contents of any of the gut sections (Fig. 8).

The fact that the purC and pyrBI mutants were diminished or washed out of the mouse intestine suggested that the unavailability of nucleotide precursors combined with the mutants’ inability to synthesize purines and pyrimidines were responsible for the reduced cell numbers of the mutants in the ecosystem. In support of the first of these assumptions the concentrations of adenine, adenosine, guanine, cytidine, cytosine and thymine were under the detection limit (< 5 µM) in small intestinal, cecal and colonic contents. However, while the concentrations of guanosine, thymidine, uracil and uridine in cecum and colon were also below the detection limit (< 5 µM), they reached considerable concentrations in the small intestine arguing at first glance against the unavailability of precursors as the cause for the washout of the pyrBI mutant and the reduction of the purC mutant. A closer look at the precursor concentrations in the small intestine revealed a high variability between individual animals for uracil, uridine, and guanosine (Fig. 9). This indicates that the E. coli mutants may encounter periods in which the concentrations of the required precursors become limiting so that their growth is hampered. In support of this notion the volume of the small intestinal contents of 19 mice was observed to vary between 0.122 and 0.860 ml (all animals killed in the morning). This is probably caused by individual differences in the time of the last feed intake. Based on these observations it is reasonable to assume that during a fasting period the growth conditions in the small intestine deteriorate because the volume of the intestinal contents and the concentrations of the nucleotide precursors are diminished due to absorption. The ability of the wild type to synthesize these nucleotide precursors is probably the reason why it outcompetes the mutants. It should also be noted that it takes several days until the mutants are washed out or reach their minimal concentration. This interpretation of the data is
also supported by a competition experiment (Fig. 10): Minimal media supplemented with 25 and 50 µM uracil, respectively, were inoculated with equal cell numbers of both wild type and pyrBI mutant. Every 12 h the cell number was determined and 0.5% of the cell culture was transferred to fresh media. Five such transfers resulted in a reduction of the mutant strain by >3 and >5 log with a supplementation of 50 and 25 µM uracil, respectively (Fig. 10).

In support of the second of the above assumptions the purC and pyrBI mutants did not grow in minimal media in the absence of nucleobases or nucleosides (data not shown). However, the purC mutant grew well on media supplemented with adenine, adenosine, guanine and guanosine, which confirms previous findings with purine auxotrophic mutants (19). Similarly, the pyrBI mutant grew on minimal media when supplemented with uracil or uridine but not when supplemented with thymine or thymidine (data not shown). It is well known that thymine and thymidine cannot serve as pyrimidine sources for pyrimidine-requiring E. coli mutants if deoxyribose-1-phosphate is missing (13). Growth experiments with the pyrBI mutant on media supplemented with 5 – 100 µM uracil showed that the wild type and the mutant did not differ in their growth rate but in their final OD (data not shown). At uracil concentrations of 100, 50, 25, 10 and 5 µM the OD reached by the mutant within 24 h was 3.3, 2.0, 1.1, 0.6, and 0.3. The unsupplemented wild type reached almost the same OD as the mutant supplemented with 100 µM uracil. Interestingly, uracil also stimulated the growth of the wild type, in particular at lower concentrations (Fig.11). The higher maximal OD reached by the wild type at any of the tested uracil concentrations may be another reason why the mutant was outcompeted by the wild type.

The OD of the pyrBI mutant after 24 h was similar to that of the mutant when it was supplemented with sufficient uracil or when the mutation was genetically complemented (data not shown).
Conclusion. The lack of purines and pyrimidines in cecum and colon, and their unsteady supply in the small intestine impede the establishment of *E. coli* in the mouse intestine when the genes for their synthesis are deleted. Hence, the ability to synthesize nucleotides appears to be a prerequisite for *E. coli* to establish in the mouse intestine.

ACKNOWLEDGMENTS:

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REFERENCES


TABLE 1. Details of chromosomal gene and operon disruptions

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<sup>a</sup>Extension lengths are given first. Numerals identify the position of the 3’ nucleotide of the extension in the E. coli genome sequence (GenBank accession no. U00096.2). C, complement; H1, homology 1; H2 homology 2.

<sup>b</sup>One primer had the H1 extension and the 3’ sequence for priming site P1 (TGTAGGCTGGAGCTGCTTCG), the other had the H2 extension and the 3’ sequence for the complement of priming site P2 (ATTCCGGGGATCCGTCGACC). Nomenclature for extensions and priming sites according to (6).

TABLE 2. Proteins with differential expression factors ≥3 in cecal samples compared to <i>in vitro</i> cultures

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<td>cheW</td>
<td>Chemotaxis protein cheW</td>
<td>3.8</td>
</tr>
<tr>
<td>P0A9Q9</td>
<td>asd</td>
<td>Aspartate-semialdehyde dehydrogenase</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>x-fold change: average of 3 biological replicates. p≤0.05 for all changes.
 FIG. 1. Time course of the competition experiment between $nanA$ mutant and wild type $E. coli$. Individually housed germfree mice were associated with equal numbers of WT (white diamonds) and mutant (black squares) bacteria. At the indicated times fecal samples were collected and analyzed by plate counting for wild type and mutant cell numbers. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with $p \leq 0.05$. 
FIG. 2. Analysis of the intestinal cell numbers at the end of the competition experiment between the nanA mutant and wild type E. coli. White bars, WT; grey bars, nanA mutant. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with p≤0.05.
FIG. 3. Time course of the competition experiment between the ydj-operon deletion mutant and wild type *E. coli*. Individually housed germfree mice were associated with equal numbers of WT (white diamonds) and mutant (black squares) bacteria. At the indicated times fecal samples were collected and analyzed by plate counting for wild type and mutant cell numbers in order to follow the colonization process through time. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with $p \leq 0.05$. 
FIG. 4. Analysis of the intestinal cell numbers at the end of the competition experiment between the ydj-operon mutant and wild type *E. coli*. White bars, WT; grey bars, ydj-operon mutant. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisk indicates a significant difference with p≤0.05.
FIG. 5. Time course of the competition experiment between the purC mutant and wild type E. coli. Individually housed germfree mice were associated with equal numbers of WT (white diamonds) and mutant (black squares) bacteria. At the indicated times fecal samples were collected and analyzed by plate counting for wild type and mutant cell numbers in order to follow the colonization process through time. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with p≤0.05.
FIG. 6. Analysis of the intestinal cell numbers at the end of the competition experiment between the purC mutant and wild type E. coli. White bars, WT; grey bars, purC mutant. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with p ≤ 0.05.
FIG. 7. Time course of the competition experiment between the pyrBI mutant and wild type E. coli. Individually housed germfree mice were associated with equal numbers of WT (white diamonds) and mutant bacteria (black squares). At the indicated times fecal samples were collected and analyzed by plate counting for wild type and mutant cell numbers in order to follow the colonization process through time. Black squares with down arrows indicate sampling points with mutant cell numbers below the detection limit of $10^3$ cfu per g dry feces. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with p≤0.05.
FIG. 8. Analysis of the intestinal cell numbers at the end of the competition experiment between the *pyrBI* mutant and wild type *E. coli*. White bars, WT. Black squares with down arrows indicate mutant cell numbers below the detection limit of $10^3$ cfu per g dry feces. Data are expressed as means ± standard deviations for an experiment with 4 mice. The asterisks indicate significant differences with $p \leq 0.05$. 

Log$_{10}$ CFU per g feces (dw)
FIG. 9. Concentrations of uracil (diamonds), uridine (squares), thymidine (circles) and guanosine (triangles) in the small intestine of monoassociated mice.
FIG. 10. Development of cell titers in a competition experiment between *E. coli* MG1655 (diamonds) and MG1655Δ*pyrBI* (triangles). Minimal media supplemented with 25 µM (open symbols) and 50 µM uracil (closed symbols), respectively, were inoculated with equal cell numbers of the two strains. Every 12 h the cell numbers were determined and 0.5% of the cell culture was transferred to fresh media.
FIG. 11. Optical densities (OD$_{420}$) of pure cultures after 24 h of growth in minimal media supplemented with the indicated concentrations of uracil. Diamonds, MG1655; triangles MG1655ΔpyrBI.