Gene expression of lactobacilli in murine forestomach biofilms

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Summary

Lactobacilli populate the gastro-intestinal tract of vertebrates, and are used in food fermentations and as probiotics. Lactobacilli are also major constituents of stable biofilms in the forestomach of rodents. In order to investigate the lifestyle of these biofilm lactobacilli in C57BL/6 mice, we applied metatranscriptomics to analyse gene expression (assessed by mRNA) and community composition (assessed by rRNA). Lactobacillales were the major biofilm inhabitants (62–82% of rRNA reads), followed by Clostridiales (6–31% of rRNA reads). To identify mRNA transcripts specific for the forestomach, we compared forestomach and hindgut metatranscriptomes. Gene expression of the biofilm microbiota was characterized by high abundance of transcripts related to glucose and maltose utilization, peptide degradation, and amino acid transport, indicating their major catabolic and anabolic pathways. The microbiota transcribed genes encoding pathways enhancing oxidative stress (glutathione synthesis) and acid tolerance. Various pathways, including metabolite formation (urea degradation, arginine pathway, γ-aminobutyrate) and cell wall modification (DltA, cyclopropane-fatty-acyl-phospholipid synthase), contributed to acid tolerance, as judged from the transcript profile. In addition, the biofilm microbiota expressed numerous genes encoding extracellular proteins involved in adhesion and/or biofilm formation (e.g. MucBP, glycosyl hydrolase families 68 and 70). This study shed light on the lifestyle and specific adaptations of lactobacilli in the murine forestomach that might also be relevant for lactobacilli biofilms in other vertebrates, including humans.

Introduction

The genus Lactobacillus encompasses a diverse group of bacteria from the Firmicutes phylum that act as starter cultures in fermented foods, are detected in the gastrointestinal tract (GIT) of humans and animals, and are applied for their health-promoting effects as probiotics (Claesson et al., 2007). It is much less known that strains of Lactobacillus are autochthonous to the proximal parts of the GIT of rodents, birds, pigs and horses forming stable biofilms (Walter, 2008). Lactobacilli adhere to the non-glandular squamous stratified epithelium lining the forestomach of rodents, the crop of birds, the oesophagus of pigs and the non-glandular stomach of horses (Savage, 1972; Fuller et al., 1978; Yuki et al., 2000; Walter et al., 2011).

One of the best characterized species of these biofilms is Lactobacillus reuteri, which is also able to persist in the human gut and is frequently recovered from cereal fermentations (Gänzle and Schwab, 2009b; Walter et al., 2011). Population genetics revealed that strains of L. reuteri cluster into host-specific clades (Oh et al., 2010; Su et al., 2012). Whole genome comparison by Frese and colleagues (2011) and Su and colleagues (2012) indicated that the genomic potential of rodent and human L. reuteri differs, and that cereal isolates have traits of both rodent and human strains. For example, only rodent isolates possess a urease cluster, while human isolates are capable of producing reuterin and 1,2-propanediol via the activity of the pdu-cri-cob-hem cluster (Frese et al., 2011; Su et al., 2012).

A similar scheme of host–microbe co-evolution was observed for L. johnsonii (Buhnik-Rosenblau et al., 2012). Mouse forestomach colonization of L. johnsonii isolates is dependent on strain origin: a rodent isolate (100–33) persisted in RLF-mice similar to L. reuteri (log 9 cfu g−1 in the forestomach), while numbers of human gut (NCC533) or blood (ATCC33220) isolates maximally reached log 7 cfu g−1 and decreased within days in conventional and antibiotic-treated mice (Denou et al., 2008; Tannock et al., 2012).
In comparison to the autochthonous strains of *L. reuteri* and *L. johnsonii*, *L. plantarum* is considered allochthonous as it does not persist in mice with complete gut microbiota after single injection and quickly transits through the GIT (Marco et al., 2007). Nevertheless, several studies on *L. plantarum* determined gene expression and identified key genes transcribed during transition through the lower GIT using microarrays or in vivo expression technology (IVET). Some genes, e.g. sugar phosphotransferase system (PTS), copper transporting ATPase, were also transcribed in vivo by *L. johnsonii NCC533* (Bron et al., 2004; Denou et al., 2007; Marco et al., 2007, 2009).

Lactobacilli-free reconstituted mice (RLF-mice, Tannock et al., 1988), complex microbiota mice pretreated with antibiotics and force-fed with the strains of interest, and germ-free re-assOCIated mice are frequently applied tools to investigate *Lactobacillus* lifestyle in the murine intestine (Bron et al., 2004; Walter et al., 2005; 2007; 2008; Denou et al., 2007; 2008; Marco et al., 2007; 2009; Frese et al., 2011; Tannock et al., 2012). In contrast, little is known about the natural autochthonous biofilm microbiota residing in the murine forestomach. Studies on natural communities can give insights into community structure and function of the forestomach biofilms and can validate the results obtained from model systems commonly applied. We, therefore, chose a metatranscriptomic approach to determine community composition and activity in the forestomach epithelium of widely used C57BL/6 mice. With this study, we aimed to find answers to the following questions: Who resides in natural biofilms on stratified squamous epithelium? What are the interactions and metabolic pathways of the community? Does host niche affect gene expression?

## Results and discussion

We applied metatranscriptomics, which was previously shown to predict activity level of bacterial communities (Helbling et al., 2012), to concurrently investigate community composition and function of autochthonous forestomach biofilms in mice. Metatranscriptomic data derived from pooled hindgut contents of caecum and colon were used to enable detection of host niche-specific impacts on gene expression. Metatranscriptomics of five individual forestomach biofilm microorganisms resulted in datasets consisting of 0.5–4.8 Mio sequences, with the majority (92.3–98.4%) being ribosomal RNA (Table S1). The metatranscriptomic datasets of the hindgut microorganisms contained between 3.2 and 13.3 Mio sequences. The utilization of two different sequencing techniques should otherwise not result in considerable data bias, as cDNA synthesis was identical, and the DNA template fragment length and the length of the generated sequences were similar due to the usage of overlapped paired-end reads for Illumina (∼160 versus ∼210 bp; Table S1). Due to the different sizes of the datasets from IonTorrent and Illumina sequencing (Table S1), we focused on transcripts that were significantly higher transcribed in the IonTorrent derived metatranscriptomes from the forestomachs. It has to be acknowledged that sequencing depth is an issue with metatranscriptomics. However, in comparison with other transcriptomic approaches (e.g. microarray and IVET), the resolution power of metatranscriptomics is high. This is, to our best knowledge, the biggest metatranscriptomic study about the lifestyle of lactobacilli in the murine intestine to date.

**The forestomach biofilm microbiota**

Community composition was determined using 16S rRNA transcripts from the metatranscriptomes. We consider this a measure of relative abundance of bacterial groups even though the rRNA content does not necessarily reflect cellular abundance and is affected in complex ways by the physiological state of the cell. *Lactobacillales* were the dominant bacterial order (62–82%) in the forestomach (FS), with the exception of FS4 where *Clostridiales* prevailed (Fig. 1). Quantitative polymerase chain reaction (PCR) targeting the 16S rRNA gene confirmed the high proportion of lactobacilli in the forestomach bacterial community (Table S2). In FS1-3 and FS5, *Clostridiales* represented between 8% and 31% of the community and consisted mainly of the families *Lachnospiraceae* (approximately 73%) and *Ruminococcaceae* (approximately 17%). Relative abundance of *Bacteroidales* fluctuated between 1% and 2%, with the exception of FS4 (9%). *Deferribacteres* and *Deferribacteriales* (mainly the genus *Mucispirillum*, up to 0.21%) were repeatedly retrieved from forestomach and were also present in the hindgut. In agreement to the complex community determined in this study, Fuller and colleagues (1978) reported a phenotypically diverse, lactobacilli-dominated biofilm in the oesophagus of pigs. In contrast to the forestomach, *Clostridiales* and *Bacteroidales* were the dominant bacterial orders in the hindgut. The proportion of *Lactobacillales* was much lower, fluctuating between 0.5% and 4% (Fig. 1).

To investigate the *Lactobacillus* species composition in more detail, we compared the identified *Lactobacillaceae* 16S rRNAs against a references database of *Lactobacillus*-type strains using BLASTN. In a prior simulation test, we validated the reference database and analytical approach (see Experimental procedures for details). On average, 58% of the *Lactobacillaceae* rRNAs could be assigned to a species, with the exception of FS5 (28%). All detected *Lactobacillus* species belonged to the
subgroups of L. johnsonii/acidophilus or L. reuteri (Fig. 2, Canchaya et al., 2006; Felis and Dellaglio, 2007). In three forestomachs (FS1-3), L. johnsonii and L. intestinalis constituted more than 70% of the classified Lactobacillus population, while in FS4 and FS5, L. vaginalis was predominant (Fig. 2). L. johnsonii and L. vaginalis were detected in all forestomachs, whereas L. reuteri, L. fermentum, L. pontis and L. intestinalis were irregularly recovered. Only Lactobacillus species detected in the forestomach were also present in the hindgut (Fig. 2).

While the co-colonization of the forestomach by L. reuteri and L. johnsonii has been reported before (Tannock et al., 2012), the presence of L. vaginalis, sometimes even outnumbering all other Lactobacillus species, was unexpected. Lactobacillus vaginalis was originally isolated from the human vagina, which is also lined by squamous stratified epithelium (Embley et al., 1989).Surprisingly, L. pontis, which was originally isolated from sourdough (Vogel et al., 1994) and belongs to the L. reuteri subgroup (Canchaya et al., 2006; Felis and Dellaglio, 2007), was frequently detected. It cannot be excluded that L. pontis is allochthonous and stemmed from feed as it has not previously been detected in mice. However, for L. reuteri, it is known that rodent and human strains can colonize both cereal and intestinal habitats (Walter et al., 2008; Gänzle and Schwab, 2009a; Su et al., 2011).

Gene expression in the forestomach and hindgut

We obtained between 22.255 and 144.454 putative mRNAs from forestomach biofilms and up to 755.736 from the hindgut (Table S1). On average, approximately 30% of these could be functionally annotated with the SEED subsystems (Mitra et al., 2011, Table S1). In FS1-3 and 5, between 44% and 87% of the mRNA reads were taxonomically assigned to Lactobacillales, whereas in FS4 lactobacilli contributed only 13% of mRNA reads (Table S1). This difference was in accordance with the rRNA-derived community composition (Fig. 1).

SEED functional category profiles differed depending on location (forestomach versus hindgut, Fig. 3) and were
generally defined by the transcription pattern of the prevailing bacterial orders (Clostridiales in hindgut and Lactobacillales in forestomach) (Fig. S1). Location within the GIT (forestomach versus hindgut) did not impact the gene expression pattern of Lactobacillales and Clostridiales to a large extent at least when analysed on the most general level of the SEED functional categories (Fig. 3). However, this might be different within more specific metabolic categories. As only two hindgut samples yielded enough Lactobacillales mRNA reads for functional analysis, we strengthened these findings including two additional samples obtained from Tyk2−/− mice on a C57BL/6 background with \( n = 1504 \) and \( n = 1071 \) Lactobacillales mRNA reads (Fig. S2).

In contrast to the results obtained here, transcription pattern of the human gut isolate L. johnsonii NCC355 varied in different segments of the GIT and was very low in the colon as determined using microarrays (Denou et al., 2007). This strain is not autochthonous to the murine intestine, which might explain the differences in gene expression. Nevertheless, L. johnsonii NCC533 (Denou et al., 2007) transcribed the highest numbers of genes in the murine forestomach, pointing at the forestomach as its preferred location during its temporary existence in the mouse GIT.

Substrate utilization and metabolic network in the forestomach

Lactobacillus johnsonii and L. reuteri lack biosynthetic pathways for amino acids, purine nucleotides and co-factors (Pridmore et al., 2004; Frese et al., 2011; Guinane et al., 2011). The higher transcript abundance of a number of proteins related to amino acid transport and degradation compared with the hindgut indicated their importance as growth requirements for the biofilm (Table 1). Additionally, Pfam domains indicative of glutamate dehydrogenases were significantly enriched in the forestomach. Glutamate dehydrogenase transforms glutamate to \( \alpha \)-ketoglutarate, which increases amino acid conversion as it acts as amino acceptor in transamination reactions (Gänzle et al., 2007).

With few exceptions, lactobacilli are also not capable of degrading and transporting oligo (\( n > 4 \)) – and polysaccharides (Gänzle and Follador, 2012). Accordingly, the high proportion of transcripts for ‘central carbohydrate metabolism’ (Fig. 4A), which are mainly associated with glucose utilization, together with the high abundance of Pfam domains for a glucose transporter, maltose phosphorylase (key enzymes of the pentose phosphate pathway) and lactate dehydrogenase (Table 1, Table S3), were indicative of glucose and maltose as preferred carbohydrate sources and the major catabolic pathways for their utilization in the forestomach biofilm microbiota.

In contrast, the higher abundance of transcripts for ‘polysaccharides’ and ‘aminosugars’ (Fig. 4A) in hindgut metatranscriptomes, including glycosyl hydrolases involved in cellulose (GH48) and mucin (GH3, GH2C) degradation (Table S3, Schwab et al., 2014), indicated mucus and cellulose as possible substrates for the hindgut microbiota. That observation agrees with the transcription of PTS systems for mannose, cellobiose and N-acetylglucosamine by L. plantarum and L. johnsonii.

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Table 1. Pfam categories that are significantly and at least tenfold more abundant in the forestomach than in the hindgut.

| Functional category          | Pfam                | Predicted function                                                                 | % Relative abundance | Fold higher than hindgut | P-value |
|-----------------------------|---------------------|------------------------------------------------------------------------------------|----------------------|--------------------------|---------|
| Metabolism                  | EscB (PF05975)      | Bacterial ABC transporter                                                          | 0.020 ± 0.013        | nd                       | 0.003   |
|                            | OprD (PF03573)      | Outer membrane porin, OprD family                                                  | 0.058 ± 0.067        | nd                       | 0.030*  |
|                            | OpuAC (PF04069)     | Compatible solute binding protein of ABD transporter                                | 0.033 ± 0.016        | 14                       | 0.001   |
| Sugar stress                | Sugar_transport (PF00083) | Putative glucose uptake                                                            | 0.038 ± 0.030        | 84                       | 0.013   |
|                            | Sugar_bind (PF04198) | Putative sugar binding domain                                                       | 0.114 ± 0.136        | 34                       | 0.037*  |
| Maltose utilization         | Glyco hydro_65C (PF03633) | Maltose phosphorylase                                                               | 0.036 ± 0.043        | 64                       | 0.04*   |
|                            | Glyco hydro_65N (PF03636) |                                                                                   | 0.168 ± 0.111        | 43                       | <0.001  |
| Other carbohydrate          | Glucosaminidase (PF01832) | Mannnosyl-glucoprotein                                                             | 0.068 ± 0.064        | 41                       | 0.031   |
| utilization                 | Glucosamine (PF01832) |                                                                                   | 0.064 ± 0.036        | 37                       | 0.002   |
| Pentose phosphate pathway   | Glyco hydro_47 (PF01532) | Alpha-mannosidase (inverting)                                                      | 0.012 ± 0.012        | nd                       | 0.030   |
|                            | G6PD_C (PF02781)    | Glucose-6-phosphate dehydrogenase                                                  | 0.044 ± 0.025        | 23                       | 0.003   |
|                            | G6PD_N (PF00479)    |                                                                                   | 0.042 ± 0.039        | 79                       | 0.03    |
|                            | XFP_C (PF3963)      | D-xylulose 5-phosphate/D-fructose                                                  | 0.372 ± 0.262        | 27                       | 0.008   |
|                            | XFP_N (PF3964)      |                                                                                   | 1.06 ± 1.163         | 50                       | 0.032*  |
| Amino acid and peptide      | AA_permease (PF00324) | Amino acid uptake                                                                  | 0.082 ± 0.083        | 16                       | 0.030   |
| uptake and metabolism       | AA_permease_2 (PF13520) | Amino acid uptake                                                                  | 0.380 ± 0.356        | 43                       | 0.029   |
|                            | Peptidease_C1_2 (PF03051) |                                                                                   | 0.669 ± 0.386        | 20                       | 0.003   |
|                            | Peptidease_C1_2 (PF03051) |                                                                                   | 0.656 ± 0.374        | 19                       | 0.003   |
|                            | Peptidease_M1 (PF01433) |                                                                                   | 0.089 ± 0.064        | 116                       | 0.007   |
|                            | Peptidease_M1_5 (PF02129) |                                                                                   | 0.018 ± 0.014        | 13                       | 0.019   |
|                            | A1_Propeptide (PF07966) | Endopeptidase propedipeptase                                                        | 0.048 ± 0.043        | nd                       | 0.022   |
|                            | Beta-lactamase2 (PF13534) |                                                                                   | 0.019 ± 0.016        | 16                       | 0.027   |
|                            | Asp (PF00026)       | Aspartyl protease                                                                  | 0.103 ± 0.115        | nd                       | 0.027*  |
| Amino acid conversion       | Bac_GDH (PF05088)   | Glutamate dehydrogenase                                                            | 0.016 ± 0.015        | nd                       | 0.028   |
| Stress tolerance            |                     |                                                                                   |                      |                          |         |
| Urea uptake and degradation | AmisS_Urel (PF02293) | Urea channel/amide transporter                                                     | 0.653 ± 0.464        | 242                       | 0.007   |
|                            | Urease_alpha (PF00449) | Urease (α, β, γ-subunit)                                                           | 0.460 ± 0.413        | 22                       | 0.027   |
|                            | Urease_beta (PF00649) |                                                                                   | 0.316 ± 0.312        | 32                       | 0.039   |
|                            | Urease_gamma (PF00547) |                                                                                   | 0.525 ± 0.436        | 41                       | 0.017   |
|                            | UreD (PF01774)      | Urease accessory protein                                                           | 0.030 ± 0.023        | 22                       | 0.015   |
| Acid stress                 | G6PD (PF00479)      | Glutaminase                                                                        | 0.086 ± 0.044        | 3                        | 0.011   |
|                            | Pyridoxal_deC (PF00282) |                                                                                   | 0.180 ± 0.137        | 10                       | 0.017   |
| Acid stress/biofilm formation | Dld_C (PF04914)   | Biosynthesis of D-lactyl-lipoteichoic acid                                          | 0.040 ± 0.023        | 368                      | 0.041*  |
|                            | Dld_M (PF04918)     |                                                                                   | 0.009 ± 0.017        | 22                       | ns      |
|                            | Dld_N (PF04915)     |                                                                                   | 0.019 ± 0.025        | nd                       | 0.044*  |
| Acid stress                 | CMAS (PF02353)      | Cyclopropane-fatty-acetyl-phospholipid synthase                                    | 0.084 ± 0.071        | 137                      | 0.017   |
| Oxygen tolerance            | Glu_cys_ligase (PF04262) | Glutamate-cysteine ligase                                                          | 0.007 ± 0.006        | 26                       | 0.020   |
|                            | GSH_S_ATP (PF02955) | Glutathione synthase                                                               | 0.006 ± 0.007        | nd                       | 0.039   |
|                            | GSH_Px (PF00255)    | Glutathione peroxidase                                                             | 0.018 ± 0.007        | 10                       | <0.001  |
| General stress              | GST_N (PF13460)     | Levanocucurase/invertase                                                           | 0.011 ± 0.012        | 50                       | 0.029*  |
| Adhesion/biofilm formation  | Usp (PF01774)       | Universal stress protein                                                           | 0.163 ± 0.062        | 14                       | <0.001  |
| Miscellaneous               | MucBP (PF06458)     | Mucus binding protein                                                              | 0.308 ± 0.287        | 169                      | 0.03    |
|                            | Rib (PF08486)       | Mucus binding protein                                                              | 0.596 ± 0.226        | 84                       | <0.001  |
|                            | PePcase (PF03317)   | Bacterial surface layer protein                                                    | 0.057 ± 0.073        | 533                      | 0.041*  |
|                            | YSIRK_signal (PF04650) |                                                                                   | 0.062 ± 0.044        | 144                      | 0.007   |
|                            | Glyco hydro_68 (PF02435) |                                                                                   | 0.011 ± 0.012        | 50                       | 0.029*  |
|                            | Glyco hydro_70 (PF02324) |                                                                                   | 0.081 ± 0.075        | 49                       | 0.029   |
|                            | DSBB (PF02600)      | Introduction of disulfide bonds (membrane bound)                                   | 0.006 ± 0.008        | nd                       | 0.043   |
|                            | PAS (PF13596)       |                                                                                   | 0.039 ± 0.024        | 360                      | 0.003   |

a. One-tailed t-test, all unmarked two-tailed t-test; nd, not detected in the hindgut; 1C-terminal domain; 2central catalytic domain; 3N-terminal domain.
NCC533 during GIT transit (Bron et al., 2004; Denou et al., 2007).

Relative abundance of Clostridiales transcripts assigned to the subcategories of ‘carbohydrate metabolism’ in the forestomach strongly correlated to proportions to those subcategories observed in the hindgut (Pearson correlation, \( P < 0.01 \)), indicating that the Clostridiales might also contribute to polysaccharide degradation in the forestomach. Clostridiales, which could be introduced to the forestomach via coprophagy, can also utilize lactate, the major fermentation product of lactobacilli, to produce butyrate (Duncan et al., 2004). Within the Clostridiales population transcriptome, we recovered few transcripts of clostridial lactate dehydrogenase converting lactate to pyruvate (Belenguer et al., 2006), as well as transcripts encoding key enzymes of the butyrate formation pathway (acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA and butyryl-CoA dehydrogenase, data not shown) (Louis and Flint, 2009; Schwab et al., 2014), strongly suggesting the formation of butyrate in the murine forestomach. Lactate cross-feeding might likewise be responsible for the presence of Desulfovibrionales, which metabolize lactate and pyruvate to acetate and \( \text{CO}_2 \) in the presence of sulfate (Odom and Singleton, 1993); however, too few transcripts

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of Desulfovibrionales prevented a verification of this speculation.

External proteins involved in adhesion and biofilm formation

The genomes of L. reuteri and L. johnsonii harbour many genes encoding large cell surface proteins putatively involved in adhesion to the epithelium and biofilm formation (Pridmore et al., 2004; Walter et al., 2005; Frese et al., 2011). In the forestomach microbiota, transcripts encoding extracellular bacterial surface layer proteins (SLAP, Boot et al., 1995) and proteins involved in mucus binding (MucBP and Rib) were significantly more abundant than in the hindgut. MucBP and Rib were most similar to extracellular surface proteins of L. reuteri and L. johnsonii (for, e.g., ZP_03073480, ZP_12484427, AAT98629, > 90% amino acid sequence identity). In vitro and in vivo experiments previously confirmed the impact of extracellular proteins in adhesion and biofilm formation. The cell surface protein MucBP of L. reuteri ATCC55368 adhered to mucus from different sources (Roos and Jonsson, 2002). Inactivation of a large surface protein (Lsp) of L. reuteri 100-23 reduced competitiveness in vivo (Walter et al., 2005).

Additionally, transcripts of proteins with GH68 and GH70 motives of fructan- and glucansucrases, respectively, were significantly more abundant in forestomach biofilm than in the hindgut lumen microbiota. Proteins with GH68 motive had the highest similarity to L. reuteri inulosucrase (CAL25302) and L. johnsonii fructosyltransferase (YP_005862512, > 94% amino acid sequence identity). Proteins with GH70 motive indicative of glucansucrases were most similar to glucansucrases of L. reuteri (AAU8004 and ABP88725, > 98% amino acid sequence identity). In analogy to the glucansucrase-dependent biofilm formation of Streptococcus mutans on the tooth surface, lactobacilli glucansucrases were imposed to be involved in forestomach biofilm formation (Walter et al., 2008; Gänzle and Schwab, 2009a). Strengthening this suggestion, reduced competitiveness was reported in two strains of L. reuteri after inactivation of extracellular fructansucrases (Walter et al., 2008; Sims et al., 2011). Similar to L. reuteri and L. johnsonii, L. vaginalis and L. pontis possess extracellular fructansucrases (GH68) (Table S4, Tiekink et al., 2003), which might be one reason for their presence in the forestomach biofilm.

Protective mechanisms in the forestomach biofilm microbiota

The murine forestomach is an open environment, and bacteria are constantly challenged by acidic pH values, varying oxygen levels and the presence of urea derived from the stomach compartment. Significantly higher abundance of transcripts related to ‘acid stress’ in the forestomach compared with hindgut (1.05 ± 0.82% versus 0.08 ± 0.06%, P < 0.05) indicated the prevailing low pH conditions. However, the biofilm community appears to be well adapted to life in this environmental niche, indicated by transcription of genes encoding a diverse array of pathways enhancing stress tolerance via the maintenance of intracellular pH homeostasis, by alterations of the environmental milieu and changes in cell wall composition (Table 1). For instance, major differences between the gene expression profiles in forestomach and hindgut lumen were observed for transcripts assigned to ‘amino acids and derivatives’ (Fig. 4B) and its subcategory ‘arginine; urea cycle, polyamines’. Latter predominantly contained transcripts assigned to ‘urea decomposition’ (38–89% of transcripts of ‘arginine, urea cycle, polyamines’) and ‘arginine and ornithine degradation’ (6–38% of transcripts of ‘arginine, urea cycle, polyamines’). The degradation of urea enhances acid resistance and facilitates bacterial life in an acidic milieu, such as the stomach. The human stomach colonizer Helicobacter pylori depends on its pH regulated urea transporters and an internal urease during colonization of the stomach to maintain a favourable periplasmic pH (Sachs et al., 2003). Among strains of L. reuteri, urease production is a unique trait of rodent and cereal isolates (Walter et al., 2011; Su et al., 2012); urea degradation was confirmed using L. reuteri sourdough and mouse isolates (Su et al., 2012). The importance of urea degradation in forestomach biofilms was highlighted by the transcription of the complete operon for urea uptake and degradation by L. reuteri (> 97% amino acid sequence identity, Table 1). In addition, high transcription of the SEED category ‘arginine and ornithine degradation’ encompassing transcripts encoding the arginine deiminase pathway and the arginine/ornithine antiporter ArcD (Fig. 4B, Table 1) indicated another pathway to generate intracellular ammonia from arginine to maintain a neutral intracellular pH in acidic environment (Rollan et al., 2003; Su et al., 2011).

The decarboxylation of glutamate, which is likely derived from glutamine by the activity of a glutaminase (Su et al., 2011), to ɣ-aminobutyrate (GABA) likewise enhances bacterial acid tolerance. In the forestomach biofilms, genes for glutamate decarboxylase and glutaminase were highly expressed (Table 1). In the rodent isolate L. reuteri 100-23, a glutaminase gene is located adjacent to glutamate decarboxylase (Su et al., 2011). Inactivation of the glutamate decarboxylase decreased acid resistance as well as competitiveness in sourdoughs (Su et al., 2011). As L. reuteri 100-23 persists well in both the intestinal tract and cereal fermentations, the impact of...
this glutamate decarboxylase on acid resistance can be likely expanded to its intestinal habitat.

Biofilm lactobacilli also highly transcribed genes encoding proteins involved in glutathione synthesis, such as glutamate-cysteine ligase, glutathione synthetase and peroxidase (Table 1). The bacterial cell is able to regulate its oxidative state through the conversion of oxidized and reduced forms of glutathione (GSSG and GSH, respectively) catalysed by glutathione reductase and glutathione peroxidase respectively (Pophaly et al., 2012). The inactivation of a glutathione reductase impaired oxygen tolerance in L. sanfranciscensis (Jänsch et al., 2007).

Beside the transcription of pathways increasing acid resistance or altering the oxidative state of the cell, we also observed expression of pathways modifying the structure of the bacterial cell wall. The dlt operon is responsible for the integration of D-alanine into cell wall teichoic and lipoteichoic acids, and has been correlated to a number of features, including acid resistance (Boyd et al., 2000; Kristian et al., 2005). Dlt domains were significantly higher expressed in the forestomach than in the hindgut. Previously, Walter and colleagues (2007) inactivated the dltA gene of L. reuteri and consequently observed reduced competitiveness in vivo; however, adherence was not affected. In contrast to the high transcription of dltA and the glutathione reductase gene observed in the forestomach, L. plantarum downregulated dltA and the glutathione reductase in the caecum (Marco et al., 2009). This observation points again at the forestomach specific adaption of members of the autochthonous biofilm community.

The high expression of a cyclopropane-fatty-acyl-phospholipid synthase indicates modification of the cell membrane. Cyclopropane-fatty-acyl-phospholipid synthase confers unsaturated fatty acids into cyclopropane derivatives leading to enhanced acid tolerance (Brown et al., 1997). In L. brevis, the expression of a cyclopropane-fatty-acyl-phospholipid synthase is increased during growth in acidic conditions, leading to chances in membrane composition (Behr et al., 2006; 2007).

Conclusions

Our study revealed via analysis of in situ gene expression the existence of a complex autochthonous forestomach biofilm community interacting via an intrinsic network of functional and metabolic features (Fig. 5). This study confirmed in situ the importance of previously identified genes encoding proteins that were shown to be involved in the adaption of strains of L. reuteri to the biofilm community (urease, extracellular proteins such as MucBP and GH68 and 70, DltA). Interestingly, transcripts for proteins, such as glutamate decarboxylase and glutathione reductase that assured competitiveness of L. reuteri in cereal fermentations, were also highly transcribed in the biofilms, strengthening again the proposed shared intestinal origin of rodent and sourdough isolates (Su et al., 2012). In contrast, some genes that were upregulated (copper binding ATPase, IgA protease, sugar PTS systems) during transit of L. plantarum and non-rodent L. johnsonii through the murine GIT (Bron et al., 2004; Denou et al., 2007) were not identified as significantly increased in the forestomach, while others were differentially regulated (dltA, glutathione reductase) (Marco et al., 2009) in line with variations in lifestyle of the Lactobacillus species (transit versus persistence) and varying environmental parameters in forestomach and hindgut (substrate availability, pH and oxygen levels). These observations strengthen the importance of considering habitat adaption when applying strains of Lactobacillus for health application, e.g. as probiotics. In addition, as comparable lactobacilli-dominated biofilm communities exist in pigs, horses, birds and the human vagina, results obtained here might be exemplary for other autochthonous Lactobacillaceae biofilms.

Experimental procedures

Animals

C57BL/6 mice of 6–8 weeks of age were sacrificed, the stomach was separated from the other parts of the gastrointestinal system, stomach contents were removed, and the forestomach was snap-frozen. For nucleic acid (NA) isolation of the forestomach biofilm, bacterial cells were recovered from the forestomach epithelium by scratching and flushing with 0.5 ml anoxic PBS, and bacterial cells were collected by centrifugation and immediately used for NA isolation. For NA isolation from the hindgut, caecum and colon were flushed together with 7 ml of PBS, flushed hindgut contents were homogenized, collected by centrifugation and snap-frozen for NA purification (Berry et al., 2012). Animal experiments were approved by the institutional ethics committee and conducted in accordance with protocols approved by the Austrian laws (BMWF-66.006/0002-II/10b/2010). FS1-3 and hindguts (CL1-3, IL1-3) were obtained from animals housed at a different facility than animals of FS4 and FS5. Samples CL1-3 and IL1-3 were derived from previous studies (Schwab et al., 2014).

NA isolation and purification of RNA and DNA and cDNA synthesis

DNA and RNA were extracted with a phenol-chloroform bead-beating procedure and kit purification (Qiagen AllPrep DNA/RNA Mini kit) as previously described (Berry et al., 2012). Total RNA was reverse-transcribed using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) with modifications as described before (Berry et al., 2012).
Sequencing of cDNA libraries

cDNA libraries generated from total RNA derived from the forestomach were sequenced by a PGM IonTorrent (LifeSciences) using 200 bp sequencing chemistry and 314, 316 or 318 chips according to instructions supplied by the manufacturer. cDNA libraries were size-selected (> 100 bp) before further processing. Double-stranded cDNA libraries from the hindgut were paired-end sequenced using an Illumina HiSeq (CSF Vienna) and were overlapped using FLASH (Magoc and Salzberg, 2011) as described before (Schwab et al., 2014).

Metatranscriptomic data analysis

Metatranscriptomic sequencing data were analysed following the double RNA analysis pipeline utilized by Urich and colleagues (2008). Community composition was determined from 100 000 rRNA reads, which were taxonomically assigned using CREST (Urich et al., 2008; Lanzén et al., 2012) (bit score = 150, top percent = 10, minimal support = 1). mRNA tags were compared against the NCBI RefSeq database using BLASTX, and functionally and taxonomically classified using MEGAN and the SEED functional classification therein (bit score = 40, top percent = 10, minimal support = 1) (Mitra et al., 2011). This resulted in between 7331 and 26 794 and up to 214 654 functionally annotated mRNAs for IonTorrent or Illumina derived samples respectively (Table S1). Metatranscriptomes were generated from five forestomachs and six hindgut contents from caecum and colon. Metatranscriptome data IL1-3 were also used in a previous manuscript with a different topic (Schwab et al., 2014). PAS (Hammer et al., 2001) was used for multivariate analysis of metatranscriptome data. Principal component analysis was done by eigenvalue decomposition of a data variance-covariance matrix. Unpaired t-test analysis in SigmaPlot 11 (Systat) was applied for statistical analysis of variance. Metatranscriptomic data is deposited at NCBI’s Sequence Read Archive under accession numbers SRP026649, SRP026292 and SRP027343.

Fig. 5. Biofilm lifestyle in the murine forestomach. Schematic diagram depicting the forestomach biofilm community, its substrate utilization and metabolite formation, and mechanism enhancing stress tolerance. Intracellular maintenance of neutral pH (activity of urease, arginine and glutamate metabolism), alterations of the environmental milieu [extracellular protonation of GABA (γ-aminobutyrate or ornithine)] and changes in cell wall composition (α-alanylation of teichoic esters, synthesis of cyclopropane fatty acids) promote acid tolerance. Glutathione contributes to oxygen tolerance of bacterial cells. AA, amino acids; GSH, reduced glutathione; GSSG, oxidized glutathione.
Lactobacillaceae community composition in biofilm and forestomach

In order to investigate the composition of the Lactobacillaceae community of forestomach and the caecum/colon, 16S rRNA transcripts extracted from MEGAN were compared against a custom-made reference database containing 153 Lactobacillus-type strains obtained from the SILVA database (Quast et al., 2013) using CREST (BLASTN, minimum bit score = 150, top percent = 1, minimal support = 20 and 50 for hindgut and forestomach derived samples respectively). To verify that assignment at the species level was possible in spite of the short 16S rRNA read length, we performed a simulation using randomly generated 16S rRNA fragments of Lactobacillus strains identified in our study. We split the 16S rRNA of L. fermentum, L. reuteri, L. oris, L. pontis, L. frumenti, L. antri, L. intestinalis, L. johnsonii and L. taiwanensis in n = 500 fragments of 170 bp, and compared these fragments against our Lactobacillus-type strain database using BLASTN. All strains were accurately assigned to the corresponding type strains with highest recovery rates for L. reuteri, L. fermentum and L. vaginalis (> 80%), medium recovery rates for L. pontis, L. intestinalis and L. johnsonii (68–50%), and low recovery rates for L. oris, L. frumenti, L. antri (47–33%) and L. taiwanensis (17%). No false-positive assignment (i.e. assignment of fragment to wrong species) was observed. This simulation, verified the validity of our approach. Due to the low recovery rates reads of L. oris, L. frumenti and L. antri were consequently only assigned at the genus level.

As the L. johnsonii and L. taiwanensis 16S rRNA genes are highly similar (99.5%, Wang et al., 2009), we omitted L. taiwanensis from our type strain database. This resulted in the improved recovery of 83% of the reads of L. taiwanensis to L. johnsonii/taiwanensis reference sequence in the simulation. We then compared all 16S rRNA Lactobacillaceae transcripts extracted from MEGAN against the modified type strain reference database (Fig. 2).

Pfam analysis

mRNA reads were translated into all six frames, each frame into separate open reading frames (ORFs), avoiding any characters marking stop codons in a resulting ORF. All ORFs equal to 30 amino acids or larger were screened for assignable conserved protein domains using reference HMMs (hidden Markov models) of the Pfam database (Punta et al., 2012; Pfam release 25, http://pfam.janelia.org/) with HMMER tools (http://hmmer.janelia.org/). All database hits with e-values below a threshold of 10^{-4} were counted. Translated reads of interest were subjected to BLASTP searches against NCBI’s nr database. Due to the different sizes of metatranscriptomes derived from Ion Torrent and Illumina sequencing (Table S1), we focused on Pfam categories that were significantly more transcribed in Ion Torrent-derived metatranscriptomes.

Quantification of selected 16S rRNA genes by quantitative PCR (qPCR)

Copies of 16S rRNA genes were quantified by qPCR using a Mastercycler ep realplex (Eppendorf). Reaction mixtures (20 μl) contained 10 μl QuantiFast SybrGreen (Qiagen), 1 μl of each of the specific primers (Table S2) at a final concentration of 0.25 μM, and 1 μl of template DNA. Running conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s, annealing for 15 s at 60°C, and 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis were performed to verify the identity of the genes of interest. Samples were run in duplicates. Standard curves were generated from linearized plasmids. Gene copies were calculated per μg DNA.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Relative abundance of all transcripts assigned to major SEED categories that were recovered from forestomachs and hindguts (A), and (B) relative abundance of transcripts of Lactobacillales and Clostridiales in forestomachs and hindguts. From the hindguts, we obtained enough mRNA reads for functional analysis (n > 400) from only two samples.

Fig. S2. Host niche-dependent gene expression. Principal component analysis based on relative abundance of SEED categories in the forestomach and the hindgut of either the entire community (hindgut, forestomach), or of Lactobacillales and Clostridiales residing in forestomach or hindgut. As only two hindgut samples of the six C57BL/6 investigated yielded enough Lactobacillales mRNA reads for functional analysis, we strengthened our analysis including two additional samples obtained from Tyk2−/− mice on a C57BL/6 background with n = 1504 and n = 1071 Lactobacillales mRNA reads.

Table S1. Metatranscriptome sequencing. Forestomach metatranscriptomes were sequenced using an Ion Torrent PGM sequencer. Double-stranded cDNA libraries derived from the hindgut were paired-end sequenced using an Illumina HiSeq (Campus Science Support Facilities GmbH, Vienna). Read pairs were overlapping used FLASH (Magoc and Salzberg, 2011). Metatranscriptomic sequencing data were analysed following an established double RNA analysis pipeline (Urlich et al., 2008; Berry et al., 2012).

Table S2. Quantitative PCR of 16S rRNA gene of lactic acid bacteria and Clostridium clusters IV and XIV. Shown are 16S rRNA gene copies μg−1 DNA of individual forestomachs (FS1-5) and the mean of six hindgut samples. To determine
the gene copies of 16S rRNA genes of *Clostridium* clusters IV and XIVa, primers targeting the respective clusters were used in separate reactions, gene copies were added and the sum was logarithmized.

Table S3. Major glycoside hydrolase (GH) families in forestomach and hindgut according to Pfam analysis.

Table S4. Selected features of the genome of *L. vaginalis* ATCC 49540 (NZ_GG693412).