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Summary
Rodent-derived strains of *Lactobacillus reuteri* densely colonize the forestomach of mice and possess several genes whose predicted functions constitute adaptations towards an acidic environment. The objective of this study was to systematically determine which genes of *L. reuteri* 100-23 contribute to tolerance towards host gastric acid secretion. Genes predicted to be involved in acid resistance were inactivated, and their contribution to survival under acidic conditions was confirmed in model gastric juice. Fitness of five mutants that showed impaired *in vitro* acid resistance were then compared through competition experiments in ex-germ-free mice that were either treated with omeprazole, a proton-pump inhibitor that suppresses acid secretion in the stomach, or left untreated. This analysis revealed that the urease cluster was the predominant factor in mediating resistance to gastric acid production. Population levels of the mutant, which were substantially decreased in untreated mice, were almost completely restored through omeprazole, demonstrating that urease production in *L. reuteri* is mainly devoted to overcome gastric acid. The findings provide novel information on the mechanisms by which *L. reuteri* colonizes its gastric niche and demonstrate that *in silico* gene predictions and *in vitro* tests have limitations for predicting the ecological functions of colonization factors in bacterial symbionts.

Introduction

A complex and diverse collection of microorganisms colonizes the gastrointestinal (GI) tract of mammals, affecting the health and immune status of the host. Among other functions, these microbial communities enhance energy absorption from ingested food, contribute to the development of their host's immune system and provide colonization resistance against pathogens (Sekirov *et al*., 2010). As a result of co-evolution, the bacteria that reside in the mammalian gut have developed a high degree of ecological fitness and specialization (Oh *et al*., 2010; Frese *et al*., 2011; O'Callaghan and O'Toole, 2013). Given the importance of the gut microbiota to the health of its host, there is currently much interest in formulating strategies that modulate its composition. However, re-modelling this complex ecosystem requires an understanding of the mechanisms by which specific gut microbes colonize the GI tract and the factors that distinguish resident autochthonous members of the microbiota from allochthonous ones (Walter, 2008).

Among the bacteria that are autochthonous to several mammalian species is *Lactobacillus reuteri* (Walter, 2008). *Lactobacillus reuteri* forms high populations in the rodent stomach that are maintained throughout the life of the animal. Colonization is achieved, in part, by the ability of the organism to adhere to the surface of the nonsecretory epithelium present in the forestomach, resulting in formation of a biofilm-like structure (Walter *et al*., 2007; Frese *et al*., 2013). The ability of *L. reuteri* strains to form these biofilms is strictly dependent on their host origin, with only rodent isolates being capable of forming biofilms (Frese *et al*., 2013). This translates to a higher ecological fitness of rodent strains when colonizing the mouse GI tract (Frese *et al*., 2011). *Lactobacillus reuteri* is therefore an example of a bacterium that maintains a tight, host-specific relationship with its mammalian host (Oh *et al*., 2010), and hence serves as a model

to study ecologically important traits that facilitate host–microbe symbiosis in mammals at the molecular level (Tannock *et al*., 2005; Frese *et al*., 2011).

A combination of comparative genomic and transcriptomic analyses has been used to identify genes that were overexpressed during gut colonization and contributed to host specificity and biofilm formation in rodent strains of *L. reuteri* (Frese *et al*., 2011; 2013; Schwab *et al*., 2014; Wilson *et al*., 2014). Several of these genes (Table 1) are predicted to be involved in acid resistance, reflecting the acidic pH in the gastric niche, which varies depending on food loading and emptying (McConnell *et al*., 2008) from pH 4 to 5.7 in the lumen (Ward and Coates, 1987), and from pH 3.5 to 4 in the forestomach (Ward and Coates, 1987; Gärtner, 2001). In particular, the presence of the urease gene cluster is mostly specific to rodent strains and its expression was induced during colonization of the mouse gut, without contributing to biofilm formation (Frese *et al*., 2011; 2013). Wilson and colleagues (2014) confirmed its induction *in vivo* and showed that the cluster contributed to ecological performance in *Lactobacillus*-free mice. In addition, genes encoding glutamate decarboxylase and glutaminase were also found to be overexpressed during stomach colonization (Schwab *et al*., 2014; Wilson *et al*., 2014). The *dlt* operon, which contributes to acid resistance through the incorporation of D-alanine esters into cell wall-associated teichoic acids, is essential for *L. reuteri* colonization of the GI tract (Walter *et al*., 2007). Several other acid resistance mechanisms (glutamate decarboxylase, glutaminase and arginine deiminase) support growth of *L. reuteri* during sour dough fermentation (Su *et al*., 2011; Teixeira *et al*., 2014).

Although it is established that gastric acid constitutes a potent barrier to bacterial pathogens (Tennant *et al*., 2008), little is known about how lactobacilli autochthonous to the stomach overcome this environmental filter. Of the genes predicted to be involved in acid resistance, only the urease cluster and the *dlt* operon have been studied in colonization experiments in mice (Walter *et al*., 2007; Wilson *et al*., 2014). However, the mechanisms by which these factors facilitate colonization have not been determined, and other functions, independent of acidity, could explain the importance of these factors *in vivo*. In addition, it is unknown which of the other acid resistance factors present in *L. reuteri* contribute to acid resistance during stomach colonization. The goal of this study was therefore to determine the ecological significance of acid resistance genes present in *L. reuteri* 100-23 during gut colonization, and to systematically determine to what degree they contribute to tolerance to host gastric acid secretion. To achieve this, we compared the ecological fitness of mutants in ex-germ-free mice treated with omeprazole, a proton-pump inhibitor that raises the pH of the stomach from approximately pH 3–5 (depending on food loading) to approximately pH 6.8–7.0 (Betton *et al*., 1988), with mice that were left untreated.

Table 1. Genes selected for functional characterization.

Gene	Protein	Description	Putative function	Reason for study
*lr*70114	UreC	Urease enzyme, α subunit	Acid resistance	Host specific (Frese *et al*., 2011; Wilson *et al*., 2014), upregulated in biofilms (Frese *et al*., 2013) and *in vivo* (Frese *et al*., 2011; Schwab *et al*., 2014; Wilson *et al*., 2014), and involved in acid resistance and critical for ecological success (Kakimoto *et al*., 1990; Cotter and Hill, 2003; Wilson *et al*., 2014).
*Ir*71325	GadB	Glutamate decarboxylase	Acid resistance	Upregulated in biofilm in *L. reuteri* 100-23 (Wilson *et al*., 2014) and involved in acid resistance (Su *et al*., 2011; Teixeira *et al*., 2014).
*Ir*69360	CgI	Cystathionine γ-lyase	Reactive oxygen resistance (Lo *et al*., 2009)	Upregulated in biofilm in *L. reuteri* 100-23 (Frese *et al*., 2013) and in acid-adapted *B. longum* biotype *longum* (Sánchez *et al*., 2007); pathway produces ammonia (Lo *et al*., 2009), which may have buffering capacity.
*Ir*71377	Adi	Arginine deiminase	Acid resistance	Upregulated in the stomach when compared with the cecum in conventional mice (Schwab *et al*., 2014) and involved in acid resistance in *L. reuteri* 100-23 (Teixeira *et al*., 2014).
*Ir*69622	LisK	Histidine sensor kinase of two-component	Two-component regulatory system regulatory system	Involved in acid response regulation in *Listeria monocytogenes* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003) and *Lactobacillus acidophilus* (Azcarate-Peril *et al*., 2005).
*Ir*69623	LisR	Response regulator of two-component regulatory system	Two-component regulatory system	Involved in acid response regulation in *Listeria monocytogenes* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003) and *Lactobacillus acidophilus* (Azcarate-Peril *et al*., 2005).
*Ir*1649-*Ir*1652	DltA	D-alanylation of lipoteichoic acids	Acid resistance, biofilm formation	Involved in acid resistance in several organisms (Boyd *et al*., 2000; Kristian *et al*., 2005; Lebeer *et al*., 2008) and strongly contributes to ecological performance in *L. reuteri* 100-23 during gut colonization (Walter *et al*., 2007).

Results

Selection of genes of L. reuteri *100-23 predicted to be involved in acid resistance*

Genes selected for this study are listed in Table 1 and included: (i) the gene encoding for the α-subunit of the urease enzyme (*ureC*). This gene cluster, which hydrolyses urea to ammonia, which increases the pH (Cotter and Hill, 2003), is mainly found in rodent strains of *L. reuteri* but is absent in other isolates and is overexpressed during gut colonization (Frese *et al*., 2011; 2013; Wilson *et al*., 2014); (ii) arginine deiminase (Adi), which increases acid resistance by intracellular consumption of protons and ammonia production (Arena *et al*., 2002; Cotter and Hill, 2003; Rollan *et al*., 2003; Vrancken *et al*., 2009; Teixeira *et al*., 2014); (iii) the glutamate decarboxylase (GadB), which is specific to *L. reuteri* strains isolated from rodents (Frese *et al*., 2011) and induced *in vivo* (Wilson *et al*., 2014), and further implicated in acid resistance during growth in sourdoughs (Su *et al*., 2011; Teixeira *et al*., 2014); (iv) the cystathionine γ-lyase (Cgl), which catalyzes several reactions transforming compounds such as L-cystine, L-cystathionine, L-homoserine, or L-cysteine (De Angelis *et al*., 2002; Wang, 2002). L-cysteine is degraded into pyruvate, hydrogen sulfide and ammonia, the latter of which could increase the pH (Wang, 2002); (v) the *dltA* gene, which is involved in D-alanyl esterification of teichoic acids associated with cell walls, is an important colonization factor of *L. reuteri* (Walter *et al*., 2007) associated with *in vitro* acid resistance in *L. reuteri* (Walter *et al*., 2007) and other organisms (Boyd *et al*., 2000; Kristian *et al*., 2005); and (vi) homologues of a two-component regulatory system consisting of a histidine sensor kinase (*lisK*, *lr*69622) and response regulator (*lisR*, *lr*69623), which has been previously shown to be involved in acid response regulation in *Listeria monocytogenes* and *Lactobacillus acidophilus* (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003; Azcarate-Peril *et al*., 2005). At the protein level, the LisR and LisK homologues have 76% and 47% similarity to the proteins in *L. acidophilus*, whereas they show less than 32% similarity to other two-component systems (*cemAKR*, *bfrKRT*, and *lr*70529/*lr*70530) described for *L. reuteri* 100-23 (Frese *et al*., 2011; Su and Gänzle, 2014). As a negative control, a mutant with an inactivated high molecular mass surface protein (*lsp* mutant) was included in our studies, as this adhesin contributes to ecological performance *in vivo* but is not predicted to be involved in acid resistance (Walter *et al*., 2005).

In vitro *characterization of putative acid resistance genes*

Isogenic mutants (Table 2) of each gene were generated by insertional mutagenesis and compared with the wildtype for survival in simulated gastric juice at pH 1.5 and 2 (Fig. 1A–F). Depending on the gene tested, the gastric fluid was supplemented with the substrate necessary for the particular pathway. The analysis revealed that the *ureC*, *adi*, *cgl*, *gadB* and *dlt* mutants were all impaired in their ability to tolerate acidic pH. For the *ureC*, *Cgl*, *gadB* and *dltA* mutants, the inhibitory effect of acidic conditions appeared to be similar at pH 1.5 and 2. Exceptions were the *adi* mutant, which was more impaired at pH 2 (Fig. 1B), and the *dlt* mutant, which was considerably more impaired in its survival at pH 1.5 than at pH 2 (Fig. 1E). The omission of urea, arginine, glutamic acid or cysteine reduced the survival rates of the wild-type to those of the respective mutants (grown with the substrates), showing that acid resistance is facilitated by these substrates. The two-component system with similarity to LisRK did not contribute to acid resistance (Fig. 1F). As expected, the *lsp* mutant was not impaired in survival in gastric juice (data not shown).

Table 2. Strains used in this study.

Strain	Relevant characteristics	Source
Lactobacillus reuteri 100-23	Isolate of rat gastrointestinal tract	Wesney and Tannock (1979)
Lactobacillus reuteri 100-23c	Plasmid-cured derivate of strain 100-23	McConnell and colleagues (1991)
Lactobacillus reuteri 100-23c *ureC* mutant	Urease α-subunit inactivated	Frese and colleagues (2013)
Lactobacillus reuteri 100-23c *lsp* mutant	Large surface protein inactivated	Walter and colleagues (2005)
Lactobacillus reuteri 100-23c *cgl* mutant	Cystathionine γ-lyase inactivated	Frese and colleagues (2013)
Lactobacillus reuteri 100-23c *gadB* mutant	Glutamate decarboxylase inactivated	This study
Lactobacillus reuteri 100-23c *lisR* mutant	Response regulator of two-component regulatory system involved in acid resistance in *Listeria monocytogenes* and *Lactobacillus acidophilus*	This study
Lactobacillus reuteri 100-23c *lisK* mutant	Histidine sensor kinase of two-component regulatory system involved in acid resistance in *Listeria monocytogenes* and *Lactobacillus acidophilus*	This study
Lactobacillus reuteri 100-23 *dlt* mutant	D-alanylation of lipoteichoic acids in the bacterial cell wall inactivated	Walter and colleagues (2007)
Lactobacillus reuteri 100-23c *adi* mutant	Arginine deiminase inactivated	This study
Escherichia coli EC1000	Contains copy of pVW01 *repA* gene	Russell and Klaenhammer (2001)

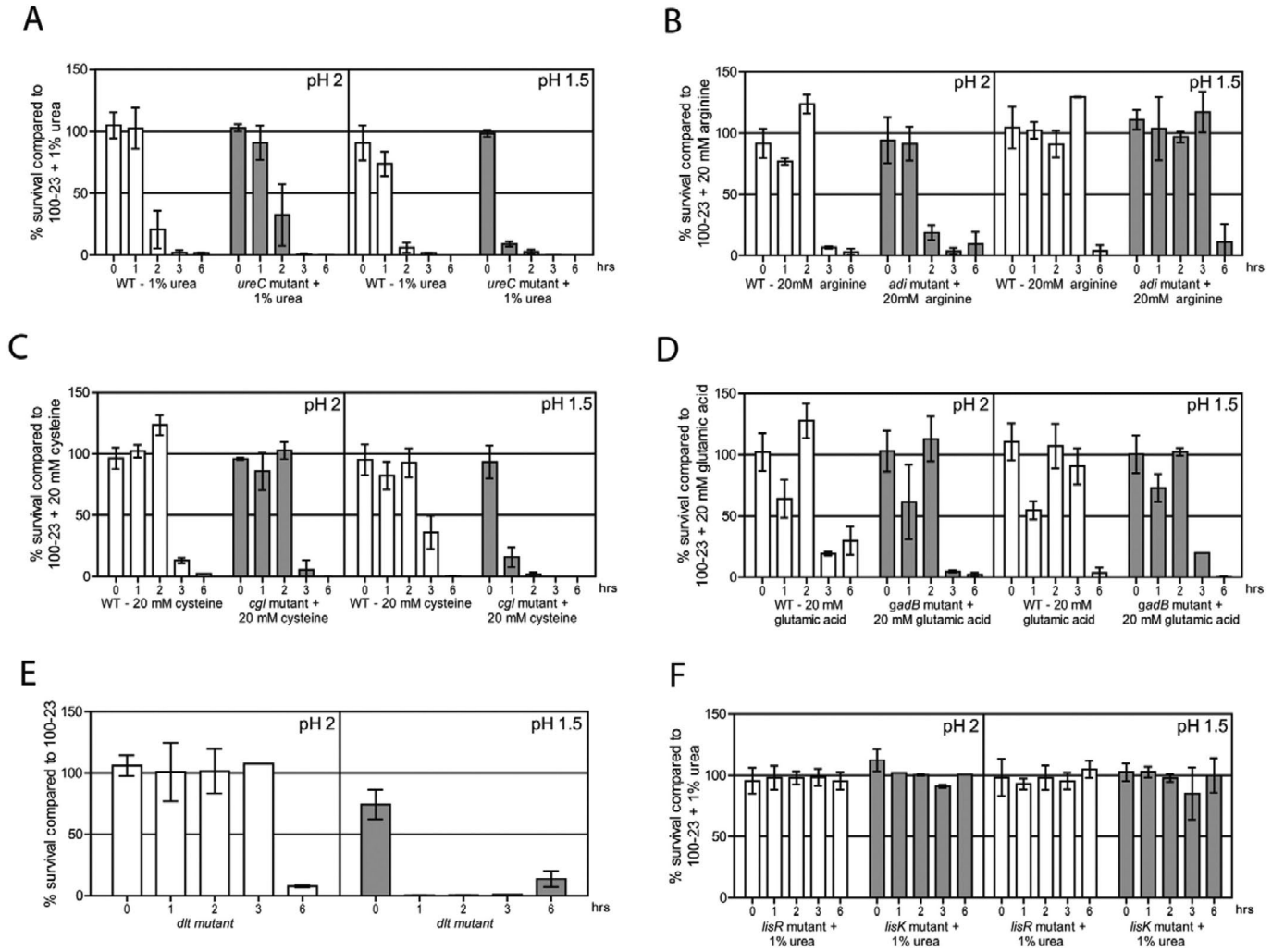

Fig. 1. Determination of the capacity of genes (Table 1) to confer survival under acidic conditions. The viability of strains was determined after incubation in artificial gastric fluid at pH 2 and 1.5 for 6 h at 37°C. Survival of (I) wild-type strains incubated without the substrate of the respective enzyme and (II) mutant strains incubated with the respective substrate are shown relative (%) to that of the wild-type strain incubated with the respective substrate. (A) *ureC*; (B) *adi*; (C) *cgl*; (D) *gadB*; (E) *dltA*; and (F) *lisR* and *lisK*. Because there is no added substrate for the *dltA*, *lisR* and *LisK* genes, only the survival of the mutants was compared with the wild-type. Data are shown as means with standard deviations of triplicate independent experiments (biological replicates).

Importance of acid resistance genes during colonization of the mouse GI tract

The ecological importance of the five genes found to contribute to acid resistance *in vitro* (see above) was subsequently tested via competition experiments of mutant and wild-type strains in germ-free mice that were treated with omeprazole or left untreated. Therefore, for genes that contribute to acid resistance *in vivo*, omeprazole would lead to an increase in competitive fitness of the mutants. Controls received either a sham treatment [containing only the dimethylsulphoxide (DMSO), polyethylene glycol and water used to dissolve the omeprazole] or no treatment (bacteria only). A schematic summary of the experimental design is depicted in Fig. 2A.

The analysis revealed that the inactivation of *ureC* had a large impact on the tolerance of *L. reuteri* 100-23c towards host gastric acid secretion (Fig. 2B). Without the neutralizing effect of omeprazole, the *ureC* mutant represented around 0.1% of the *L. reuteri* population in the gut after 8 days of colonization. Omeprazole treatment restored the population of the *ureC* mutant to 29.8% ± 11 of the total lactobacilli population detected in the forestomach and 50.2% ± 15 in the cecum. The omeprazole solvent, polyethylene glycol, has weak buffering capacity, which likely is responsible for the increase of mutant abundance in mice on the sham treatment (Fig. 2B).

The *adi* mutant was only slightly impaired *in vivo*. When in direct competition with the wild-type strain, the mutant represented 26.9% ± 13 of the total lactobacilli in the forestomach and 35.7% ± 17 in the cecum. No significant differences

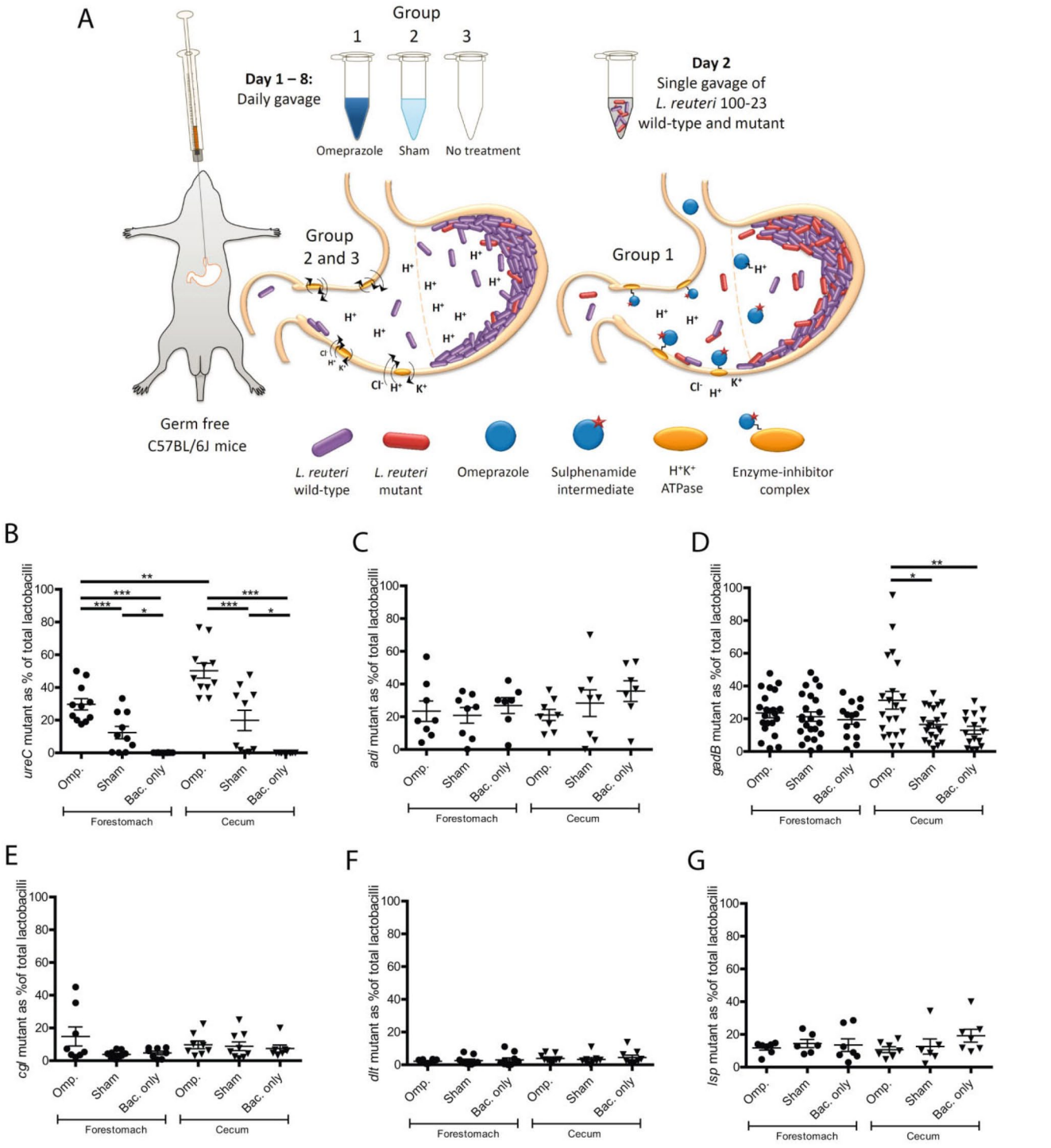

Fig. 2. Mouse competition experiment with mutant and wild-type strains in ex-germ-free C57BL/6J mice treated with omeprazole, sham or no treatment.

A. Conceptual summary of the experimental design for mouse experiments. Mice were divided into three groups. Group 1 mice were treated daily with omeprazole, whereas group 2 mice were gavaged with the polyethylene glycol, water and DMSO sham. Group 3 mice served as the control animals and received no treatment. All treatments were administered for 8 days. On day two, all mice received a single gavage with a 1:1 mixture of wild-type and mutant. The proportions of total lactobacilli composed of each mutant in the forestomach and cecum of mice co-inoculated with wild-type and mutant strains were shown.

B–G. *In vivo* competition experiment between wild-type and *ureC* mutant (B); wild-type and *adi* mutant (C); wild-type and *gadB* mutant (D); wildtype and *cgl* mutant (E); wild-type and *dltA* mutant (F); wild-type and *lsp* mutant (G). Data are shown as means with standard errors of the mean. Significance of $P \leq 0.05$ is denoted by a single asterisk (*), $P \leq 0.01$ as two asterisks (**), and $P \leq 0.001$ by three asterisks (***). Circles and triangles represent the forestomach and cecum, respectively, of a single mouse.

were observed for omeprazole or sham treatment (Fig. 2C). The inactivation of *gadB* also only led to a slight impairment *in vivo* (Fig. 2D), with the mutant comprising 19.5% ± 11 and 13.0% ± 10 of the total lactobacilli population in forestomach and in the cecum, respectively. Omeprazole treatment had no detectable effect in the forestomach (23.5% ± 14), but significantly enhanced survival in the cecum (31.3% ± 25).

The *cgl* mutant showed a high degree of impairment when competing with the wild-type in both the forestomach (4.8% ± 3) and the cecum (7.5% ± 6), demonstrating that this gene is ecologically relevant *in vivo* (Fig. 2E). Similar findings were obtained for the *dlt* mutant, which was highly impaired (Fig. 2F), an observation consistent with previous findings in *Lactobacillus*-free mice (Walter *et al*., 2007). In both mutants, omeprazole did not influence the ecological performance. Thus, it appears that both *cgl* and *dltA* encode for ecologically relevant colonization factors that are not involved in providing resistance to gastric acid secretion.

The *lsp* mutant lacks a putative adhesin that is not involved in acid resistance. We included this mutant to determine unspecific effects of omeprazole on the competiveness of mutant strains in general. As shown previously (Walter *et al*., 2005), the *lsp* mutant was impaired *in vivo*, forming around 10% of the population. However, as expected, no difference between the three treatments was observed (Fig. 2G).

Altogether, these experiments demonstrate that the urease gene cluster is the only factor that mediates resistance against gastric acid secretion in *L. reuteri* 100-23 during stomach colonization, and that no other acid resistance factor was able to compensate for its loss under the given experimental and dietary conditions.

Urease activity is regulated by pH

The *in vivo* competition experiments demonstrated the importance of the urease gene cluster as an acid-related colonization factor for *L. reuteri* 100-23 in the rodent forestomach. To characterize the regulation of this cluster, the wild-type strain was grown in mMRS broth supplemented with 1% urea and growth and urease activity was monitored for 24 h (Fig. 3A). The addition of urea caused a slightly decreased growth rate, but led to a rapid increase in pH after 12 h of incubation.

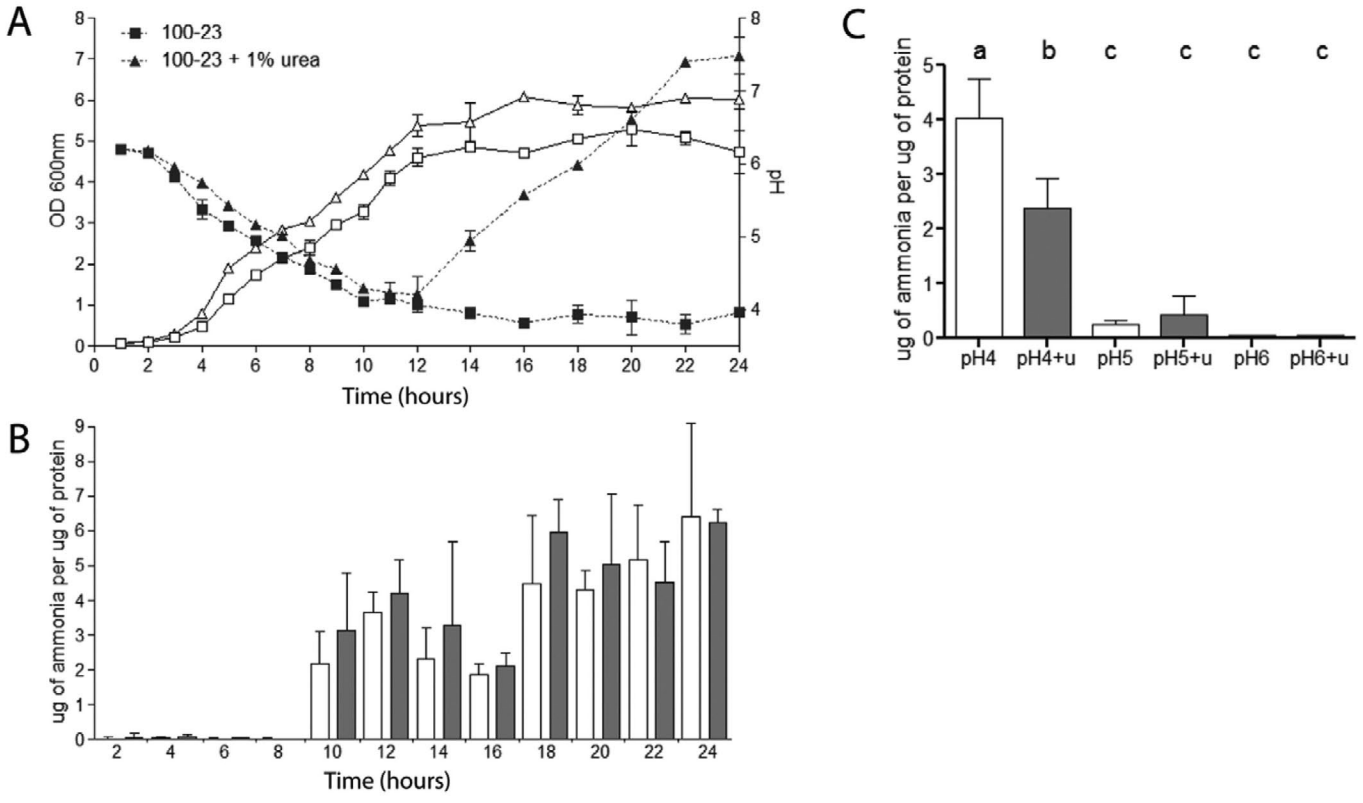

Fig. 3. A. Bacterial growth (OD_{600}) of *L. reuteri* 100-23 (continuous line, empty symbols, left ordinate axis) and the pH of the media (dotted line, full symbols, right ordinate axis) with (triangle symbol) and without urea (square symbol). B. Urease activity over time in cell lysates of wild-type strain 100-23 grown with and without 1% urea in mMRS media, gray and white bars respectively. C. Urease activity in cell lysates of wild-type strain 100-23 in induction experiment. mMRS media adjusted to pH 4, 5 or 6 with and without 1% urea supplementation, gray and white bars respectively. $n = 3$, means and standard deviations are shown. Treatments with different letters (a, b, c) are significantly different from one another ($P \leq 0.05$).

The final pH after 24 h was 7.4 with urea supplementation, compared with pH 3.9 without urea in the media ($P < 0.0001$). This alkalization of the supernatant was not observed for the *ureC* mutant (data not shown). There was no detectable urease activity in the first 8 h. However, urease activity became detectable after 10 h when the pH approached pH 4 (Fig. 3A). Urea supplementation had no significant effect on the urease activity at any time point. These findings suggest that urease activity in *L. reuteri* was not induced by the substrate but by rather acidic conditions.

To confirm that induction of urease activity in *L. reuteri* 100-23 occurs via acidity and not urea, cells were grown for 6 h in mMRS, centrifuged and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6. Cells were incubated for another 2 h before urease activity was measured. Cells after 6 h of growth were used for these experiments as *L. reuteri* did not show any urease activity until 10 h of growth (Fig. 3A), allowing the determination of conditions that induce urease activity. This experiment demonstrated that urease activity was induced at pH 4, unaffected at pH 5 and not detectable at pH 6 ($P < 0.01$). The presence of urea did not enhance urease activity. Transcript analysis revealed that expression of *ureC* was 124 times higher at pH 4 compared with pH 6 independently of the presence of urea. These findings demonstrate that the urease activity in *L. reuteri* is regulated on the transcriptional level.

Two-component systems are commonly used by lactic acid bacteria for environmental sensing and signal transduction and are often involved in the acid stress response (Cotter and Hill, 2003). *Lactobacillus reuteri* 100-23 possess homologues to the LisRK system, which has been implicated in mediating acid resistance in *L. monocytogenes* (Cotter *et al*., 1999) and *L. acidophilus* (Azcarate-Peril *et al*., 2005). To test if the *lisRK* genes are involved in regulating urease activity, we compared culture supernatant pH of the *lisK* and *lisR* mutants during growth in the presence of urea. The *lisR* mutant was also tested in the same pH induction experiment described above. Neither the *lisK* nor the *lisR* mutation had an effect on the buffering capacity during growth in the presence of urea, and urease activity was still induced by low pH in the *lisR* mutant in the pH induction experiment (data not shown). Hence, it was concluded that this two-component regulatory system is not involved in the regulation of the urease gene cluster, which is consistent with the finding that the *lisR* and *lisK* mutants were not impaired in simulated gastric juice (Fig. 1K). Therefore, it is currently unknown how *L. reuteri* senses acidic pH and induces gene expression of the urease cluster.

Discussion

The rodent stomach consists of two parts: forestomach and corpus. The forestomach represents about two thirds of the total stomach volume and is lined by a squamous stratified epithelium. The corpus is lined by a glandular and secretory epithelium covered by a mucus layer (Gärtner, 2001) and harbors the H^+/K^+ proton-pumps responsible for the low pH in the stomach (Fig. 2A). *Lactobacillus reuteri* colonizes the forestomach epithelium, but it is also found throughout the digestive tract, including the cecum, where pH values are closer to neutral. However, the spatial patterns of *L. reuteri* populations throughout the mouse digestive tract suggest that cells in the cecum are likely allochthonous to this site and originated from cells colonizing the stomach (Walter, 2008). This notion was supported by the findings of this study; cecal mutant proportions always mirrored those of the forestomach, independently of gene function (Fig. 2B–G). This agrees with previous findings concerning the *dlt* (Walter *et al*., 2007), *gtfA*, *inu*, (Walter *et al*., 2008), *lsp, msrB* (Walter *et al*., 2005) and *ftf* (Sims *et al*., 2011) mutants. The forestomach of mice is therefore the primary habitat of *L. reuteri*, which makes acid resistance a key factor for successful colonization.

Accordingly, several pathways and factors have been identified and functionally characterized to contribute to acid resistance in *L. reuteri* (Fig. 4). However, our experiments in omeprazole-treated mice identified the urease gene cluster as the predominant factor necessary for *L. reuteri* 100-23 to tolerate host gastric acidic secretion (Figs 2B and 4A). Inactivation of the *ureC* gene resulted in the lowest levels of colonization (around 0.1%) of all mutants tested here. This finding, consistent with observations in *Lactobacillus*-free mice (Wilson *et al*., 2014), demonstrated the paramount ecological importance of the urease cluster. Restoration of mutant proportions to around 30% and to 50% with omeprazole in the forestomach and cecum, respectively, indicated that host acid secretion is the main ecological factor decreasing mutant levels, and that urease production of *L. reuteri* is almost completely devoted towards resistance to host gastric acid production. Furthermore, the percentage of mutant strains was significantly lower in the forestomach in omeprazole treated mice compared with the cecum. One could speculate that *ureC* has a residual function in the forestomach that is unrelated to host acid production. Instead, *ureC* may contribute to resistance against the build-up of acidic metabolic end-products in the biofilm generated through bacterial fermentation. Overall, our findings show that host acid secretion exerts a substantial selective pressure on the *L. reuteri* population, even in the non-secretory forestomach, and that urease production serves as an adaptive phenotype to overcome this pressure.

Although the other four genes (*gadB*, *cgl*, *adi* and *dltA*) evaluated here also contributed to both survival in the *in vitro* gastric model and ecological performance in mice, our findings indicate that they do not contribute to tolerance of host gastric acid secretion during forestomach colonization. Mutants for two of the genes, *gadB* and *adi*, were only marginally impaired, comprising >20% of the population in competition experiments. The *gadB* gene has been identified as the most important mechanism of acid resistance in *Escherichia coli* (Feehily and Karatzas, 2013) and was previously shown to contribute to acid resistance (Teixeira *et al*., 2014) and ecological performance of *L. reuteri* 100-23 during growth in

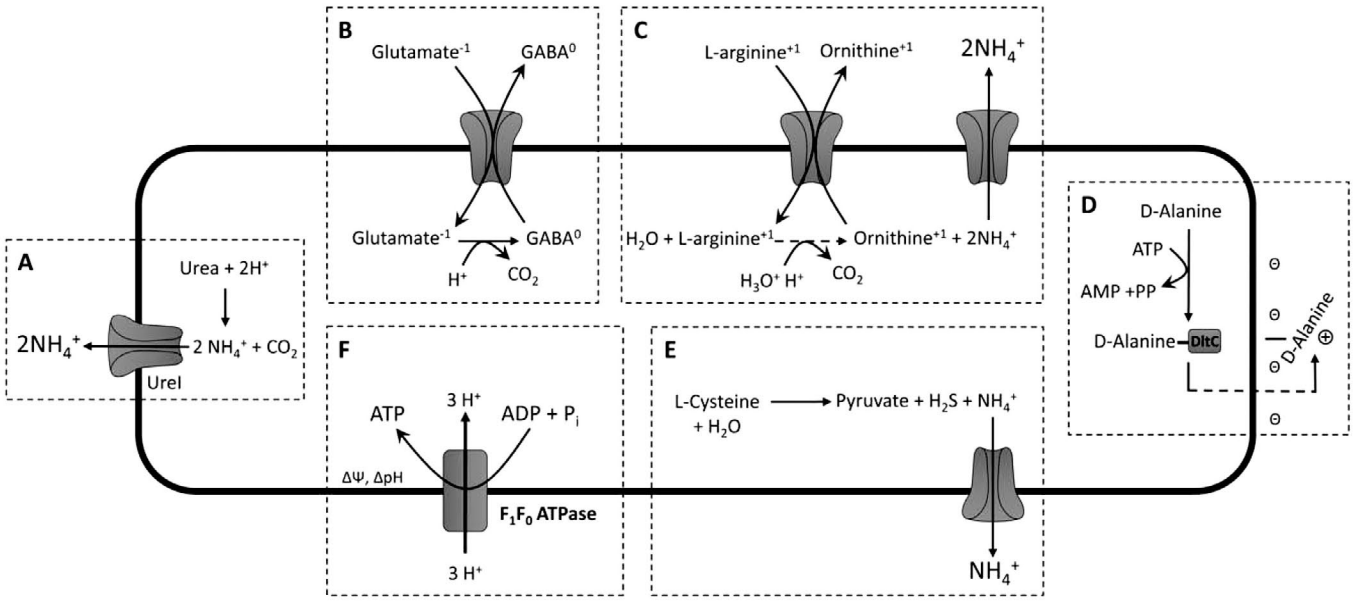

Fig. 4. Overview of metabolic pathways of genes assessed in this study.

A) The urease gene converts urea to ammonia and CO_2. Ammonia is exported from the cytoplasm using the UreI transporter, thus buffering the cell from its surrounding environment.

B) Glutamate is imported into the cell by an antiport system and converted to GABA using the glutamate decarboxylase pathway while generating a ΔΨ and ΔpH. H^+ consumption raises the intracellular pH. Adapted from Su and colleagues (2011) and Price and colleagues (2012).

C) L-arginine is imported using an L-arginine–ornithine antiporter and converted into citrulline and ammonia by the arginine deiminase enzyme. Citrulline is further catalyzed to ornithine and ammonia, while consuming H^+. Ammonia is exported from the cytoplasm potentially using the UreI transporter.

D) D-Alanine is coupled to a DltC carrier protein, exported across the cytoplasmic cell membrane and used for esterification of teichoic acids associated with the cell wall. This esterification results in a positive charge of the cell wall. Adapted and simplified from Peschel and colleagues (1999).

E) Cysteine is converted to ammonia, hydrogen sulfide and pyruvate by the cystathionine γ-lyase. Ammonia is exported from the cytoplasm using the potentially via the UreI transporter, thus buffering the cell from its surrounding environment.

F) F_1-F_0-ATPase-producing ATP using a ΔΨ and ΔpH, which is generated, in part, by the glutamate decarboxylase pathway. Dashed arrows indicate that more than one step is involved in the pathway.

sourdough (Su *et al*., 2011). In our experiments, omeprazole treatment did lead to small but significantly higher levels of the *gadB* mutant in the cecum, suggesting that the gene contributed to acid survival during transit into the cecum but not the forestomach. These findings may be explained by GadB of *L. reuteri* being active primarily at pH 2.5 (Teixeira *et al*., 2014), a feature that may confer a survival benefit when the stomach lumen becomes very acidic. Expression of *gadB* is required for the conversion of glutamate to CO_2 and g-aminobutyric acid (GABA) (Su *et al*., 2011); this function is independent of its role in acid resistance. When glutamate is exchanged with GABA by an antiporter system, a ΔΨ and ΔpH are generated. This proton motive force generated by GadB provides a mechanism for conserving ATP that would otherwise be required to fuel the F_1F_0 ATPase (Su *et al*., 2011) (Fig. 4B and F). The loss of the proton motive force may also explain the impairment of this mutant during transit because less energy may be available for the cells to launch a stress response towards the acidic conditions in the stomach lumen.

The arginine deiminase pathway is widely distributed among bacteria (Senouci-Rezkallah *et al*., 2011), triggered in *L. reuteri* CRL 1098 by low pH (Rollan *et al*., 2003), and over-expressed in the *Lactobacillus* population colonizing the stomach of conventional mice when compared with the cecum (Schwab *et al*., 2014). This pathway consumes intracellular protons and raises the cytoplasmatic pH when converting L-arginine and H_2O to ammonia and citrulline, which is further catalyzed to ornithine, ammonia and CO_2 (Konings, 2002; Teixeira *et al*., 2014) (Fig. 4C). *In vivo*, however, the *adi* mutant was only marginally impaired, and the gene did not confer resistance against host acid secretion.

In contrast to the *gadB* and *adi* mutants, the ecological performance of the *dlt* and *cgl* mutants was substantially impaired *in vivo*. Contrary to the consistent involvement

of the *dltA* gene in acid resistance of *L. reuteri* and other organisms *in vitro* (Boyd *et al*., 2000; Kristian *et al*., 2005; Walter *et al*., 2007; Lebeer *et al*., 2008), host acid production was not the factor that reduced mutant populations in mice. An alternative function of the *dlt* operon is to increase resistance to cationic antimicrobial peptides by generating a positive net charge of the cell surface (Kristian *et al*., 2005; Walter *et al*., 2007). This positive charge leads to a decreased binding of positively charged antimicrobial peptides, e.g. defensins, which may result in increased cell lysis and impaired ecological performance *in vivo* (Walter *et al*., 2007) (Fig. 4D). A recent study showed that a reduction of the negative cell surface charge through Lipid A dephosphorylation mediates resistance to antimicrobial peptides in the Gram-negative *Bacteroides thetaiotaomicron* (Cullen *et al*., 2015). The *dlt* operon could have a similar function in *L. reuteri*.

Although bacterial cystathionine γ-lyases have not been associated with acid resistance, the *cgl* mutant of *L. reuteri* 100-23c was impaired in the *in vitro* acid resistance assays (Fig. 1C). Among other reactions, these enzymes catalyze the transformation of L-cysteine and water to hydrogen sulfide, pyruvate and NH_4^+ (Wang, 2002; Lo *et al*., 2009) (Fig. 4E). Although expression of the *cgl* gene is upregulated in *L. reuteri* 100-23 growing in biofilms *in vitro* (Frese *et al*., 2013) and in acid-adapted *B. longum* subsp. *longum* (Sánchez *et al*., 2007), our mouse experiments did not support a role for *cgl* in overcoming gastric acid secretion. In *L. reuteri* BR11, this pathway was shown to improve oxidative stress defense and is required for thiol production (Lo *et al*., 2009); it could be important during forestomach colonization. This study establishes the *cgl* gene as an important colonization factor of *L. reuteri* 100-23, but further research is needed to elucidate the mechanism by which this gene contributes to gut colonization.

Together with our previous phylogenetic and comparative genomic studies on *L. reuteri* (Oh *et al*., 2010; Frese *et al*., 2011), this work provides novel insight into the ecology and evolution of a vertebrate gut symbiont, and the mechanisms by which a host-specific lifestyle can emerge. Urease is commonly used by bacteria from different phyla to tolerate stomach acidity (e.g. in *Helicobacter pylori*) and in some pathogens (e.g. *Clostridium perfringens* and *Yersinina enterocolitica)*, urease is considered a virulence factor that facilitates survival during gastric transit (Mora and Arioli, 2014). *Lactobacillus reuteri* has acquired the urease cluster, which is extremely rare in the genus *Lactobacillus* (Zheng *et al*., 2015), by horizontal gene transfer (Frese *et al*., 2011). The cluster has then been stably maintained within rodent lineages of the species (Walter *et al*., 2011). The findings presented here now provide an explanation for the conservation of this trait among rodent strains – it constitutes an essential colonization factor that provides a key adaptation to the gastric niche in rodents. During the evolutionary process, it appears that *L. reuteri* has tailored transcriptional regulation of the cluster towards the environmental conditions of the murine stomach. Transcriptional expression of the urease cluster is strictly regulated by pH (Fig. 3), allowing the organism to respond to the variation in gastric pH and only produce urease when the habitat becomes too acidic. Urea, in contrast, is always present as it enters the stomach by diffusion and through the saliva (Burne and Chen, 2000), and it was therefore not required for *L. reuteri* to evolve a mechanism of substrate induction. Substrate availability through the rodent host is also a likely reason why urease formation evolved to become more important than GadB and Adi, as the latters' substrates (glutamate and arginine) must be provided in the diet where supply is not reliable. Urea hydrolysis is therefore a key facet of host adaptation (and potentially even co-evolution) in the *L. reuteri*–rodent symbiosis, and the absence of the phenotype in most non-rodent strains (Walter *et al*., 2011) is likely an important reason for their low ecological performance in the mouse GI tract (Oh *et al*., 2010; Frese *et al*., 2011).

In conclusion, the findings obtained during this study demonstrated that urease production is essential and sufficient for *L. reuteri* 100-23 to cope with host gastric acid secretion. Other genes, such as *adi*, *clg*, *dltA* and *gadB*, and genes encoding for glutaminase [which were overexpressed in acid resistance tests *in vitro* (Teixeira *et al*., 2014) and in the forestomach (Wilson *et al*., 2014) but were not studied here due to the presence of several copies in the genome] might contribute to resistance against acidic bacterial metabolic end-products, or may become more important in a different dietary context. However, in the experiments conducted here, none of these genes was able to complement the loss of *ureC* in mediating resistance to host gastric acid secretion, which appears to exert a major selective pressure. This study provides a better understanding of the phenotypic adaptations of vertebrate gut symbionts that contribute to both a highly successful lifestyle and specialization towards a particular host. Most importantly, it demonstrates that gene annotations and *in vitro* tests have limitations to predict the exact ecological functions of colonization factors of bacterial gut symbionts.

Experimental procedures

Ethics statement

All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska (Project ID 731).

Bacterial strains and media used in the study

All strains of *L. reuteri* and *E. coli* are listed in Table 2. *Lactobacilli* were grown anaerobically at 37°C in de Man, Rogosa and Sharpe (MRS) medium (Difco™; Le point-de-Claix, France) supplemented with 10 g l^{-1} maltose and 5 g l^{-1} fructose (referred to as mMRS). For gene inactivation in *L. reuteri* 100-23c (plasmid-free derivative of strain 100-23), *E. coli* EC1000 was used as a cloning vector and grown aerobically

in Luria–Bertani media (Difco™; Sparks, MD, USA) at 37°C. Erythromycin (200 μg ml^{-1} for *E. coli*, 5 μg ml^{-1} for lactobacilli), kanamycin (40 μg ml^{-1} for *E. coli*) and chloramphenicol (7.5 μg ml^{-1} for lactobacilli) were used for the propagation of recombinant strains.

Determination of genes predicted to be involved in acid resistance of L. reuteri *100-23*

Several different approaches were used to select genes of interest for this study. First, we identified genes that were specific to rodent strains of *L. reuteri* (Frese *et al*., 2011) and predicted to be involved in acid resistance. Second, putative acid resistance genes that were upregulated *in vivo* compared with *in vitro* cultures were identified (Frese *et al*., 2013; Wilson *et al*., 2014). Third, genes coding for metabolic pathways that produce ammonia (Lo *et al*., 2009) and two-component systems involved in acid resistance in other bacteria were also considered (Kallipolitis and Ingmer, 2001; Cotter and Hill, 2003). One additional criterion for the selection of genes was that the gene had to be a single copy gene to generate the knock-out mutants according to the method described by Walter and colleagues (2005).

Derivation of mutants

Genes of interest were inactivated by insertional mutagenesis by site-specific integration of the plasmid pORI28 into the target sites in the *L. reuteri* 100-23c genome (Walter *et al*., 2005). Internal regions of the genes of interest were amplified using the primers in Table S1 for each mutant. Each knock-out mutation was confirmed by polymerase chain reaction (PCR) using primers flanking the target region of each gene. Strains were routinely maintained in mMRS medium containing 5 μg ml^{-1} of erythromycin, unless the mutant was used for the *in vitro* acid survival assay. Growth curves showed no growth impairments in any of the mutants (data not shown).

In vitro *acid survival assay*

To simulate the acidic conditions in the mouse stomach, an artificial gastric fluid developed by Cotter and colleagues (2001) was used. The experiment was performed with wild-type *L. reuteri* 100-23c and all mutants; bacterial survival was monitored over time. To evaluate acid resistance, lactobacilli were grown in mMRS (pH 6.5) for 12–16 h, harvested by centrifugation and washed in PBS. Pre-warmed gastric fluid was adjusted to pH 1.5 and 2 with HCl, and inoculated with approximately 10^8 cells ml^{-1}. Samples were incubated at 37°C and quantified by serial plating after 0, 1, 2, 3 and 6 h. To assess the importance of the *ureC*, *adi*, *gadB* and *LisK/R* genes, assays were performed in gastric fluid with and without 1% urea, 20 mM arginine, 20 mM glutamic acid or 20 mM cysteine respectively. The role of *lsp* and *dlt* genes was assessed without supplementation of the gastric fluid. To gain an insight on the effect of gene inactivation on survival and to allow for a better comparison between experiments, cell numbers of the wild-type strain plus supplement was set to 100%. Based on that value the cell numbers of wild-type without the respective substrate and mutant strains with the substrate was expressed as per cent of those obtained with the wild-type incubated with the substrate. Experiments were done in triplicate of biological replicates.

Determination of genes' role in in vivo *acid resistance*

Germ-free C57BL/6J mice (males and females) were bred and reared in flexible film isolators and maintained under gnotobiotic conditions at the University of Nebraska-Lincoln and were randomly assigned to one of three treatment groups. Mice in group 1 received a daily oral gavage of 400 μmol of omeprazole kg^{-1} (6-methoxy-2-[(4-methoxy-3,5- dimethylpyridin-2-yl)methanesulfinyl]-1*H*-1,3-benzodiazole; Sigma) for 8 days (Tennant *et al*., 2008). Omeprazole was dissolved in 50 μl of a DMSO-polyethylene glycol solution (90% DMSO, 4.5% polyethylene glycol and 5.5% water) and was filter sterilized (Zavros *et al*., 2002). Mice in group 2 were orally gavaged daily with the DMSO–polyethylene glycol vehicle and otherwise treated the same way as group 1 animals. Mice in group 3 did not receive any treatment. On day two, each mouse was inoculated with 10^6 cells in a 1:1 ratio of 100-23c wild-type and a mutant strains in a single oral gavage. The inoculum was also plated on mMRS plates with and without erythromycin (5 μg ml^{-1}) to confirm equal representation of the two strains. Mice had access to food and water *ad libitum*. After 8 days, mice were euthanized and forestomach and cecum contents were serially diluted and plated on mMRS with and without erythromycin (5 μg ml^{-1}) to determine the ratio of the wild-type and mutant strains in the samples. A total of 6–11 mice per each group (omeprazole, sham, control) were used per experiment. The experiment was repeated twice with the *gadB* mutant because the first experiment showed a trend towards a higher survival rate due to omeprazole treatment compared with the sham in the forestomach. However, this tendency was not confirmed (Fig. 2D). It should be noted that polyethylene glycol possesses weak buffering capacity, which may therefore impact acid exposure to the lactobacilli. Therefore, the amount of solution was kept as low as possible (50 μl total). For all analyzed gene clusters, it was assumed that the corresponding substrates, i.e. glutamic acid, arginine, urea etc., were present in the forestomach as they are supplied by the diet, or in the case of urea, enter the stomach by diffusion (Burne and Chen, 2000).

Determination of pH regulation of urease activity

Lactobacillus reuteri 100-23c was grown for 6 h in mMRS at 37°C, centrifuged and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6 with HCl (before sterile filtration). Cells were incubated for another 2 h at 37°C, and subsequently 10 ml of *L. reuteri* 100-23c culture was centrifuged for 5 min at 15,000 × *g* and stored in 10% glycerol at −20°C until determination of urease activity.

Measurement of urease activity

Cell solutions were thawed on ice, washed twice with citrate buffer (pH 4) and disrupted with 0.3 g sterile silica beads (0.5 mm) at maximum speed in a cell mill (Mini-Beadbeater Biospec product) for three 1 min intervals. Tubes were cooled on ice for 2 min between intervals to prevent overheating. Samples were centrifuged at 10,000 × *g* for 2 min. Supernatant was collected and stored at –20°C until the assays were performed. Urease activity in the supernatant was determined by conversion of urea to ammonia, as described previously (Chaney and Marbach, 1962). Citrate buffer (pH 4) containing 167 mM urea was mixed in equal volumes with cell supernatant and incubated at 30°C for 30 min. Ammonia was quantified by the Berthelot reaction (Chaney and Marbach, 1962). To determine protein, cell pellets were washed twice with 10 mM Tris (pH 8) and disrupted as described above. Protein concentration was determined according to Lowry and colleagues (1951). Urease activity is expressed as microgram of ammonia formed per microgram of protein.

RNA extraction from L. reuteri *cell cultures*

Lactobacillus reuteri 100-23 was grown for 6 h in mMRS media at 37°C, and cells were collected by centrifugation for 10 min at 3214 × *g* and re-suspended in fresh mMRS media adjusted to pH 4, 5 or 6 with HCl. Cells were incubated for another 30 min at 37°C, and subsequently mixed with RNAprotect bacterial reagent (Qiagen, Valencia, CA, USA) at a ratio of 1 to 5. The solution was incubated for 5 min at room temperature, centrifuged and stored at −80°C until used for RNA isolation. Total RNA was isolated after the cell pellet was washed with RNase-free PBS buffer and re-suspended in 100 µl of lysis buffer (30 mM Tris–HCl; 1 mM EDTA, pH 8.0; 15 mg ml^{-1} lysozyme; 10 U ml^{-1} mutanolysin; and 100 µg ml^{-1} Proteinase K). Samples were treated as previously described (Rattanaprasert *et al*., 2014) and subsequently transferred to an RNeasy Mini spin column (Qiagen, Hilden, Germany). Mixtures were centrifuged for 15 s at 14 000 × *g* and the eluate discarded. 350 µl of Buffer RW1 was added and centrifuged as before. There was 80 µl of DNase I incubation mix applied to the RNeasy column and incubated at room temperature for 15 min. And, 350 µl of RW1 buffer was added and centrifuged as described above. The flow-through was discarded, 500 µl of Buffer RPE added and centrifuged. 500 µl of Buffer RPE was added again and centrifuged for 2 min at 14 000 × *g*. RNeasy column was placed in a new 2.0 ml collection tube and centrifuged for 1 min at 14 000 × *g*. RNeasy column was placed in a new 1.5 ml collection tube and RNA eluted with 50 µl of RNase-free water. Samples were centrifuged for 1 min at 12 000 × *g*. According to the manufacturer's protocol (Applied Biosystems/Ambion, Austin, TX, USA) the purified RNA was subsequently treated with the TURBO DNA-free kit. RNA was quantified using the Qubit® RNA BR Assay kit (Invitrogen, Carlsbad, CA, USA), and RNA integrity was validated on a 1% agarose gel. The absence of DNA contamination was confirmed by real-time PCR.

Determination of gene expression by quantitative reverse transcription PCR (qRT-PCR)

The purified RNA was reverse transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions with minor modifications as described by Frese and colleagues (2013). qRT PCR was performed using an Eppendorf Mastercycler Realplex2 machine (Eppendorf AG, Hamburg, Germany) and Quanti-Fast SYBR Green PCR kits (Qiagen, Valencia, CA, USA). The *ureC* and glyceraldehyde 3-phosphate dehydrogenase primers (Table S1) were previously validated using serial 10-fold dilutions of pooled cDNA to determine specificity and efficiency (Frese *et al*., 2013). For each 25 µl qRT-PCR reaction, 12.5 µl of 2x Quantifast SYBR Green Mastermix, 1 µl of cDNA and 10 µMol of each primer were used. The DNA was denatured at 95°C for 5 min and followed by 40 two-step cycles of 10 s at 95°C, then 30 s at 60°C. Each PCR product was validated on an agarose gel and by inspection of their melting curves. Gene transcripts of the urease α-subunit were quantified relative to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene, and relative quantification was performed using the method by Pfaffl (2001).

Statistical analysis

Data are expressed as means ± standard deviations unless otherwise stated. Statistical analyses were carried out using GRAPHPAD PRISM 5 (GraphPad Software, California, USA). If only two groups were compared, Student's *t*-tests were performed. ANOVA and Tukey's post-tests were used for multiple comparisons. Significance of $P \leq 0.05$ is denoted by a single asterisk (*), $P \leq 0.01$ as two asterisks (**), and $P \leq 0.001$ by three asterisks (***).

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Supporting information — Table S1. Primers used — follows the References.

Table S1: Primers used

Strain	Primer Sequence (5' -> 3')
L. reuteri 100-23c *ureC* mutant	F' AACTAACCCATATTGTAAGAACA; R' AGAAGTCATCATACTAAGGGCA
L. reuteri 100-23c *gadB* mutant	F' ACTTATATGGAGCCAAAAGCAA; R' CTGTCCGTAGCTCAACATCAA
L. reuteri 100-23c *lisR* mutant	F' AAACTGTGGTGAACTTCGCAA; R'ACGAACCGTTTGGATGTAGC
L. reuteri 100-23c *lisK* mutant	F' AAGCGACCGGTAATTAAGGAA; R' CAATGGTCGAAGGAGGAAAA
L. reuteri 100-23c *adi* mutant	F' CCAATGCCAAATGCATAC; R' AGAACTAATTCATCTCCAC
L. reuteri 100-23 glyceraldehyde 3-phosphate dehydrogenase gene	F' CGGATTCACGAACTTAACACAA; R' CCTTACCATCAACAACAATAC
L. reuteri 100-23 16S rRNA	F' GTACGCACTGGCCCAA; R' ACCGCAGGTCCATCCCAG