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C4-dicarboxylates and L-aspartate utilization by *Escherichia coli* K-12 in the mouse intestine: L-aspartate as a major substrate for fumarate respiration and as a nitrogen source

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Summary

C4-dicarboxylates, such as fumarate, L-malate and L-aspartate represent substrates for anaerobic growth of *Escherichia coli* by fumarate respiration. Here, we determined whether C4-dicarboxylate metabolism, as well as fumarate respiration, contribute to colonization of the mammalian intestinal tract. Metabolite profiling revealed that the murine small intestine contained high and low levels of L-aspartate and L-malate respectively, whereas fumarate was nearly absent. Under laboratory conditions, addition of C4-dicarboxylate at concentrations corresponding to the levels of the C4-dicarboxylates in the small intestine (2.6 mmol kg^{-1} dry weight) induced the *dcuBp-lacZ* reporter gene (67% of maximal) in a DcuS-DcuR-dependent manner. In addition to its role as a precursor for fumarate respiration, L-aspartate was able to supply all the nitrogen required for anaerobically growing *E. coli*. DcuS-DcuR-dependent genes were transcribed in the murine intestine, and mutants with defective anaerobic C4-dicarboxylate metabolism (*dcuSR*, *frdA*, *dcuB*, *dcuA* and *aspA* genes) were impaired for colonizing the murine gut. We conclude that L-aspartate plays an important role in providing fumarate for fumarate respiration and supplying nitrogen for *E. coli* in the mouse intestine.

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Introduction

C4-dicarboxylates (C4-DCs) such as succinate, fumarate, L-malate and L-aspartate, play an important role in the energy metabolism of *Escherichia coli* and other enteric bacteria. The metabolic routes for growth on the C4-DCs differ under aerobic and anaerobic conditions. In aerobic growth, the C4-DCs are oxidized to CO_2 by the use of the citric acid cycle (TCA) in combination with the pyruvate bypass (Gottschalk, 1986; Unden *et al*., 2016). L-Aspartate is a high-quality nitrogen source under aerobic conditions (Schubert *et al*., 2020). L-Aspartate is transported into the bacterial cell by transporter DcuA which catalyses an L-aspartate/fumarate exchange resulting in the net uptake of ammonia (Strecker *et al*., 2018). The ammonia of L-aspartate is able to satisfy all requirements for nitrogen in the growth of *E. coli* (Strecker *et al*., 2018; Schubert *et al*., 2020). It is assimilated by aspartase AspA, glutamine synthetase and the GOGAT pathway. The activity of AspA is regulated by the central regulator GlnB of nitrogen assimilation, integrating the ammonia assimilation in the nitrogen assimilatory network (Schubert *et al*., 2020).

Under anaerobic conditions, the C4-DCs are used for fumarate respiration (Guest, 1979; Unden *et al*., 2016) where fumarate is reduced by fumarate reductase to succinate (Guest, 1979; Cole *et al*., 1985; Unden *et al*., 2014; Unden *et al*., 2016). L-Malate and L-aspartate serve the same function after uptake and conversion to fumarate by fumarase FumB and aspartase AspA, respectively. Transport of the C4-DCs is achieved under anaerobic conditions by DcuB and DcuA that couple C4-DC uptake to succinate excretion (C4-DC/succinate antiport) (Engel *et al*., 1992; Engel *et al*., 1994; Six *et al*., 1994; Zientz *et al*., 1996; Janausch *et al*., 2002).

DcuB is used preferentially for the antiport fumarate/succinate or L-malate/succinate, and DcuA for the L-aspartate/succinate antiport (Six *et al*., 1994; Zientz *et al*., 1996; Strecker *et al*., 2018). Expression of *dcuB* is stimulated under anaerobic conditions and by the presence of C4-DCs by the transcriptional regulator FNR and the DcuS-DcuR two-component system, respectively (Golby *et al*., 1998; Zientz *et al*., 1998; Golby *et al*., 1999). During anaerobic growth DcuB is the major antiporter, it has a high affinity for the C4-DCs (K_m 90–130 μM) (Engel *et al*., 1994). DcuA, on the other hand, has a high preference for L-aspartate (K_m = 43 μM) (Strecker *et al*., 2018). DcuA and DcuB are able to replace each other in C4-DC/succinate antiport for fumarate respiration when one of the carriers is inactivated (Six *et al*., 1994). However, deletion of *dcuA* specifically inhibits L-aspartate/succinate antiport, and the deletion of DcuB weakens fumarate/succinate antiport (Six *et al*., 1994), indicating that DcuA is the preferred antiporter when L-aspartate is the substrate for fumarate respiration. Expression of *dcuA* is constitutive under aerobic and anaerobic conditions and independent of DcuS-DcuR (Golby *et al*., 1998; Strecker *et al*., 2018).

The intestine of mice and other animals represents an important habitat for *E. coli* and enteric bacteria. Mutants of *E. coli* deficient of fumarate reductase have colonization defects in the intestine in the streptomycin-treated mouse model (Jones *et al*., 2007; Jones *et al*., 2011). The data indicate that fumarate and related compounds are important anaerobic substrates and electron acceptors in contrast to dimethyl sulfoxide, nitrite and trimethylamine *N*-oxide. Nitrate, which is a preferred electron acceptor, was present only in low levels in the intestine under homeostatic conditions (Jones *et al*., 2011). Under conditions of inflammation, nitrate is generated through reactive nitrogen species metabolism and contributes to the bloom of *E. coli* during inflammatory diseases of the intestinal tract (Winter *et al*., 2013).

Here, we investigated the availability of C4-DCs in the mouse intestine to better understand the role and significance of C4-DCs and of fumarate respiration for the physiology of *E. coli*. First, the levels of the C4-DCs fumarate, succinate, L-malate and L-aspartate and their progression from the small intestine to the colon were determined. After learning the C4-DC levels in the mouse intestine, we analysed whether the respective levels of C4-DCs are suitable to induce expression of the DcuS-DcuR-regulated fumarate respiratory system. Thus, the mRNA levels of marker genes *frdA*, *dcuB*, *dcuA* and *aspA* of fumarate respiration were determined in samples of the intestine. *In vitro*, the expression of the *dcuB* gene as a reporter for DcuS-DcuR dependent gene expression was tested in anaerobic cultures with increasing concentrations of C4-DCs to confirm expression under concentrations relevant for the intestine. For verifying the role of anaerobic C4-DC metabolism for the fitness of *E. coli* and colonization of the mouse intestine, the survival of mutants deficient of C4-DC metabolism was studied in competition experiments. Finally, after demonstrating the role of L-aspartate as a major C4-DC in the mouse intestine, we tested *in vivo* whether L-aspartate can be used as a source of nitrogen under anaerobic similar to aerobic growth conditions (Schubert *et al*., 2020), in addition to its function as an electron acceptor in fumarate respiration (Six *et al*., 1994; Zientz *et al*., 1996; Golby *et al*., 1998).

Results

C4-dicarboxylates in the mouse intestine

Initially, we quantified the C4-DC contents in the mouse intestine (Fig. 1). Animals were fed a standard breeding diet. Extracts were obtained from four locations of the small intestine, cecum, and colon of male mice, and C4-DC levels were quantified by mass spectrometry. The proximal part of the small intestine contained high levels of L-aspartate (4.36 mmol kg^{-1}), intermediate levels of L-malate (188 μmol kg^{-1}) and low levels of fumarate (<50 μmol kg^{-1}). The levels of these C4-DCs decreased in lower sections of the intestine, in particular between the distal small intestine and the colon. The progression of the succinate levels was different. Succinate was intermediate in the proximal and increased in the distal small intestine to 496 μmol kg^{-1}, before it dropped again in the cecum. In the colon, succinate was the major C4-DC. The distribution of C4-DCs in the murine gut is compatible with the assumption that fumarate, L-malate and L-aspartate are converted by fumarate respiration to succinate as the end product. However, the increase of succinate by 203 μmol kg^{-1} did not equal the decrease in fumarate, L-malate and L-aspartate levels, a total of 4186 μmol kg^{-1}, suggesting that large amounts of the C4-DCs are used for other processes. The levels of C4-DCs are low in the cecum and colon, with succinate representing the major C4-DC. The data suggest that L-aspartate, L-malate and fumarate are converted in parts to succinate through fumarate respiration, but additionally other processes result in their consumption, such as microbial cell mass synthesis or resorption in the small intestine. Furthermore, the decrease in succinate levels in the cecum and colon can be caused by succinate fermentation, oxidative degradation using low levels of diffused oxygen, or intestinal gluconeogenesis (de Vadder *et al*., 2016; Connors *et al*., 2018).

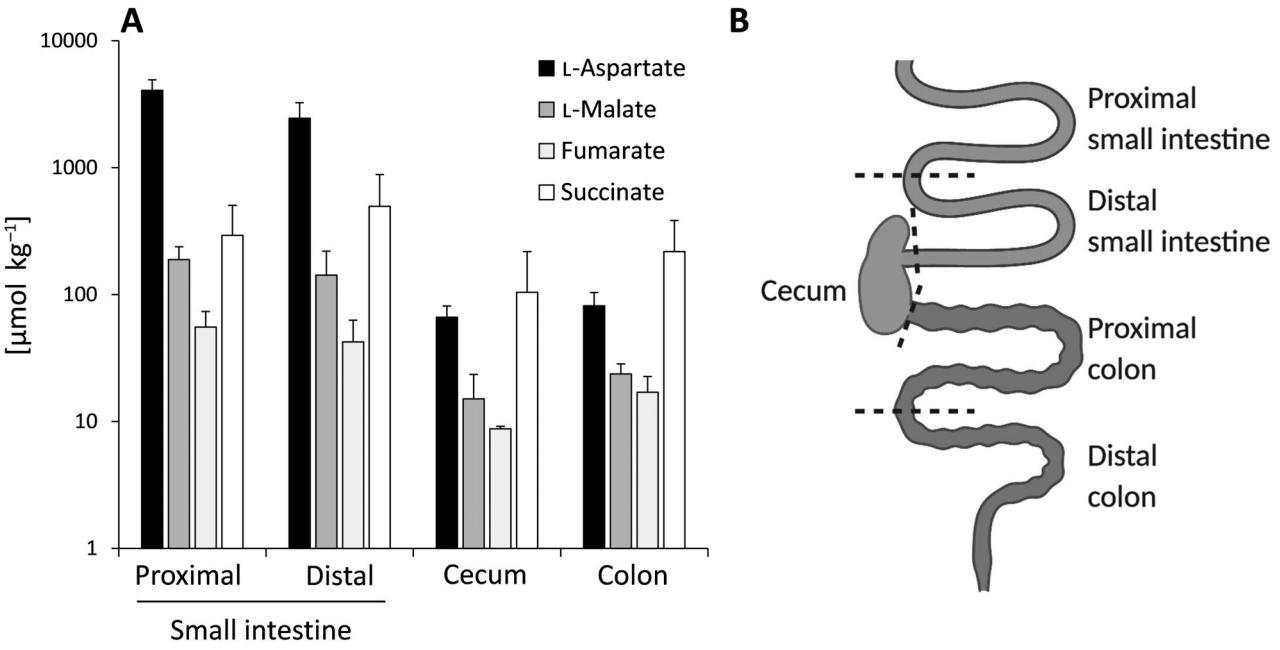

Fig 1. Concentration of C4-DCs L-aspartate, L-malate, fumarate and succinate in the intestinal contents of male mice (A). Three replicates were analysed for each position of the intestine shown in (B).

Induction of DcuS-DcuR-regulated genes by the C4-DC levels in the mouse intestine

Our quantification of C4-DCs in the mouse intestine allows for an estimate on the expression levels of DcuS-DcuR-regulated genes. The sensitivity of DcuS is similar for most C4-DCs, including L-aspartate, with a K_D of approx. 2.0 mM (Kneuper *et al*., 2005), whereas that for succinate is even lower (3.0 mM). The C4-DCs acting as strong inducers of DcuS-DcuR (L-malate, fumarate, L-aspartate) add up to 4.6 and 2.6 mmol kg^{-1} in the proximal and the distal small intestine respectively. The combined levels of the C4-DCs suggest significant and more than half-maximal induction of DcuS-DcuR-regulated genes in the small intestine. For direct testing, the expression of the *dcuBp-lacZ* reporter gene was studied in laboratory media in response to increasing levels of C4-DCs (Fig. 2). Maleate was used as a representative C4-DC since maleate stimulates DcuS-DcuR with similar sensitivity as physiological substrates (Kneuper *et al*., 2005) but is not or only slowly metabolized and maintained at constant levels throughout the experiment. The expression of *dcuBp-lacZ* started to increase at maleate concentrations above 100 µM (Fig. 2) and at 1.5 mM maleate, half-maximal activity was observed, similar to earlier studies (Kneuper *et al*., 2005). The stimulation was similar in exponentially growing bacteria and bacteria of the late logarithmic growth state. At 2.6 mM

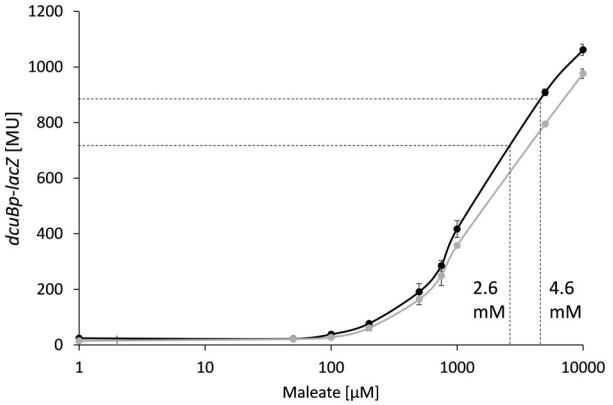

Fig 2. Expression of *dcuBp-lacZ* by *E. coli in vitro* in the presence of increasing concentrations of maleate. *Escherichia coli* IMW237 (*dcuBp-lacZ*) was grown anaerobically in eM9 medium with glycerol (50 mM), DMSO (20 mM) and the concentrations of maleate as indicated. The activity of the β-galactosidase produced from *dcuBp-lacZ* expression was measured from the cultures at OD = 0.5 (-○-) and in the early stationary (-●-) growth phase. Activities were measured in triplicate from three independent biological repeats each.

maleate, roughly equivalent to the C4-DC contents of 2.6 mmol kg^{-1} dry weight in the distal small intestine, 67% of maximal expression levels can be estimated. In the cecum and colon with approx. 0.1 mM of the combined C4-DCs (without succinate), the expression (3.6% of the maximum) was still measurable. As such, the C4-DC levels found in the small intestine can induce

dcuB and other DcuS-DcuR-regulated genes of fumarate respiration to high levels, but in the cecum and colon only to low levels.

L-Aspartate as a nitrogen source for the growth of E. coli under anaerobic conditions

L-Aspartate serves under anaerobic conditions as a substrate for fumarate respiration (Six *et al*., 1994), which requires uptake into the bacterial cell and conversion to fumarate by aspartase AspA. L-Aspartate may also represent a source of nitrogen for the growth under anaerobic conditions, using the constitutively produced DcuA for an L-aspartate/fumarate antiport (Strecker *et al*., 2018) and AspA for the release of ammonia (Schubert *et al*., 2020). The low amounts of succinate in the cecum and colon (Fig. 1) suggest that L-aspartate, or succinate derived from L-aspartate, is used for further metabolic processes. We, therefore, tested whether L-aspartate can serve as a source of nitrogen for cell synthesis under anaerobic conditions (Fig. 3). The bacteria were grown anaerobically in mineral medium deficient of a nitrogen source, with glycerol + fumarate which allows fumarate respiration, but no fermentation. Without further nitrogen source, no growth was observed (Fig. 3A). Inclusion of ammonia (Fig. 3B) or L-aspartate (Fig. 3C) in addition to glycerol + fumarate supported the growth of the wild type to high levels, demonstrating that ammonia or L-aspartate can be used as nitrogen sources. Similar growth was obtained when only L-aspartate was supplied in addition to the glycerol (Fig. 3D). L-Aspartate had to serve then as the nitrogen source and as an acceptor for fumarate respiration. When *aspA* was deleted, growth was almost completely lost under growth conditions where L-aspartate was used as a nitrogen source (Fig. 3C), or both as nitrogen source and electron acceptor (Fig. 3D), but not when ammonia was the nitrogen source (Fig. 3B). Therefore, L-aspartate can be used both as a source of fumarate for fumarate respiration as shown earlier (Six *et al*., 1994), and as a source of ammonia for nitrogen assimilation.

The observation that the *dcuA dcuB* double mutant shows substantial growth when fumarate is the electron acceptor and ammonia the nitrogen source (Fig. 3B) is consistent with earlier data showing that DcuC is able to take over fumarate/succinate antiport when DcuA and DcuB are missing (Six *et al*., 1994; Zientz *et al*., 1999). However, when L-aspartate was used as the nitrogen source instead of ammonia, growth dropped significantly in the *dcuA dcuB* mutant (Fig. 3C). This finding indicates that DcuC is not able to supply the L-aspartate for nitrogen assimilation. Similarly, growth is virtually lost in the double mutant when L-aspartate is used both as the nitrogen source and for fumarate respiration (Fig. 3D). Overall, the data show that L-aspartate can be used as a nitrogen source under anaerobic conditions in addition to its role in fumarate respiration, and that AspA, DcuA and DcuB are essential for the use of L-aspartate as a nitrogen source and in fumarate respiration.

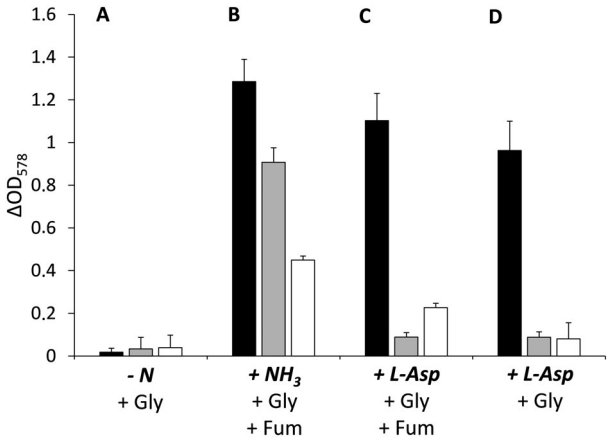

Fig 3. Anaerobic growth of *E. coli* K-12 strains by fumarate respiration using L-aspartate as a nitrogen source, electron acceptor, or both. Wild-type *E. coli* BW25113 (black bars) or mutants with deletions in *aspA* (strain JW4099, grey bars) or *dcuA dcuB* (strain JRG 2814, white bars) were used. The bacteria were grown in eM9 medium deficient of a nitrogen source with the following additions: (A) Glycerol; (B) Glycerol + fumarate + ammonia; (C) Glycerol + fumarate + L-aspartate; (D) Glycerol + L-aspartate. Ammonia or L-aspartate were added as the nitrogen sources in B and C (20 mM), and fumarate or L-aspartate as the electron acceptors (20 mM). L-Aspartate supplied in exp. (D) serves both functions.

DcuS-DcuR-regulated genes contribute to gut colonization

Next, we sought to investigate whether DcuS-DcuR and DcuS-DcuR-regulated genes contribute to colonization of the murine intestinal tract. Since *E. coli* K-12 strains only poorly colonize the murine intestinal tract, we chose to use a murine *E. coli* isolate, MP1 (Lasaro *et al*., 2014), for our initial experiments. Pretreatment of mice with antibiotics, such as streptomycin, is commonly used to promote colonization. We, therefore, colonized two groups of mice, one pre-treated for 1 day with 20 mg of streptomycin and one mock-treated control group, with an equal ratio of the MP1 wild-type and an isogenic *dcuSR* mutant. After 3 days, the bacterial load in the intestine was determined (Fig. 4A). In mock-treated animals, MP1 poorly colonized the intestinal tract. In contrast, streptomycin treatment greatly enhanced *E. coli* colonization. Compared with the MP1 wild-type strain, the *dcuSR* mutant was recovered in 25-fold, 34-fold and 39-fold lower numbers from the content of the small intestine, the cecum and the colon respectively, suggesting that DcuS-DcuR and DcuS-DcuR-regulated genes contribute to

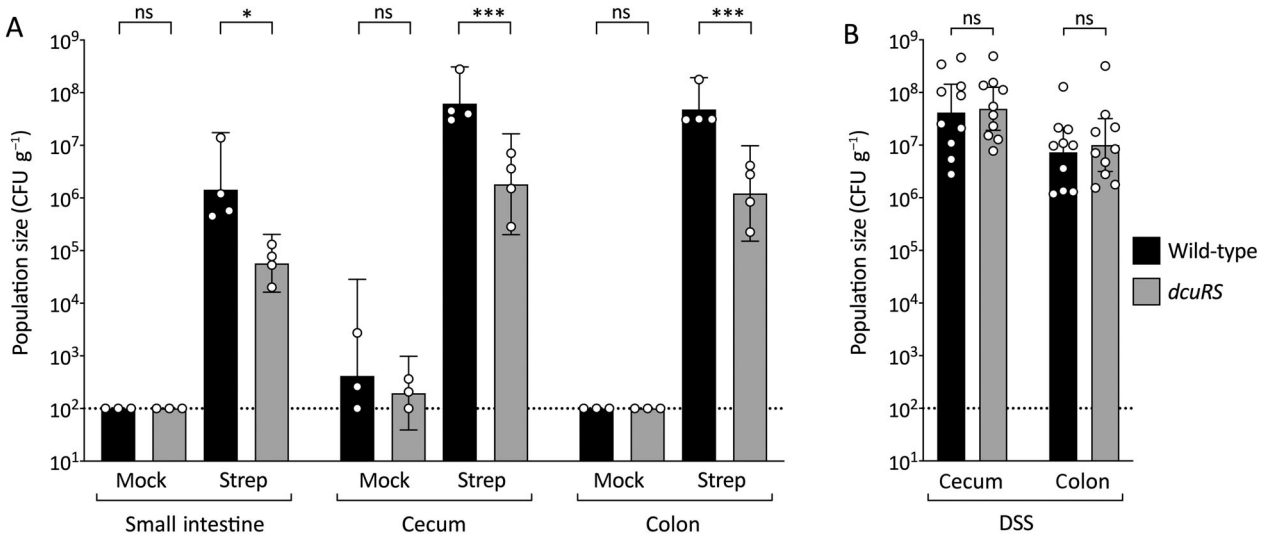

Fig 4. Contribution of DcuS-DcuR to fitness of *E. coli* in the murine gut.
A. Groups of C57BL/6 mice received 20 mg of streptomycin or were mock-treated with sterile water. After 24 h, animals were intragastrically inoculated with an equal ratio of the MP1 wild-type harbouring pWSK129 (black bars) and the MP1 *dcuRS* mutant (MW473) harbouring pWSK29 (grey bars). Three days later, the abundance of each strain in the content of the small intestine, cecum and colon was determined by plating on selective media.
B. C57BL/6 mice were treated with dextran sulfate sodium (3% wt./vol.) in the drinking water for 4 days and then intragastrically inoculated with *E. coli* as described above. After 5 days, the abundance of each *E. coli* strain in the cecum and colon was determined. Bars represent the geometric mean, error bars represent the 95% confidence interval and each dot represents data from one animal. *$P < 0.05$; ***$P < 0.001$; ns, not statistically significant. The dotted line indicates the limit of detection.

colonization of the intestinal tract in mice. This finding is consistent with a previous report (Lasaro *et al*., 2014).

In *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), the DcuS-DcuR system is required for efficient colonization of the intestinal tract (Nguyen *et al*., 2020). *S*. Typhimurium causes mucosal inflammation to alter nutrient availability, generate a replicative niche and outcompete commensal microbes in the gut lumen (Stecher *et al*., 2007). To determine whether the DcuS-DcuR system provides a fitness advantage to *E. coli* in the context of mucosal inflammation, we determined the fitness of a *dcuSR* mutant in comparison to the wild-type strain in a murine model of chemically induced colitis. After the onset of colitis, animals were intragastrically inoculated with an equal ratio of the MP1 wild type and an isogenic *dcuSR* mutant and the bacterial load in the intestine was determined after 5 days (Fig. 4B). Both strains were recovered in similar quantities, indicating that DcuS-DcuR are dispensable for fitness of *E. coli* during gut inflammation.

We then asked whether the DcuS-DcuR-regulated genes *aspA*, *dcuB* and *frdA*, and C4-DC metabolism enhance fitness of *E. coli* in the streptomycin-treated mouse model. Since *E. coli* K-12 strains colonize well in this animal model (Myhal *et al*., 1982), we colonized groups of streptomycin-treated mice with *E. coli* K-12 BW25113 and various isogenic mutants as described above. No colonization of the small intestine was observed (data not shown). In contrast, the large intestine was robustly colonized (on average 4.5×10^6 CFU g^{-1} in the cecum and 2.9×10^6 CFU g^{-1} in the colon content). Compared with the wild-type strain, the *frdA* mutant was recovered in significantly lower numbers in the colon and cecum content; similarly, the *dcuB* mutant was recovered in significantly lower numbers from the colon content (Fig. 5). The *aspA* mutant colonized the large intestine to a lesser extent than the wild-type strain; however, these differences were not statistically significant. Taken together, these data suggest that DcuB-mediated uptake of L-aspartate and other C4-DCs contribute to colonization of the murine intestine. Notably, the fitness defect of the *frdA* mutant was significantly more pronounced than the phenotypes of the *aspA* and *dcuB* mutants, suggesting that DcuB and AspA can be replaced by other enzymes or pathways.

DcuS-DcuR-regulated genes are transcribed in the intestinal lumen

We then quantified the expression of *aspA*, *dcuA*, *dcuB* and *frdA in vitro* and *in vivo*. To mimic conditions found in the intestinal tract, we cultured *E. coli* K-12 anaerobically in No-carbon E medium supplemented with 0.2% porcine type II mucin, with and without fumarate. Furthermore, we colonized two groups of streptomycin-treated mice

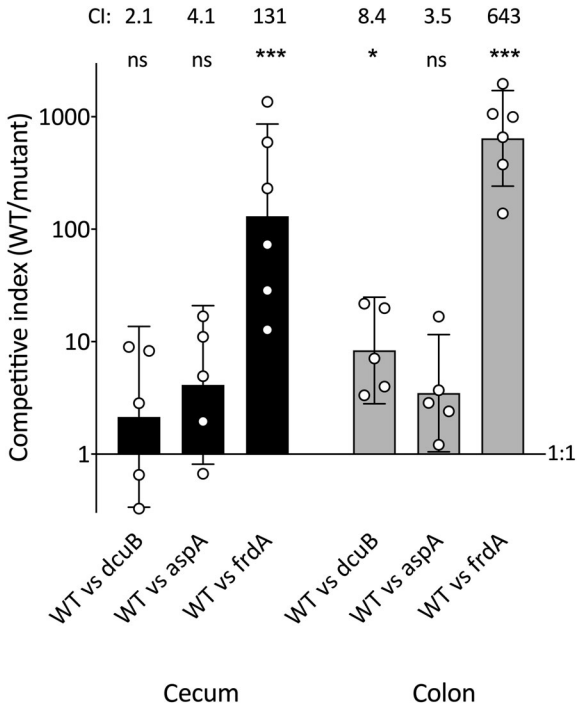

Fig 5. Contribution of C4-DC metabolism to fitness of *E. coli* K-12 in the streptomycin-treated mouse model. Three groups of BALB/C mice, pre-treated with 20 mg of streptomycin 1 day prior, were intragastrically inoculated with an equal ratio of the K-12 wild-type strain (BW25113) and the indicated mutant (JW4099, JW4084, JW4115), all harbouring pWSK29. After 3 days, the abundance of each strain in the cecum (black bars) and colon content (grey bars) was determined by plating on selective media. The competitive index (CI) was calculated as the ratio of the wild-type strain divided by the mutant strain in the intestinal content, corrected by the corresponding ratio in the inoculum. Bars represent the geometric mean, error bars represent the 95% confidence interval, each dot represents data from one animal, and the number above each bar shows the CI. To calculate statistical significance, a two-way ANOVA analysis, with a Šidák multiples comparisons test, was applied to ln-transformed populations of wild-type and mutant bacteria; $^{*}P < 0.05$; $^{***}P < 0.001$; ns, not statistically significant.

with the *E. coli* K-12 wildtype strain and a *dcuR* mutant, and after 2 days, we isolated total RNA from the cecal content. mRNA levels were determined by qRT-PCR using *gmk*, encoding guanylate kinase, as a housekeeping gene (Fig. 6). No *dcuR* mRNA was detected in the *dcuR* mutant. Transcription of *frdA* was significantly reduced in the *dcuR* mutant compared with the wild-type strain (Fig. 6A), indicating that this gene is regulated by the DcuS-DcuR system in the murine gut. Supplementation of mucin broth with fumarate was sufficient to induce transcription of *frdA* and *dcuB*, while no or only marginal effects were noted for *dcuA*, *dcuC*, *dcuD*, *dcuR*, and *aspA* (Fig. 6B–H). When comparing mRNA levels of these genes under laboratory conditions and in the murine intestine, transcription of *frdA*, *aspA*, *dcuB*, and *dcuC* was significantly elevated in the gut (Fig. 6B–H). These data provide further evidence that C4-DC metabolism occurs in *E. coli* growing in the mammalian gut. Furthermore, elevated transcription in the mouse model raises the possibility that factors other than DcuS-DcuR contribute to transcriptional regulation of fumarate respiration genes.

The aminogram of the mouse intestine and the role of lrp *for the expression of fumarate respiration genes*

The proximal small intestine of mice contains high levels of the proteinogenic amino acids. Assuming yields for the extraction and detection of the amino acids are comparable to those for the C4-DCs allowed approximation of their absolute contents (Fig. 7). Most amino acids were present in the range of 4 to 17 mmol kg^{-1} dry weight, with L-Ala possessing the highest levels, whereas those for L-cysteine and L-asparagine were very low. Relative levels are similar for most of the amino acids, with the exception of L-Cys and L-Gln, to the relative contents of the amino acids in standard proteins (Bruice, 2004), suggesting that the amino acids originate from the degradation of nutritional proteins.

In the colon the contents for most of the amino acids were decreased to very low levels (< 2 mmol kg^{-1} dry weight) without preference of individual amino acids (Fig. 7B), only for L-Ala higher levels were retained. The data are consistent with the notion that the amino acids are rapidly resorbed by the host, degraded by bacterial fermentation or used for assimilation by the bacteria (Bröer *et al*., 2018; Diether and Willing, 2019).

The complex nutrient composition of the mouse intestine could be responsible for multiple regulation of gene expression in the intestine, including transcriptional regulation of the genes of C4-DC metabolism by FNR and DcuS-DcuR in response to electron acceptors, anaerobiosis and C4-DCs. The remarkable increase of expression of the genes for C4-DC metabolism in the intestine (Fig. 6B) indicates regulation by additional factors. The global transcriptional regulator Lrp responds to L-leucine, L-methionine, L-alanine and other amino acids and controls the expression of a large number of genes in *E. coli* (Hart and Blumenthal, 2011). Lrp is thought to function by interaction with other regulators (Kroner *et al*., 2019). The involvement of Lrp was tested with bacteria carrying the *dcuBp-lacZ* reporter gene fusion (Fig. 8). The bacteria were grown anaerobically in a mineral medium that was supplemented with LB broth. Inactivation of *lrp* decreased expression of *dcuBp-lacZ*, especially fumarate stimulation was affected. The effect of Lrp on *frdAp-lacZ* expression was significant but lower than for *dcuBp-lacZ*. On the other hand, anaerobic expression of *dcuCp-lacZ* was not decreased in the *lrp* mutant. Considering transcriptional regulation of *dcuB*, *frdA and dcuC*, each gene requires anaerobic conditions and FNR-activation for

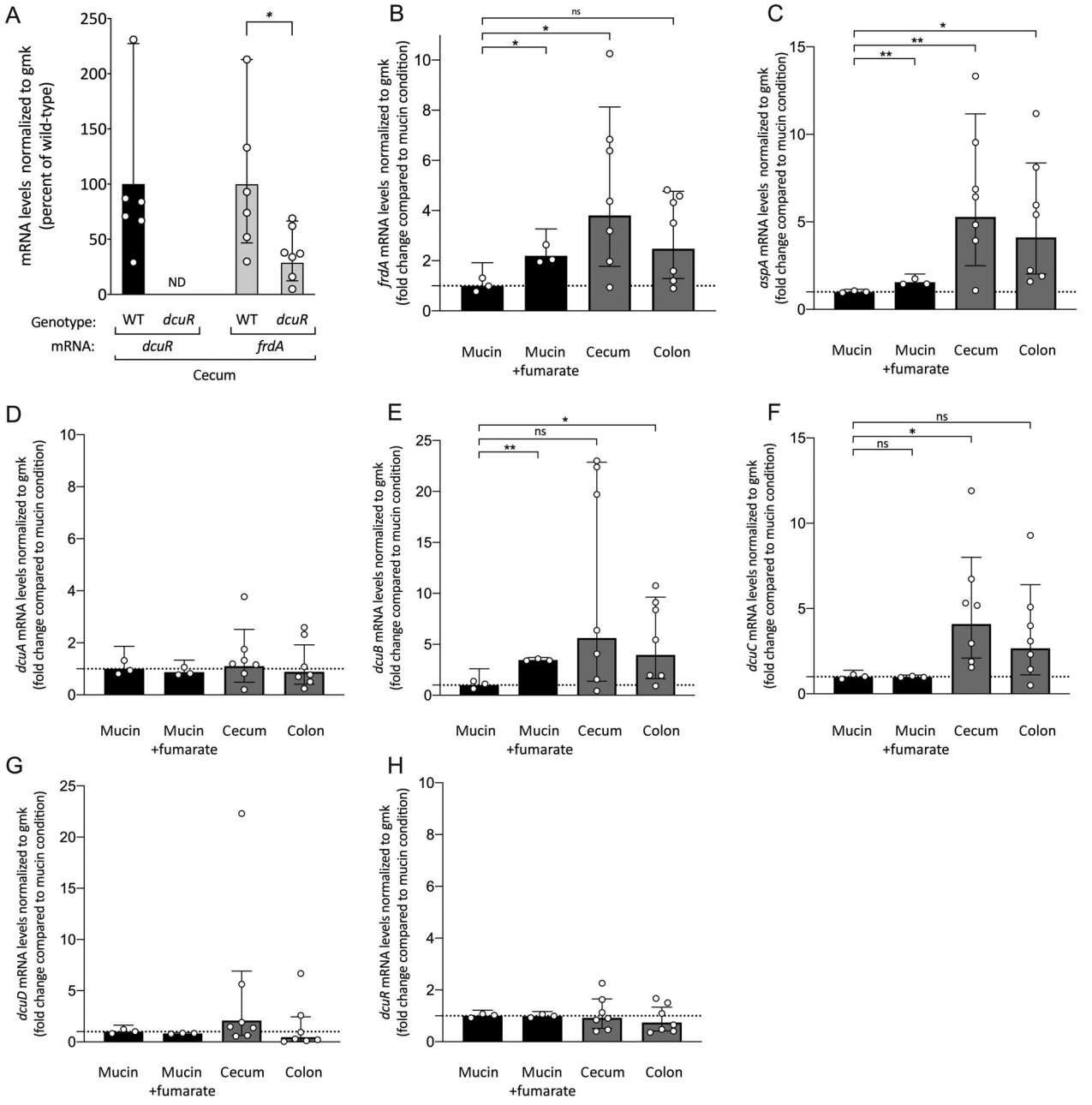

Fig 6. Comparison of mRNA levels of C4-DC metabolism genes in the murine cecum and in mucin broth.
A. Groups of BALB/C mice, pre-treated with 20 mg of streptomycin 1 day prior, were intragastrically inoculated with the K-12 wildtype strain (BW25113) or a *dcuR* mutant (JW4085). Two days later, RNA was extracted from the cecal content and the mRNA levels of *dcuR* and *frdA* were determined and normalized to the housekeeping gene *gmk* determined by RT-qPCR.
B–H. NCE media containing porcine mucin (0.05% wt./vol.), supplemented with fumarate (20 mM) as indicated, was inoculated with the K-12 wildtype strain (BW25113), anaerobically incubated for 3 h, and RNA extracted. The mRNA levels of *frdA* (B), *aspA* (C), *dcuA* (D), *dcuB* (E), *dcuC* (F), *dcuD* (G) and *dcuR* (H) in samples obtained from mucin broth (black bars) or the murine cecum and colon (grey bars) were quantified by RT-qPCR. Values are normalized by the housekeeping gene *gmk*. Bars represent the geometric mean and error bars represent the 95% confidence interval. Each dot represents data from one biological replicate or one animal. ND, none detected. $^{*}P < 0.05$; $^{***}P < 0.001$; ns, not statistically significant.

expression. They differ, however, in their need for DcuS-DcuR which is high for *dcuB*, intermediate for *frdA* and absent for *dcuC* (Zientz *et al*., 1996; Zientz *et al*., 1998; Golby *et al*., 1999; Zientz *et al*., 1999). Thus, there appears to be a link between the Lrp effect and DcuS-DcuR regulated gene expression.

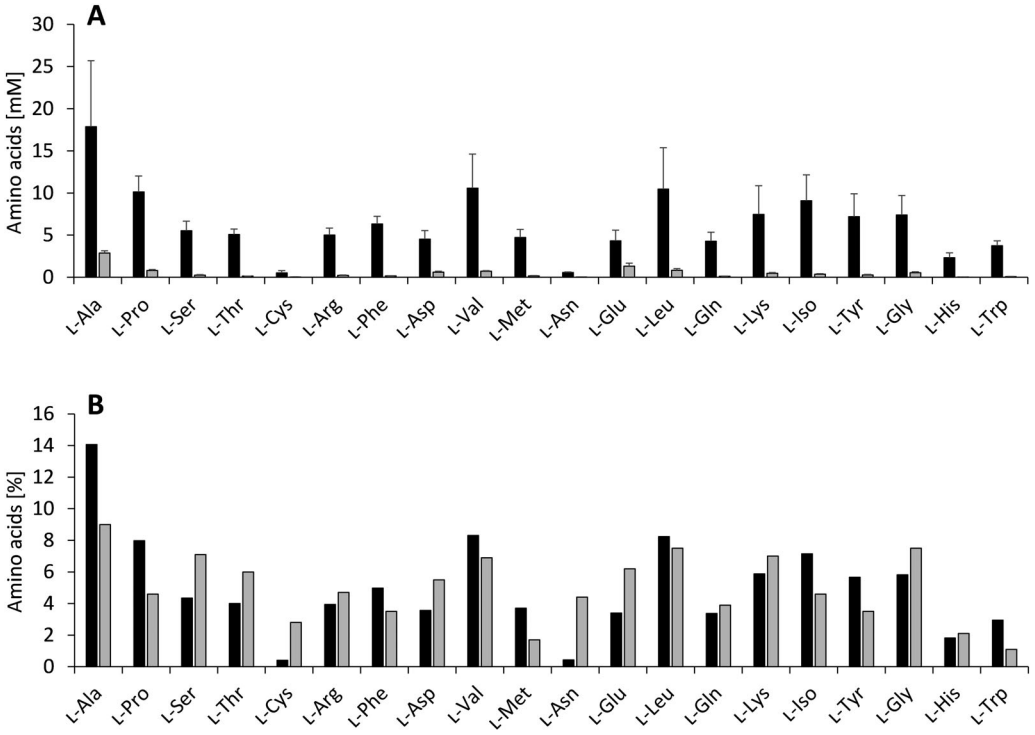

Fig 7. Aminogram of the proximal small intestine and of the colon.
A. Aminogram of the proximal small intestine (black) and the colon (grey). The amino acids of the respective intestinal part were analysed by mass spectrometry while absolute quantities were approximated with a small set of reference substrates.
B. Comparison between the distribution of amino acids in the proximal small intestine (black bars) and the distribution of amino acids in human proteins (grey bars) (Bruice, 2004). Each amino acid is shown as the percentage of all 20 amino acids from the respective group.

Discussion

L-Aspartate as a preferred substrate for fumarate respiration in the murine intestine

The TCA cycle is transformed under anaerobic conditions to a non-cyclic form (Guest, 1992). The reductive branch of the cycle then produces fumarate, which is reduced to succinate by fumarate reductase. Approx. 0.1 mol succinate per mol glucose is formed and excreted in this way. Fumarate is not significant as an external electron acceptor due to the very low levels of fumarate in the environment. In environments with decomposed fruits and plants, L-malate is available in significant levels and can be used for fumarate respiration. The transporter DcuB and fumarase FumB that are produced anaerobically, are highly efficient for the uptake of L-malate and its conversion to fumarate (Woods and Guest, 1987; Guest, 1992; Engel *et al*., 1994; Six *et al*., 1994). L-Aspartate represents an alternative substrate for growth by fumarate respiration (Six *et al*., 1994), which requires the function of DcuA for the uptake (Six *et al*., 1994; Strecker *et al*., 2018) and AspA for the conversion of L-aspartate to fumarate (Woods and Guest, 1987; Woods *et al*., 1988; Schubert *et al*., 2020). The role of L-aspartate as a substrate for fumarate respiration has received little attention despite its use for fumarate respiration in the bovine intestine (Bertin *et al*., 2018). The data shown here demonstrate, however, that L-aspartate is the major substrate for fumarate respiration in the mouse intestine, and both the capacity for L-aspartate metabolism and for fumarate respiration are important for the fitness of *E. coli* in the intestine.

Notably, Enterobacteriaceae family members other than *E. coli* depend on C4-DC utilization for host colonization as well. *S*. Typhimurium mutants lacking DcuR or DcuS were less efficient in colonizing the murine gut lumen than the wild-type strain in mice with a low-complexity microbiota (Nguyen *et al*., 2020). Furthermore, to establish intestinal colonization in mice, *Salmonella* Typhimurium utilizes hydrogen as the electron donor for fumarate respiration (Maier *et al*., 2013). In this mouse infection model, L-aspartate and L-malate are imported into *S*. Typhimurium and converted to fumarate (Nguyen *et al*., 2020). *Salmonella* Typhimurium is also able to use succinate accumulation in macrophages and mice as a cue for induction of the type III secretion system and survival (Rosenberg *et al*., 2021). The response depends on the presence of DcuB, representing another example of the significance of C4-DCs and their metabolism for virulence. Moreover, fumarate reductase is

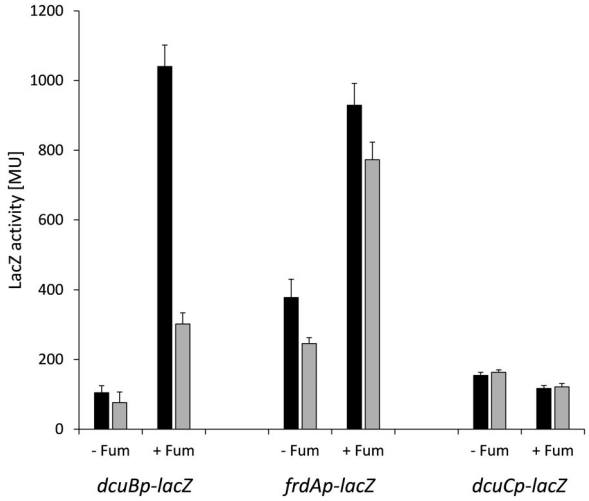

Fig 8. Effect of Lrp on the expression of genes of anaerobic C4-DC metabolism (*dcuB*, *frdA*, *dcuC*). The β-galactosidase activity of *dcuBp-lacZ*, *frdAp-lacZ* and *dcuCp-lacZ* fusions (chromosomal insertions) was measured in strains IMW237, MC4100λJ100 and IMW240 and the corresponding *lrp* inactivation strains. The bacteria were cultivated anaerobically at 37°C in minimal M9 medium, supplemented with LB broth (5 ml L^{-1}), plus glycerol (50 mM) with either DMSO or fumarate (each 20 mM). The control activities are shown in black bars and the reporter gene expression in the lrp mutant as grey bars. The β-galactosidase assay was performed in the exponential growth phase (OD_{570} = 0.5) in triplicate for three independent replicates each.

essential for colonization of the mouse stomach by the ε-proteobacterium *Helicobacter pylori* (Ge *et al*., 2000), extending the significance of anaerobic C4-DC metabolism for virulence to bacteria outside the γ-proteobacteria.

L-Aspartate as a high-quality substrate for nitrogen assimilation under anaerobic conditions

L-Aspartate has been identified recently as a high-quality nitrogen source for *E. coli* which is able to satisfy the complete nitrogen requirement for growth including amino acids, nucleotides and amino sugars (Strecker *et al*., 2018; Schubert *et al*., 2020). Similarly, for the bovine small intestine, L-aspartate was shown to be used to synthesize uridyl monophosphate UMP in addition to supporting fumarate respiration (Bertin *et al*., 2018). AspA which releases the ammonia for assimilation in *E. coli* is integrated into the GlnB (or PII) system for nitrogen assimilation regulation (Schubert *et al*., 2020). The *aspA* and *dcuA* genes that code for the specific proteins of the route before it leads into the general L-glutamine synthetase pathway, are expressed constitutively in the murine intestine, suggesting their role as housekeeping genes, and they support colonization of the intestine by *E. coli*. Therefore, there is strong evidence that L-aspartate is used in the intestine as a nitrogen source, and accordingly, *E. coli* growing in defined cultures was able to grow with L-aspartate as the nitrogen source under anaerobic conditions.

Gene regulation of C4-DC utilization in the intestine

Fumarate respiration is driven by C4-DCs from the environment. This process requires transcriptional stimulation by DcuS-DcuR (Zientz *et al*., 1998; Golby *et al*., 1999; Unden *et al*., 2016). In the murine small intestine, L-aspartate exceeds the levels of L-malate by up to 23.2-fold. The levels of L-aspartate are able to induce expression of the fumarate respiratory genes strongly compared with low-level induction by L-malate. In the colon, the stimulation by the C4-DCs and DcuS-DcuR is lost mostly due to the low C4-DC levels. Fumarate respiration, however, can be maintained at intermediate levels by the use of the constitutive DcuA and the basal synthesis of FrdABCD which is decreased to approx. 40.7% of the maximum in the absence of C4-DCs (Fig. 8). Curiously, we found that the mRNA levels of *frdA* and *dcuB* of fumarate respiration were increased during growth in the intestine compared with laboratory conditions that mimic the intestinal tract. Subsequent experimentation implicated the global transcriptional regulator Lrp, responding to L-leucine, L-methionine, L-alanine, and other amino acids is responsible for this type of regulation. Lrp controls the expression of a large number of genes and is thought to function by interaction with other regulators (Hart and Blumenthal, 2011; Kroner *et al*., 2019). The data suggest that DcuB is a target of Lrp as well. Further investigation is required to elucidate the molecular details of this regulation.

Catabolism of amino acids by Enterobacteriaceae in the mammalian gut is context-dependent. In a mouse model of inflammation (DSS colitis), invasive *E. coli* and *Citrobacter rodentium* depend on the uptake of L-serine (Kitamoto *et al*., 2020). Removing L-serine from the diet decreased colonization of the gut lumen by these organisms. Curiously, depleting L-aspartate from the diet had no effect on *E. coli* burden (Kitamoto *et al*., 2020). We found that DcuS-DcuR was dispensable for *E. coli* fitness in the DSS colitis model, that is inflammatory conditions, when terminal electron acceptors such as nitrate are generated as byproducts of the host reactive nitrogen species and reactive oxygen species metabolism (Winter *et al*., 2013). Nitrate which represses fumarate respiration and transcription of *frdABCD* (Jones and Gunsalus, 1985) can be envisaged to have a negative effect on C4-DC utilization (Hughes *et al*., 2017). Under homeostatic conditions, C4-DC utilization and fumarate respiration, which is driven essentially by L-aspartate availability, contribute to gut colonization as shown by the present study and earlier (Jones *et al*., 2011). Oxygen tension in the mid small intestine and mid colon of mice

amounts to approx. 5.9 Torr or 7.8 mbar (He *et al*., 1999) which allows near half-maximal expression of FNR-regulated fumarate and nitrate respiration (Becker *et al*., 1996; Tseng *et al*., 1996) and maximal expression of the microaerobic oxidase encoded by *cydAB* (Tseng *et al*., 1996). Fumarate respiration and the capacity for aerobic respiration are required for efficient colonization of the mouse colon (Jones *et al*., 2007; Jones *et al*., 2011, this work). Given the data from the physiological, expression and colonization studies a picture emerges that microaerobic and fumarate respiration coexist and function in parallel under the physiological conditions of the homeostatic mouse intestine. Nitrate, which is found generally in low levels in homeostatic mice, can be used at the same time.

Experimental procedures

Bacterial strains and growth conditions. The *E. coli* K-12 strains and the plasmids are listed in Table 1. All molecular methods, including cloning, phage P1 transduction, construction of *lacZ* promoter fusions, DNA isolation and manipulations were performed according to standard procedures (Jones and Gunsalus, 1987; Sambrook *et al*., 1989; Miller, 1992; Zientz *et al*., 1998; Kleefeld *et al*., 2009). The oligonucleotide primers are shown in Table 1. The MP1 *dcuRS* mutant (MW473) was generated by cloning the upstream and downstream regions of *dcuRS* into pGP706 (Gillis *et al*., 2018). The resulting plasmid pMW56 was introduced into MP1 wild-type strain via conjugation using S17-1λ*pir* as the donor strain. Exconjugants were subjected to sucrose selection (8 g L^{-1} nutrient broth, 5% wt./vol. sucrose, 15 g L^{-1} agar) and the double cross-over event confirmed by PCR. Bacteria were grown anaerobically at 37°C in minimal medium M9 (Miller, 1992), where NH_4Cl was omitted, unless stated otherwise. Glycerol (50 mM) and L-aspartate or NH_4Cl (each 20 mM) were added as the carbon and nitrogen sources respectively. Growth studies were performed in Müller–Krempel bottles that were degassed and infused with N_2 (1.2 atm; purity 99.999%). The substrates were supplemented as stated for the individual experiments. Growth was measured as the optical density at 578 nm (OD_{578}).

β-Galactosidase assay. Expression of *dcuB*, *frdA* and *dcuC* was studied in transcriptional promotor-*lacZ* fusions (*dcuBp-lacZ*, *frdAp-lacZ*, *dcuCp-lacZ*), where *p* stands for promotor. The promotor fragment included the intergenic region between the first three codons of *dcuB*, *frdA*, or *dcuC* and the start of the upstream gene *dcuR*, *epmA*, *or pagP* respectively. Bacteria carrying chromosomal *dcuBp-lacZ*, *frdAp-lacZ* and *dcuCp-lacZ* reporter gene fusion were incubated anaerobically in 96-deep-well plates at 37°C overnight in eM9 medium (Kim *et al*., 2007), glycerol (50 mM), varying maleic acid concentrations and 5 g L^{-1} lysogeny broth (LB). Expression of *dcuBp-lacZ*, *frdAp-lacZ* and *dcuCp-lacZ* was measured in terms of the β-galactosidase activity expressed in Miller-Units (MU) (Miller, 1992; Monzel *et al*., 2013). Activities are the mean of at least two independent experiments and four replicates each.

Intestinal contents of mice and quantification of C4-DC by LC–MS/MS. C57BL/6J mice were maintained on 12 h/12 h light (200 lx)/dark cycle, with Atromin breeding diet for rats and mice (Atromin, Germany) and water *ad libitum*. Association for Research in Vision and Ophthalmology statements and institutional guidelines for animal care were followed. Nine-week-old mice (five male and five female) were euthanized by CO_2 followed by cervical dislocation. The gut was removed from the abdominal cavity. Subsequently, the intestinal lumen contents in the distal part of the jejunum (Fig. 1), the Ileum as well as proximal and distal colon were collected. The contents of each intestinal section were transferred to an Eppendorf reaction vessel containing 500 µl TBS buffer and weighed. The samples were then vortexed for 2 min to break up the pellet. The solids and cells were centrifuged (Eppendorf, benchtop centrifuge; 6000 rpm; 5 min). The supernatant was aliquoted and deep-frozen at −80°C until use. The relative C4-DC contents were determined by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). An Agilent Poroshell 120 PFP (2.1 × 100 mm, 2.7 µm particle size) column was used for liquid chromatography with ammonium formate (10 mM) and formic acid (0.1% vol./vol.) in water as mobile phase. The flow rate was 0.15 ml min^{-1} for 5 min and no gradient was applied. The injection volume was 3 µl. The MS/MS parameters were described previously (Guder *et al*., 2017). The absolute C4-DC contents were quantified enzymatically for L-aspartate and L-malate (Bergmeyer *et al*., 1983). The schematic in Fig. 1B was created using BioRender (BioRender.com).

Assessment of fitness in the murine intestinal tract. C57BL/6 and BALB/c mice, reared under specific pathogen-free conditions at UT Southwestern, were on a 12 h light/dark cycle and had access to food and water *ad libitum*. One group of mice received 20 mg streptomycin dissolved in sterile water by gavage, the other group received sterile water. After 24 h, animals were intragastrically inoculated with an equal ratio of the indicated *E. coli* strains. After 3 days, samples of the small intestine, cecum and colon content were obtained. After weighing, serial dilutions were spread on selective LB agar plates to quantify colonization with each strain. In some experiments, the competitive index was calculated

Table 1. Strains of *E. coli*, plasmids and oligonucleotides used in this study.

Strain	Genotype	References
BW25113	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514*	Baba *et al*. (2006)
JW5735	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*dcuA764*::KanR	Baba *et al*. (2006)
JW4099	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*aspA765*::KanR	Baba *et al*. (2006)
JW4084	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*dcuB749*:: KanR	Baba *et al*. (2006)
JW4085	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*dcuR750*:: KanR	Baba *et al*. (2006)
JW4115	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*frdA781*::KanR	Baba *et al*. (2006)
JW0872	F⁻ Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ⁻, *rph-1* Δ*(rhaD-rhaB)568*, *hsdR514* Δ*lrp-787*::KanR	Baba *et al*. (2006)
MP1	*E. coli* murine isolate	Lasaro *et al*. (2014)
MW473	MP1 Δ*dcuRS*	This work
AN387	*E. coli* K-12 wild-type strain	Wallace and Young (1977)
JRG2814	AN387 *dcuB*::KanR *dcuA*::SpcR	Six *et al*. (1994)
MC4100	F-*araD139 D(argF-lac)U169 rpsL150 DlacZ relA1 flbB530 deoC1 ptsF25 rbsR*	Silhavy *et al*. (1984)
IMW237	MC4100 *dcuBp-lacZ*, AmpR	Zientz *et al*. (1998)
IMW683	IMW237 but *lrp*::KanR, AmpR	This work
MC4100λJ100	MC4100 *frdAp-lacZ*, AmpR	This work
IMW682	MC4100λJ100 but *lrp*::KanR, AmpR	This work
IMW240	MC4100, *dcuCp-lacZ*	Zientz *et al*. (1999)
IMW691	IMW240 but *lrp*::*kan*	This work
DH5α λ*pir*	F⁻ endA1 hsdR17 ($r^- m^+$) supE44 thi-1 *recA1 gyrA relA1* Δ(*lacZYA-argF)U189 Φ80lacZ*ΔM15 λ*pir*	Kolter *et al*. (1978)
S17-1 λ*pir*	*zxx*::*RP4 2*-(TetR::Mu) (KanR::Tn7) λ*pir recA1 thi pro hsdR* ($r^- m^+$)	Simon *et al*. (1983)
Plasmid	**Relevant Properties**	**References**
pWSK29	*ori*(pSC101) AmpR	Wang and Kushner (1991)
pWSK129	*ori*(pSC101) KanR	Wang and Kushner (1991)
pGP706	*ori*(R6K) *mobRP4 sacB* KanR	Gillis *et al*. (2018)
Oligonucleotides for mutagenesis	**Sequence (5′-3′)**	**References**
dcuRS (MP1)	ctagaggtaccgcatgCTGAA GGTGGAAGACGAAG ggaagctgCGGATCGCTGGTTATCTG gcgatccgCAGCTTCCTTG TGTGACAAATTTC agctcgatatcgcatgcATCG CTAAATTCTCTCCGG	This study
Oligonucleotides for real time PCR	**Sequence (5′-3′)**	**References**
dcuA	ACCAGGAAGGACATCACCAG GGCATCAGCTACCTCCATCT	This study
dcuB	GCCGAAGGATGGACGCAGGT GATACCGCGACGCTGCTGGA	This study
dcuC	GGAGCAGTGGTGGTGATTTC GCTTCCGAAATGTCGCTGAT	This study
dcuD	CCTGATGCTCGGCTCGCTGT CGCCAATACCGCCCAGACCA	This study
dcuR	GCAGGACAGGCAGTAAATCG CAGGCTTTCAATGCTGTGGA	This study
aspA	AAACTGCGATACGGAAACCG GGTCTGGAACTGATGGGTCA	This study
frdA	GTTGAACACGCTGGAAGTGT CCTGGCTATGGAAGAAGGCT	This study
gmk	CGCAAGCTGTTGCAGAAATG GGCGCGAATAATGGTCTTCA	Chanin *et al*. (2020)

by dividing the number of wild-type bacteria by the number of mutant bacteria in each sample, corrected by the ratio of wild-type bacteria to mutant bacteria in the inoculum. To induce colitis, dextran sulfate sodium (DSS) was added to the drinking water of mice at a concentration of 3%. After 4 days, animals were intragastrically inoculated with the indicated *E. coli* strains. Five days after inoculation, the abundance of the experimentally introduced *E. coli* strains was determined as described above. These mouse experiments were reviewed and approved by the Institute of Animal Care and Use Committee at UT Southwestern.

Transcription of C4-DC genes in vitro *and* in vivo. Type II hog mucin was sterilized in 70% ethanol and the solvent removed under vacuum. No-carbon E medium (3.9 g L^{-1} KH_2PO_4, 5.0 g L^{-1} anhydrous K_2HPO_4 and 3.5 g L^{-1} $NaNH_4HPO_4 \cdot 4\ H_2O$) was supplemented with mucin (0.05% wt./vol.) and fumarate (20 mM), as indicated. Five milliliters of media was inoculated with 0.1 ml of the *E. coli* overnight culture and incubated in an anaerobic chamber (5% hydrogen, 5% CO_2, 90% nitrogen) for 3 h. RNA was extracted from 2 ml of culture using the Aurum Total RNA extraction kit (Bio-Rad) according to the instructions of the manufacturer. Groups of BALB/c mice were pre-treated with streptomycin and colonized with the *E. coli* K-12 wild-type strain BW25113 as described above. After 2 days, the entire cecal content was collected and RNA extracted using TRI reagent (Molecular Research Center). One microgram of RNA, obtained from cultured bacteria or mouse samples, was reverse transcribed using commercially available components (Taqman; Life Technologies). Real-time PCR was performed using SYBR Green mix (Life Technologies), 250 nM of the primers listed in Tables 1 and 0.02 ml cDNA in a reaction volume of 0.12 ml. Raw data were acquired and processed using a QuantStudio 6 Flex instrument (Life Technologies). Data were further analysed by the comparative C_T method using *gmk* as the housekeeping gene. This mouse experiment was reviewed and approved by the Institute of Animal Care and Use Committee at UT Southwestern.

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