Comparative evaluation of the genomes of three common *Drosophila*-associated bacteria

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**ABSTRACT**

*Drosophila melanogaster* is an excellent model to explore the molecular exchanges that occur between an animal intestine and associated microbes. Previous studies in *Drosophila* uncovered a sophisticated web of host responses to intestinal bacteria. The outcomes of these responses define critical events in the host, such as the establishment of immune responses, access to nutrients, and the rate of larval development. Despite our steady march towards illuminating the host machinery that responds to bacterial presence in the gut, there are significant gaps in our understanding of the microbial products that influence bacterial association with a fly host. We sequenced and characterized the genomes of three common *Drosophila*-associated microbes: *Lactobacillus plantarum*, *Lactobacillus brevis* and *Acetobacter pasteurianus*. For each species, we compared the genomes of *Drosophila*-associated strains to the genomes of strains isolated from alternative sources. We found that environmental *Lactobacillus* strains readily associated with adult *Drosophila* and were similar to fly isolates in terms of genome organization. In contrast, we identified a strain of *A. pasteurianus* that apparently fails to associate with adult *Drosophila* due to an inability to grow on fly nutrient food. Comparisons between association competent and incompetent *A. pasteurianus* strains identified a short list of candidate genes that may contribute to survival on fly medium. Many of the gene products unique to fly-associated strains have established roles in the stabilization of host-microbe interactions. These data add to a growing body of literature that examines the microbial perspective of host-microbe relationships.

**KEY WORDS:** *Drosophila*, Intestine, Microbiota, Host-microbe

**INTRODUCTION**

Environmental, microbial, and host factors act at mucosal barriers to establish a unique microclimate that shapes the lives of all participant species (Spor et al., 2011). For example, expression of a host genotype in gastrointestinal tissues works in concert with extrinsic factors to determine microbial associations (Donaldson et al., 2015). The metabolic outputs of the gastrointestinal microbiota influence critical events in the host such as education of immune phenotypes (Hooper et al., 2012; Round and Mazmanian, 2009), development of intestinal structures (Kamada et al., 2013), and access to essential micronutrients (Hacquard et al., 2015). Given the intertwined relationship between host genotype and microbial genotype, it is of some surprise that hosts often tolerate extensive alterations to their microbiota in response to environmental shifts, such as changes in diet (David et al., 2014). However, alterations to the gastrointestinal microbiota are not invariably without consequence, and intestinal dysbiosis may lead to chronic, debilitating, and occasionally deadly diseases within the host (Belkaid and Hand, 2014; Lee et al., 2011; Schwabe and Jobin, 2013; Wen et al., 2008; Wu et al., 2010). Our appreciation of the holobiont as an intricate network of biochemical and genetic transactions between multiple participants mandates a thorough evaluation of the microbial genomes that shape host physiology. Unfortunately, such studies are tremendously complex in conventional mammalian models due to the size of the microbiome, and also the lack of laboratory techniques for the isolation and manipulation of many mammalian commensals.

The simple invertebrate *Drosophila melanogaster* is an excellent model holobiont (Buchon et al., 2013; Ma et al., 2015). From a developmental perspective, the *Drosophila* posterior midgut shares a number of important similarities with the small intestine of more complex mammalian counterparts (Buchon et al., 2013). Both organs are endodermal in origin, and are surrounded by a sheath of mesodermal visceral muscle (Spence et al., 2011; Tepass and Hartenstein, 1994). The mammalian small intestine and *Drosophila* posterior midgut are maintained by regularly spaced, basal intestinal stem cells that generate transitory progenitor cells (Barker et al., 2008; Jiang and Edgar, 2012; Takashima and Hartenstein, 2012); the non-proliferative enteroblasts of *Drosophila*; and the transient-amplifying cells of mammals. In both systems, signals along the Notch-Delta axis promote differentiation of transitory progenitors into secretory enteroendocrine cells or absorptive enterocytes (Buchon et al., 2013; Peterson and Artis, 2014). In contrast to mammals, *Drosophila* lacks specialized basal paneth cells for the release of antimicrobial peptides. Nonetheless, the fly genome encodes antimicrobial peptides that actively contribute to the control of intestinal symbionts and pathogens (Ryu et al., 2008), indicating the release of such factors into the *Drosophila* intestinal lumen. In both the mammalian small intestine and *Drosophila* midgut, host factors and biogeography favor association with members of the *Lactobacillaceae* family (Donaldson et al., 2015; Matos and Leulier, 2014). In return, metabolites from *Lactobacilli* activate host response pathways that promote intestinal stem cell proliferation and reactive oxygen species generation (Jones et al., 2013). Combined with the genetic accessibility of flies and their suitability for longitudinal studies of large populations in carefully defined environments, these attributes establish *Drosophila* as an excellent system to decipher the forces that determine genetic interactions within a holobiont (Buchon et al., 2013; Charroux and Royet, 2012; Ferrandon, 2013).
In contrast to conventional vertebrate models, the *Drosophila* microbiome consists of a small number of aerotolerant bacterial species that are easily isolated and cultured (Broderick and Lemaître, 2012). The adult *Drosophila* intestine hosts little to no bacteria immediately after emergence from the pupal case and the microbiobial population grows in number over time (Clark et al., 2015). Several studies established that environmental factors and host genotype influence the diversity of the microbiota (Chandler et al., 2011; Ryu et al., 2008; Wong et al., 2011). It is unclear if bacteria establish stable associations with the host gut, or if they cycle from the intestine to the environment and back (Blum et al., 2013; Broderick et al., 2014). Nonetheless, lab-raised and wild *Drosophila* frequently associate with representatives of the genera *Lactobacillus* and *Acetobacter*. These data suggest that the intestinal lumen of an adult fly favors the survival of specific bacteria, and that such bacteria encode the necessary factors to survive or proliferate within a *Drosophila* intestine.

Consistent with a long-term association between the fly intestine and specific microbes, many *Drosophila* phenotypes are influenced by individual *Lactobacillus* or *Acetobacter* species. For example, several strains of *Lactobacillus plantarum*, a common *Drosophila*-associated microbe, promotes larval development via regulation of the TOR signal transduction pathway and induction of intestinal peptidases (Erkosar et al., 2015; Storelli et al., 2011), while *Acetobacter pomorum* regulates host insulin growth factor signals to promote development and metabolic homeostasis (Shin et al., 2011). In addition, members of the *Acetobacter* and *Lactobacillus* populations regulate levels of essential nutrients in the host (Chaston et al., 2015; Huang and Douglas, 2015; Wong et al., 2014). Combined, these data present a compelling argument that *Lactobacilli* and *Acetobacter* are important members of the *Drosophila*-microbe holobiont.

Despite our advances in the elucidation of *Lactobacillus* and *Acetobacter* influences on their *Drosophila* host, it is unclear if the individual species encode factors that permit survival during passage through the adult *Drosophila* intestine. We prepared whole genome sequences of three bacterial species that regularly associate with *Drosophila* – *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Acetobacter pasteurianus*. These sequences included those for a *Lactobacillus plantarum* strain isolated from our lab-raised flies, and a separate strain isolated from a wild *Drosophila* strain, with approximately 500,000 nucleotides more, and an additional 500 coding sequences (Table 2).

### Table 1. Bacterial strains used in this study

| Bacteria     | Strain | Source | Reference                  |
|--------------|--------|--------|----------------------------|
| *Lactobacillus brevis* | ATCC 367 | Silage | (Makarova et al., 2006)    |
| *Lactobacillus brevis* | EW | *Drosophila* | (Kim et al., 2013a)        |
| *Lactobacillus brevis* | EF | *Drosophila* | This study                  |
| *Acetobacter pasteurianus* | NBRC 101655 | Pineapple | (Matsutani et al., 2012)   |
| *Acetobacter pasteurianus* | ATCC 33445 | Beer   | (Matsutani et al., 2012)   |
| *Acetobacter pasteurianus* | AD | *Drosophila* | This study                  |
| *Lactobacillus plantarum* | JOJTO1.1 | *Drosophila* | (Newell et al., 2014)      |
| *Lactobacillus plantarum* | ATCC 14917 | Pickled *Cabbage* | (Orla-Jensen, 1919)       |
| *Lactobacillus plantarum* | WJL | *Drosophila* | (Kim et al., 2013b)        |
| *Lactobacillus plantarum* | KP | *Drosophila* | This study                  |
| *Lactobacillus plantarum* | DF | *Drosophila* | This study                  |

### General genomic features

*Lactobacillus brevis* and *Lactobacillus plantarum*

*L. brevis* is a common member of the *Drosophila* intestinal microbiota, and the whole genome sequence of a fly-associates strain, *L. brevis* EW is available (Kim et al., 2013a). We prepared a whole-genome sequence of an additional *L. brevis* strain (*L. brevis* EF) that we isolated from the intestines of wild-type adult *Drosophila* from our lab. For comparative purposes, we extended our study to include the genome of the environmental ATCC 367 strain. We plated homogenates from flies ten days after feeding a mono-culture of the ATCC 367 isolate and found that *L. brevis* ATCC 367 retained an association with wild-type adult *Drosophila*, confirming that the ATCC 367 strain is association-competent (Fig. 1A). Genome-to-genome distance calculations suggest that the *Drosophila*-associated EW and EF strains are more closely related to each other than to the ATCC 367 strain (Table 2). The genomes of *Drosophila*-associated strains are also larger than the environmental strain, with approximately 500,000 nucleotides more, and an additional 500 coding sequences (Table 2).

**RESULTS**

The intestine contains structural and chemical barriers that typically inhibit bacterial growth or viability. In response, the intestinal microbiota express factors that overcome host defenses to permit bacterial survival. Here, we examined the genomes of *L. brevis*, *L. plantarum* and *A. pasteurianus*, which are all common members of the *Drosophila* intestinal community. For each species, we studied whole-genome sequences of bacterial strains that we isolated from adult *Drosophila* intestines, and compared them to related strains isolated from the environment. Details on the respective genomes characterized in this study are presented in Table 1.

We processed each genome in a similar manner. Where necessary, we used genomic databases to identify the bacterial species of newly sequenced genomes. We then annotated each genome with RAST, used PHAST to scan each genome for intact prophages, and searched for possible CRISPR arrays in the respective genomes. We scrutinized the annotated genomes for functions that might facilitate microbial survival within the intestinal lumen, with a focus on genes involved in signal transduction, transcriptional responses, orchestration of stress responses, or induction of virulence factors. Finally, we compared environmental and *Drosophila*-associated genomes for each species to identify bacterial factors that are unique to *Drosophila*-associated genomes. We present the results for each genus below.
remained associated with wild-type *Drosophila* L. brevis isolated from pickled cabbage. Similar to the ATCC 367 strain of bacterial colony-forming units per fly. (D, E) Liquid cultures results of three separate measurements, and association was measured as OD600 of 50 and 200, respectively (columns 5 and 6). Each column shows the (column 1) flies, germ-free (column 2) flies, gnotobiotic flies that were fed Quantification of *Lactobacillus* plantarum ATCC 14917 (B), 10 days after the initial feeding. Each plate contains the equivalent of 1% of the homogenate of an entire fly. (C) Environmental ATCC 14917 strain. For comparative studies of *A. pasteurianus* we noticed that the ATCC 14917 strain of *A. pasteurianus* ATCC 33445 (E) were added to fly food, incubated at association with conventionally reared *A. pasteurianus* strain AD at OD600 of 50 and 200, respectively (columns 3 and 4), or gnotobiotic flies that were fed *A. pasteurianus* strain ATCC 33445 at OD600 of 50 and 200, respectively (columns 5 and 6). Each column shows the results of three separate measurements, and association was measured as bacterial colony-forming units per fly. (D, E) Liquid cultures *A. pasteurianus* AD (D) and *A. pasteurianus* ATCC 33445 (E) were added to fly food, incubated at 29°C for 1 week, rinsed in MRS and re-plated on selective plates.

For comparative studies of *L. plantarum*, we focused on the environmental ATCC 14917 strain. *L. plantarum* ATCC 14917 was isolated from pickled cabbage. Similar to the ATCC 367 strain of *L. brevis*, we noticed that the ATCC 14917 strain of *L. plantarum* remained associated with wild-type *Drosophila* ten days after feeding (Fig. 1B). We compared the ATCC 14917 strain to four *Drosophila*-associated genomes: WJL, DMCS_001, DF and KP. WJL and DMCS_001 were isolated from *Drosophila* raised in geographically separate labs (Kim et al., 2013b; Newell et al., 2014). We isolated the KP strain from the intestines of our lab-raised wild-type strain, and the DF strain from an isofemale wild *Drosophila melanogaster* line that we captured in Edmonton, Canada in the summer of 2014. The DF and KP genomes encode one chromosome and three closely related plasmids each (Fig. 2). While all five genomes are closely related, genome-to-genome distance calculators suggest a greater degree of identity among the *Drosophila*-associated KP, DF, WJL and DMCS_001 strains (Table 2). In general, the environmental genome is smaller than the *Drosophila*-associated genomes, encodes fewer RNAs and coding sequences, and contains fewer phage-associated proteins (Table 2).

**Environmental response factors**

We then examined genetic regulatory networks within the individual *Lactobacillus* strains to determine if *Drosophila*-associated strains encode distinct regulatory components that permit adaptation to the harsh environment of an adult intestine. For these studies, we paid particular attention to two-component systems, transcription factors and additional DNA-binding proteins within the respective genomes. We did not observe substantial differences between *Drosophila*-associated and environmental genomes for either *L. brevis* or *L. plantarum* (Table 2). Likewise, we only observed slight differences between *Drosophila*-associated and environmental strains when we considered genes dedicated to signal transduction, stress responses, or virulence (Table 2).

**Prophages and CRISPR responses**

Comparisons between environmental and *Drosophila*-associated *Lactobacillus* genomes uncovered a propensity for prophage accumulation within the *Drosophila*-associated genomes. For example, we detected an average of four intact prophage genomes in *Lactobacillus* strains isolated from flies, and a maximum of two prophage genomes in environmental strains. The EW and EF *L. brevis* genomes include four intact temperate prophages, compared to an absence of prophages from the environmental *L. brevis* strain (Table 2). We found CRISPR sequences that target a common *Lactobacillus* phage within all three genomes, while the environmental strain encoded a separate CRISPR array that targets a *Lactobacillus* plasmid (Table 2). These results suggest an ongoing interaction between prophages and CRISPR defenses in the genomes of *Drosophila*-associated *L. brevis* strains. Similar to our observations with *L. brevis* genomes, we observed a greater number of intact prophage genomes in *Drosophila*-associated *L. plantarum* strains than in the environmental strain (Table 2). The main difference between the *Drosophila*-associated *brevis* and *plantarum* strains is that the *plantarum* strains do not appear to encode CRISPR-dependent anti-phage defenses within their genomes.

**Function-based comparisons of *Drosophila*-associated and environmental *Lactobacillus* strains**

In general, the data above suggest very minor differences between the genomes of *Drosophila*-associated and environmental strains of *Lactobacilli*. To characterize these differences in greater detail, we performed a function-based comparison of the 185 genes that are common to *Drosophila*-associated *L. brevis* genomes, but absent from the environmental strain. This set of 185 genes describes thirteen distinct functional categories, with forty-seven unique roles (Table 2). Unsurprisingly, phage and CRISPR-associated gene
products account for two of those categories, and cover eleven of the forty-seven unique roles.

Of the remaining gene products, the dominant functional categories are dedicated to roles that appear suited for survival within an intestine. These include a biochemical cascade that converts $\alpha$-D-glucose-1-phosphate to dTDP-4-dehydro-6-deoxy-L-mannose, an exopolysaccharide that contributes to prokaryotic survival within a host intestine (Ruas-Madiedo et al., 2006; Zivkovic et al., 2015), and gene products that contribute to the formation of rhamnose-containing glycans, a cell membrane component of acid-fast bacteria that affects several host-microbe interactions, such as adhesion, recognition, and biofilm formation (Martinez et al., 2012).

We also identified gene products within Drosophila-associated L. brevis genomes that facilitate nutrient acquisition from different sources. Bacteria frequently respond to limitations in nutritional environments through activation of the cAMP receptor protein, a transcription factor that we did not identify in the environmental strain of L. brevis, but found in both Drosophila-associated strains. The cAMP receptor protein controls, among other things, the expression of gene products that coordinate metabolism of citrate (Meyer et al., 2001), a function that is also enriched among associated Drosophila-associated L. brevis genomes. In lactic acid bacteria, citrate lyase is activated in acidic environments such as those found in the gut, and increases carbon utilization and energy generation by blocking the inhibitory effects of the Lactobacillus fermentation product lactate (Magni et al., 1999). Finally, we detected an enrichment of genes involved in the transport and degradation of pectin in Drosophila-associated L. brevis genomes. Pectin is an abundant source of energy and carbon for bacteria that grow on plant and vegetable surfaces, and microbial consumption of pectin accelerates the decay of organic matter.

When we looked at the thirty-five genes exclusively observed in the genomes of DF, KP, WJL and DMCS_001 L. plantarum strains, the majority (nineteen) were prophage genes, and an additional five were hypothetical proteins of unknown function. Rather strikingly, several of the remaining genes encode products that actively suppress the growth of competing microbes. These include the PlnMNO operon that encodes a bacteriocin and cognate immunity protein (Diep et al., 1996), and 1,3-propanediol dehydrogenase, an enzyme that converts propane-1,3,2-diol to 3-hydroxypropanal. 3-hydroxypropanal, also known as reuterin, is a Lactobacillus reuteri metabolite that exerts broad-spectrum microbialidal effects on intestinal microbes in other animals (Jones and Versalovic, 2009).

### Table 2. Details on Lactobacillus genomes described in this study

|                      | L. brevis EF | L. brevis EW | L. brevis ATCC #367 |
|----------------------|--------------|--------------|---------------------|
| Number of contigs or scaffolds | 32 | 38 | 3 |
| Genome to genome distance (Prob. DDH >70%) | 100 | 98.3 | 89.92 |
| Genome size          | 2,864,530    | 2,885,101    | 2,340,228           |
| CG content (%)       | 45.3         | 45.3         | 46.1                |
| Number of RNAs       | 80           | 82           | 79                  |
| Predicted CDS        | 2808         | 2830         | 2284                |
| Assigned function    | 1961         | 1969         | 1786                |
| Uncharacterized      | 13           | 13           | 8                   |
| Conserved hypothetical| 11           | 9            | 5                   |
| Unknown function     | 31           | 31           | 32                  |
| Hypothetical         | 673          | 682          | 431                 |
| Phage-associated proteins | 119        | 126          | 22                  |
| Two-component systems| 22           | 22           | 20                  |
| Transcription Factors| 203          | 204          | 158                 |
| Other DNA binding proteins | 14        | 16           | 18                  |
| Number of Prophages  | 4            | 4            | 0                   |
| Number of CRISPRs    | 1            | 1            | 2                   |

|                      | L. plant KP | L. plant DF | L. plant WJL | L. plant JOJT01.1 | L. plant ATCC 14917 |
|----------------------|-------------|-------------|--------------|-------------------|--------------------|
| Number of contigs or scaffolds | 4           | 4           | 102          | 83                | 36                 |
| Genome to genome distance (Prob. DDH >70%) | 100         | 98.22       | 97.74        | 97.04             | 96.99              |
| Genome size          | 3,692,742   | 3,697,306   | 3,477,495    | 3,194,687         | 3,198,761          |
| CG content (%)       | 44          | 44.5        | 44.2         | 44.5              | 44.5               |
| Number of RNAs       | 104         | 104         | 85           | 65                | 65                 |
| Predicted CDS        | 3569        | 3574        | 3365         | 3063              | 3061               |
| Assigned function    | 2403        | 2400        | 2344         | 2227              | 2246               |
| Uncharacterized      | 9           | 8           | 9            | 9                 | 9                  |
| Conserved hypothetical| 13          | 12          | 10           | 10                | 11                 |
| Unknown function     | 72          | 72          | 74           | 69                | 70                 |
| Hypothetical         | 884         | 894         | 797          | 694               | 668                |
| Phage-associated proteins | 180        | 188         | 131          | 54                | 57                 |
| Two-component systems| 27          | 27          | 29           | 25                | 25                 |
| Transcription Factors| 252         | 252         | 244          | 230               | 230                |
| Other DNA binding proteins | 20        | 20          | 18           | 13                | 13                 |
| Number of Prophages  | 5           | 6           | 4            | 2                 | 2                  |
| Number of CRISPRs    | 0           | 0           | 0            | 0                 | 0                  |
Acetobacter pasteurianus

General genomic features

Although Acetobacter frequently associate with Drosophila in the wild and in the lab, we are unaware of any whole-genome sequences of A. pasteurianus strains derived from the intestines of adult Drosophila. To address this shortcoming, we completed a whole-genome sequence of an A. pasteurianus strain (A. pasteurianus AD) that we isolated from the intestines of wild-type Drosophila. For comparative purposes, we examined the available genomic sequences of the NBRC 101655 strain, and the ATCC 33445 strain. Our initial attempts to generate gnotobiotic flies, suggested that the ATCC 33445 strain fails to associate with Drosophila, something we subsequently confirmed (Fig. 1C). These data suggest that the ATCC 33445 isolate is either incapable of survival within the fly gut, or incapable of growth on fly culture medium. To distinguish between these possibilities, we examined the viability of the ATCC 33445 isolate on fly food in the absence of Drosophila. The AD strain isolated from Drosophila survives culture on fly food (Fig. 1D), however, we found that the ATCC 33445 strain failed to do so (Fig. 1E).

The different viability profiles of the different strains prompted us to compare the AD, NBRC 101655, and ATCC 33445 genomes. At first glance, we did not observe substantial differences between the respective genomes. Each genome is approximately 3 MB in length, with similar GC content and similar numbers of RNA, and predicted coding sequences (Table 4). From an evolutionary perspective, A. pasteurianus AD appears more closely related to the NBRC 101655 strain than the ATCC 33445 strain (Table 4). Consistent with a greater evolutionary distance to the ATCC strain, we found that the ATCC 33445 genome encodes 112 unique proteins, while the NBRC 101655 and AD strains share 112 genes that are absent from the ATCC 33445 genome (Fig. 3).

Environmental response factors

As with L. brevis, we first compared the Drosophila-associated and environmental genomes for distinctions in gene products that respond to environmental factors. Specifically, we looked at signaling factors, stress response factors, and virulence factors (Fig. 3). Across this series of comparisons, the most pronounced differences were commensurate with a closer relationship of the AD strain to the NBRC 101655 strain than to the ATCC 33445 strain (Table 4). Consistent with this, the AD strain contains fewer genes that respond to environmental factors, while the NBRC 101655 strain contains more such genes (Table 4).

**Fig. 2. Illustrations of the genomes for L. plantarum strains KP and DF.** GC skew is indicated in purple, and GC content is indicated in black. All positive strand ORFs are shown in blue, and negative strand ORFs are shown in yellow.
| Category                        | Subcategory | Subsystem                                                                 | Role                                                                 |
|--------------------------------|-------------|---------------------------------------------------------------------------|----------------------------------------------------------------------|
| Amino Acids and                | Arginine; urea cycle, polyamines | Arginine Biosynthesis – gjo                                              | Acetylamotiline deacetylase (EC 3.5.1.16)                             |
| Derivatives                    | Lysine, threonine, methionine, and cysteine | Threonine degradation                                                  | Threonine dehydrogenase and related Zn-dependent dehydrogenases     |
| Carbohydrates                  | No subcategory | Conserved cluster around inner membrane protein gene yghQ, probably involved in polysaccharide biosynthesis | Conserved hypothetical TPR repeat protein, clustered with yghQ       |
| Cell Wall and Capsule          | Capsular and extracellular polysaccharides | Rhamnose containing glycans                                            | Capsular polysaccharide biosynthesis protein                         |
| Clustering-based subsystems    | No subcategory | CBSS-316273.3.peg.2378                                                   | FIG006126: DNA helicase, restriction/modification system component YeeB |
| Clustering-based subsystems    | No subcategory | CBSS-316273.3.peg.2378                                                   | FIG045374: Type II restriction enzyme, methylase subunit YeeA         |
| Clustering-based subsystems    | No subcategory | CBSS-316273.3.peg.2378                                                   | YeeC-like protein                                                     |
| Cofactors, Vitamins,           | Riboflavin, FMN, FAD | Riboflavin, FMN and FAD metabolism                                        | 3,4-dihydroxy-2-butanoone 4-phosphate synthase (EC 4.1.12.12)        |
| Prosthetic Groups, Pigments    | Tetrapyrroles | Cobalamin synthesis                                                      | Cobalamin biosynthesis protein CbiG                                  |
| DNA Metabolism                 | No subcategory | DNA structural proteins, bacterial Restriction-Modification System    | DNA-binding protein HU Type I restriction-modification system, specificity subunit S (EC 3.1.21.3) |
| Fatty Acids, Lipids, and       | Fatty acids | Fatty Acid Biosynthesis FASII                                             | Enoyl-[acyl-carrier-protein] reductase [NADPH] (EC 1.3.1.10)          |
| Isoprenoids                    | No subcategory | Ton and Tol transport systems                                            | TolA protein                                                          |
| Miscellaneous                  | No subcategory | Broadly distributed proteins not in subsystems                          | Putative oxidoreductase YncB                                          |
| Nitrogen Metabolism            | Denitrification | Denitrifying reductase gene clusters                                     | Respiratory nitrate reductase alpha chain (EC 1.7.99.4)              |
| Nitrogen Metabolism            | Denitrification | Denitrifying reductase gene clusters                                     | Respiratory nitrate reductase beta chain (EC 1.7.99.4)               |
| Nitrogen Metabolism            | Denitrification | Denitrifying reductase gene clusters                                     | Respiratory nitrate reductase delta chain (EC 1.7.99.4)              |
| Nitrogen Metabolism            | Denitrification | Denitrifying reductase gene clusters                                     | Respiratory nitrate reductase gamma chain (EC 1.7.99.4)              |
| Nitrogen Metabolism            | No subcategory | Nitrate and nitrite ammonification                                        | Assimilatory nitrate reductase large subunit (EC:1.7.99.4)            |
| Nitrogen Metabolism            | No subcategory | Nitrate and nitrite ammonification                                        | Nitrate ABC transporter, nitrate-binding protein                      |
| Nitrogen Metabolism            | No subcategory | Nitrate and nitrite ammonification                                        | Nitrate/nitrite transporter                                           |
| Phages, Prophages,             | No subcategory | Nitrate and nitrite ammonification                                        | Nitrate reductase [NAD(P)]H large subunit (EC 1.7.1.4)                |
| Transposable elements,         | Phages, Prophages | Nitrate and nitrite ammonification                                        | Response regulator NasT                                              |
| Plasmids                       | Phage tail proteins | Phage tail proteins                                                     | Phage tail length tape-measure protein                                |
| Phages, Prophages,             | Phages, prophages | Phage tail proteins                                                     | Phage tail tube protein                                               |
| Transposable elements,         | Phages, prophages | Phage tail proteins                                                     | Phage tail/DNA circulation protein                                    |
| Plasmids                       | Phages, prophages | Phage tail proteins                                                     | Phage tail/DNA circulation protein                                    |
| Protein Metabolism             | Protein biosynthesis | tRNAs                                                                    | tRNA-Ser-CGA                                                          |
| Protein Metabolism             | Protein biosynthesis | tRNAs                                                                    | tRNA-Ser-GGA                                                          |
| Protein Metabolism             | Protein processing and modification | N-linked glycosylation in bacteria                                       | N-linked glycosylation glycosyltransferase PglG                       |
| Regulation and Cell signaling  | Programmed cell death and toxin-antitoxin systems | Phd-Doc, YdE-YdcD toxin-antitoxin (programmed cell death) systems        | Prevent host death protein, Phd antitoxin                            |

Continued
survive passage on Drosophila medium, and the ATCC 33445 genome that fails to do so.

Function-based comparisons of individual strains of Acetobacter pasteurianus

Our fortuitous identification of an environmental A. pasteurianus strain that fails to grow on fly food under experimental conditions that permit growth of all other Lactobacillus and Acetobacter strains tested allowed us to explore A. pasteurianus genomes for factors that may permit survival within a Drosophila-friendly environment. We reasoned that the AD and NBRC 101655 genomes encode biochemical functions absent from ATCC 33445 that permit survival on fly food, or that the ATCC 33445 genome encodes biochemical functions absent from the other strains that prevent survival on fly food. This prompted us to identify biological subsystems shared exclusively by the AD and NBCR 101655 (Table 5), or unique to the ATCC 33445 genome (Table 3).

In this comparative analysis, we noted four subsystems exclusive to the AD and NBCR 101655 genomes that may explain their ability to survive on fly food. Both strains encode polyamine metabolism factors that are frequently associated with cellular growth and survival, and have established roles in the formation of biofilms (Di Martino et al., 2013). The association-competent genomes also encode factors necessary for the conversion of urea to ammonium and carbon dioxide. A similar system operates in Helicobacter pylori where it raises the gastric pH to generate a more hospitable environment for microbial survival (Scott et al., 1998). We also detected the redox-sensitive transcriptional activator SoxR in both association-competent genomes. SoxR promotes microbial survival by countering the antibacterial actions of superoxide anions (Imlay, 2015). Finally, we detected several genes that contribute to organic sulfur assimilation in association-competent genomes. These gene products may allow A. pasteurianus AD and NBRC 101655 to use alkanesulfonates as a source of sulfur during sulfate or cysteine starvation and may provide both strains a competitive advantage if sulfur is limiting.

The association-incompetent ATCC 33445 strain also encodes products that may contribute to generation of ammonia. However, the ATCC 33445 strain apparently relies on respiratory nitrate reductase and nitrite reductase to generate ammonia, as well as assimilatory nitrate reductase to access nitrate for metabolic growth. This represents an entirely different strategy to use nitrogen as a fuel for metabolic energy and growth. We also observed two toxin-antitoxin systems unique to the association-incompetent ATCC 33445 genome – an addiction module toxin that ensures propagation of plasmids to progeny cells (Engelberg-Kulka and Glaser, 1999), and a MazE/MazF type toxin-antitoxin (Masuda et al., 1993). The MazE/MazF system induces programmed cell death in prokaryotic cells in response to stressful environments.

| Category | Subcategory | Subsystem | Role |
|----------|-------------|-----------|------|
| Regulation and Cell signaling | Programmed cell death and toxin-antitoxin systems | Phd-Doc, YdcE-YdcD toxin-antitoxin (programmed cell death) systems | Programmed cell death antitoxin MazE like |
| Regulation and Cell signaling | Programmed cell death and toxin-antitoxin systems | Phd-Doc, YdcE-YdcD toxin-antitoxin (programmed cell death) systems | Programmed cell death toxin MazF like |
| Regulation and Cell signaling | Programmed cell death and toxin-antitoxin systems | Toxin-antitoxin replicon stabilization systems | RelE/StbE replicon stabilization toxin |
| RNA Metabolism | RNA processing and modification | ATP-dependent RNA helicases, bacterial | Cold-shock DEAD-box protein A |
| Stress Response | No subcategory | Flavohaemoglobin | Nitric-oxide reductase (EC 1.7.99.7), quinol-dependent probable dibenzothiophene desulfurization enzyme |
| Sulfur Metabolism | Organic sulfur assimilation | Alkanesulfonate assimilation | Arsenic efflux pump protein |
| Virulence, Disease and Defense | Resistance to antibiotics and toxic compounds | Arsenic resistance | Arsenic resistance protein ArsH |

Table 4. Details on Lactobacillus brevis genomes described in this study

| | A. pasteurianus AD | A. pasteurianus ATCC 33445 | A. pasteurianus NBRC 101655 |
|-----------------|-------------------|--------------------------|--------------------------|
| Number of contigs or scaffolds | 161 | 306 | 294 |
| Genome to genome distance (Prob. DDH >=70%) | 100 | 90.67 | 98.3 |
| Genome size | 2,830,055 | 2,888,200 | 3,018,312 |
| CG content (%) | 52.7 | 53.1 | 52.7 |
| Number of RNAs | 42 | 44 | 42 |
| Predicted CDS | 2673 | 2797 | 2834 |
| Assigned function | 1845 | 1866 | 1923 |
| Uncharacterized | 10 | 9 | 10 |
| Conserved hypothetical | 8 | 9 | 8 |
| Unknown function | 12 | 15 | 11 |
| Hypothetical | 770 | 854 | 852 |
| Phage-associated proteins | 28 | 44 | 30 |
to the observed a small number of genetic pathways that were exclusive environmental strains of the same species. For each species, we flies. To this end, we compared fly-associated genomes to factors that could permit survival within the intestines of adult particularly interested in the identification of candidate bacterial host-microbe interactions, raising the possibility these products associated pathways encode products with established roles in biochemical events that permit bacterial survival within a fly gut lumen. To facilitate such studies, we are developing protocols for genetic manipulation of our lab isolates of Lactobacillus strains. These studies are particularly important given the strain-specific effects of individual Lactobacillus plantarum strains on host phenotypes (Erkosar et al., 2015; Schwarzer et al., 2016; Storelli et al., 2011).

### Lactobacilli

For our studies of Lactobacillus genomes, we prepared whole-genome sequences of L. brevis or L. plantarum strains that we isolated from lab-raised wild-type flies, and an L. plantarum strain that we isolated from a wild Drosophila. These genomes formed the cornerstones of a comparative study that included three previously reported Drosophila-associated genomes (Kim et al., 2013a,b; Newell et al., 2014), and the genomes of environmental strains that successfully associate with the intestines of wild-type Drosophila. In this manner, we identified bacterial functions that are unique to the Drosophila-associated genomes of L. brevis and L. plantarum covered in this study. The functions fall into four broad categories: antibacterial, structural, metabolic, and phage-related.

The most striking feature common to all four Drosophila-associated L. plantarum genomes was the presence of broad-spectrum bactericidal factors. For example the DF, KP, WJL and DMCS_001 genomes all encode a complete PnLMNO operon, which encodes a bacteriocin and a corresponding immunity protein (Diep et al., 1996). Bacteriocins are produced by many lactic acid bacteria to kill neighboring bacteria, while the immunity protein protects L. plantarum from collateral damage (Cotter et al., 2005). In addition, the Drosophila-associated L. plantarum genomes encode the enzymatic capacity to generate 3-hydroxypropionaldehyde to suppress the growth of other commensals. Combined, these bactericidal molecules have the potential to counter the growth of competing bacteria inside a Drosophila host, and favor expansion of L. plantarum. The putative competitive advantages conferred by the PnLMNO operon and 3-hydroxypropionaldehyde may explain why L. plantarum is frequently reported in studies that characterize the intestinal microbiota of Drosophila.

The Drosophila-associated genomes of L. plantarum and L. brevis also encode structural components that may stabilize associations with their fly host. For example, we detected metabolic pathways for modifications to cell walls that permit host-microbe interactions and biofilm formation. These include the construction of Drosophila with the individual strains.

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**DISCUSSION**

The last decade witnessed a proliferation of elegant studies that uncovered critical host responses to microbial factors in the Drosophila intestine [reviewed in Buchon et al. (2013)]. Bacterial cues promote larval growth (Shin et al., 2011; Storelli et al., 2011), direct innate immune responses (Broderick et al., 2014; Erkosar et al., 2014), orchestrate the proliferation of intestinal stem cells (Buchon et al., 2009a,b), and regulate the uptake and storage of nutrients (Wong et al., 2014). Despite the importance of the intestinal microbiota for Drosophila health and development, there are gaps in our understanding of the biochemical events that permit bacterial survival within the hostile terrain of a fly intestine. Recent studies identified microbial metabolism and stress response pathways that mediate interactions between intestinal bacterial and their Drosophila host (Chaston et al., 2014; Newell et al., 2014). In this study, we examined the genomes of Drosophila-associated strains of L. brevis, L. plantarum, and A. pasteurianus. We were particularly interested in the identification of candidate bacterial factors that could permit survival within the intestines of adult flies. To this end, we compared fly-associated genomes to environmental strains of the same species. For each species, we observed a small number of genetic pathways that were exclusive to the Drosophila-associated genomes. Many of the Drosophila-associated pathways encode products with established roles in host-microbe interactions, raising the possibility these products may facilitate association of Drosophila with the individual strains.

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**Caveats**

Interpretation of the data presented in this study should be influenced by several important caveats. The experimental design in this study does not distinguish between true colonization of an adult intestine and simple passage through the gut. To date, there are no studies that have identified Lactobacillus strains that fail to associate with the adult intestine of Drosophila. We also observed that environmental strains of L. brevis and L. plantarum form stable associations with Drosophila. The rather indiscriminate associations between flies and Lactobacilli confound attempts to identify fly-specific response factors within a bacterial genome. Indeed, it cannot be excluded that core elements of Lactobacillus genomes are sufficient for survival during transit through a fly intestine. In contrast, we have identified an A. pasteurianus strain that appears incapable of growth on fly food. This strain is a useful starting point for identification of Acetobacter genes that are required for association with Drosophila and we present several potential candidates within this report. As a next step, it is important to perform mutagenesis studies on candidate genes to identify the specific bacterial factors that permit survival within a fly gut lumen.

Fig. 3. Distribution of unique gene functions in the genomes of A. pasteurianus strains AD, ATCC 33445 and NBRC 101655. All data are based on gene function annotations within RAST and exclude gene products with unknown functions.

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**TABLE**

| GENOME          | SIGNALING          | STRESS            | VIRULENCE         |
|-----------------|--------------------|-------------------|-------------------|
| A. pasteur. AD  | A. pasteur. ATCC 33445 | A. pasteur. AD     | A. pasteur. ATCC 33445 |
| 1               | 0                  | 1                 | 0                 |
| 1709            | 0                  | 1                 | 0                 |
| 112             | 0                  | 1                 | 0                 |
| 33              | 0                  | 1                 | 0                 |

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**Biology Open**
| Category | Subcategory | Subsystem | Role |
|----------|-------------|-----------|------|
| Amino Acids and Derivatives | Alanine, serine, and glycine | Glycine biosynthesis | Low-specificity L-threonine aldolase (EC 4.1.2.5) |
| Amino Acids and Derivatives | Arginine; urea cycle, polyamines | Polyamine metabolism | 4-aminobutyraldehyde dehydrogenase (EC 1.2.1.19) |
| Amino Acids and Derivatives | Arginine; urea cycle, polyamines | Polyamine metabolism | Spermidine Putrescine ABC transporter permease component PotB (TC 3. A.1.11.1) |
| Amino Acids and Derivatives | Arginine; urea cycle, polyamines | Polyamine metabolism | Spermidine Putrescine ABC transporter permease component PotC (TC 3. A.1.11.1) |
| Clustering-based subsystems | Clustering-based subsystems | CBSS-292415.3.peg.2341 | Major facilitator superfamily (MFS) transporter |
| Clustering-based subsystems | No subcategory | Conserved gene cluster associated with Met-tRNA formyltransferase 16S rRNA (cytosine(967)-C(5))-methyltransferase (EC 2.1.1.176) | Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3) |
| Cofactors, Vitamins, Prosthetic Groups, Pigments | Biotin | Biotin biosynthesis | Long-chain-fatty-acid-CoA ligase (EC 6.2.1.3) |
| Cofactors, Vitamins, Prosthetic Groups, Pigments | Folate and pterines | 5-FCL-like protein | Butyryl-CoA dehydrogenase (EC 1.3.8.1) |
| DNA Metabolism | DNA repair | DNA repair, bacterial UvrD and related helicases | ATP-dependent DNA helicase UvrD/PcrA, proteobacterial paralog |
| DNA Metabolism | Iron acquisition and metabolism | DNA structural proteins, bacterial Hemin transport system | DNA-binding protein HBsu, Outer membrane receptor proteins, mostly Fe transport |
| Membrane Transport | Cation transporters | Magnesium transport | Mg(2+) transport ATPase protein C, HoxN/HupN/NixA family nickel/cobalt transporter |
| Membrane Transport | Cation transporters | Transport of Nickel and Cobalt | |
| Metabolism of Aromatic Compounds | Peripheral pathways for catabolism of aromatic compounds | Benzoate degradation | Benzoate 1,2-dioxygenase alpha subunit (EC 1.14.12.10) |
| Metabolism of Aromatic Compounds | Peripheral pathways for catabolism of aromatic compounds | Benzoate degradation | Benzoate 1,2-dioxygenase beta subunit (EC 1.14.12.10) |
| Metabolism of Aromatic Compounds | No subcategory | Aromatic amin catabolism | Nitrilotriacetate monoxygenase component B (EC 1.14.13.-) |
| Metabolism of Aromatic Compounds | No subcategory | Aromatic amin catabolism | Phenylacetalddehyde dehydrogenase (EC 1.2.1.39) |
| Phosphorus Metabolism | No subcategory | Phosphate metabolism | Soluble pyridine nucleotide transhydrogenase (EC 1.6.1.1) |
| Protein Metabolism | Protein biosynthesis | tRNA aminoacylation, Pro Urease accessory protein UreD | tRNA proofreading protein STM4549 |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease accessory protein UreE |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease accessory protein UreG |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease gamma subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Protein Metabolism | Protein processing and modification | G3E family of P-loop GTPases (metallocenter biosynthesis) | Urease beta subunit (EC 3.5.1.5) |
| Respiration | Electron accepting reactions | Anaerobic respiratory reductases | Vanillate O-demethylase oxidoreductase (EC 1.14.13.-) |
| Respiration | No subcategory | Carbon monoxide dehydrogenase maturation factors | Aerobic carbon monoxide dehydrogenase molybdenum cofactor insertion protein CoxF |
| RNA Metabolism | No subcategory | Group II intron-associated genes | Retron-type RNA-directed DNA polymerase (EC 2.7.7.49) |
| Stress Response | Oxidative stress | Oxidative stress | Redox-sensitive transcriptional activator SoxR |
of exopolysaccharides by *L. brevis* and the regulation of sialic acid by *L. plantarum*. Sialic acid is a comparatively rare microbial metabolite, but has been observed on microbes that associate with deuterostomes. Bacteria use sialic acid as a nutrient, but they also use it to mask detection by host immune responses. While the role of sialic acid in *L. plantarum* association with *Drosophila* requires further investigation, we feel that these elements merit consideration as host-microbe interaction factors.

The *Drosophila*-associated genomes of *L. plantarum* and *brevis* also include gene products that may address nutritional requirements. Functional annotation of the respective genomes suggests that these gene products may enhance access to limited resources such as methionine by *L. plantarum* and utilization of citrate as an energy source by *L. brevis*. We were particularly struck by the presence of pectin metabolism factors within the genomes of *Drosophila*-associated strains of *L. brevis*. Pectin is an excellent source of carbon for bacteria that grow on plants; however, bacterial utilization of pectin accelerates the ripening and decay of the same plants (Abbott and Boraston, 2008). Thus, *Drosophila*-associated *L. brevis* genomes express factors that contribute to the decay of organic substrates. We consider this noteworthy, as *Drosophila* preferably consumes decayed matter as a source of nutrients. The ability of *L. brevis* to generate meals for their *Drosophila* host provides a possible explanation for the fact that *Drosophila* frequently associate with *L. brevis*. As *L. brevis* generates palatable meals for fly hosts, we speculate that their chances of association with flies in the wild are greater than those for many other bacteria. This host-microbe relationship is similar to a proposed mechanism for association of *Erwinia carotovora* with *Drosophila* in the wild (Basset et al., 2000). Our lab raised fly strains are fed a meal that contains yellow cornmeal, a potential source of pectin, possibly explaining the persistence of pectin metabolism genes in *L. brevis* strains isolated from flies.

The final difference we noted between environmental and *Drosophila*-associated *Lactobacillus* genomes was an accumulation of temperate prophage genomes throughout *Drosophila*-associated *Lactobacilli*. Intestinal stresses such as high levels of reactive oxygen species are known to trigger lyogenic induction of temperate prophages (DeMarini and Lawrence, 1992). Thus, it is feasible that bacterial strains that pass through the fly intestine will release and integrate greater numbers of lytic prophages, explaining the increased numbers of prophage genomes in *Lactobacillus* strains that associate with adult *Drosophila*.

### Acetobacter

In this study, we report the first genome of a *Drosophila*-associated strain of *A. pasteurianus*, and identified an *A. pasteurianus* strain that cannot grow on fly food. Unfortunately, our genomic comparisons are limited by the fact that only one *Drosophila*-associated genome is available for study. Nonetheless, our study yields a comparatively short list of candidate functions that may regulate growth of *Acetobacter* on nutrient medium for *Drosophila*.

### MATERIALS AND METHODS

#### Drosophila husbandry

All *Drosophila* assays were performed with virgin *w*1118* males and female flies raised on standard corn-meal medium (Nutri-Fly Bloomington Formulation, Genesee Scientific) in a humidified incubator at 29°C. To generate germ-free flies, we transferred freshly eclosed (0-16 h old) adult flies to standard medium that we supplemented with an antibiotic cocktail (100 μg/ml ampicillin, 50 μg/ml vancomycin, 100 μg/ml neomycin and 100 μg/ml metronidazole dissolved in ethanol). This mixture has been described previously (Ryu et al., 2008). To generate gnotobiotic flies, we raised adult flies on the antibiotic cocktail for five days, starved flies for two hours, and transferred the flies to a vial containing an autoclaved fly vial cotton plug soaked with the respective bacteria. Bacterial cultures were prepared to OD600 of 50 in 5% sucrose/PBS. Twelve flies per vial were then associated with 1 ml of commensal bacteria suspension on cotton plugs. We fed the flies the bacterial meal for 16 h and transferred the flies to vials of freshly autoclaved food. Flies were raised on the initial vial for one week and transferred to fresh vials weekly thereafter. To test association, we plated fly homogenates on bacterial medium selective for *Acetobacter* (GYC agar) or *Lactobacilli* (MRS-agar) every two weeks. For *A. pasteurianus*, colony forming units were determined by independent quantification of three replicates of five flies/replicate. Flies were sterilized in 50% bleach, 75% ethanol and rinsed in water. Sterilized flies were homogenized in MRS broth (Fluka Analytical) and serial dilutions of the homogenate were plated on GYC agar plates. To test the survival of *A. pasteurianus* on fly food, bacteria were grown from 2 days at 29°C with shaking. A bacterial culture of an OD 50 was prepared in 5% sucrose in PBS. From the OD, 50 culture serial dilutions down to 10−7 were prepared. 50 μl of each of the serial dilutions was added to autoclaved fly food. Vials were gently rotated to spread out the bacterial culture. Vials were plugged and incubated at 29°C for one week. Vials were rinsed with 1 ml of MRS and of the 1 ml rinse 50 μl was plated on GYC plates and incubated for 2 days at 29°C. The images shown in panels D and E of Fig. 1 correspond to the 10−7 dilutions.

#### Bacterial isolation and sequencing

We plated homogenates of 15-day-old adult *Drosophila* on GYC and MRS culture plates. We found that *L. brevis* colonies are easily distinguished from *L. plantarum* colonies on MRS-agar medium. We isolated individual colonies of *A. pasteurianus*, *L. brevis* and the KP strain of *L. plantarum* and grew them statically at 29°C in liquid MRS (*L. brevis* and *L. plantarum*), or shaking in liquid (*A. pasteurianus*). The DF strain of *L. plantarum* was
isolated from a wild, mated isofemale *Drosophila melanogaster* captured on a rotting strawberry in the kitchen of EF in Edmonton, Canada. Bacterial DNA was isolated with the Microbial DNA Isolation kit from MO BIO Laboratories Inc. (catalog number: 12224-250) according to its instructions. The genomes of *L. plantarum* strains DF and KP were sequenced and assembled at the McGill University and Génome Québec Innovation Centre on the PacBio platform. The genomes of *A. pasteurianus* (strain AD) and *L. brevis* (strain EF) were sequenced at The Applied Genomics Core of the University of Alberta. For the latter genomes, we prepared Nextera XT libraries from the isolated microbial DNA according to Illumina’s protocol and sequenced the libraries with using the V3-600 cycle Kit (Illumina). Whole genome sequences were then assembled using Lasergene software (DNASTAR).

**Genome assembly and annotation**

For each sequencing project, we confirmed the individual species with the SpeciesFinder 1.2 algorithm (Larsen et al., 2014) and calculated genome to genome distances with the genome to genome distance calculator of the SpeciesFinder 1.2 algorithm (Larsen et al., 2014) and calculated genome to genome distance.

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