Experimental Evaluation of Host Adaptation of *Lactobacillus reuteri* to Different Vertebrate Species

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ABSTRACT The species *Lactobacillus reuteri* has diversified into host-specific lineages, implying a long-term association with different vertebrates. Strains from rodent lineages show specific adaptations to mice, but the processes underlying the evolution of *L. reuteri* in other hosts remain unknown. We administered three standardized inocula composed of strains from different host-confined lineages to mice, pigs, chickens, and humans. The ecological performance of each strain in the gastrointestinal tract of each host was determined by typing random colonies recovered from fecal samples collected over five consecutive days postadministration. Results revealed that rodent strains were predominant in mice, confirming previous findings of host adaptation. In chickens, poultry strains of the lineage VI (poultry VI) and human isolates from the same lineage (human VI) were recovered at the highest and second highest rates, respectively. Interestingly, human VI strains were virtually undetected in human feces. These findings, together with ancestral state reconstructions, indicate poultry VI and human VI strains share an evolutionary history with chickens. Genomic analysis revealed that poultry VI strains possess a large and variable accessory genome, whereas human VI strains display low genetic diversity and possess genes encoding antibiotic resistance and capsular polysaccharide synthesis, which might have allowed temporal colonization of humans. Experiments in pigs and humans did not provide evidence of host adaptation of *L. reuteri* to these hosts. Overall, our findings demonstrate host adaptation of *L. reuteri* to rodents and chickens, supporting a joint evolution of this bacterial species with several vertebrate hosts, although questions remain about its natural history in humans and pigs.

IMPORTANCE Gut microbes are often hypothesized to have coevolved with their vertebrate hosts. However, the evidence is sparse and the evolutionary mechanisms have not been identified. We developed and applied an experimental approach to determine host adaptation of *L. reuteri* to different hosts. Our findings confirmed adaptation to rodents and provided evidence of adaptation to poultry, suggesting that *L. reuteri* evolved via natural selection in different hosts. By complementing phylogenetic analyses with experimental evidence, this study provides novel information about the mechanisms driving host-microbe coevolution with vertebrates and serve as a basis to inform the application of *L. reuteri* as a probiotic for different host species.

KEYWORDS *Lactobacillus reuteri*, host adaptation, probiotics, symbiosis

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Vertebrates have gained access to a number of metabolic functions absent in their genomes by virtue of symbiosis with microbes (1). The crop and the cecum are evident anatomical adaptations to exploit the enzymatic activity of trillions of symbiotic microbes to access nutrients from otherwise indigestible diets (2). In addition to nutrient provision, gut microbes exclude pathogens and aid in development of the host's immune system (3). It is clear that vertebrates benefit from symbiotic associations with microbes. However, core concepts on how symbioses are formed and maintained over evolutionary time scales are not well understood.

The taxonomic profile of the vertebrate microbiota is largely host specific (3, 4) and, in some cases, congruent with the evolution of the host species (5). This apparent relatedness between microbial community composition and host phylogeny has been interpreted as evidence for coevolution (6–8). Much of this information has been derived from analysis of 16S rRNA gene sequences, which have evolved too slowly to provide information on evolutionary relationships over relevant time scales, especially given the more recent diversification of contemporary vertebrate species compared to their bacterial symbionts (9). Examples of codiversification of specific bacterial lineages with mammalian hosts have been discovered by analyzing fast-evolving gene phylogenies (10, 11), suggesting cospeciation of some symbiotic microbes alongside their vertebrate hosts. However, even in the few established cases, the mechanisms by which these microbes evolved and the outcomes that arose from the evolutionary process remain undefined.

Symbiotic gut microbes that remain stably associated with particular vertebrate species are predicted to evolve host-specific adaptations and, as a result, display enhanced ecological performance in their cognate host (12). Determining the rate of colonization success of individual symbionts in naive (aposymbiotic) hosts in combination with phylogenetic analyses thus provides a platform to infer the evolutionary mechanisms by which host-microbe symbioses evolve. Such experimental approaches have been successfully applied to study the evolutionary consequences of bacterial symbioses in insects (13, 14) and in invertebrates (15, 16), but rarely in vertebrates.

The bacterial species *Lactobacillus reuteri* inhabits the gastrointestinal tracts of a variety of vertebrates and has diversified into distinct phylogenetic lineages that are coherent with host origin (17). A series of phylogenetic, phylogenomic, and experimental studies in mice have established this species as a paradigm for host adaptation of a nonpathogenic symbiont of the vertebrate gut microbiota (9). Rodent isolated strains display elevated fitness in mice (17, 18), and biofilm formation in the forestomach is restricted to strains from rodent lineages (19). Together, these results show that specialized adaptations to the gut environment of the host underlie the evolution of *L. reuteri* with rodents. In contrast, the mechanisms by which *L. reuteri* has evolved with other vertebrate host species have not been determined. In this respect, it is important to point out that the presence of host-specific lineages in itself does not provide evidence for natural selection, as clusters can arise by neutral processes such as genetic drift (17, 20). Furthermore, clustering of both human and poultry isolates in the same phylogenetic lineage (lineage VI) has raised questions regarding the evolutionary history of this lineage. Therefore, the goals of this study were to test for host adaptation of *L. reuteri* to nonrodent hosts and to resolve outstanding questions regarding the evolution of phylogenetic lineage VI. We developed and validated an experimental method to systematically compare the ecological performance of strains isolated from different hosts and assigned to distinct phylogenetic lineages found in the gastrointestinal tracts of chicken, pigs, and human volunteers. We then complemented these studies with comparative genomic analyses to gain insight into genome evolution of poultry and human strains of the lineage VI.

RESULTS

Evolutionary relationships of *L. reuteri* strains determined via whole-genome phylogenetic analysis. To achieve a higher resolution in the analysis of the evolutionary relationships of strains belonging to lineage VI, we sequenced the genomes of 3

TABLE 1 *L. reuteri* genomes used for phylogeny reconstruction and comparative genomics

Strain (alternative name)	Origin	Lineage ^a	Source ^b
mlc3	Mouse	III	JGI 2506381016
100-23	Rat	III	JGI 2500069000
LTH2584	Sourdough	I	JGI 2534682349
TMW1.112	Sourdough	III	JGI 2534682347
TMW1.656	Sourdough	III	JGI 2534682350
I5007	Pig	IV	JGI 2554235423
ATCC 53608	Pig	IV	EMBL LN906634
Ip167-67	Pig	IV	JGI 2599185361
ZLR003	Pig	ND	JGI 2687453552
pg-3b	Pig	IV	JGI 2599185334
3c6	Pig	V	JGI 2599185333
20-2	Pig	V	JGI 2599185332
TD1	Rat	I	JGI 2554235439
lpuph1	Mouse	I	JGI 2506381017
LTH5448	Sourdough	I	JGI 2571042361
JCM1112 (DSM20016 ^T /F275)	Human	II	JGI 640427118
MM4-1a (ATCC PTA-6475)	Human	II	JGI 2502171170
MM2-3 (ATCC PTA-4659)	Human	II	JGI 2502171171
CSF8	Chicken	VI	JGI 2684623009
1366	Chicken	VI	JGI 2684623010
JCM1081	Chicken	VI	JGI 2684623011
CF48-3A	Human	VI	JGI 2502171173
M27U15	Human	VI	JGI 2687453659
MM34-4A	Human	VI	JGI 2660238834
SD2112 (ATCC 55730)	Human	VI	JGI 650716048

^aData were obtained using the method described in reference 17. ND, not determined.

^bFurther information on the listed source sequences can be found at the Joint Genome Institute (JGI) genome portal (<http://genome.jgi.doe.gov>) or the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) portal (<http://www.ebi.ac.uk>).

strains originating from chickens and 2 from humans and included these genomes in a whole-genome phylogenetic analysis with an additional 20 *L. reuteri* genomes available in public databases (Table 1). As shown in Fig. 1, the tree showed clear separation of *L. reuteri* isolates into six host-defined phylogenetic lineages in complete agreement with a previous multilocus sequencing analysis (17) and whole-genome phylogeny analysis (21). Our core-genome phylogenetic analysis also confirmed that human and poultry isolates clustered in lineage VI, which we refer to here as the human VI and poultry VI strains, respectively. This analysis also revealed important differences in genome diversity among lineage VI strains, with the genomes of human VI clustering tightly while poultry VI strains showed high diversity (Fig. 1).

Introduction of *L. reuteri* to the digestive tract of different vertebrate hosts. We developed an experimental approach to study host adaptation of *L. reuteri* strains in different hosts (Fig. 2). We designed three inocula, each containing six *L. reuteri* strains representing lineages I, II, III, V, and VI, with isolates originating from rodents (mice or rats), pigs, chickens, and humans, including one poultry VI strain and one human VI strain in each inoculum (see Table 3). We administered the same three inocula at standardized doses to germfree mice and germfree pigs, *Lactobacillus*-free (LF) chickens, and human volunteers (with low backgrounds of lactobacilli), and we determined cell numbers before (day 0) and daily for 5 days after administration via quantitative culture on *L. reuteri* isolation medium (LRIM) plates. As shown in Fig. 3, *L. reuteri* became detectable between 6.0 and 8.0 log₁₀ CFU/g of feces (or per swab) in all hosts within 1 day of oral administration. In ex-germfree mice, ex-LF chickens, and ex-germfree pigs, *L. reuteri* established populations reaching numbers comparable to those reached by *Lactobacillus* in conventional animals (22–24). In addition, in all three animal models, *L. reuteri* populations remained stable and reached around 10⁸ cells per gram of feces by day 5 postadministration. The fact that the numbers of bacterial cells recovered from the animals exceeded the numbers that were administered implies that the bacteria actively colonized the gastrointestinal tract. This was expected, given that animals were

FIG 1 Neighbor-joining phylogenetic tree of *Lactobacillus reuteri* based on the core genome alignment (900 genes) of 25 strains. Tips of the branches are color coded by lineage, and cohesive clades are labeled.

either germfree (mice and pigs) or treated with antibiotics (chickens), which is associated with reduced colonization resistance (25). In contrast, *L. reuteri* became detectable in human fecal samples at around $7.5 \log_{10}$ CFU/g at days 1 and 2 and subsequently continuously declined, decreasing to numbers that were slightly above the baseline by day 5. Therefore, it can be concluded that in humans *L. reuteri* shows high survival but only temporal persistence. These results were expected, as similar temporal patterns were previously observed for the persistence of *L. reuteri* in humans (26, 27).

Rodent isolates become enriched in mice. Host adaptation of *L. reuteri* strains to mice has been previously established (17–19). Therefore, *L. reuteri* strains from rodents were expected to become enriched in this model. As shown in Fig. 4, by day 2 (inocula A and B) or day 3 (inoculum C), the relative abundance of rodent strains was significantly higher ($P < 0.05$) than was the abundance of nonrodent lineages. Notably, in those animals receiving inoculum A, nearly all of the colonies typed from days 2 to 5 were identified as the rat isolate CR from rodent lineage I. The relative amounts of colonies typed per individual mouse by day 5 are shown in the adjacent bar graphs in Fig. 4. As expected, the strain CR was significantly enriched ($P < 0.05$) in mice that received inoculum A. Furthermore, 66% of the colonies recovered on day 5 from mice administered inoculum B belonged to rodent lineages and, compared to human II, poultry VI, and porcine V strains, the rodent I strain lpuph1 was significantly enriched ($P < 0.05$). Following the same trend, the rat isolate N2D of inoculum C represented 67% of the colonies typed on this day, being significantly higher ($P < 0.05$) than strains from nonrodent lineages. Together, these results demonstrated that administration of a mixture of strains and subsequent molecular typing (by sequencing the *leuS* gene) of random colonies from fecal cultures allowed us to determine which *L. reuteri* strains became enriched under competitive conditions. Thus, this experimental approach can be used to make accurate inferences about the ecological performance of different strains *in vivo*.

FIG 2 Graphic representation of the experimental design. Eighteen strains of different host origins and phylogenetic lineages were grouped into 3 different inocula containing 6 strains each. To facilitate differentiation, strains within the same inoculum were selected to carry distinct *leuS* alleles. Standardized inocula were prepared to contain equivalent cell numbers of each strain and administered to germfree mice ($n = 5$ per inoculum group), *Lactobacillus*-free chickens ($n = 5$ per inoculum group), germfree pigs ($n = 3$ per inoculum group), and humans with a low background of lactobacilli ($n = 5$ per inoculum group). Bacteria were cultured from the inocula and from fecal samples collected between days 1 and 5 after administration, and strain composition was determined by randomly typing colonies.

The chicken host. Molecular typing of *L. reuteri* colonies grown from cloacal swabs of chickens that received inoculum A indicated that, from days 3 to 5, the relative abundance of the poultry VI strain CSF8 was significantly higher ($P < 0.05$) than that of strains belonging to all other lineages. Similarly, in animals that received inoculum B, the poultry VI strain 1366 represented between 50 and 70% of the colonies typed from days 2 to 5. At day 3, the relative abundance of strain 1366 was significantly higher ($P < 0.05$) than that of all other strains except for human VI strain CF48-3A. Although the poultry VI strain JCM1081 did not become significantly enriched, it represented between 33 and 55% of the colonies recovered from chickens administered inoculum C. By day 5, strain CSF8 represented a significantly higher ($P < 0.05$) percentage of the colonies typed per animal. Similarly, strain 1366 was found significantly more frequently ($P < 0.05$) than the human II and rodent I and III strains. In animals that received inoculum C, the relative abundance of JCM1081 at day 5 (53%) was significantly higher ($P < 0.05$) than most other lineages, except human lineage II.

Overall, this analysis revealed that chicken VI strains were enriched during colonization of the chicken gut. Interestingly, we also found that human VI strains were good colonizers of this host, especially during the early colonization phase. For example, in birds receiving inoculum A, the relative abundances of poultry VI and human VI were not significantly different, and together these strains accounted for 80% (42.5% CSF8 and 37.5% M27U15) of the colonies typed on day 1 and 90% (55% and 36%) on day 2. No significant difference between the relative abundance of poultry VI strain 1366 and that of human VI strain CF48-3A of inoculum B was found at days 1, 3, or 5. Additionally,

FIG 3 Cell numbers of *L. reuteri* in fecal samples (mice, pigs, and humans) or cloacal swabs (chickens), determined by quantitative culture. Data are presented as the $\log_{10}$ CFU. Each data point represents a sample from individual animals or human volunteers, and horizontal bars represent means $\pm$ standard deviations.

at day 1 the relative abundance of the human VI isolate MM34-4A from inoculum C was slightly higher (43%) than that of the poultry isolate JCM1081 (33%).

The pig host. Contrary to results in mice and chickens, strains of pig origin (porcine VI) did not become significantly enriched ($P < 0.05$) in pigs at any point during the experimental period (Fig. 4). Although some rodent isolates became significantly enriched, this trend was not consistent across inocula, ruling out a competitive advantage of rodent isolates. For example, the strain CR from inoculum A was found in significantly larger amounts ($P < 0.05$) from days 2 to 5, but the strains Ipuph1 (inoculum B) and N2D (inoculum C) were significantly increased ($P < 0.05$) only at day 2. Overall, these results indicate that porcine strains do not possess a competitive advantage in their original host.

The human host. Human VI strains were present in the feces of human volunteers during the 5-day postinoculation period (Fig. 4) but were not significantly enriched. The only strains reaching significant enrichment ($P < 0.05$) were of porcine origin; however, a competitive advantage of these strains can be ruled out, as the trend was not

FIG 4 Stacked bar plots show the relative abundance of *L. reuteri* strains in the inocula and feces of mice, pigs, and humans and cloacal swabs of chickens at baseline and during days 1 to 5 after oral administration of 3 different inocula (A, B, and C). An asterisk denotes when a strain from a host-specific lineage was significantly more abundant ($P < 0.05$) than all strains from other lineages in the native host. A white star denotes when the percentages of poultry VI and human VI strains were not significantly different ($P < 0.05$) in chickens. A triangle indicates when a strain became significantly enriched in a nonnative host. Adjacent bar graphs show the means and standard errors of the relative strain abundance of each strain from the colonies typed at day 5. Individual data points represent the percent colonies typed in each animal or human volunteer. Groups labeled with different letters are significantly different ($P < 0.05$). Statistical significance was determined by a one-way ANOVA ($\alpha = 0.05$).

consistent across inocula and was only observed for 1 or 2 days. For example, the porcine strain jw2015 was enriched only at day 5 in volunteers that consumed inoculum A, and strain ATCC 53608 was enriched only on days 1 and 2 in volunteers that consumed inoculum C. Notably, human VI strains were essentially outcompeted in the human host. These findings indicated that *L. reuteri* strains originating from humans, regardless of their lineage, did not show elevated levels of persistence in the human gut.

Evolution and genome characteristics of lineage VI strains. To gain a deeper understanding of the evolution of lineage VI strains, we reconstructed the ancestral states within the *L. reuteri* phylogeny (Fig. 5). This analysis revealed that rodent lineages date back as far as 2 million years (event 0), predating all other lineages by at least 800,000 years. Human lineage II emerged 1.5 million years ago from a rodent ancestor (event 1). A host switch approximately 1 million years ago resulted in the emergence of the poultry lineage VI and porcine lineage IV (event 2). The porcine lineage V appeared much more recently, 96,000 years ago (event 3). Most importantly, all isolates

FIG 5 Inferred evolutionary history of *L. reuteri*-host associations. Ancestral states were inferred on the bacterial phylogeny, modified from a previous study (17). The tree is a maximum-likelihood reconstruction of a concatenated set of 7 single-copy genes from 116 strains. Colors represent host state on the tips of the tree and inferred states on ancestral nodes. Equivocal ancestral states are represented by mixed colors in the circle. Four host-switching events are highlighted as enlarged circles (labeled 1 to 4). The time scale is on a scale of thousands of years (kyrs), and estimates were obtained by using a Bayesian phylogenetic analyses.

of lineage VI share an ancestor associated with poultry, with the human isolates emerging more recently, less than 61,000 years ago (event 4) (Fig. 5).

Next, we determined the genomic characteristics of lineage VI strains by analyzing the genomes of three strains that originated from chickens (1366, CSF8, and JCM108) and four strains of human origin (SD2112, MM34-4A, M27U15, and CF48-3A). As shown in Fig. 6, all seven analyzed lineage VI strains shared 1,433 predicted orthologous genes. Beyond this core genome, poultry VI strains possessed an open pan-genome with large numbers of strain-specific genes (197 genes in strain 1366, 215 in CSF8, and 484 in JCM1081) and an average nucleotide identity (ANI) between 98.77% and 99.06%. This is in stark contrast to human VI strains, which showed very few unique genes and essentially a closed pan-genome (between 3 and 6 genes per strain) and an ANI between 99.92% and 99.99%. These findings are in line with the tight clustering of human VI strains in the phylogenetic tree shown in Fig. 1.

We then sought to identify genes specific to (i.e., not present in other *L. reuteri* genomes) and conserved in the genomes of all seven strains in lineage VI. We found only 3 genes, which encoded an aspartate racemase (EC 5.1.1.13) and two transcriptional regulators of the XRE and DeoR families. Next, we identified 28 genes that were conserved in the poultry VI strains and absent in the genomes of human VI strains. Of those, 10 were annotated as hypothetical proteins, 4 as phage related, 3 as c-di-GMP activation proteins via the GGDEF-EAL transduction system involved in biofilm formation (28), and 1 as a transcriptional regulator of the HxIR family. The remaining encoded

FIG 6 Core- and strain-specific gene content of *L. reuteri* lineage VI strains. The ovals represent the genomes of poultry VI (yellow) and human VI (orange) strains. The core gene set (genes unique to each strain) is indicated by the number in the center, and the value below it in parentheses is the number of unique lineage VI genes, compared with other *L. reuteri* strains (i.e., the value obtained after subtracting genes found in other *L. reuteri* strains).

proteins were involved in vitamin biosynthesis or sugar transporters/transferases. Notably, none of these genes appeared to be exclusive to poultry VI strains in relation to other *L. reuteri* genomes (Table 2).

Next, we examined whether human VI strains possessed genes not present in poultry VI strains. Of the 190 proteins identified through this process, 92 were annotated as hypothetical proteins and 61 as transposases, endonucleases, or phage related. Genes with a functional annotation that did not fall in those categories are listed in Table 2. Interestingly, 13 of the genes are colocalized in a putatively horizontally acquired gene cluster (flanked by transposase genes and with a 10% higher GC content than the genome average) that encodes membrane glycosyl transferases and transmembrane transporters predicted to be involved in capsular polysaccharide biosynthesis. Another interesting human VI-specific gene included a tetracycline resistance cassette, carried by plasmid pLR581 and previously described for strain *L. reuteri* SD2112 (29). Human VI strains also harbored three additional plasmids carrying genes for proteins involved in lincomycin resistance (pLR585), cadmium resistance (pLR584), and uncharacterized functions (pLR580). Not all the genes carried by these plasmids are exclusive to human VI strains. However, the replication proteins are conserved in all of the human VI genomes surveyed (Table 2), and the presence of these plasmids was confirmed via extraction and agarose gel visualization (data not shown).

DISCUSSION

In this study, we devised an experimental approach that allowed us to directly assess the competitive interactions among *L. reuteri* strains in the gastrointestinal tracts of different vertebrate hosts. By testing the relative enrichment of a specific strain, the assay allowed us to directly compare the abilities of strains to propagate under the ecological conditions of the gut. Since population growth directly relates to ecological fitness of microbes (12), an enrichment of strains from a particular phylogenetic lineage, in our models, does provide direct evidence for adaptedness. An interpretation of the findings can therefore be used to infer the evolutionary history of a phylogenetic lineage and the underlying mechanism that drove its diversification.

We tested this approach and confirmed host adaption of rodent isolates to the mouse gut. As shown previously (17, 18), colonization phenotypes were in agreement with phylogeny, as both mouse and rat strains showed similar ecological performance in mice. This suggests that rodent lineages are not evolutionarily confined to a specific host species but are adapted to specific gastrointestinal conditions that are expected to be very similar in mice, rats, and possibly other rodents (17). Similar trends have been observed for the bumble bee symbiont *Snodgrassella alvi*, which appears to have adapted to the host genera (*Bombus*) and not the species (30). Although much remains

TABLE 2 Genes specific to poultry VI and human VI strains

Lineage and gene ID no. ^a	Gene name or function
Poultry VI	
20805	GGDEF c-di-GMP synthetase
20806	EAL c-di-GMP phosphodiesterase
20821	Cytochrome heme/steroid domain
21815	Butanediol dehydrogenase
21816	Sugar phosphate permease
21916	UDP-glycosyltransferase
22288	Xanthine/uracil/VitC permease
22433	Thiamine phosphate synthase
22434	Hydroxymethylpyrimidine kinase
22959	HxlR transcriptional regulator
22963	Sugar transferase, LPS biosynthesis
23007	Hydroxyethylthiazole kinase
Human VI	
64061	dTDP-glucose 4,6-dehydratase/epimerase
64063	CDP-glycerol phosphotransferase
64064	Glycosyltransferase
64065	Wzx flippase
64066	Transmembrane protein
64067	Glycosyltransferase
64068	Glycosyltransferase
64069	Glycosyltransferase
64070	1,6-Galactosyltransferase
64071	Glycosyltransferase
64072	Glycosyltransferase
64073	Glycosyltransferase
64074	Nucleoside diphosphate sugar epimerase
64355	O-Acyltransferase
64358	Glycosyltransferase
64361	UDP-galactofuranosyltransferase
64432	SNF2 family helicase
64483	ABC transporter ATP-binding protein
64484	ABC transporter
64485	LytTR transcriptional regulator
64759	Glucan sucrose
65314	PglZ alkaline phosphatase
65474	Lantibiotic protection ABC transporter
65587	MarR transcriptional regulator
66329	CAAX amino protease (plasmid pLR585)
66330	MerR transcriptional regulator (pLR585)
66332	Replication protein (pLR585)
66333	Replication-associated protein (pLR585)
66334	Replication-associated protein (pLR585)
66336	Replication protein (pLR580)
66348	Tetracycline resistance protein Tet (pLR581)
66357	Initiator RepB protein (pLR581)
66364	Replication Rep protein (pLR584)
66370	Lysophospholipase family protein (pLR584)
66374	Transcriptional repressor CopY (pLR584)

^aThe Gene ID number is that assigned by JGI to *L. reuteri* SD2112 and *L. reuteri* JCM1081 for the genes specific to poultry VI and human VI, respectively. Gene IDs shown in boldface are exclusive to human VI strains, and genes in italics are colocalized at the Wzx-dependent capsular polysaccharide synthesis cluster. For nonchromosomal genes, the name of the harboring plasmid is shown in parentheses.

unknown regarding the dynamic evolutionary patterns and adaptive paths of *L. reuteri* strains within rodents, our findings in mice indicate that competition experiments with standardized mixtures of strains are appropriate to make accurate assessments of host adaption and therefore allow insight on the evolutionary outcomes of host-microbe interrelationships.

Lineage VI strains, even if isolated from humans, show elevated fitness in chickens. In agreement with previous studies (17, 31), the genome-wide phylogenetic analysis of *L. reuteri* showed that strains isolated from poultry cluster together with a subset of human isolates into one cohesive phylogenetic lineage (lineage VI). Although

we previously speculated about the natural history of this lineage (17), interpretation of phylogenies alone can lead to incorrect conclusions (32). Our experiments here now demonstrate that poultry VI strains are host adapted to chickens and that human VI strains also perform well in this host, while showing extremely low performance in humans. Accordingly, the genomic analysis revealed that human VI isolates are likely to possess all the colonization factors necessary to colonize the chicken gut, as only very few genes were present in poultry VI strains but absent from human VI strains (Table 2). Both poultry and human lineage VI strains possess lineage-specific putative response regulators, suggesting an important role of environmental sensing in the colonization of the chicken gastrointestinal tract. In this regard, a single regulatory gene has been shown to alter host specificity in *Vibrio fischeri* (33), and rodent-specific two-component systems are known to regulate biofilm formation of *L. reuteri* in mice (18, 19). These findings indicate that human VI isolates, like poultry VI isolates, share an evolutionary history with poultry. Accordingly, the immediate ancestral node of human VI strains was inferred to be associated with poultry (Fig. 5).

The above-mentioned findings beg the question of why have lineage VI strains been isolated from humans if the lineage has maintained stringent evolutionary ties with poultry and is host adapted? One possibility is that *L. reuteri* switched from poultry to humans and became permanently associated with this new host. However, in our human experiment, human VI strains were completely outcompeted, thus ruling out an adaptation to the human gastrointestinal tract. In addition, the majority of the human VI isolates originate from extraintestinal sources (breast milk, vagina, mouth), for which the species *L. reuteri* has not been described as a significant member (34, 35), which is contrary to the situation in poultry, where *L. reuteri* is autochthonous and an abundant member of the gut microbiota (23). Based on these considerations, we propose that specific strains from lineage VI can become transiently associated with humans. Microbial exchange between birds and humans is not only possible but frequent, as demonstrated by the 2.5 million cases per year of foodborne illnesses in the United States that arise from the transmission of pathogens from poultry, meat, and eggs (36). Interestingly, our genomic analysis revealed that poultry VI strains possess a large and adaptable pan-genome, while human VI strains show very little genomic variation. These findings suggest that essentially one single clone (<375 single nucleotide polymorphisms [SNPs] compared to *L. reuteri* SD2112) among lineage VI has been repeatedly isolated from humans. Similar phenomena have been observed for globally spread monomorphic pathogens (37), such as *Yersinia pestis*, in which the acquisition of a few horizontally acquired traits was sufficient to enable switching hosts (38). Under this scenario, and given the high number of human VI-specific mobile genetic elements found in the genomes of human VI strains, it is possible that this clone acquired specific traits that allowed a temporary migration to humans. The Wzx-dependent capsular polysaccharide biosynthetic gene cluster and the plasmid-carried antibiotic resistance cassette, which are both likely to be horizontally acquired, might represent key traits that allow transfer to humans. The ability of these strains to grow in the presence of tetracycline was confirmed experimentally (data not shown), and capsular molecules can induce or suppress host immune responses (39, 40). From this perspective, it is tempting to speculate about a scenario in which a specific *L. reuteri* clone was able evade the immune system and temporarily colonize the human host, presumably after a course of antibiotics.

Human and porcine isolates do not show elevated ecological fitness in their respective hosts. Human II strains cluster separately from all other *L. reuteri* strains. This lineage is both remarkably homogeneous and specific to humans (9, 17). However, our experiment did not provide sufficient evidence of adaptation of these strains to humans, even though they persisted longer than human VI strains. This finding suggests that the evolution of the human II lineage was not driven by specialized adaptations to the human gut. Another possible explanation is that these isolates have a nonhuman niche (i.e., environment, food, other hosts). However, the phylogenetic cohesiveness of these strains argues strongly against this scenario. It is widely docu-

mented that food- and plant-derived *Lactobacillus* strains are commonly isolated from human fecal samples, but these strains, unlike *L. reuteri* human II strains, are neither phenotypically nor phylogenetically related (41–43). We cannot rule out that the human resident microbiota could have prevented the experimental *L. reuteri* strains from becoming established. However, recent work provided evidence that a gut microbe can engraft in the human gut if an open niche is available (44). In this respect, it is important to point out that *L. reuteri* was commonly detected as a member of the human gut microbiota in studies conducted between 1950 and 1960 (45), while it is rarely detected among the human gut microbiota today (46–48). This suggests that *L. reuteri* might have been displaced as a dominant member of the human gut microbiota due to environmental changes (e.g., antibiotic usage, hygiene, and dietary practices) associated with modern lifestyles (9), as has been proposed for other members of the microbiota (49). Although speculative, this idea is supported by a recent study in which *L. reuteri* was found to be a dominant member of the microbiota of rural Papua New Guineans (50). From this perspective, the absence of the niche in which human II *L. reuteri* evolved would have prevented these strains from becoming enriched in our human experiment. Further work will be required to resolve outstanding questions regarding the evolution of *L. reuteri* with humans.

Contrary to the situation in modern humans, *L. reuteri* is a core member and one of the most abundant *Lactobacillus* species of the porcine microbiota (24), suggesting *L. reuteri* is a symbiont in pigs. However, a recent genome-wide comparative study did not find any host-specific genomic signatures among porcine isolates that clustered into two pig-confined phylogenetic clades (17, 21). This finding suggests that clustering of these strains could be driven by neutral evolution or ecological factors not directly related to the porcine host. Our data agree with this notion, as we found no evidence for host adaptation in pigs. Nevertheless, it is important to point out that our pig model is not exempt of limitations that could explain these observations. For example, it is conceivable that complex interactions with the microbiota (both cooperative and antagonistic) might underlie the adaptation of *L. reuteri* to pigs. *L. reuteri* is one member of a multispecies biofilm that forms on the squamous keratinized lining of the pig's pars esophageal tissue (22). In this sense, the germfree pig model might fail to recapitulate adaptive interactions between *L. reuteri* and coexisting members of the biofilms, such as *L. amylovorus* and *L. johnsonii*, which are the dominant *Lactobacillus* species in pigs (24). Overall, our findings suggest a direct coevolution of *L. reuteri* with rodents and chicken (potentially as a primary colonizer in the biofilms), while in pigs, the species may evolve in a tripartite interrelationship with the host and other microbes.

Another important factor to consider is that dietary glycans can have a direct effect on the composition of the pig's microbiota, including the abundance of lactobacilli (51). The infant formula fed to piglets contained galactooligosaccharides (GOS) that can be utilized by almost all *L. reuteri* strains independent of host origin (52). It is possible that porcine *L. reuteri* strains have evolved to utilize oligosaccharides in the milk of pigs and that this adaptation resulted in the host-confined phylogenetic clusters. A formula in which these carbohydrates are replaced by the nonselective GOS would have rendered insignificant strain-specific differences in the ability to utilize milk carbohydrates, potentially removing the ecological advantage of porcine isolates. Additional animal experiments will ultimately be necessary to derive clear conclusions on the existence of host adaptation of *L. reuteri* to pigs and the mechanisms by which the porcine-specific lineages arose.

Limitations of this study. It is extremely difficult to experimentally replicate the ecological conditions under which bacteria evolve, which are often dynamic and subject to change. The animal host is an excellent replication of the natural habitat of a gut symbiont, but as described above, both germfree and conventional models have limitations. Germfree models fail to replicate the interrelationships between members of the community, which might be especially relevant in the evolution of *L. reuteri* as a component of biofilms. On the other hand, in hosts with a conventional microbiota,

TABLE 3 *L. reuteri* strains used in the host adaptation assay

Inoculum group and strain	Host of isolation	Lineage ^a	<i>leuS</i> allelic profile ^a
Inoculum A			
Cf46g	Human	II	4
mlc3	Mouse	III	31
CR	Rat	I	14
CSF8	Chicken	VI	15
M27U15	Human	VI	11
jw2015	Pig	IV	3
Inoculum B			
MM4-1a (ATCC PTA6475)	Human	II	4
r2lc	Rat	III	35
lpuph1	Mouse	I	9
1366	Chicken	VI	6
CF48-3A	Human	VI	11
lpa1	Pig	IV	3
Inoculum C			
sr11	Human	II	4
N2D	Rat	III	34
6799jm1	Mouse	I	9
JCM1081	Chicken	VI	24
MM34-4A	Human	VI	11
ATCC 53608	Pig	IV	3

^aLineage and *leuS* type were determined as described in reference 17.

such as the humans in our study, the niche might already be occupied by more resilient taxa, thus preventing the establishment of external strains. Future studies should be devoted to apply this experimental system to more refined models, such as gnotobiotic animals, especially those containing species that coexist with *L. reuteri* in natural settings.

Conclusion. Results from this study contribute to our understanding of the evolutionary history of *L. reuteri*, a vertebrate gut symbiont for which specific adaptations have now been experimentally proven in different hosts. This work also expands our knowledge about the various lifestyles and the array of selective pressures shaping the evolution of *Lactobacillus* species. For example, the adapted lifestyle of *L. reuteri* sharply contrasts with that of the generalists *L. plantarum* (43), *L. paracasei* (41), and *L. rhamnosus* (53). This aspect can be particularly important in the selection of probiotics, as functional attributes can be directly related to the evolution of particular strains and the nature of the symbiotic relationship maintained with different hosts (31). Survival and persistence in the digestive system might also be desirable traits for some probiotic applications. Therefore, our findings also provide an ecological and evolutionary basis for the selection of strains for probiotic applications in different hosts.

MATERIALS AND METHODS

Strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 3 and were grown at 37°C in deMan Rogosa Sharpe medium supplemented with 10 g/liter maltose and 5 g/liter fructose (mMRS). To ensure selective cultivation from feces, an LRIM was devised based on the recipe of the Rogosa medium (54). The LRIM contained, per liter, 15 g of raffinose, 15 g of sodium acetate, 15 g of agar, 10 g of tryptone, 6 g of KH₂PO₄, 5 g of yeast extract, 2 g of ammonium acetate, 1.32 ml of glacial acetic acid, 1 g of Tween 80, 0.57 g of MgSO₄, 0.12 g of MnSO₄, 0.003 g of FeSO₄. Since only a limited number of *Lactobacillus* species grow on raffinose (55), this medium allowed for a sufficiently selective culture of *L. reuteri* in the presence of a background fecal microbiota. To narrow the number of *Lactobacillus* species able to grow in LRIM, incubations were performed under anaerobic conditions (<0.1% O₂, ≥15% CO₂) at 45°C for 48 h (55).

Preparation of strain mixtures to prepare inocula. Eighteen *L. reuteri* strains originating from different hosts and assigned to separate phylogenetic lineages were selected and assigned to one of three inoculum groups (Table 3). To facilitate differentiation, strains within the same inoculum were selected to carry distinct *leuS* alleles. Inocula for host experiments were prepared by growing individual strains overnight in mMRS, followed by subculture (with 1% inoculum each) twice in previously boiled (100°C for 30 min) food-grade DE-PHAGE medium (Cargill) supplemented with 20 g/liter of malt. Cell numbers of each individual strain after growth for 16 h in DE-PHAGE medium were determined in at least

six replicate experiments. This information was used to prepare standardized inocula with equivalent proportions of each strain and adjusted to contain approximately 3×10^5 cells of total *L. reuteri* per gram of host body weight (BW). In order to determine if these conditions were met, cell numbers of each individual strain were determined by quantitative culture on mMRS prior to generating the inocula.

Mouse experiment. Germfree C3H/HeN mice (~30 g BW) were maintained at the University of Nebraska Gnotobiotic Mouse Facility. Groups of mice ($n = 5$) were assigned to receive one of the three inocula and subsequently moved from sterile isolators into individual ventilated biocontainment cages. To prepare the inocula, *L. reuteri* strains grown for 16 h on DE-PHAGE medium were harvested by centrifugation ($3,000 \times g$ for 10 min) and washed twice with sterile phosphate-buffered saline (PBS; pH 7.0). Each mouse received via gavage 100 μ l of PBS suspension containing a total of 3×10^5 cells per gram of BW. *L. reuteri* was enumerated on LRIM from fresh fecal pellets collected immediately prior and daily for 5 days after gavage. To ensure gnotobiotic conditions were maintained during the experiments, three mice were gavaged with sterile PBS and housed in separate biocontainment cages located in the same ventilator rack as the experimental mice. Fecal pellets from control mice were plated daily on brain heart infusion (BHI) and mMRS media and checked for aerobic and anaerobic growth. All procedures were conducted with approval from the Institutional Animal Care and Use Committee of the University of Nebraska (project 731).

Chicken experiment. Specific-pathogen-free Leghorn chickens were hatched in the Poultry Research Facility at the University of Alberta and transported the same day to the Animal Research Facility of the same institution. Birds were randomly assigned to an inoculum group ($n = 5$ per group) or PBS control (4 birds). Chickens were housed in pairs or groups of three and maintained in biocontainment cages. In order to obtain LF chickens, penicillin was added to the drinking water at a concentration of 0.6 g/liter. Four days after penicillin treatment commenced, the absence of lactobacilli was confirmed by plating cloacal swabs on Rogosa medium (Difco) and LRIM. Antibiotic administration was removed 18 h prior to LF chickens (~30 g BW) undergoing gavage with 300 μ l of PBS containing a standardized *L. reuteri* inoculum with a total of 3×10^5 cells per gram of BW. Cloacal samples were obtained from each animal immediately prior to (day 0) and for 5 days after gavage. Upon collection, cloacal samples were transferred into 1.5-ml tubes containing sterile PBS with 10% (vol/vol) glycerol and immediately processed by dilution plating on LRIM. All procedures were carried out in accordance with protocol AUP00000003, approved by the University of Alberta's Animal Care and Use Committee.

Pig experiment. Nine germfree piglets were delivered from a full-term pregnant sow by sterile hysterectomy following the methods described by Miniats and Jol (56) and aseptically transferred into one of three sterile polyvinyl flexible isolators. Animals in the same isolator ($n = 3$) were kept in separate stainless steel compartments with false floors to collect excreta. Isolators were maintained under positive pressure at an ambient temperature of 35°C and ventilated with sterilized filtered air prewarmed to the same temperature. Piglets were fed commercially sterile infant formula throughout the experiment. At 10 days of age, piglets (~3 kg BW) in the same isolator were administered either inoculum A, B, or C containing *L. reuteri* at 3×10^5 cells per gram of BW and which was suspended in the feeding infant formula. Cell counts of *L. reuteri* were determined by quantitative LRIM culture of fecal samples obtained directly from the pig's rectum using sterile plastic loops and collected before treatment (day 0) and at days 1 to 5 postadministration. Fecal samples were also plated on BHI to detect any contamination. Aerobic growth on BHI was detected in the feces of one of the treatment groups on day 3 to 5 postinoculation. However, *L. reuteri* counts were not different from those of pigs in the other groups. All procedures were conducted with approval of the Institutional Animal Care and Use Committee of the University of Nebraska (project 939).

Human subjects. Fecal samples from 20 human subjects were screened for growth on LRIM. Of those subjects, 15 (8 females, 7 males) produced less than 10^4 CFU per gram of feces and were considered eligible for the study. Subjects were then randomly assigned to receive one of the three inocula ($n = 5$ per inoculum group). All subjects were between the ages of 18 and 55 years, abstained from using probiotic and prebiotic products, had not consumed oral antibiotics within 3 months before the study, and considered themselves healthy.

Inocula were prepared in the food laboratory of the Department of Agricultural, Food, and Nutritional Science at the University of Alberta. Briefly, *L. reuteri* strains were grown for 16 h on DE-PHAGE medium and mixed to contain a total of 3×10^5 cells per gram of BW (based on an average BW of 75 kg). Cells were harvested by centrifugation at $3,000 \times g$ for 5 min and suspended in bottled spring water immediately prior to consumption. Subjects were instructed to drink contents of the solution in a single setting. Fecal samples were collected daily from day 0 (before consumption) and during days 1 to 5 postconsumption. Samples were processed within 2 h of deposition, and *L. reuteri* was cultured by quantitative dilution plating on LRIM. Human studies were completed at the University of Alberta in accordance with protocol Pro00051493, approved by The Health Research Ethics Board biomedical panel.

Strain typing and identification. Sixteen colonies were randomly selected from LRIM plates for each fecal sample (or cloacal swab) cultured from day 1 to day 5 postinoculation and typed by colony PCR. The *leuS* gene was directly amplified from each colony with the primers *leuS*-F, TACGACGCGGCAGATAC, and *leuS*-R, ATAGAGATCAACTGGTGACC. PCR conditions were described previously (17) and were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1 min, with a final extension of 7 min at 72°C. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using Sanger technology. Sequences were assigned to a strain by BLASTn search against a local nucleotide database implemented in Bioedit (57).

Genome sequencing and annotation. Genomic DNA from the strains *L. reuteri* 1366, CSF8, JCM1081 (poultry VI) and MM34-4A and M27U15 (human VI) was obtained using the Qiagen DNeasy blood and tissue kit with some modifications as described by Oh et al. (17). Genomes were sequenced to draft status at The Applied Genomics Centre (TAGC; University of Alberta) by using Illumina MiSeq paired-end technology. Reads were assembled into scaffolds using SPAdes (58) available in the PATRIC program (59), annotated using the Joint Genome Institute (<http://jgi.doe.gov>) pipeline and deposited.

Comparative genomic and phylogenetic analyses. Comparative analysis of genome sequences was performed in EDGAR (60) based on an all-against-all comparison of the predicted proteomes (GenBank) downloaded from the JGI. Unique and lineage-specific genes were determined using the “Singleton” and “Calculate genesets” functions in EDGAR version 2.2 (60) and confirmed with the IMG phylogenetic profiler (61). Single nucleotide polymorphisms were detected with Mauve (62) and the average nucleotide identity (ANI) was calculated in EDGAR (60). The core genome was calculated as the set of orthologous genes present in all strains by bidirectional best BLAST hits. Phylogeny was constructed by aligning 900 core orthologous genes present in all the genomes sequenced in this study and others available in the public databases (Table 1). Concatenated sequences were used to calculate a distance matrix, which provided the input for the neighbor-joining method in the PHYLIP package as implemented in EDGAR (60). The tree was drawn and annotated using Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Ancestral state analysis. To infer the order of emergence of host lineages, the sequences of seven genes from 116 strains used in previous multilocus sequence analysis (17) were analyzed using Mesquite 3.2 (63) as previously described (64). *Lactobacillus fermentum* was used as an outlier. The host of each strain was assigned as rodent, swine, poultry, or human based on the origin of isolation (17). Ancestral states were inferred using parsimony. Host switch events were identified when two or more equally parsimonious ancestral state reconstructions were found, or when the host state of the immediate ancestral node was different from the offspring. The putative dates of host switch events were estimated using Bayesian phylogenetic analyses in BEAST v.2.4.3 (65) with the HKY85 substitution model, an estimated clock rate of 10^{-8} , and the calibrated Yule model.

Statistical analysis. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test ($\alpha = 0.05$) implemented in the statistical package Prism version 6.0 (GraphPad Software, La Jolla, CA, USA).

Accession number(s). The genome sequences of *L. reuteri* sequenced in this study were deposited in the Integrated Microbial Genomes system (IMG) (61). Genome IDs are provided in Table 1. Genome sequences are also available in GenBank under BioProject ID [PRJNA380292](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA380292).

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REFERENCES

- Moran NA. 2006. Symbiosis. *Curr Biol* 16:R866–R871. <https://doi.org/10.1016/j.cub.2006.09.019>.
- Stevens CE, Hume ID. 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiol Rev* 78:393–427.
- Dethlefsen L, McFall-Ngai M, Relman DA. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449:811–818. <https://doi.org/10.1038/nature06245>.
- Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JL. 2008. Evolution of mammals and their gut microbes. *Science* 320:1647–1651. <https://doi.org/10.1126/science.1155725>.
- Ochman H, Worobey M, Kuo C-H, Ndjanga J-BN, Peeters M, Hahn BH, Hugenholtz P. 2010. Evolutionary relationships of wild hominids recapitulated by gut microbial communities. *PLoS Biol* 8:e1000546. <https://doi.org/10.1371/journal.pbio.1000546>.
- Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JL. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol* 6:776–788. <https://doi.org/10.1038/nrmicro1978>.
- Fraune S, Bosch TCG. 2010. Why bacteria matter in animal development and evolution. *Bioessays* 32:571–580. <https://doi.org/10.1002/bies.200900192>.
- Rosenberg E, Zilber-Rosenberg I. 2016. Microbes drive evolution of animals and plants: the hologenome concept. *mBio* 7:e01395-15. <https://doi.org/10.1128/mBio.01395-15>.
- Walter J, Britton RA, Roos S. 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. *Proc Natl Acad Sci U S A* 108(Suppl):4645–4652. <https://doi.org/10.1073/pnas.1000099107>.
- Falush D, Wirth T, Linz B, Pritchard JK, Stephens M, Kidd M, Blaser MJ, Graham DY, Vacher S, Perez-Perez GI, Yamaoka Y, Mégraud F, Otto K, Reichard U, Katzowitsch E, Wang X, Achtman M, Suerbaum S. 2003. Traces of human migrations in *Helicobacter pylori* populations. *Science* 299:1582–1585. <https://doi.org/10.1126/science.1080857>.
- Moeller AH, Caro-Quintero A, Mjunga D, Georgiev AV, Lonsdorf EV,

- Muller MN, Pusey AE, Peeters M, Hahn BH, Ochman H. 2016. Cospeciation of gut microbiota with hominids. *Science* 353:380–382. <https://doi.org/10.1126/science.aaf3951>.
12. Garcia JR, Gerardo NM. 2014. The symbiont side of symbiosis: do microbes really benefit? *Front Microbiol* 5:510. <https://doi.org/10.3389/fmicb.2014.00510>.
13. Dale C, Moran NA. 2006. Molecular interactions between bacterial symbionts and their hosts. *Cell* 126:453–465. <https://doi.org/10.1016/j.cell.2006.07.014>.
14. Kwong WK, Engel P, Koch H, Moran NA. 2014. Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc Natl Acad Sci U S A* 111:11509–11514. <https://doi.org/10.1073/pnas.1405838111>.
15. Wollenberg MS, Ruby EG. 2012. Phylogeny and fitness of *Vibrio fischeri* from the light organs of *Euprymna scolopes* in two Oahu, Hawaii populations. *ISME J* 6:352–362. <https://doi.org/10.1038/ismej.2011.92>.
16. Bongrand C, Koch EJ, Moriano-Gutierrez S, Cordero OX, McFall-Ngai M, Polz MF, Ruby EG. 2016. A genomic comparison of 13 symbiotic *Vibrio fischeri* isolates from the perspective of their host source and colonization behavior. *ISME J* 10:2907–2917. <https://doi.org/10.1038/ismej.2016.69>.
17. Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, Walter J. 2010. Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. *ISME J* 4:377–387. <https://doi.org/10.1038/ismej.2009.123>.
18. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, Zhang M, Oh PL, Heng NCK, Patil PB, Juge N, MacKenzie DA, Pearson BM, Lapidus A, Dalin E, Tice H, Goltsman E, Land M, Hauser L, Ivanova N, Kyrpides NC, Walter J. 2011. The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. *PLoS Genet* 7:16. <https://doi.org/10.1371/journal.pgen.1001314>.
19. Frese SA, Mackenzie DA, Peterson DA, Schmaltz R, Fangman T, Zhou Y, Zhang C, Benson AK, Cody LA, Mulholland F, Juge N, Walter J. 2013. Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. *PLoS Genet* 9:e1004057. <https://doi.org/10.1371/journal.pgen.1004057>.
20. Doolittle WF, Zhaxybayeva O. 2009. On the origin of prokaryotic species. *Genome Res* 19:744–756. <https://doi.org/10.1101/gr.086645.108>.
21. Wegmann U, MacKenzie DA, Zheng J, Goessmann A, Roos S, Swarbrick D, Walter J, Crossman LC, Juge N. 2015. The pan-genome of *Lactobacillus reuteri* strains originating from the pig gastrointestinal tract. *BMC Genomics* 16:1023. <https://doi.org/10.1186/s12864-015-2216-7>.
22. Tannock GW. 1992. The lactic microflora of pigs, mice and rats, p 21–48. *In* Wood BJB (ed), *The lactic acid bacteria*, vol 1. The lactic acid bacteria in health and disease. Springer US, Boston, MA.
23. Abbas Hilmi HT, Surakka A, Apajalahti J, Saris PEJ. 2007. Identification of the most abundant *Lactobacillus* species in the crop of 1- and 5-week-old broiler chickens. *Appl Environ Microbiol* 73:7867–7873. <https://doi.org/10.1128/AEM.01128-07>.
24. Leser TD, Amenuvor JZ, Jensen TK, Lindecrone RH, Boye M, Møller K. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microbiol* 68:673–690. <https://doi.org/10.1128/AEM.68.2.673-690.2002>.
25. Lawley TD, Walker AW. 2013. Intestinal colonization resistance. *Immunology* 138:1–11. <https://doi.org/10.1111/j.1365-2567.2012.03616.x>.
26. Frese SA, Hutkins RW, Walter J. 2012. Comparison of the colonization ability of autochthonous and allochthonous strains of lactobacilli in the human gastrointestinal tract. *Adv Microbiol* 2:399–409. <https://doi.org/10.4236/aim.2012.23051>.
27. Rattanaprasert M, Roos S, Hutkins RW, Walter J. 2014. Quantitative evaluation of synbiotic strategies to improve persistence and metabolic activity of *Lactobacillus reuteri* DSM 17938 in the human gastrointestinal tract. *J Funct Foods* 10:85–94. <https://doi.org/10.1016/j.jff.2014.05.017>.
28. Gjermansen M, Ragas P, Tolker-Nielsen T. 2006. Proteins with GGDEF and EAL domains regulate *Pseudomonas putida* biofilm formation and dispersal. *FEMS Microbiol Lett* 265:215–224. <https://doi.org/10.1111/j.1574-6968.2006.00493.x>.
29. Rosander A, Connolly E, Roos S. 2008. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Appl Environ Microbiol* 74:6032–6040. <https://doi.org/10.1128/AEM.00991-08>.
30. Kwong WK, Moran NA. 2015. Evolution of host specialization in gut microbes: the bee gut as a model. *Gut Microbes* 6:214–220. <https://doi.org/10.1080/19490976.2015.1047129>.
31. Spinler JK, Sontakke A, Hollister EB, Venable SF, Oh PL, Balderas MA, Saulnier DMA, Mistretta T-A, Devaraj S, Walter J, Versalovic J, Highlander SK. 2014. From prediction to function using evolutionary genomics: human-specific ecotypes of *Lactobacillus reuteri* have diverse probiotic functions. *Genome Biol Evol* 6:1772–1789. <https://doi.org/10.1093/gbe/evu137>.
32. Moran NA, Sloan DB. 2015. The hologenome concept: helpful or hollow? *PLoS Biol* 13:e1002311. <https://doi.org/10.1371/journal.pbio.1002311>.
33. Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG. 2009. A single regulatory gene is sufficient to alter bacterial host range. *Nature* 458:215–218. <https://doi.org/10.1038/nature07660>.
34. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 108(Suppl 1):4680–4687. <https://doi.org/10.1073/pnas.100261107>.
35. Jost T, Lacroix C, Braegger C, Chassard C. 2013. Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *Br J Nutr* 110:1253–1262. <https://doi.org/10.1017/S0007114513000597>.
36. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM. 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerg Infect Dis* 19:407–415. <https://doi.org/10.3201/eid1903.111866>.
37. Achtman M. 2012. Insights from genomic comparisons of genetically monomorphic bacterial pathogens. *Philos Trans R Soc B Biol Sci* 367:860–867. <https://doi.org/10.1098/rstb.2011.0303>.
38. Achtman M, Zurth K, Morelli G, Torrea G, Guiryoulle A, Carniel E. 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* 96:14043–14048. <https://doi.org/10.1073/pnas.96.24.14043>.
39. Comstock LE, Kasper DL. 2006. Bacterial glycans: key mediators of diverse host immune responses. *Cell* 126:847–850. <https://doi.org/10.1016/j.cell.2006.08.021>.
40. Willis LM, Whitfield C. 2013. Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. *Carbohydr Res* 378:35–44. <https://doi.org/10.1016/j.carres.2013.05.007>.
41. Smokvina T, Wels M, Polka J, Chervaux C, Brisse S, Boekhorst J, Vlieg JET van, Siezen HRJ. 2013. *Lactobacillus paracasei* comparative genomics: towards species pan-genome definition and exploitation of diversity. *PLoS One* 8:e68731. <https://doi.org/10.1371/journal.pone.0068731>.
42. Siezen RJ, Tzeneva VA, Castioni A, Wels M, Phan HTK, Rademaker JLW, Starrenburg MJC, Kleerebezem M, Molenaar D, van Hylckama Vlieg JET. 2010. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. *Environ Microbiol* 12:758–773. <https://doi.org/10.1111/j.1462-2920.2009.02119.x>.
43. Martino ME, Bayjanov JR, Caffrey BE, Wels M, Joncour P, Hughes S, Gillet B, Kleerebezem M, van Hijum SAFT, Leulier F. 2016. Nomadic lifestyle of *Lactobacillus plantarum* revealed by comparative genomics of 54 strains isolated from different habitats. *Environ Microbiol* 18:4974–4989. <https://doi.org/10.1111/1462-2920.13455>.
44. Maldonado-Gómez MX, Martínez I, Bottacini F, O'Callaghan A, Ventura M, van Sinderen D, Hillmann B, Vangay P, Knights D, Hutkins RW, Walter J. 2016. Stable engraftment of *Bifidobacterium longum* AH1206 in the human gut depends on individualized features of the resident microbiome. *Cell Host Microbe* 20:515–526. <https://doi.org/10.1016/j.chom.2016.09.001>.
45. Reuter G. 2001. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr Issues Intest Microbiol* 2:43–53.
46. Walter J. 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Appl Environ Microbiol* 74:4985–4996. <https://doi.org/10.1128/AEM.00753-08>.
47. Martínez I, Müller CE, Walter J. 2013. Long-term temporal analysis of the human fecal microbiota revealed a stable core of dominant bacterial species. *PLoS One* 8:e69621. <https://doi.org/10.1371/journal.pone.0069621>.
48. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto J-M, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen

- K, Pedersen O, Parkhill J, Weissenbach J, et al. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464:59–65. <https://doi.org/10.1038/nature08821>.
49. Blaser MJ, Falkow S. 2009. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* 7:887–894. <https://doi.org/10.1038/nrmicro2245>.
50. Martínez I, Stegen JC, Maldonado-Gómez MX, Eren AM, Siba PM, Greenhill AR, Walter J. 2015. The gut microbiota of rural Papua New Guineans: composition, diversity patterns, and ecological processes. *Cell Rep* 11: 527–538. <https://doi.org/10.1016/j.celrep.2015.03.049>.
51. Frese SA, Parker K, Calvert CC, Mills DA. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* 3:28. <https://doi.org/10.1186/s40168-015-0091-8>.
52. Goin CD. 2010. Growth of lactic acid bacteria: influence of protozoan cooperation, bacteriophage infection, and prebiotic carbohydrates. Master's dissertation. University of Nebraska—Lincoln, Lincoln, NE.
53. Douillard FP, Ribbera A, Kant R, Pietilä TE, Järvinen HM, Messing M, Randazzo CL, Paulin L, Laine P, Ritari J, Caggia C, Lähdeinen T, Brouns SJJ, Satokari R, von Ossowski I, Reunanen J, Palva A, de Vos WM. 2013. Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain GG. *PLoS Genet* 9:e1003683. <https://doi.org/10.1371/journal.pgen.1003683>.
54. Rogosa M, Mitchell JA, Wiseman RF. 1951. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J Bacteriol* 62:132–133.
55. Pot B, Felis GE, Bruyne De K, Tsakalidou E, Papadimitriou K, Leisner J, Vandamme P. 2014. The genus *Lactobacillus*, p 249–353. In Holzapfel WH, Wood BJB (ed), *Lactic acid bacteria*. John Wiley & Sons, Ltd., Chichester, United Kingdom.
56. Miniats OP, Jol D. 1978. Gnotobiotic pigs: derivation and rearing. *Can J Comp Med Rev* 42:428–437.
57. Hall T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98.
58. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Pribelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 19:455–477. <https://doi.org/10.1089/cmb.2012.0021>.
59. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, Gillespie JJ, Gough R, Hix D, Kenyon R, Machi D, Mao C, Nordberg EK, Olson R, Overbeek R, Pusch GD, Shukla M, Schulman J, Stevens RL, Sullivan DE, Vonstein V, Warren A, Will R, Wilson MJC, Yoo HS, Zhang C, Zhang Y, Sobral BW. 2014. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Res* 42:D581–D591. <https://doi.org/10.1093/nar/gkt1099>.
60. Blom J, Kreis J, Spänig S, Juhre T, Bertelli C, Ernst C, Goesmann A. 2016. EDGAR 2.0: an enhanced software platform for comparative gene content analyses. *Nucleic Acids Res* 44:W22–W28. <https://doi.org/10.1093/nar/gkw255>.
61. Markowitz VM, Szeto E, Palaniappan K, Grechkin Y, Chu K, Chen I-MA, Dubchak I, Anderson I, Lykidis A, Mavromatis K, Ivanova NN, Kyrpides NC. 2008. The integrated microbial genomes (IMG) system in 2007: data content and analysis tool extensions. *Nucleic Acids Res* 36:D528–D533. <https://doi.org/10.1093/nar/gkm846>.
62. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. <https://doi.org/10.1371/journal.pone.0011147>.
63. Maddison WP, Maddisson DR. 2017. Mesquite: a modular system for evolutionary analysis, version 3.2. <http://mesquiteproject.org>.
64. Sachs J, Skophammer R, Regus J. 2011. Evolutionary transitions in bacterial symbiosis. *Proc Natl Acad Sci U S A* 108:10800–10807. <https://doi.org/10.1073/pnas.1100304108>.
65. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput Biol* 10:e1003537. <https://doi.org/10.1371/journal.pcbi.1003537>.