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Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm

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Vertebrates engage in symbiotic associations with vast and complex microbial communities that colonize their gastrointestinal tracts. Recent advances have provided mechanistic insight into the important contributions of the gut microbiome to vertebrate biology, but questions remain about the evolutionary processes that have shaped symbiotic interactions in the gut and the consequences that arise for both the microbes and the host. Here we discuss the biological principles that underlie microbial symbiosis in the vertebrate gut and the potential of the development of mutualism. We then review phylogenetic and experimental studies on the vertebrate symbiont *Lactobacillus reuteri* that have provided novel insight into the ecological and evolutionary strategy of a gut microbe and its relationship with the host. We argue that a mechanistic understanding of the microbial symbiosis in the vertebrate gut and its evolution will be important to determine how this relationship can go awry, and it may reveal possibilities by which the gut microbiome can be manipulated to support health.

vertebrate symbiont | microbiota | mutualism

Prokaryotic microorganisms arose more than 3 billion y ago and have diversified to occupy virtually all environments that became available. Multicellular eukaryotes appeared later, and their bodies provided new habitats for microbial exploitation. The microbial communities that inhabit the digestive tracts of vertebrates are especially impressive both in sheer number and complexity. The largest populations are found in the digestive tracts of mammals, which can contain 10^{10} to 10^{12} cells/mL (e.g., in the rumen and large intestines), the highest cell numbers recorded for any known microbial ecosystem (1). These communities are comprised of thousands of species, and their diversity and metabolic capacity is specific for a particular host animal and gut segment. Technical advances in the molecular characterization of the gut microbiota and work with animal models have begun to provide insight into the diversity of these communities and their interactions with the host (2–6). Although most gut microbes are not pathogenic, it is now widely appreciated that they are of significant importance for the health and performance of their vertebrate host (7, 8).

To gain a deeper understanding of the microbial populations in the vertebrate digestive tract and their interactions with the host, it is instructive to consider the biological principles that underlie these partnerships in an evolutionary context. In this review, we will attempt such an approach by first discussing the basic characteristics of microbial symbiosis in the vertebrate gut before we use findings obtained with the Gram-positive bacterium *Lactobacillus reuteri* as a paradigm to gain insight into the ecology, evolution, and biological role of vertebrate gut microbes.

Symbiosis with Trillions of Partners

At birth, the vertebrate digestive tract is sterile but becomes rapidly colonized by a microbial population that, after a period of initial fluctuations, remains remarkably stable and resilient over time (9). This relationship can be referred to as symbiosis (from Greek *sym* "with" and *biosis* "living"), a term that describes close and long-term interactions between unlike organisms (10). Once considered a rare phenomenon, microbial symbiosis is gaining recognition as a ubiquitous feature in animal life (11). According to the fitness effects on the host, symbiotic relationships can be everything from beneficial to detrimental. This broad definition of symbiosis is not universally agreed on, and some researchers prefer to reserve the term solely for mutualistic interactions. However, when Anton de Bary introduced the term in the mid-nineteenth century, he characterized symbiosis as "specific cases of parasitism and mutualism" (10).

In this article we will follow de Bary's original definition and refer to symbiosis as an umbrella term for mutualistic, commensal, or parasitic relationships, including all of the interactions for which the full spectrum of effects on the host is simply not known. Using the term in this context is appropriate when referring to individual members of the gut microbiota because there is currently no scientific consensus on which microbial taxa constitute the mutualistic and pathogenic components within this community. Although many scientists have attempted to make such categorizations, they remain hypothetical (and indeed difficult to prove). One has also to consider that symbiotic relationships in the vertebrate gut exist on a continuum between mutualism and parasitism dependent on the host's genetic background and environmental factors. The net effect of the gut microbiota, however, is beneficial, and of critical importance for vertebrate biology.

Gut microbes were pivotal in the emergence of herbivorous lifestyles in mammals and birds (12). Vertebrate genomes harbor a very limited repertoire of glycosylhydrolases, and it is the microbes that confer metabolic traits to extract energy from the fibrous portion of plants, such as leaves, petioles, and stems (13). The energy contributions through microbial metabolism, which are to a large degree through the provision of short chain fatty acids (SCFA), is significant in many vertebrate species, ranging from ≈70% in ruminants, 20–30% for several omnivorous animals, and 10% for humans (14). Another important attribute conferred by the gut microbiota is the capacity to prevent enteric disease by pathogenic microorganisms, a trait referred to as colonization re-

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sistance or microbial interference (9, 15). Vertebrate gut microbes further contribute to epithelial barrier function, the provision of vitamins, detoxification of xenobiotic compounds, angiogenesis, and the development and maturation of the immune system (7). The significant benefits provided by the gut microbiota demonstrate that it is conceptually questionable to dismiss this symbiosis as mere commensalism.

To Live and Let Live: The Evolution of Mutualism

In symbiotic relationships, selection pressure on the host has the potential to lead to the improvement of beneficial traits in both partners (11). Such mutualistic relationships are extremely well understood in vertically transmitted symbionts of insects, such as *Buchnera aphidicola* in aphids (16). These symbionts have been stably associated with their host species over evolutionary time, as indicated by concurrent phylogenetic trees (17–20). In most cases, the microbes produce essential nutrients for the insects, whereas the latter have evolved specialized cells or organs to house them and to facilitate vertical transmission (18–20). This evolutionary process often results in strong interdependencies and can be described as coevolution in the sense that both parties have evolved so as to sustain their mutualistic relationship (11, 16).

It is easy to envision how the crucial contributions of the gut microbiota to vertebrate fitness (e.g., nutrient provision, pathogen exclusion, and immune maturation) would constitute phenotypic traits on which host selection could act (21). There are many features of vertebrates and their microbes that give testimony for an evolutionary alliance. Vertebrates possess specialized organs (foreguts, hindguts, ceca, enlarged crops in herbivorous birds) that facilitate microbial fermentation of plant materials (13). Furthermore, an extensive gut-associated mucosal immune system has evolved to regulate and maintain beneficial microbial communities (22, 23). Further evidence for human evolution with gut microbes arises from the presence of a large array of complex oligosaccharides in breast milk. These structures have no obvious nutritional value to the infant, but have likely emerged to support the growth of bacteria that benefit the infant (24). In parallel, gut bacteria have evolved elaborate systems that facilitate their own survival but which also benefit the host. One example is the ability of microbes to ferment complex polysaccharides to SCFA, which are then absorbed by the host and fulfill trophic functions (24–26). In addition, SCFA and other allelopathic compounds produced by gut bacteria benefit the host by inhibiting pathogens (15, 27). Finally, symbionts have evolved more specific factors, such as the polysaccharide A (PSA) of *Bacteroides fragilis*, that drive the maturation of the immune system (28).

Although vertebrate gut microbes provide clear benefits to their hosts, the development of bacterial traits that support the partnership poses a series of challenging evolutionary questions. Why would selection on the host favor microbes that provide a service rather than "cheaters" present within the community that accept benefits but provide nothing in return (29)? Microbial traits that evolve specifically to benefit the host but impose a fitness cost to the bearer create a conflict and the potential for "cheating" (30). In a microbial community like the gut microbiota, "bottom-up" selective pressures to compete with other microbes present in the same niche would always prevent such costly cooperative investments. However, traits that contribute to the fitness of the microbes and incidentally benefit the host (by-product benefits) result in a no-cost mutualism that does not generate a conflict (30). Many beneficial traits of gut microbes (SCFA, competition with pathogens) fall within this category. When the host receives such automatic by-products, selection pressure on the host can shape these traits to maximize the benefits (31). In addition, partner-fidelity feedbacks could accrue and promote positive selection for evolutionary events that are advantageous to the host but neutral to the microbe's fitness, favoring cooperation without generating an opportunity for cheating. This process could be highly relevant in the evolution of mutualism, because the majority of evolutionary changes within an organism are selectively neutral (32). The host could then further select actively, through its adaptive immune functions or the evolution of specific attachment sites, for beneficial microbes (21).

The evolutionary process described herein would result in a win-win situation in which the host provides the habitat for gut microbes (which are often extremely rare in the environment) while the microbes provide benefits such as access to fibrous diets and prevention of enteric infections (13, 15). Unfortunately, we have no empirical data on the evolutionary outcomes of vertebrate symbiosis in terms of measurable fitness benefits for the host. Research with gnotobiotic animals has provided clear evidence for the significant contributions of the microbiota to colonization resistance and nutrient utilization (2, 15), but we do not know to what degree these attributes are adaptive or coincidental. We also lack a general theory about the ecological and evolutionary factors that favor mutualism in gut ecosystems. In other symbiotic systems, vertical transmission over evolutionary time has been shown experimentally to promote traits that enhance partner performance (30, 33). Repeated interactions appear important for cooperation to evolve, which argues that mutualism will be favored when the partners stay together in stable associations and align their fitness interests (29). If we also assume this to account for the evolution of mutualism in vertebrates, then our interpretation of symbiotic interactions would benefit from phylogenetic studies that provide predictions about the evolutionary relationships of gut microbes with their hosts.

Evolutionary Strategies of Vertebrate Symbionts

The phylogenetic patterns of the human and mouse gut microbiota are characterized by a high level of strain and species variation but far fewer intermediate and deep lineages, and a very low diversity at the phylum level when compared with other microbial habitats such as soil and sea water (34). Ley et al. (34) argued that this genetic "shallowness" and "fan-like" phylogenetic architecture suggests a pattern of recent adaptive radiations, where a small initial community that became associated with animals gave rise to a diverse array of descendants. It is often postulated that this process involved coevolution of individual microbial lineages with vertebrates, which is supported by the presence of phylotypes that are specific to particular hosts (21). However, clear evidence for stable associations and codiversification of microbial lineages with vertebrates has not been provided by 16S rRNA data. Patterns of community similarity provide evidence for codiversification of entire gut communities with their hosts, which suggests that there are in fact host-specific evolutionary interactions between mammals and their microbiomes (12).

It is important to point out that coevolution is just one possible mechanism by which microbes evolve with animal hosts (17), and there is little reason to assume that there will be a universal pattern of evolutionary dynamics that applies to all vertebrate gut microbes. Many microbial lineages and species, such as *Escherichia coli*, are found in many different vertebrates, and these organisms could follow a promiscuous lifestyle (12, 35). It is further likely that many gut microbes have occasionally switched hosts. Such dynamic patterns of evolutionary transmission are illustrated by facultative symbionts of insects, which are often erratically distributed and resemble invasive pathogens in that they spread through various host lineages (16). It will require appropriate phylogenetic approaches to reveal the exact evolutionary relationships between microbes and vertebrates. Because of their slow evolution, 16S rRNA sequences have a significant limitation in such studies. The average substitution rate of bacterial 16S rRNA genes has been calculated as ≈1% per 50 million y, and the closely related species *Escherichia coli* and *Salmonella enterica* are predicted to have separated more than 100 million y ago (36, 37). Although such estimates have to be taken with

caution, it is likely that most of the lineages detected in contemporary vertebrates have diversified before they became associated with their vertebrate hosts. Therefore, evolutionary studies of gut symbionts will require, in many cases, more sensitive population genetic approaches. Techniques such as multilocus sequence analysis (MLSA) proved very valuable in studying evolution of bacterial pathogens and environmental microbes (38–40).

The only vertebrate gut microbe that was intensively studied with population genetic approaches is *E. coli*, and this body of work was recently summarized in a review by Tenaillon et al. (35). The population structure of this organism consists of well-supported phylogenetic groups that have no clear association with particular vertebrate hosts. Therefore, the data does not suggest that *E. coli* lineages form stable evolutionary relationships with particular animals. Instead, *E. coli* seems to use dynamic and diverse adaptive strategies that are driven by both host and environmental factors, and it appears that the organism has evolved to occupy niches within a broad host range and also in secondary habitats in the environment (35).

Lactobacillus reuteri as a Model Gut Symbiont

The Gram-positive bacterium *L. reuteri* is an excellent model for basic studies on ecological and evolutionary mechanisms of a vertebrate gut symbiont. *L. reuteri* is found in the digestive tract of mammals such as humans, pigs, hamsters, mice, rats, dogs, sheep, cattle, and different birds (41). In pigs, rodents, and chickens, *L. reuteri* is one of the most abundant species present in the gut and can be detected in a large subset of animals (42–45). In contrast, the prevalence of *L. reuteri* is much lower in humans, where the species is only occasionally found (46). For example, Molin et al. (47) reported that only 4% of the human subjects harbored *L. reuteri* on the GIT mucosa. Nevertheless, the type strain of *L. reuteri*, DSM 20016^T, could be continuously isolated from a human subject over several months, and the species has been considered autochthonous to the human digestive tract (41, 48). There is some evidence that the prevalence of *L. reuteri* in human fecal samples was higher in the middle of the past century. Gerhard Reuter and Tomonari Mitsuoka, who in the 1960s and 1970s intensively studied the *Lactobacillus* biota of the human digestive tract, reported that *L. reuteri* was then one of the dominant lactobacilli and regularly detected (41, 48). The low prevalence in humans in more recent studies suggests a reduction of the *L. reuteri* population size during the past 50 y. Although we need further data to confirm such a decline, a reduction in prevalence within the past decades has also been observed for other microbial lineages (35, 49). In this respect, it is of considerable relevance that a very recent population bottleneck is also supported by the clonal nature of human *L. reuteri* strains (see following).

The ecological strategies of *L. reuteri* are fundamentally different in humans and animals (46). Mice, rats, pigs, and chicken contain thick cell layers of lactobacilli that line parts of their upper digestive tract, and *L. reuteri* is a large component of these biofilms (50–52). *L. reuteri* adheres directly to cells of the stratified squamous epithelium present at these sites (the murine forestomach, porcine pars esophagus, and chicken crop; Fig. 1) (53, 54). Several surface proteins of *L. reuteri* that are involved in biofilm formation and the binding to epithelia, epithelial cells, or mucus have been functionally characterized (Table 1). Proteins such as Mub and Lsp contain LPXTG cell wall binding motifs, are extremely large, contain multiple repeated motifs, and resemble adhesins of pathogenic microbes (55, 56). In contrast, stratified squamous epithelia are absent in the human gut, and epithelial cell layers rich in lactobacilli equivalent to those found in the above-mentioned animals have not been described (46).

L. reuteri strains, like other lactobacilli, are fastidious and rely on the availability of easily fermentable sugars, amino acids, vitamins, and nucleotides. If these factors are provided, the organisms grow very fast (duplication times of less than an hour), and *L. reuteri* can use several external electron acceptors (fructose, glycerol, nitrate) to gain additional energy and increase growth rates (57–59). The growth requirements of *L. reuteri* are satisfied in the proximal digestive tracts of rodents, pigs, and chickens as substrates get supplied through the diet. However, easily accessible nutrients are in low supply in the human colon due to their prior absorption in the small intestine. The ability of *L. reuteri* to use 1,2-propanediol as an energy source (which is also a common trait in *Enterobacteriaceae*) might therefore constitute an important colonization factor in the human gut (60). The enzyme for 1,2-propanediol utilization, diol dehydratase, is vitamin B_{12} dependent, and the encoding genes are organized in the same genomic context as the vitamin B_{12} synthesis operon (61). The enzyme is also involved in the utilization of glycerol as an electron acceptor and reuterin formation (60). This gene cluster is therefore likely to play several important roles in the biology of *L. reuteri*.

Transmission from generation to generation is a key factor for the success of a vertebrate symbiont, and it is not entirely clear to what degree vertebrate symbionts are transmitted vertically or horizontally. Similar to other GIT bacteria, *L. reuteri* may be transferred to the newborn child or animal during birth via vaginal transmission (62). *L. reuteri* has been experimentally shown to be maternally transmitted in humans and pigs (63, 64). Interestingly, *L. reuteri* is present in low numbers in milk from humans, pigs, and dogs, and thus transmission to the next generation might be facilitated by inoculation during lactation (65–67).

Sticking Together: Evolution of *L. reuteri* with Its Host

To gain insight into the evolution of *L. reuteri*, we have recently characterized the population genetic structure and phylogeny of

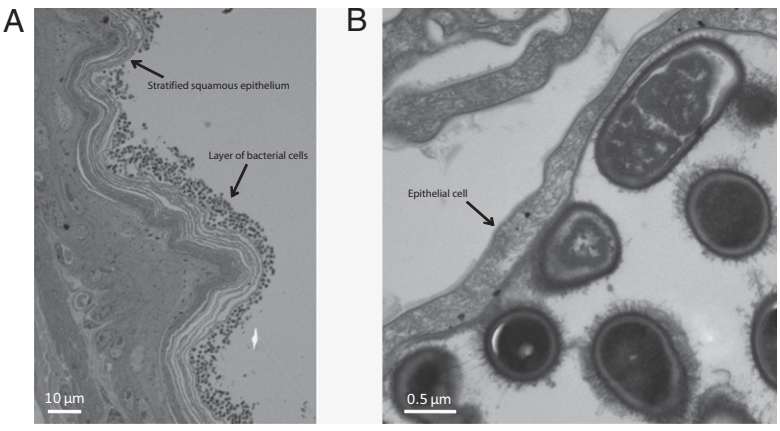

Fig. 1. Associations of *L. reuteri* strain 100-23 with the forestomach epithelium of an ex-*Lactobacillus*-free mouse 7 d after inoculation. The micrographs were produced by transmission electron microscopy as described by Walter et al. (99). (*A*) Biofilm formed on the stratified squamous epithelium present in the forestomach. (*B*) *L. reuteri* cells that adhere directly to stratified cells.

Table 1. Proteins of *L. reuteri* involved in adherence to epithelia and/or biofilm formation

Protein	Full name	Strain(s)	Origin	Putative function in the gastrointestinal tract	Refs.
CnBP/MapA	Collagen-binding protein	DSM20016^T, RC-14, 104R	Human, hamster, pig	Binding to epithelial cells or mucus	100–102
Mub	Mucus-binding protein	1063	Pig	Binding of mucos and/or IgA	55, 103
Lsp	Large surface protein	100-23	Rat	Adherence to forestomach epithelium	56
GtfA/ Inu	Glycosyl-transferases	*L. reuteri* TMW1.106	Food fermentation	Cell aggregation, biofilm formation	104

strains isolated from six different hosts (human, mouse, rat, pig, chicken, and turkey) from global geographic locations (68). Although the 16S rRNA genes of the isolates used in the study were >99% identical, there was considerable genetic heterogeneity within the *L. reuteri* population that could be resolved by amplified fragment length polymorphism and MLSA. Most importantly, both techniques detected the presence of phylogenetic groups with a high reflection of host origin but not geographic location. Figure 2 shows the reconstruction of genealogies of the *L. reuteri* population based on MLSA data using the ClonalFrame software. The phylogeny that is now available for *L. reuteri* allows a prediction of the evolutionary and ecological strategies of this species. The presence of lineages that track with host origin indicates a stable association of *L. reuteri* lineages with particular vertebrates over a long evolutionary time-span and host-driven diversification. However, the population structure also indicates that evolution was not specific to the host genus, because isolates from rodents (mice and rats) and poultry (chickens and turkeys) form joint clades. This suggests that *L. reuteri* lineages evolved with groups of related vertebrates and occasional horizontal transfer between these hosts (68).

The population genetic analysis indicates that *L. reuteri* employs a markedly different lifestyle and adaptive strategy than commensal *E. coli*. Significant secondary habitats outside the hosts have not been identified for *L. reuteri*, and the high host specificity of genetic clusters indicates that this species is composed of subpopulations that have become host adapted. Host specialization is indeed reflected by the phenotypic characteristics of strains. Several experiments in animals showed that indigenous strains of *L. reuteri* outperform exogenous strains when competing in the gastrointestinal tract (63, 68–71). Furthermore, the ability of *L. reuteri* strains to adhere to epithelia and epithelial cells in the proximal gut is to a large degree host-specific (53, 54, 72). Strains originating from the forestomach of rodents adhere to epithelial cells of mice and rats, but do not adhere to crop epithelial cells. Conversely, isolates from poultry do not adhere to epithelial cells from the rodent forestomach or the pars oesophagea of pigs.

To gain additional insight into the phenotypic diversification within *L. reuteri*, we have investigated 32 *L. reuteri* strains isolated from different hosts for their ability to produce the enzyme urease and the antimicrobial compound reuterin. These factors were chosen as they are likely to play an important role in the gut, potentially contributing to acid resistance (urease) and reuterin formation/propanediol fermentation (58, 73, 74). Our analyses revealed that all rodent strains (n = 9) produced urease and harbored the *ureC* gene (encoding the urease alpha subunit), whereas only one of the 23 strains isolated from other hosts was positive for these traits (Fig. 2*A*). In contrast, only one of the rodent strains produced reuterin and possesed the *pduC* gene (encoding a subunit of diol/glycerol dehydratase, the first enzyme in the propanediol fermentation/reuterin formation pathway), whereas all human and poultry strains possessed these traits. The phylogenetic distribution of these phenotypic factors indicates that they evolved to access specialized niches in respective hosts.

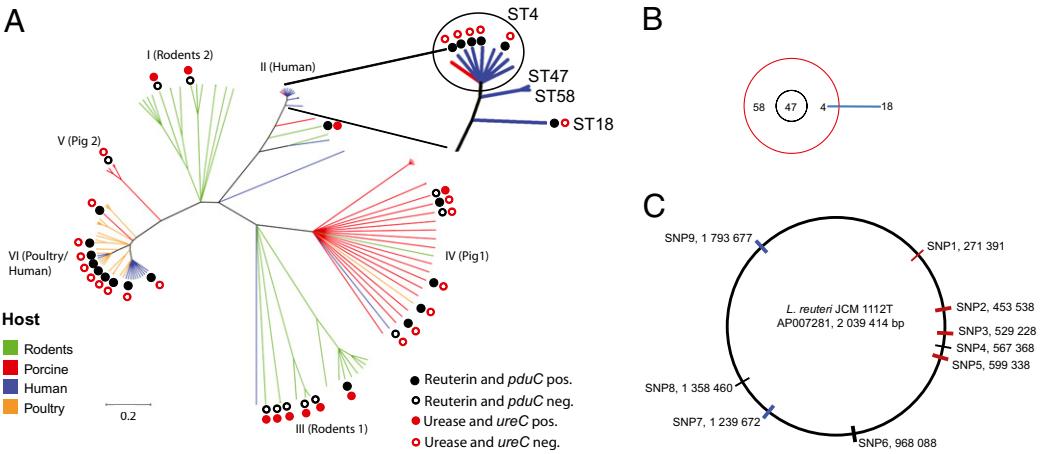

Fig. 2. Phylogenetic and genomic analysis of *L. reuteri* isolates originating from different vertebrate hosts. (*A*) Genealogy of 116 *L. reuteri* strains as inferred from MLSA sequences using the ClonalFrame software as described by Oh et al. (68). The branches in the tree are color coded by host origin, and cohesive subpopulations are labeled. The human cluster II is enlarged, and the four sequence types (STs, strains with seven identical housekeeping genes) represented in this cluster are indicated. Strains that produce reuterin and posses the *pduC* gene (large subunit of glycerol/diol dehydratase) are marked by closed black circles; strains that do not produce reuterin or possess the *pduC* gene are marked with open black circles. Strains that produce urease and posses the *ureC* (urease alfa subunit) are marked with closed red circles; strains that do not produce urease or the *ureC* gene are labeled with open red circles. (*B*) Human isolates of the *L. reuteri* cluster II form one clonal complex (CC). Allelic profiles were analyzed by eBurst, and CCs were defined as sets of related strains sharing identical alleles at five of the seven MLSA loci with at least one other member of the group. The figure shows the clonal grouping among the human *L. reuteri* strains of cluster II, which is comprised of four STs. The black circle in the middle indicates the putative founder (ST47). (*C*) Visualization of SNPs in the genomes of the human *L. reuteri* strains JCM 1112^T (DSM 20016^T), ATCC PTA 4659, ATCC PTA 5289, and ATCC 6475. SNPs with red markings are found solely in ATCC PTA 4659, SNPs with blue markings are found solely in ATCC PTA 5289, and SNPs with black markings are found in ATCC 4659, 5289, and 6475. Nonsynonymous SNPs are represented with thick markings and synonymous SNPs with thin markings.

The population structure and phenotypic characteristics of the isolates described herein identify the host environment as the major factor in the evolution of *L. reuteri*. Although we do not yet know the precise ecological forces that drive diversification, the population genetic structure indicates marked differences between hosts. The genetic heterogeneity is much higher in the two rodent lineages when compared with other clusters, and recombination played an important role in generating this diversity (68). In contrast, genetic variation and the impact of recombination in the clusters from pigs, poultry, and humans are much lower. The cluster with the lowest genetic homogeneity is the human cluster II. Most human isolates in this subpopulation fall into one single clonal complex (CC), meaning that they share at least five of the seven MLSA loci (Fig. 2*B*). To gain additional insight into the genetic diversity within the human cluster II, we have sequenced the genomes of three strains that belong to the most common sequence type (ST4) by Illumina sequencing and compared the sequences to the genome of *L. reuteri* DSM20016^T (JCM 1112^T), which is a member of ST4. This analysis revealed a total of only nine single-nucleotide polymorphisms (SNPs) in the four genomes (Fig. 2*C* and Table S1). This is remarkable, as these strains have been isolated in Germany, Finland, and Japan over a time span of almost 40 y. This data indicates a recent selective sweep or a population bottleneck in the human *L. reuteri* population. We do not know if this bottleneck was caused by a recent change in the human environment, but such an event would explain the decreased prevalence of *L. reuteri* in humans.

Making an Impact: Beneficial Effects Conferred by *L. reuteri*

The phylogenetic patterns detected for *L. reuteri* indicate a stable evolutionary relationship with the host, which, in theory, has the potential for the development of mutualistic interactions (21, 29, 30). As described previously, we lack empirical data that would provide direct evidence for such a process. Nevertheless, the beneficial attributes of *L. reuteri* have been researched intensively during the past three decades because of the common use of different strains as probiotics (Table 2). Although these experiments were not designed to study symbiotic interactions per se, they still suggest beneficial attributes of *L. reuteri* in both humans and animals.

The effects of *L. reuteri* on the host were studied in animal models using rodents, turkeys, chickens, and pigs. For example, intestinal resistance to the eukaryotic pathogen *Cryptosporidium parvum* was increased by *L. reuteri* in a murine model of acquired immunodeficiency syndrome (75). In addition, Casas and Dobrogosz (63) have found that administration of *L. reuteri* reduced mortality in chickens and turkeys upon infection with *Salmonella*. The mechanisms that underlie this protection have not been clearly identified, but might include an increase in competitive exclusion. Various *L. reuteri* strains produce an array of antimicrobial compounds that inhibit pathogens in vitro (76, 77). The best characterized of those, reuterin, is a mixture of different forms of beta-hydroxypropionaldehyde (3-HPA) that have bactericidal and bacteriostatic activity against a wide range of bacterial pathogens (77). Although many bacteria contain the pathway to reduce glycerol, *L. reuteri* is unique as it secretes high levels of reuterin. The role of reuterin in competitive exclusion has not been addressed directly, but it has been shown to decrease *E. coli* population in an in vitro model of colonic fermentation (78). In addition, *L. reuteri* strains are much more resistant to reuterin than most other bacteria, indicating that the antimicrobial activity of reuterin is of ecological and evolutionary significance (79).

Arguably the most intriguing feature of *L. reuteri* and a likely underpinning of its probiotic effect is the ability to modulate the host's immune system. Empirical evidence for an immunoregulatory effect was achieved in several experimental models of colitis, where *L. reuteri* was highly efficient in reducing inflammation (70, 71, 80–82). Immunomodulation has also been shown in humans, where *L. reuteri* ATCC 55730 has been shown to temporarily colonize the stomach and small intestine of healthy subjects and increase CD4$^+$ T lymphocytes in the ileum (83). The physiological implications of these immune effects are not yet established in humans, but immune modulation might contribute to the reduction in the duration and severity of diarrhea and the prevention of sensitization and IgE-associated eczema in children (84–87).

Several trials have shown that *L. reuteri* does confer health benefits in humans. In a double-blind, placebo-controlled, randomized trial, *L. reuteri* ATCC 55730 was shown to improve the health of infants in a daycare setting (88). Children receiving *L. reuteri* supplementation had a reduced number of sick days, antibiotic prescriptions, diarrheal episodes, and duration of diarrhea. *L. reuteri* has also been shown to improve symptoms of infant colic (89). Colic is a poorly understood syndrome in which

Table 2. Beneficial attributes of *L. reuteri* studied in human and animal trials and with cell cultures

Function	Observation	Refs.
Humans		
Prevention of diarrhea	Reduced duration and severity of diarrhea caused by rotavirus in children; reduced incidence of diarrhea in infants	86–88
Reduction of infant colic	Reduced colicky symptoms in 95% of infants; improved gastric emptying and reduced crying time in premature infants	89, 90
Reduction of IgE-associated eczema and sensitization	Reduction of IgE associated eczema in 2-y-olds; reduced levels of TGF-B2	84, 85
Immune stimulation	Short-term survival of *L. reuteri* in the stomach and small intestine. Stimulation of CD4 lymphocytes	83
Animals		
Immune stimulations	Transient increase in proinflammtory cytokines and chemokines in the intestinal tract.	97
Immune regulation	Increased levels of regulatory T cells upon colonization of *Lactobacillus*-free free mice with *L. reuteri*	96
Prevention of experimental colitis	Reduced levels in animal models of colitis	70, 71, 80–82
Immune cells		
Modulation of immune reactions in cultured macrophages, dendritic cells, and T cells	Reduction in TNF-α production in activated macrophages; reduced production of proinflammatory cytokines in dendritic cells, induction of regulatory T cells	53, 81, 93, 94, 96, 105

infants, from just after birth to 6 mo, have uncontrollable crying spells that last for at least 3 h at a time. *L. reuteri* was compared with simethicone treatment for efficacy in treating colic in a prospective controlled trial. After 28 d of treatment with *L. reuteri* 95% of infants were deemed responders and found to have significantly reduced their daily crying times, compared with only 7% of infants receiving simethicone. The mechanism by which *L. reuteri* reduces colic symptoms is not yet understood but may be linked to stimulation of gastric emptying (90).

L. reuteri Contributes to Tolerance in the Gut

Recently, Edwards (91) proposed an important role for tolerance strategies for the evolution, maintenance, and breakdown of mutualism in symbiotic relationships. In the vertebrate gut, the establishment of tolerance to the microbes is a key requirement for peaceful coexistence (7). Strict compartmentalization by confining symbiotic bacteria to the gut lumen is essential, but signals from the microbiota are also involved by influencing the differentiation of T cells and the induction of regulatory T cells (Tregs) that suppress excessive immune responses. The significance of immune homeostasis in the gut becomes evident in inflammatory bowel diseases (92).

Recent studies revealed that *L. reuteri* might play a key role in the induction of tolerance in the vertebrate gut (a summary of the immune effects is provided in Fig. 3). Christensen et al. (93) showed that *L. reuteri* had the ability to inhibit induction of proinflammatory cytokines interleukin (IL)-12, IL-6, and TNF-α in murine dendritic cells (DCs). The priming of DCs by *L. reuteri*, which was initiated by the binding of C-type lectin DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), resulted in an induction of regulatory T cells in vitro (94). A similar down-regulation of proinflammatory cytokines (e.g., TNF-α) by *L. reuteri* was also observed with macrophages, lipopolysaccharide-activated monocytes, and primary monocyte-derived macrophages from children with Crohn's disease (81, 95). The bacterial molecule(s) responsible for down-regulating TNF-α in antigen-presenting cells have not been identified to date but appear to function by inhibiting the activation of c-Jun and AP-1 (95).

The physiological relevance of the immune effects of *L. reuteri* was recently demonstrated in vivo using *Lactobacillus*-free (LF) mice (96, 97). In these animals, administration of *L. reuteri* resulted in a transient activation of proinflammatory cytokines and chemokines produced by intestinal epithelial cells in the jejunum and ileum (97). However, the inflammatory response was transient and proinflammatory cytokine levels completely returned to normal after 21 d, although high numbers of lactobacilli continued to be present in the gut. This process could be explained by elevated levels of IL-10, IL-2, and TGF-β in supernatants from immune cells recovered from the mice, as well as increased levels of Foxp3-positive regulatory T cells (96). The induction of immune tolerance in LF-mice by *L. reuteri* is a remarkable finding, as these mice have a complex microbiota that is functionally equivalent to that of conventional mice (98). It suggests that *L. reuteri* contributes to the immune regulation in the gut by modulating antigen-presenting cells toward favoring tolerance. The ability of *L. reuteri* to prevent experimental colitis in animal models indicates that the immunoregulatory effects of this organism can have a significant benefit for the host (70, 71, 80–82).

Conclusions and Perspective

Researchers have just begun to unravel the complex features of microbial symbiosis in vertebrates, and research on the evolution of gut microbes is clearly in its infancy. Studies on the bacterium *L. reuteri* have provided unique insight into the evolutionary mechanisms of a gut microbe that maintains a close symbiotic relationship with its vertebrate host. Despite the inevitable dissemination through feces, *L. reuteri* lineages share an evolutionary history with particular vertebrate animals and became host adapted. This evolutionary strategy is in striking contrast to that of commensal *E. coli*, which have a broad host range and follow more diverse adaptive strategies (35). Although we lack a general theory about the consequences of such distinct evolutionary patterns in vertebrate gut symbionts, it is intriguing that the phenotypes of *L. reuteri* and *E. coli* in terms of their impact on the host are in accordance with both theoretical considerations and observations in other symbiotic systems (29, 30, 33). Specifically, the beneficial attributes of *L. reuteri* might be a direct consequence of its shared evolutionary fate (and potentially coevolution) with groups of vertebrate hosts. In contrast, the dynamic relationship of *E. coli* with vertebrates might account for the emergence of the well-known human pathogens within this species, which contain virulence factors that may have evolved coincidentally because of their role as colonization factors in other hosts (35). If analogous principles do apply to gut microbes in general, we could use evolutionary studies to better interpret their symbiotic interactions within the vertebrate gut microbiota.

Breakthroughs in our understanding of the roles of microbes in the vertebrate digestive tract and the biological principles that apply are likely to arise from studies that are informed by ecological and evolutionary theory. This will require integrative studies spanning all scales, from molecules, individual microbes, microbial communities, and populations of vertebrate hosts, to answer the open questions. What are the evolutionary strategies of members of the gut microbiota other than *L. reuteri* and *E. coli*? Are there host-specific reciprocal evolutionary interactions between specific microbes and their vertebrate hosts that could be described as coevolution? What effect does selection pressure have on the host in terms of the evolution of the gut microbiome? Are symbiotic interactions and beneficial effects of microbes host-

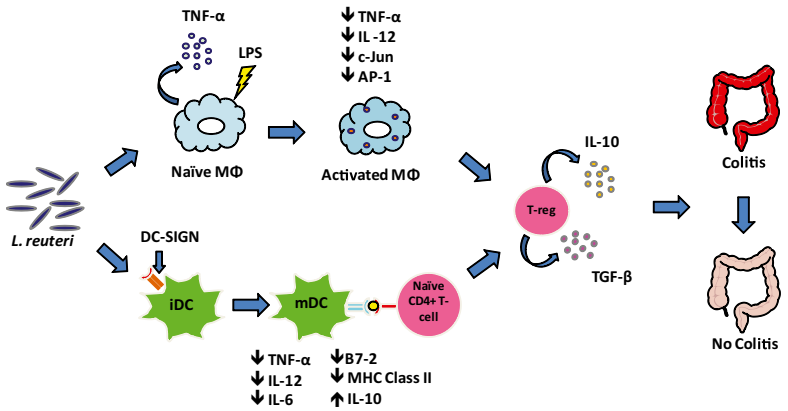

Fig. 3. Effects of *L. reuteri* on immune cells that contribute to tolerance in the gut. *L. reuteri* has been shown to suppress the production of proinflammatory cytokines such as TNF-α and IL-12 in macrophages, monocytes, and dendritic cells. The modulation of dendritic cells by *L. reuteri* has been shown to be mediated through DC-SIGN and promote development of regulatory T cells producing high amounts of IL-10 and TGF-β. This suppression of immune responses is likely to underlie the ability of *L. reuteri* to reduce intestinal inflammation in several murine colitis models. Please see text for details and references.

specific? How do environmental factors affect both evolution and functionality of the gut microbiota? It is important to realize that the study of symbiotic interactions in humans might already be hampered, as features of modern lifestyle are almost certain to have introduced bottlenecks to symbiont transmission (49). Research using animal models is therefore especially important. The elegant approaches that have been used to study microbial symbiosis of invertebrates can clearly serve as a paradigm for similar research in vertebrates (16–19).

The phylogenetic and phenotypic characterization of *L. reuteri* is supportive of the notion that some gut microbes form an intrinsic symbiotic relationship with vertebrates that is significant for health. A disruption of these ancient partnerships through modern lifestyle could have contributed to the recent increase in diseases in westernized societies. If there is indeed a decrease of ancestral microbial lineages that is linked to disease, as suggested by Blaser and Falkow (49), such microbes could be restored to provide the same beneficial functions that they have evolved as members of the microbiome. However, such beneficial lineages would have to be identified first to reach a scientific consensus on what composes a healthy gut microbiota, and it is naïve to think that we can modulate ancient symbiotic relationships and generate a benefit without an understanding of their evolution, the ecological forces that shape them, and how they function (21). Therefore it is highly unfortunate that most of the probiotic and prebiotic strategies that have been developed to date are not based on ecological or evolutionary criteria. In the future, selection of probiotic strains and prebiotic targets could be based on criteria such as their evolutionary relationships with the host. It is a logical working hypothesis that symbionts that share an evolutionary fate with their host are more likely to possess adaptive traits that provide benefits.

Materials and Methods

Strains were screened for the production of urease and reuterin and the presence of the *pduC* and *ureC* genes by standard phenotypic tests and PCR. The genome sequences of *L. reuteri* strains ATCC PTA 4659 (previously MM2-3, isolated in Finland in 1997), 5289 (FJ1, isolated in Japan, around 2002), and 6475 (MM4-1a, isolated in Finland in 1997) were determined by Illumina sequencing of genomic DNA, and the sequences were subjected to SNP analysis. Detailed methods for the procedures are provided in *SI Materials and Methods*.

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Supporting information

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SI Materials and Methods

Determination of Urease Activity and Reuterin Production. Urease activity was tested by suspending the bacteria from a 10-mL overnight culture (MRS broth; Oxoid) in 10 mL sterile filtered 2% urea solution. The pH was measured at the beginning and after 16 h, and an increase of more than two pH units indicated production of urease. To detect reuterin production, the bacteria were grown for 48 h on MRS agar plates (inoculated as streaks) in anaerobic atmosphere. The plates were then overlaid with 500 mM glycerol agar (1% agar) and incubated at 37 °C for 30 min. Reuterin was detected by the addition of 5 mL 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl). After 3 min incubation, the solution was poured off and 5 mL 5 M KOH was added. Red zones around the bacterial streaks demonstrate the presence of reuterin.

Detection of *ureC* and *pduC*. The genes encoding urease alpha subunit (*ureC*) and the large subunit of the glycerol dehydratase (*pduC*) were detected by PCR analyses. The bacteria were cultivated on an MRS agar (Oxoid) plate and grown for 16 h at 37 °C in anaerobic atmosphere. With a sterile plastic loop, 1 μL bacterial cells was collected and suspended in 100 μL sterile water. The PCRs were performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare) and primers (0.4 μM of each) for detecting *ureC*, ureCF (5′-GAAAGTCTTTTTGGTGGTGG-3′), and ureCR (5′-AACGTCGTCAGGAATCTTAG-3′); and *pduC*, pduCF (5′-CCTGAAGTAAAYCGCATCTT-3′), and pduCR (5′-GAAACYATTTCAGTTTATGG-3′). Bacterial suspension (0.5 μL) was added to the PCR mix (in total 25 μL), and the PCR was performed by running the program 95 °C, 5 min; 30× (95 °C, 30 s; 53 °C, 30 s; 72 °C, 30 s); 72°, 10 min. The PCR products were separated and visualized by using standard agarose gel electrophoresis.

Illumina Sequencing of *L. reuteri* Genomes. *L. reuteri* strains used in this study were ATCC PTA 4659 (previously MM2-3, isolated in Finland in 1997), 5289 (FJ1, isolated in Japan, around 2002), and 6475 (MM4-1a, isolated in Finland in 1997), grown in MRS media (Difco) and genomic DNA prepared by using the Qiagen Genomic-Tip System. DNA was fragmented by 20 min sonication (130 W) to obtain an average fragment size of 500 bp, then further purified and concentrated with QIAquick PCR Purification Spin Columns (Qiagen). Treatment to remove 3′ overhangs and fill in 5′ overhangs resulted in blunt-ended genomic fragments. An adenine residue was added by terminal transferase to the 3′ end, and the resulting fragments were ligated to Solexa adapters. The products were separated by agarose gel electrophoresis, and the band between 150 and 200 bp was excised from the gel. The DNA fragments were extracted from the agarose slice using a QIAquick Gel Extraction Kit (Qiagen). Adapter-modified DNA fragments were enriched by an 18-cycle PCR using Solexa universal adapter primers. The DNA fragment library was quantitated, then diluted to a 10-nM working stock for cluster generation. Adapter-ligated fragments (2 nM) were denatured in 0.1 M NaOH for 5 min, then further diluted to a final 9 pM concentration in 1 mL of prechilled hybridization buffer, and introduced onto the Solexa flow cell using the Cluster Station. Following isothermal amplification, clusters were made single-stranded by 0.1 M NaOH denaturation, metered across the flow cell by the Solexa Cluster Station. A sequencing primer complementary to one Solexa adapter was added to prime the single strands of each cluster. Once hybridized and with excess primer removed by a wash, the flow cell was ready for sequencing. The Solexa Genome Analyzer II was programmed to provide up to 36 sequential flows of fluorescently labeled, 3′-OH blocked nucleotides and polymerase to the surface of the flow cell, thus producing a fixed 36-bp read length. After each base incorporation step, the flow cell surface was washed to remove reactants and then imaged by microscope objective. The experiments collected 300 tiled images ("tiles") per flow cell lane, each containing on average 30,000 clusters.

SNP Analysis. The three lanes' sequencing results were mapped onto the reference genome *L. reuteri* JCM 1112^{T} (GenBank accession no AP007281) separately. The mapping software Maq version 0.6.6 (http://maq.sourceforge.net/maq-man.shtml) was used to perform the mapping (default parameters). SNPs were identified and validated by the MAQ software, and classified into coding SNP and intergenetic SNPs. Coding SNPs were identified as synonymous and nonsynonymous. The SNPs were finally verified by PCR amplification of the surrounding region, followed by Sanger sequence determination.

Table S1. Single-nucleotide polymorphisms (SNPs) in human *L. reuteri* strains compared with *L. reuteri* JCM 1112T (GenBank AP007281)

SNP	Strain	Position (in genome JCM 1112^T)	Coding	Gene in genome JCM 1112^T	Annotation	Nonsynonymous	GenBank accession no.
1	4659	271 391	+	LAR_0226	Conserved hypothetical protein	–	GU563996
2	4659	453 538	+	LAR_0387	Chloride channel protein	+	GU563997
3	4659	529 228	+	LAR_0456	ATP synthase gamma subunit	+	GU563998
4	4659, 5289, 6475	567 368	+	LAR_0489	Thiamine biosynthesis protein ThiI	–	GU563999
5	4659	599 338	+	LAR_0512	DNA mismatch repair protein HexB	+	GU564000
6	4659, 5289, 6475	968 088	+	LAR_0849	Two-component sensor histidine kinase	+	GU564001
7	5289	1 239 672	+	ORF between LAR_1080 and 1081	Conserved hypothetical protein	+	GU564002
8	4659, 5289, 6475	1 358 460	+	LAR_1203	Penicillin-binding protein 2A	–	GU564003
9	5289	1 793 677–8	+	LAR_1579	Multidrug ABC transporter ATP-binding and permease components	+	GU564004