Title

Extensive Horizontal Gene Transfer in Cheese-Associated Bacteria

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Abstract

Acquisition of genes through horizontal gene transfer (HGT) allows microbes to rapidly gain new capabilities and adapt to new or changing environments. Identifying widespread HGT regions within multispecies microbiomes can pinpoint the molecular mechanisms that play key roles in microbiome assembly. We sought to identify horizontally transferred genes within a model microbiome, the cheese rind. Comparing 31 newly-sequenced and 134 previously sequenced bacterial isolates from cheese rinds, we identified over 200 putative horizontally transferred genomic regions containing 4,844 protein coding genes. The largest of these regions are enriched for genes involved in siderophore acquisition, and are widely distributed in cheese rinds in both Europe and the US. These results suggest that horizontal gene transfer (HGT) is prevalent in cheese rind microbiomes, and the identification of genes that are frequently transferred in a particular environment may provide insight into the selective forces shaping microbial communities.

Introduction

Great strides have been made in characterizing the composition of microbiomes, and in understanding their importance in the ecology of natural systems, in agriculture and in human health. However, despite these advances, the forces that shape the diversity, structure, and function of microbiomes remain poorly understood (Widder et al. 2016). Investigating these underlying mechanisms *in situ* is difficult, since observational and sequenced-based analysis rarely enables causal conclusions (Nemergut et al. 2013). Replicating microbial communities *in vitro* is also an enormous challenge, due to high levels of diversity and the difficulties in establishing pure cultures of most bacterial species. These obstacles significantly hamper our ability to move from observations of microbial diversity to the unravelling of the underlying

molecular mechanisms shaping key processes such as species interactions and microbial evolution.

Horizontal gene transfer (HGT) is a major force in microbial evolution and can lead to the wholesale acquisition of novel functions. In some cases, these novel functions can have significant adaptive consequences, such as in the transfer of antibiotic resistance genes (Ochman, Lawrence, and Groisman 2000). HGT also allows rapid adaptation to new niches (Wiedenbeck and Cohan 2011), since ecologically relevant genes may be acquired by species not previously adapted to a particular niche (Tasse et al. 2010; Hehemann et al. 2010). The movement of microbes to new environments has been shown to increase both the rate and impact of HGT, and HGT is most frequent for genes under positive selection (Niehus et al. 2015). In moving to a new environment, microbes can face novel abiotic conditions (temperature, moisture, salinity, pH, and nutrients) and novel biotic challenges and opportunities due to the presence of microbial neighbors.

Evaluating HGT within the context of microbial communities has the potential to uncover new insights concerning the extent, mechanisms, and ecological impact of this important process. Advances in genome sequencing have begun to provide a glimpse into HGT within environmentally, medically and economically important microbial communities (McDaniel et al. 2010; Andam, Carver, and Berthrong 2015). For example, extensive gene sharing has been observed throughout the commensal human microbiome ((Smillie et al. 2011; Hehemann et al. 2010)), including genes that enable nutrient acquisition from novel food sources (Smillie et al. 2011; Hehemann et al. 2010), and pathogenicity islands and antibiotic resistance genes in pathogenic microbes (McCarthy et al. 2014; Hiramatsu et al. 2001; Forsberg et al. 2012). While these studies offer valuable insights into the spectrum of genes that can be transferred in microbial communities, the complexity of these systems makes further examination of the effects of these HGT events on their evolution and ecology difficult.

The microbial communities of fermented foods experience strong selection as a result of growing in novel, human-made environments. Previous work has demonstrated that HGT can be a major driver of adaptation in food systems and other human-managed environments (Andam, Carver, and Berthrong 2015; Rossi et al. 2014). During the aging of cheese, bacteria and fungi form a multi-species biofilm called the rind (Button and Dutton 2012). We have previously shown that these communities can be used to examine community-based processes,such as succession and interspecies interactions, within an experimentally tractable system (Wolfe et al. 2014; Kastman EK, Kamelamela N, Norville JW, Cosetta CM, Dutton RJ, Wolfe BE. 2016). Given that biofilms such as these are high density communities, and microbes in cheese rinds are under strong selection to obtain limited nutrients (e.g. free amino acids, iron) as well as tolerate cell stress (Monnet et al. 2015), we predicted that HGT might be widespread in cheese rind microbiomes.

Prior analysis of microbial species from cheese have revealed several instances of HGT in this environment. Lactic acid bacteria (LAB) such as *Lactobacillus* and *Lactococcus*, which are used in the initial fermentation of milk, are known to harbor antibiotic resistance genes and may be

reservoirs for transfer to pathogenic enterococci (Wang et al. 2006; Mathur and Singh 2005) and other pathogenic microbes. Other food-associated bacteria may also contribute to antibiotic resistance gene transfer (Cocconcelli, Cattivelli, and Gazzola 2003; Delorme 2008; Flórez, Delgado, and Mayo 2005). In yogurt, another dairy ferment utilizing LAB, HGT of metabolic genes between protocooperative species *L. bulgaricus* and *S. thermophilus* has been observed (Li et al. 2013; Liu, Siezen, and Nauta 2009). Sequencing of *Penicillium* species isolated from the cheese-making environment identified HGT of large genomic islands between these key fungal inhabitants of cheese (Cheeseman et al. 2014; Ropars et al. 2015; Gibbons and Rinker 2015). However, a more global investigation of HGT, spanning the full diversity of microbes in cheese microbiomes, is lacking.

We sought to determine the diversity, distribution, and functional content of HGT in bacterial species isolated from cheese rinds. Specifically, we predicted that 1) HGT would be widespread, 2) that HGT genes would be enriched for functions related to survival in cheese environment, and 3) that there would be uneven distribution of HGT events across taxa. We analyzed the genomes of newly isolated and sequenced cheese-associated bacterial species (31 genomes) and those available in public databases (134 additional genomes). We present data which suggests that there has been extensive HGT in cheese-associated bacteria. The regions of DNA identified appear to encode a number of functions which would be expected to provide adaptive advantages within the cheese environment. In particular, we identified three large multi-gene islands that are shared within multiple Actinobacteria, Proteobacteria and Firmicutes species respectively. These genomic regions are not related, but appear to have analogous functions involving iron acquisition, and are widely distributed in geographically distant cheeses. This work provides foundational knowledge in an experimentally-tractable system in which future work can help to provide insight on the causes and consequences of HGT within microbiomes.

Results

Identification of putative horizontally transferred regions

To establish a diverse database of cheese-associated bacterial genomes, we isolated species from cheese samples collected as part of previous work (Wolfe et al. 2014). A total of 31 isolates, representing 4 bacterial phyla and 11 genera, were selected for genome sequencing using Illumina and PacBio (Supplementary Table 1). Recently, a large collection of cheese-associated bacterial genomes were sequenced (Almeida et al. 2014), which allowed us to include additional genomes in our analysis. Our isolates were mainly from cheeses produced in the United States while the Almeida et al. collection was almost exclusively from France. We also included genomes from the NCBI reference sequence (RefSeq) database that are associated with cheese, for a total of 165 bacterial genomes.

We next developed a computational pipeline for the identification of putative horizontally transferred genes adapted from work on the human microbiome (Smillie et al. 2011). We built a

central BLAST database containing all ORFs from all cheese-associated genomes. For each gene in each genome, we performed BLAST against this database, and compiled a list of hits (Supplementary Figure 1, Materials and Methods). For each hit, we examined the length and percent identity of aligned regions. Closely related species will have many nearly identical genes due to vertical inheritance. To avoid capturing these genes in our analysis, we determined the pairwise average nucleotide identity (ANI) between species within the same genus (Chan et al. 2012; Rodriguez-R and Konstantinidis 2016). ANI provides a measure of the overall similarity of two genomes. We tested varying thresholds for length and ANI in order to examine the effects of these parameters on the results (Supplementary Figure 2). Higher maximum ANI cutoffs and shorter lengths are more likely to yield false positives, since closely related species are more likely to share short stretches of high nucleotide identity. At the same time, a lower maximum ANI cutoff may exclude legitimate HGT events, especially considering that closely related species are also more likely to engage in HGT. Based on our most conservative gene identity parameters (minimum 99% identity over 500 nucleotides), we identified at least one putative horizontally transferred gene in 130/165 cheese-associated species in the analysis, for a total of 4,844 genes (Figure 1A, Supplementary Table 3). At least one putative HGT protein coding gene was found in 130 out of 165 species (78.8%). Because this analysis depends on the species included for comparison, this list of HGT is almost certainly an underestimate.

Since multiple genes can be transferred in a single HGT event, we next assembled the putative HGT genes into groups based on genomic proximity. Individual coding sequences (CDS) for each species were grouped into islands if they were found within 5000 nucleotides of one another on the same contig. These islands were then clustered with islands in other species if they shared at least one CDS in common. The 4,844 genes clustered into 259 individual groups (Figure 1B, Supplementary Table 3). Mobile elements such as transposons complicate our method of group clustering, since non-contiguous islands may be grouped together if they share a common transposon. Indeed, this appears to have occurred with Group 1, which contains genes from several disparate genomic regions. In other cases, a single species may have genes in a single group spread across multiple contigs (Supplementary Figure 3B), but this may accurately represent a single HGT event.

Most HGT groups we identified (234, 90%) contain only members of the same phylum, or even a single genus (180 or 69%, Supplementary Table 4). This supports previous studies that suggest that HGT is most prevalent among closely related species (Ravenhall et al. 2015). However, we uncovered several notable exceptions. For example, *Alkaibacterium kapii* FAM208.38, a Firmicute, has a substantial (~8kb) fraction of Group 1, which is predominantly found in *Actinobacteria* species. Groups 2 and 3 each have hits found in 3 different phyla (though both are predominantly found in Actinobacteria).

Functions encoded in HGT regions

HGT enables rapid evolution of microbes entering a new environment, and genes that are under positive selection are observed more frequently (Wiedenbeck and Cohan 2011). Identifying the

Figure 1

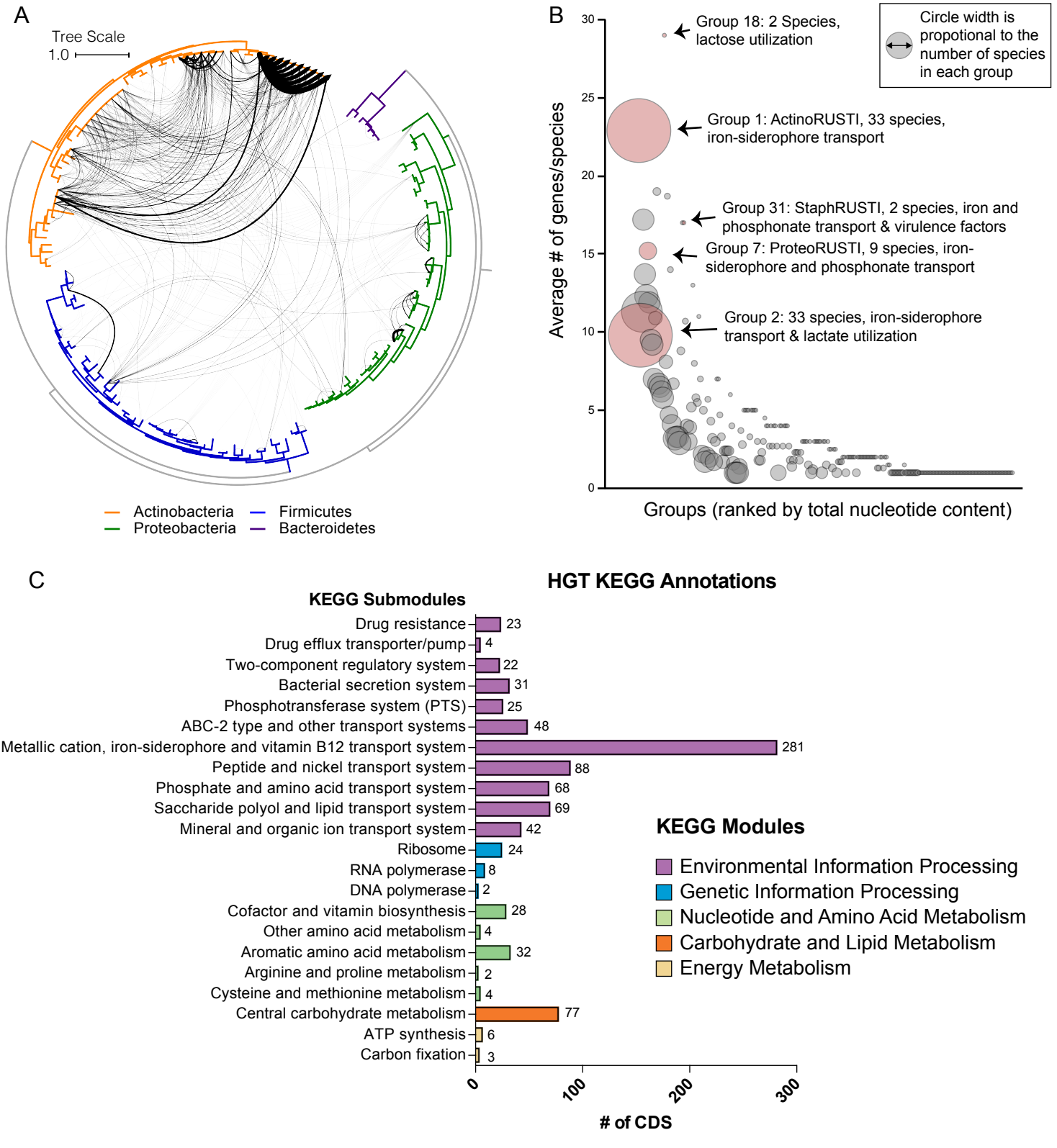

Figure 1: Extensive Horizontal Gene Transfer in the Cheese Microbiome
(A) All HGT events in analyzed cheese-associated bacteria. Connection thickness is scaled to # of shared protein coding sequences. Phylogenetic tree based on 16S RNA alignment using Ribosomal Database Project (RDP). (B) Mean number of genes per species in HGT groups. Diameter of each circle is proportional to the total number of species in the group. Groups highlighted in red are described further in the text. (C) Quantification of KEGG modules and submodules for protein coding genes (CDS) identified as horizontally transferred. Annotations were generated by BLAST-Koala. Genes without function prediction are not depicted.

functions of genes that are frequently transferred may provide a window into the selective forces that are most important for adapting to the cheese rind environment. Across all of the genes identified in our analysis, the most abundant gene functions are transposases, conjugal transfer genes, phage-related proteins and other mobile elements (673/4844 or 14% of all protein coding sequences). Nearly a third (75/259) of all HGT groups contain mobile elements. These genetic elements are likely involved in either direct or indirect mobilization and transfer of DNA.

In order to determine if gene functions other than mobile elements are enriched in identified HGT regions, we used BlastKOALA (Kanehisa, Sato, and Morishima 2016) to assign KEGG functional annotations (Figure 1C, Figure 2, Supplemental Table 3). Approximately half (52.4%) of genes could not be assigned KEGG annotations. Of the KEGG-annotated genes, the most frequent module (281/2264 or 11%) was “metal ion, iron siderophore and vitamin B12 transport systems”. Five of the ten largest HGT groups as measured by total number of genes (Groups 1, 2, 3, 7 and 8) contain siderophore transport systems (K02013-K02016). Iron is known to be limiting on cheese for several bacterial species (Monnet, Back, and Irlinger 2012; Monnet et al. 2010). Previous work has also shown that genes involved in iron acquisition are present in higher numbers in cheese-associated species compared to closely related species from other environment (Monnet et al. 2010; Walter et al. 2014).

Many other horizontally transferred genes (267/2264 or 12% of KEGG annotated genes) are also involved in the transport of nutrients relevant for growth in the cheese environment. Lactate is an abundant carbon source in freshly-made cheese, since the initial stages of cheesemaking involve the fermentation of lactose to lactate by lactic acid bacteria (Button and Dutton 2012). We observe a large number of genes (63/2264 or 2.8% of KEGG annotated genes) involved in lactate import (lactate permease - K03303) or lactate metabolism. Lactate dehydrogenase (K00101), which reduces lactate to pyruvate, represented nearly 1% of all horizontally transferred protein coding sequences. Since pyruvate is central to many biosynthetic pathways and may be efficiently converted to energy in oxidative phosphorylation, the ability to synthesize it from an abundant metabolite such as lactate could be particularly adaptive on cheese.

Apart from lactate, the primary source of energy for microbial growth in cheese would be derived from metabolism of the abundant lipids and proteins, particularly casein (Monnet et al. 2015). Glutamate importers (43/2264 or 1.9%, eg. K12942, K10005-K10008) and short peptide/nickel transporters (88/2264 or 3.9% eg. K03305) were identified, suggesting pathways for utilization of casein degradation products. Transporters for micronutrients, including phosphonate (K05781, K06163-K06165), molybdate (K02017, K02019, K02020, K03750, K03750, K03639), and metal ions like zinc and manganese were also identified.

HGT of drug resistance genes is of particular concern, since mobile resistance genes from food-associated microbes may be transferred to animal- and human-associated microbes (Rossi et al. 2014). Cheese rind communities frequently contain filamentous fungi including *Penicillium* species and other microbes that could produce antimicrobial compounds and thus select for antibiotic resistance in co-occurring species. Yet surprisingly, less than 1% of KEGG-annotated genes in this dataset are related to drug resistance. A tetracycline resistance gene was

Figure 2

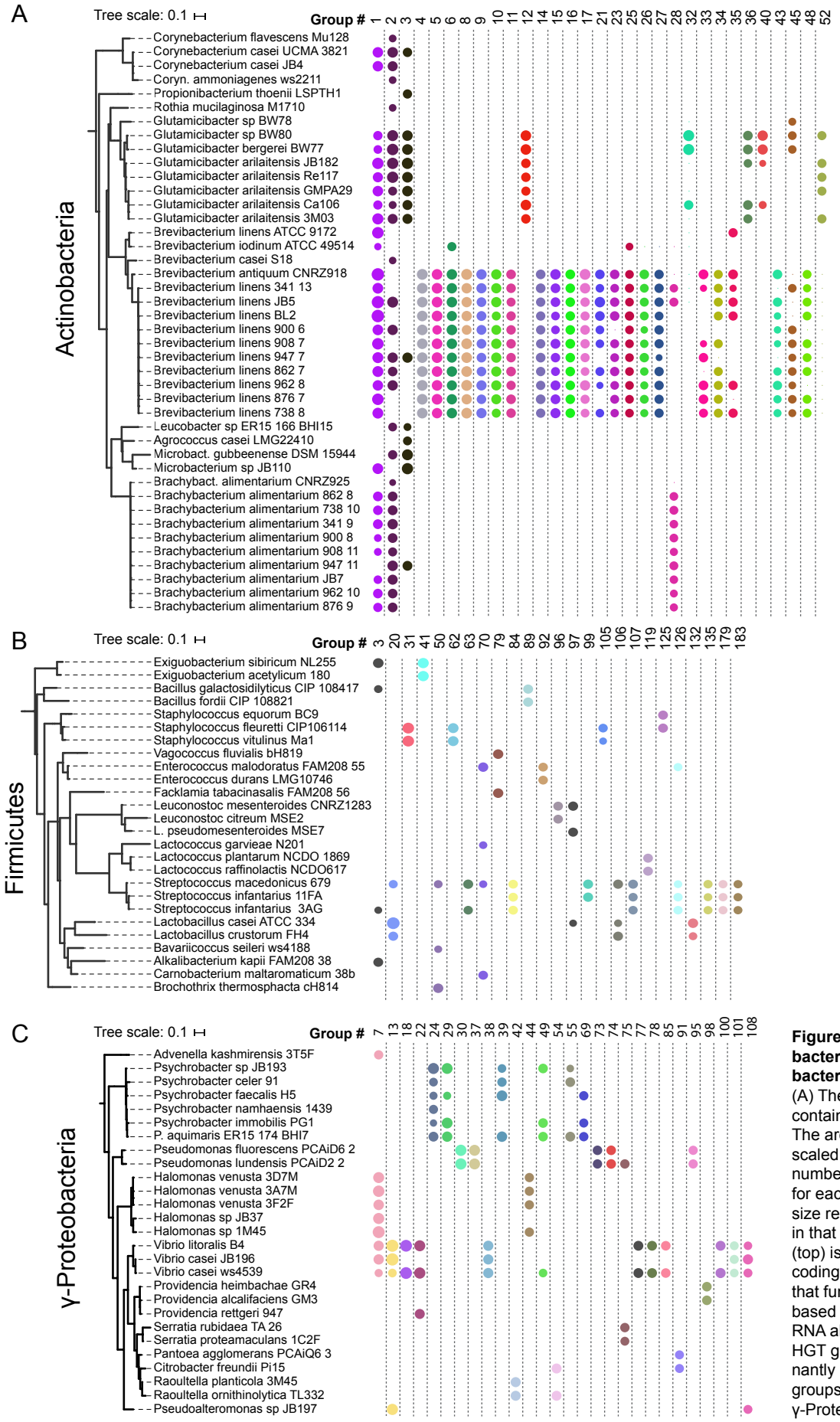

Figure 2: HGT Groups in Actinobacteria, Firmicutes, and γ-Proteobacteria Groups
(A) The 31 largest HGT groups that contain predominantly Actinobacteria. The areas of colored circles are scaled to log2(n), where n is the total number of nucleotides in that group for each species. The largest circle size represents the largest HGT group in that phylum. Protein function matrix (top) is shaded if at least one protein coding sequence in the group has that function. Phylogenies (left) are based on small subunit ribosomal RNA alignment. (B) The 25 largest HGT groups that contain predominantly Firmicutes. (C) The 28 Largest groups that contain predominantly γ-Proteobacteria.

identified in 8 *Brevibacterium* species (group 10) and a tripartite multidrug resistance system (K03446, K03543) in 3 *Pseudomonas* species (group 37).

We also noticed a small number of genes that should be part of the core genome and not expected to be horizontally transferred. For example, group 27 is found in all 10 strains of *B. linens* in this dataset, as well as the closely related *B. antiquum* CNRZ918, and contains the SSU ribosomal protein S1p, as well as DNA polymerase 1. We considered the possibility that these results are false positives since *B. linens* and *B. antiquum* have an ANI ~88%, and these genes are typically more highly conserved than average. At the same time, other ribosomal genes that should also be highly-conserved protein coding genes have substantially lower homology between these species than S1p (Supplementary Table 5). Further, another gene in this HGT group (SAM-dependent methyltransferase) is not typically highly conserved, but nevertheless is >99% identical between these *Brevibacterium* species. We cannot exclude the possibility that this is a false positive, but this may be an example of homologous recombination facilitated by the high sequence identity of the ribosomal protein gene. Several other groups also contain ribosomal proteins (42 - L5p and S3p, 180 - L4p, 219 - S3p), but these groups do not contain any other protein coding genes, and they are clustered with other ribosomal protein coding genes which is a more typical arrangement.

Iron Acquisition HGT

The abundance of iron acquisition genes identified as HGT suggests that iron is a driving force in the adaptation to growth on cheese. The largest HGT region we identified in cheese-associated bacteria, Group 1, the largest contiguous island of which comprises ~47 kbp (~1% of the genome of *B. linens* JB5) and 34 genes, and is found in whole or in part in 15 different species in 5 different Actinobacterial genera (*Brachybacterium, Brevibacterium, Corynebacterium, Microbacterium,* and *Glutamicibacter,* formerly *Arthrobacter*), and one Firmicute (*Alkalibacterium*). The core of this region, flanked by AraC-like transcriptional regulators (eg Ga0099663_102740 and Ga0099663_102753 from JB182), contains several genes predicted to form a siderophore import complex, including two cell-surface associated substrate binding protein genes (Ga0099663_102743-44), two membrane permease genes (Ga0099663_102745-46), and an ATPase subunit (Ga0099663_102747). A siderophore reductase (Ga0099663_102741) is present immediately downstream of the AraC regulator, but has less than 99% identical between the species we analyzed (Figure 3A, Supplementary Table 3). We named this region i**R**on **U**ptake/**S**iderophore **T**ransport **I**sland (RUSTI).

Horizontally transferred genes are not always expressed in the recipient genome, due to possible incompatibilities in promoter sequence (Ochman, Lawrence, and Groisman 2000). Since iron is a limiting resource in cheese (Monnet, Back, and Irlinger 2012; Kastman EK, Kamelamela N, Norville JW, Cosetta CM, Dutton RJ, Wolfe BE. 2016), we reasoned that if RUSTI is a functional operon, it would likely have increased expression in the presence of additional competition for iron. In order to assess whether RUSTI genes are regulated in the presence of competition, we grew *G. arilaitensis* JB182 alone or in the presence of *Penicillium* and performed RNA sequencing (RNA-seq) to monitor gene expression. In addition, we cultured

Figure 3

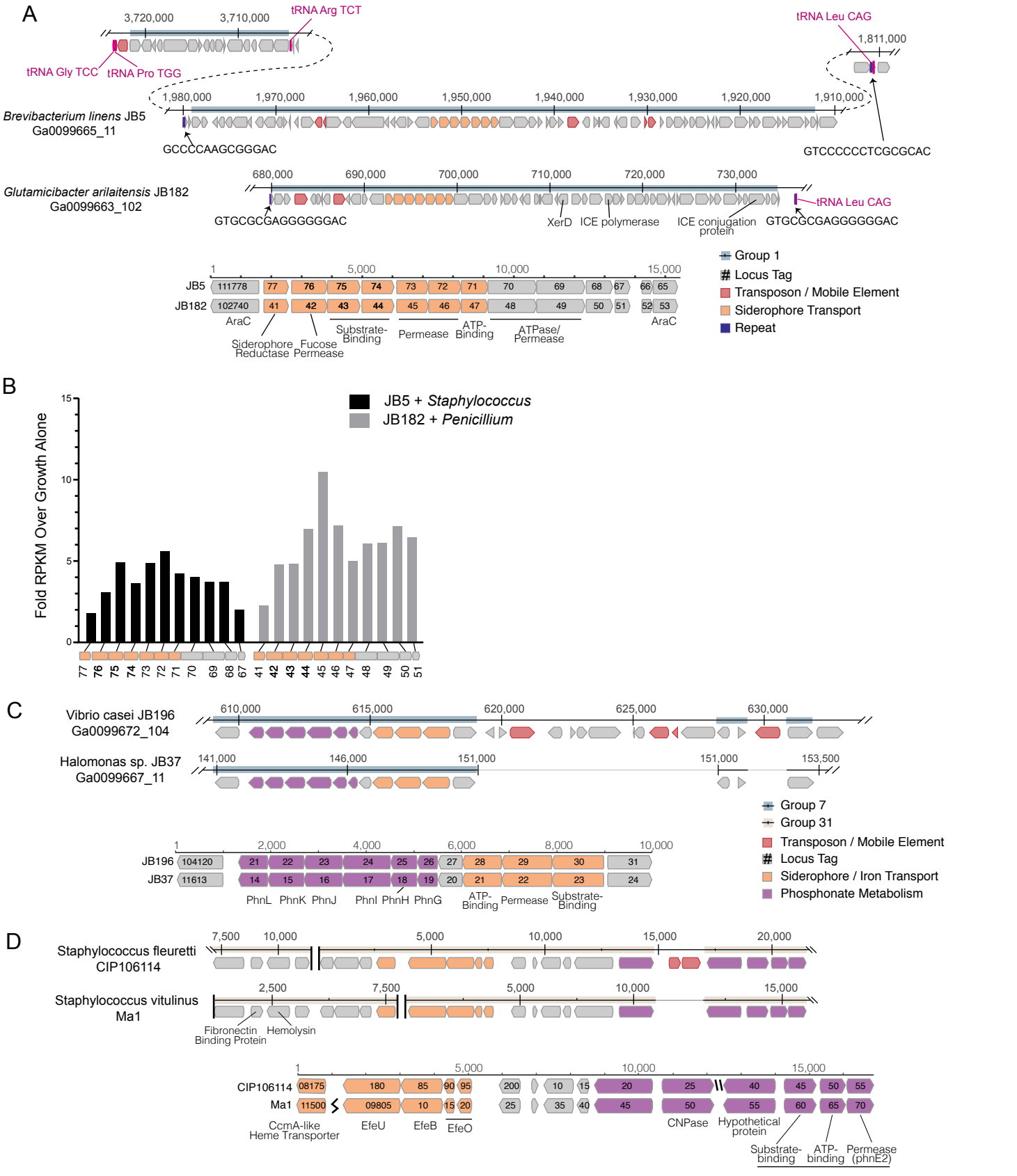

Figure 3: Structure of RUSTI Islands

(A) At-scale schematics for genomic context of HGT Group 1 (top) for B. linens JB5 and G. arilaitensis JB182 and alignment of RUSTI operon (bottom). Nucleotide position values (top) refer to contigs Ga0099665_11 and Ga0099663_102 respectively. Dotted line for JB5 depicts regions of the contig that are not shown. Nucleotide position values (bottom) refer to operon starting from stop codon of leading AraC coding sequence. (B) At scale schematics for genomic context of HGT Group 7 for Halomonas sp. JB37 and V. casei JB196 (top) and alignment of iron and phosphonate metabolism genes (bottom). Nucleotide position values (top) refer to contigs Ga0099667_11 and Ga0099672_104 respectively. Grey lines for JB196 depicts gaps in the alignment due to insertions in JB37. Nucleotide position values (bottom) refer to **operon starting from stop codon of leading protein coding**

B. linens JB5 alone or in the presence of a competing bacterium, *Staphylococcus succinus* BC15. In both cases, the genes in RUSTI were significantly upregulated in the presence of a competing microbe relative to growth alone (Figure 3B), suggesting that this horizontally transferred region is transcriptionally active and responds to competition for limited iron in cheese.

Hundreds of different siderophores have been identified belonging to three major classes: hydroxamate, catecholate and α-hydroxycarboxylate (Hider and Kong 2010). In order to predict the function of the RUSTI transporters, we compared their protein sequences to the Transporter Classification Database (TCDB) (Saier et al. 2016) using BLAST (Supplementary Table 6). The two genes annotated as permease subunits and one gene annotated as ATP binding subunits each share substantial homology to the catechol ferric enterobactin transport system (FepD, FepG and FepC respectively) in *E. coli (Elkins and Earhart 1989; Shea and McIntosh 1991; Chenault and Earhart 1991)*. Two genes annotated as substrate binding proteins have weak homology to vibriobactin and iron(III) dicitrate binding proteins from *Vibrio cholerae* and *E. coli* respectively.

Siderophore-related genes are also well-represented in γ-Proteobacterial HGT groups. Like Group 32 in Actinobacteria, Group 39 contains both siderophore acquisition and siderophore biosynthesis genes and is found in 3 species of *Psychrobacter*. The HGT group with the most protein coding genes that we identified in γ-Proteobacteria (group 7) is found in several *Vibrio* and *Halomonas* species, and like ActinoRUSTI contains an ABC siderophore transport system with a individual substrate-binding, permease and ATP-binding domains (Figure 3C). Though this group appears to have analogous function in the acquisition of iron with RUSTI from Actinobacteria, this ProteoRUSTI does not appear to be related. TCDB analysis suggests homology to hemin transporters in *Yersinia pestis* and *Bordetella pertussis* (Supplementary Table 6).

The same gene island also contains genes related to the Phn family involved in phosphonate import and metabolism (Jochimsen et al. 2011). Phosphonate metabolism genes have previously been associated with iron siderophore acquisition in acidic environments (Osorio et al. 2008)), and cheese is typically close to pH5 during the initial periods of rind community growth. Interestingly, BLAST of this region against the NCBI RefSeq database reveals that several uropathogenic *E. coli* strains share identical DNA sequences (Supplementary Table 7). Highly similar sequences are found in *Oligella urethralis*, another gram negative pathogen of the urogenital tract, and *Vibrio harveyi*, a bioluminescent ocean-dwelling microbe. Iron sequestration by animals is a common defense against pathogens (Parrow, Fleming, and Minnick 2013) and enhanced iron acquisition is commonly associated with virulence. Mammals produce lactoferrin in milk for the same reason (Ellison 1994), and these data suggest that the same genes would be adaptive in both pathogenesis and growth on cheese.

The convergence of strategies for both pathogenesis and growth on cheese is also demonstrated in the Firmicutes (Figure 3D). Two species of cheese-associated *Staphylococcus* (*S. fleuretti* CIP106114 and *S. vitulinus* Ma1) share a large (~20kb) cluster of genes (Group 31)

Figure 4

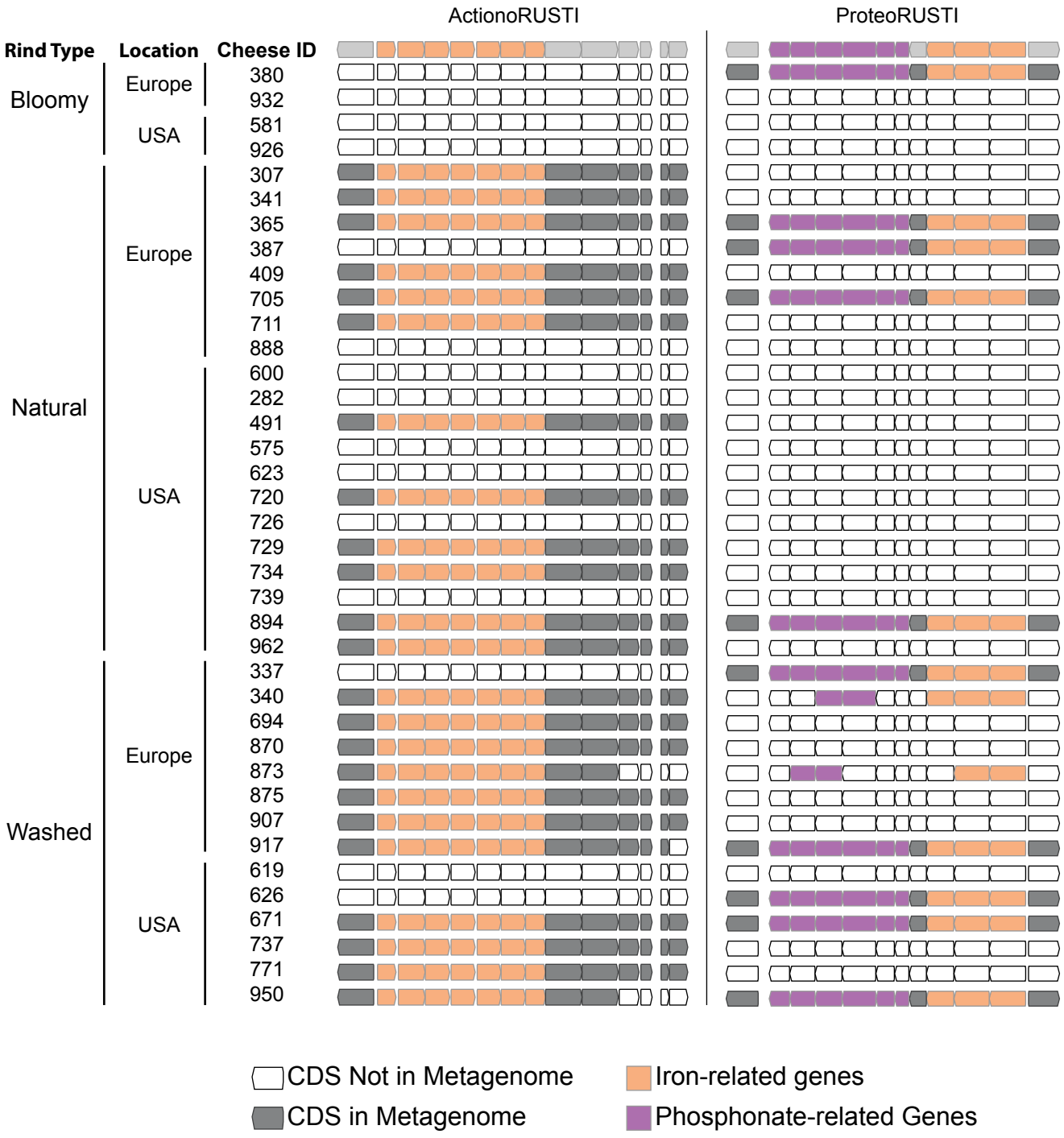

Figure 4: Presence of RUSTI in Cheese Metagenomes
Genes in ActinoRUSTI (G. arilaitensis JB182) and ProteoRUSTI (V. casei JB196) regions were compared to 32 assembled metagenomes from the US and Europe. Filled CDS represents positive (>97% identical nucleotides) hit in that metagenome.

that includes hemolysin and fibronectin binding protein (FnBP), which are involved in virulence in *S. aureus (Sinha et al. 1999; Cheung and Ying 1994)*. Hemolysin (also known as alpha toxin) forms pores in cell membranes, and is so-named due to its ability to lyse red blood cells. FnBP enables binding to and invasion of cells, and has been implicated in the formation of biofilms in methicillin resistant *S. aureus (McCourt et al. 2014)*. It is unclear if these genes could provide a selective advantage to cheese-associated Staphylococci, but Group 31 also contains genes for iron acquisition. These genes are homologous to the EfeUOB systems in *E. coli* and *Bacillus subtilis* and FepABC system in *S. aureus,* which are active in low-pH conditions (Cao et al. 2007; Miethke et al. 2013; Turlin et al. 2013). These iron acquisition genes are also found in association with hemolysin and FnBP *S. aureus* and the animal-associated *S. sciuri*, though at only ~80% nucleotide identity (supplementary Table 7) (Kloos and Schleifer 1976).

The genomes sequenced are from a limited number of cheeses from Europe and the United States. In order to determine the distribution of RUSTI across a more expansive sampling of cheese microbiomes, we used BLAST searches against assembled metagenomic data from 38 different cheeses using representative Proteo-, Actino-, and Staph-RUSTI sequences (Figure 4). Gene islands at least 97% identical to ActinoRUSTI were readily identified in 23 (61%) of these metagenomes, in both natural and washed rind cheeses from the United States and Europe. Though less common (26% of metagenomes), ProteoRUSTI was also identified in diverse cheeses in both the US and Europe. StaphRUSTI could not be found in any of the metagenomes we analyzed. These data demonstrate that siderophore-associated HGT islands are widespread in cheese rind microbiomes. Whether independent HGT events are happening within each cheese production and aging facility, or if they happened before the widespread distribution of these microbes across cheese production regions is unknown.

A potential mechanism of transfer and source of the Actinobacterial RUSTI

To begin to understand potential mechanisms which could mediate HGT in cheese-associated bacteria, we analyzed the sequences surrounding the RUSTI region of *Glutamicibacter* JB182. Conjugative elements are a common way for HGT to occur (Wozniak and Waldor 2010). Integrative and conjugative elements (ICEs) can in part be identified by the presence of signature proteins associated with core functions of integration into and excision from the host genome (recombinase), replication as an extrachromosomal element (polymerase), and conjugation from the host to recipient cell (conjugation) (Ghinet et al. 2011). Analysis of the *Glutamicibacter* JB182 RUSTI region reveal homologs of each of these protein classes (Figure 3A). A recombinase of the site-specific tyrosine recombinase XerD family (Ga0099663_102762) (Subramanya et al. 1997), a hexameric ATPase conjugation protein of the VirD4/TraG/TraD family (Ga0099663_102784) (Hamilton et al. 2000), and a homolog of the bi-functional primase-polymerase DNA replication protein family (Ga0099663_102766). Interestingly, Actinobacterial ICE systems typically utilize a conjugation apparatus belonging to the SpoIIE/FtsK family, which allows transfer of double-stranded DNA (te Poele, Bolhuis, and Dijkhuizen 2008; Bordeleau, Ghinet, and Burrus 2012). However, the conjugation machinery

here is more reminiscent of Gram-negative and Firmicute systems of single-stranded transfer (Burrus and Waldor 2004).

ICE integration is site-specific, and frequently occurs at the 3' end of tRNA genes (Ghinet et al. 2011). Immediately downstream of the RUSTI region in *Glutamicibacter* is a Leucine tRNA. The 3' end of the tRNA forms an imperfect repeat with the region immediately upstream of the RUSTI region, which strongly suggests that the tRNA-Leu is used at the integration site (*att* site) for this ICE. In order to determine whether this ICE is active, we performed PCR using primers within and flanking the putative integration site (Figure 5A). We were able to detect PCR products which suggest that at least a portion of the cells within the population have lost the RUSTI ICE from their chromosome, and it is present as an extrachromosomal circular form (Figure 5B). Sequencing of the PCR product (primers 1+6) that spans the predicted excision site matched the predicted remaining sequence, containing Repeat element B (Supplementary Figure 4A). Sequencing of the PCR product (primers 2+5) that spans the predicted circularization site matched the predicted sequence, containing Repeat element A (Supplementary Figure 4B).

There are several possible explanations for the widespread distribution of of nearly identical ActinoRUSTI. Initial transfer events may have occurred in a single location, on the surface of a cheese or in livestock, and subsequently been dispersed to many separate cheesemaking facilities. The continued mobility of the ICE in JB182 raises the alternate possibility that it may be continually introduced to many cheeses from a common source. Cheese producers often use commercially-available "starter" cultures that contain desirable species, including many Actinobacteria (Robinson 2005). We tested 5 common starter cultures for the presence of ActinoRUSTI by PCR, and positively identified it in 1 of them (Figure 5C). This culture is known to contain 2 species of Actinobacteria, *Brevibacterium linens* and *Glutamicibacter nicotinae*. In order to identify which species may be the RUSTI donor, we plated the starter culture and isolated 4 distinct strains based on colony morphology. SSU sequencing revealed that two isolates were *Glutamicibacter* and two were *Brevibacterium*. One of the *Brevibacterium* isolates tested positive for ActinoRUSTI by PCR (Figure 5C). These data are consistent with the hypothesis that a HGT from a starter culture could explain some of the dissemination of ActinoRUSTI.

Discussion

In this paper, we provide evidence of extensive horizontal gene transfer in cheese-associated bacteria. Many of the transferred regions are large multi-gene islands, and are shared by numerous species. Genes involved in nutrient acquisition, especially iron and lactate, are particularly abundant, suggesting that HGT may provide a selective advantage in the iron and sugar limited environment of cheese. The largest HGT we identified appears to be an active ICE and is found in a starter culture, raising the possibility that we are observing contemporary processes that may have ongoing importance. These data support previous studies that show HGT is an important factor in the evolution of microbial communities (Wiedenbeck and Cohan

Figure 5

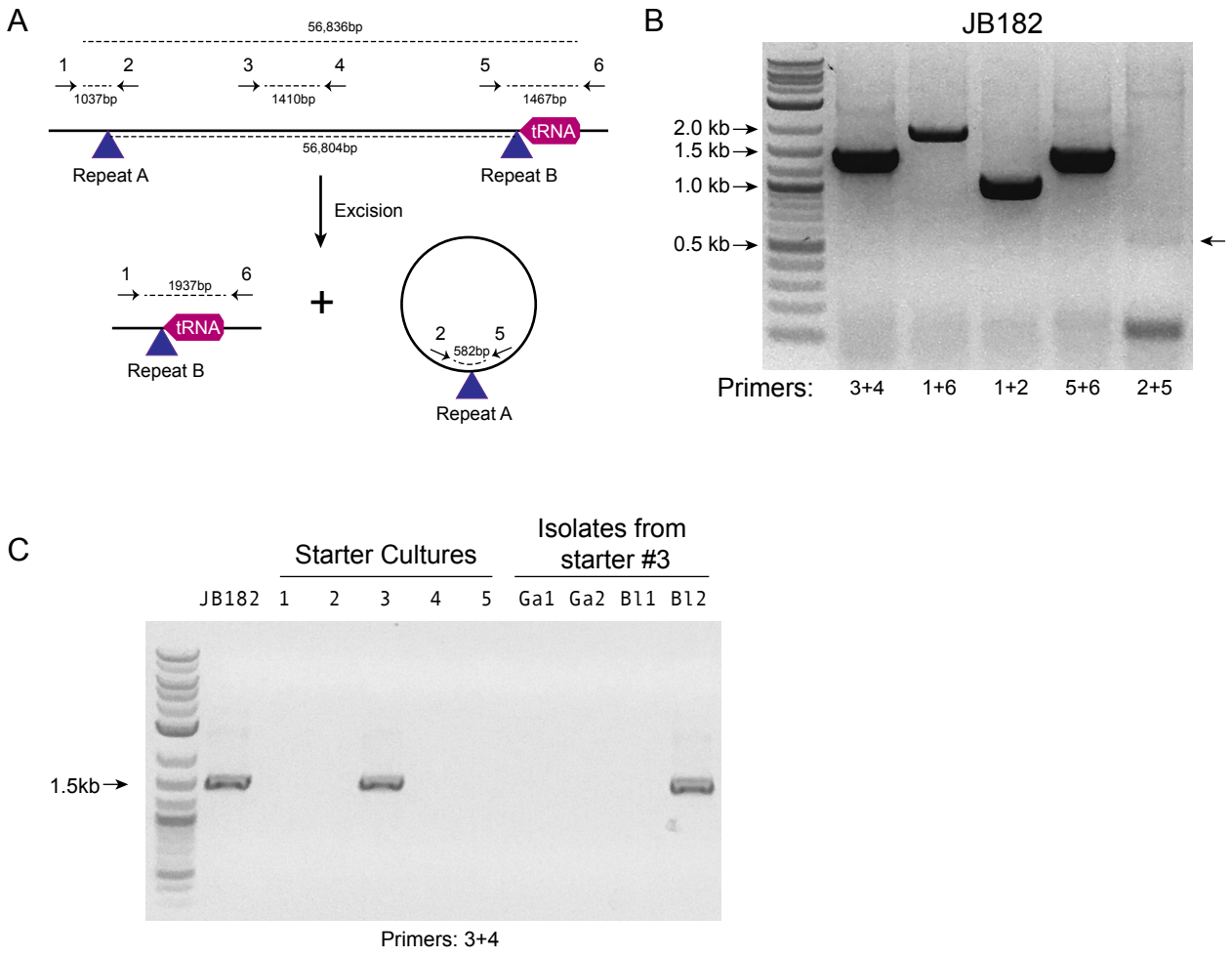

Figure 5: Mobility of RUSTI

(A) Schematic for PCR primer design - see materials and methods for details. (B) PCR testing for the presence of RUSTI and for the excision of the ICE in an overnight culture of G. arilaitensis JB182. (C) DNA was extracted from 5 commercially available starter cultures and tested for the presence of RUSTI using PCR with primers specific for the HGT region (Materials and Methods). Starter culture 3 was plated on PCAMS media, and 4 isolates selected based on colony morphology were also tested. The expected size for the amplicon is ~1.4kb. Sequencing of the 16S ribosomal RNA genes for these isolates suggested that two isolates are Glutamicibacter arilaitensis and two are Brevibacterium linens. G. arilaitensis. JB182 was used as a positive control.

2011) and suggest that cheese rind communities may be a useful model for studying this process in greater detail.

For this study, we focused on bacterial members of the cheese microbiome, but many cheeses also contain numerous fungal species. Indeed, HGT has been previously documented in cheese-associated *Penicillium* species (Cheeseman et al. 2014). HGT between bacteria and fungi has also been documented in other environments, though is thought to be rare (Keeling and Palmer 2008). Evaluating bacterial-fungal gene transfer in cheese could provide additional insights into the extent and importance of gene exchange in microbial communities. Further sequencing of bacterial genomes from cheese could also continue to reveal HGT.

We were able to show that ActinoRUSTI in *G. arilaitensis* JB182, is likely contained within an integrative and conjugative element, although many other mechanisms for transfer are likely at play. Indeed, based on our genome assembly, RUSTI in *Corynebacterium casei* JB7 appears to be on an extrachromosomal plasmid. Further, the appearance of phage-related genes, transposases and other mobile elements in many HGT groups suggests that we are observing the results of multiple methods for mobilizing DNA.

Our method for identifying HGT does not permit us to determine the direction of gene flow, and indeed it seems likely that the original sources of many of these genetic elements are not present in our dataset. In some cases, we can infer a possible origin, such as the *Brevibacterium* strain in a starter culture that may be the source of RUSTI in multiple cheese species around the world. However, this does not enable us to identify where this species acquired them. Further characterization of cheese-associated microbes, as well as those found in dairy farms or in cheese caves may provide a more complete picture, but the evidence that at least some of these genetic elements are found in human pathogens and ocean dwelling bacteria suggests that genes are shared across diverse environments.

Though previous studies demonstrated that iron is limiting for *Glutamicibacter, Brevibacterium,* and *Corynebacterium* species growing on cheese (Monnet et al. 2010; Monnet, Back, and Irlinger 2012), the preponderance of siderophore and other iron acquisition genes we observe being horizontally transferred suggests that the same is true across bacterial phyla. Limiting iron is a deliberate strategy on the part of mammalian hosts to block the growth of infectious microbes, and this strategy influences the composition of milk due to the presence of lactoferrin (Ellison 1994). Interestingly, convergent strategies for acquiring iron are utilized by pathogens and by cheese-associated microbes and we observe that in some cases these disparate species appear to have shared genes through horizontal transfer. The presence of these same genes in a microbe found in ocean habitats suggests that these genes have broad utility for the common challenge of iron limitation.

We have yet to demonstrate the functional consequences of these genes on individual species or on the community as a whole. Given that iron is limiting in cheese, and that ActinoRUSTI genes are upregulated in response to other species (Figure 4C), it is likely that these genes are functional and may play an important role in competition. The prevalence of siderophore import,

but not siderophore synthesis pathways may suggest that species may cheat by scavenging the biosynthetic products of others (Cordero et al. 2012).

Though we and others observe a large number of HGT events in microbial species (Ravenhall et al. 2015), it remains unclear how frequently HGT occurs, the biotic and abiotic conditions that affect this frequency, and what effects HGT may have on the community. A model system to study HGT in a community context is particularly important, since sequence-based characterization of complex communities has particular limitations when it comes to HGT. Further, even if complete characterization *in situ* were possible, many microbial communities are difficult to experimentally manipulate *in vitro*.

By contrast, cheese rind-associated bacteria are readily isolated and cultured, and model communities may enable identification of features of microbial communities and their environments that alter frequency and extent of HGT. The cheese rind model system may provide an opportunity to observe HGT as it happens and to investigate how community composition affects the frequency of transfer and the persistence of genes. The *in vitro* cheese system enables experimental manipulation to investigate the role of community composition in driving HGT. Further, since many gene products may only have survival benefits in the context of community competition and cooperation, investigating the role of RUSTI and other horizontally transferred genes on microbial growth in the context of their natural community is critical.

In addition to its use as a model, the identification of widespread sharing of genes in cheese microbial communities has important implications in its own right. In particular, the possibility that a starter culture is the source of mobile gene elements suggests that the genomic content, rather than just specific species must be considered when designing microbial supplements. In addition to starter cultures used for fermented foods, living microbial supplements (“probiotics”) are increasingly being adopted in agriculture (Verschuere et al. 2000; Chaucheyras-Durand and Durand 2010) and for a wide range of human health conditions (Cuello-Garcia et al. 2015; Onubi et al. 2015; McKenzie et al. 2016) and even as cosmetics (Whitlock, Jamas, and Weiss 2016). The need to screen for clinically relevant elements such as antibiotic resistance genes is widely recognized (Sanders et al. 2010), but other mobile gene elements from these organisms may also enter native microbial populations with unknown consequences.

Understanding the extent of HGT in the cheese microbiome is the first step towards addressing how the movement of genes shapes and is shaped by a microbial community. Using cheese rinds as a model system, we hope to understand the factors that influence the frequency of HGT, and how it impacts competition and cooperation within a community. It may also be possible to observe the impact of HGT on the adaptation of bacteria to a new environment. Many species of cheese-associated bacteria have close relatives in the soil, in the ocean or in other foods that may be poorly adapted to growth on cheese but may be able to acquire genes from better adapted relatives (Wiedenbeck and Cohan 2011). Horizontal acquisition of iron-uptake genes has been documented in numerous environments including the oceans and in human pathogens (Gyles and Boerlin 2014; Richards et al. 2009), indicating that study of the

cheese microbiome may provide broader utility. Experimental evolution of poorly adapted strains in the presence of microbes with known mobile elements like RUSTI could provide insight into the role of HGT in the formation and stability of microbiomes.

Methods

Sequencing and Genome Assembly

Bacterial strains JB4, 5, 7, 37, 110, 182, 196, and 197 were isolated from cheeses in a single geographic region and sequenced using a combination of Illumina short-read (100bp, paired end) and Pacbio long-read sequencing. DNA was extracted using Genomic Tip 100/G (Qiagen, USA) or Power Soil (MoBio, USA). Illumina library preparation and sequencing were performed at Harvard University by the Bauer Core facility. Pacbio library preparation and sequencing were performed by the University of Massachusetts Medical School Deep Sequencing Core. De novo hybrid assembly was performed using SPAdes (v3.5.0) (Bankevich et al. 2012). Genomes were annotated using the Integrated Microbial Genomes Expert Review (IMG/ER) annotation pipeline (Markowitz et al. 2012). In addition, we also sequenced 8 additional rind isolates of *Brachybacterium* (strains 341.9, 738.10, 862.8, 876.9, 900.8, 908.11, 947.1, 962.10) and *Brevibacterium* (strains 341.13, 738.8, 862.7, 876.7, 900.6, 908.7, 947.7, 962.8) and 3 additional isolates of *Glutamicibacter* (strains BW77, 78, 80) from different cheeses in a broad geographic distribution. For these isolates, we prepared draft genomes using Illumina short-read sequencing and assembled with CLC genomics workbench.

PCR

PCR reactions were performed using Q5 Hot Start Mastermix (New England Biolabs). Where JB182 RUSTI is integrated in the chromosome, PCR using primer 1 (CAACTGTGCCACGCAATTCA) and primer 2 (CGGCTACTTCTCGGATGGTC) are expected to produce a 1037bp product that includes the 5' ICE repeat. Primer 3 (CGCAATCGTGGTGTATCTGC) and primer 4 (GACGGGATCAGGAACGACG) should produce a 1410bp product, while primer 5 (GCCGCATCTACCTCGATGAA), and primer 6 (CCAAATCGCGACGCATTGAT) are expected to form a 1467bp product. Primers 1 and 6 are separated by approximately 59kb when RUSTI is present and are not expected to form a PCR product, but should form a 1937bp product if RUSTI is excised. Primers 2 and 5 should not form a PCR product when RUSTI is integrated, but would form a 500bp product if the excision circle is present.

Phylogenetic trees

16S sequences were retrieved from the sequenced genomes and aligned using the structure-based aligner, Infernal v1.1rc4(Nawrocki and Eddy 2013), as implemented in the Ribosomal Database Project release 11 (Cole et al. 2009). The alignment was imported into

Geneious v9 (Biomatters, LTD), and a tree was calculated using the maximum likelihood method PHYML (GTR model)(Guindon and Gascuel 2003). The tree was rooted using *Thermus thermophilus*. The tree was then uploaded to Interactive Tree of Life (iTOLv3)(Letunic and Bork 2016) to enable mapping of HGT data (connections and group abundance profiles).

RNAseq

Four replicate transcriptomes from two treatments were sequenced: 1) *G. arilaitensis* alone and 2) *G. arilaitensis* + *Penicillium*. We used a strain of *Penicillium solitum* that was isolated from a natural rind cheese and was used for experiments in Wolfe et al. 2014. For each experimental unit, approximately 80,000 CFUs of *Glutimicibacter arilaitensis* were spread across the surface of a 100mm Petri dish containing 20 mL of cheese curd agar (10% freeze-dried fresh cheese, 3% NaCl, 1.7% agar, 0.5% xanthan gum) (Wolfe et al. 2014). For the + *Penicillium* treatment, approximately 100,000 CFUs were co-inoculated onto the plates with the Arthrobacter. Plates were incubated in a bin with moist paper towel (> 90% relative humidity) at 24 °C for 5 days.

Rind biofilms were then harvested by scraping the cheese curd surface (*G. arilaitensis*) or from liquid cheese culture (2% freeze-dried fresh cheese, 3% NaCl) (*B. linens*) and stored in RNAProtect Reagent (Qiagen) to stabilize mRNA frozen at -80°C. RNA was extracted using a standard phenol-chloroform protocol used for many different fungal and bacterial species and has been adopted from transcriptomics work in gut microbiomes, (see(David et al. 2014)). This protocol uses a standard bead-beating step in a lysis buffer to release cell contents from biofilms stored in RNAProtect. DNA was removed from the samples using a TURBO DNA-free kit (Life Technologies), and 5S, tRNA and large rRNA was depleted using MEGAClear (Life Technologies) and RiboZero (Illumina) kits, respectively. To remove both fungal and bacterial large rRNA, we used an equal mixture of Ribo-Zero Yeast and Bacteria rRNA Removal Solution. To confirm that the samples are free of DNA contaminants, a PCR of the 16S rRNA gene was with standard primers (27f and 1492r). Overall quantity and quality of the RNA preps were confirmed by Nanodrop and Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit.

RNA-seq libraries were constructed from purified mRNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) where each library received a unique 6 base pair barcode for demultiplexing after the sequencing run. Each library was run on an Agilent 2100 Bioanalyzer High Sensitivity DNA chip to confirm that primer dimers and adapter dimers are not present in the sample and to determine the size of the library. Final libraries were standardized to 10 nM each after quantification with a Qubit dsDNA HS Assay Kit (Life Technologies) and the pooled in equal amounts to get similar sequencing coverage across all libraries. The pooled library samples were be sequenced using paired-end 100bp reads on an Illumina HiSeq Rapid Run by the Harvard Bauer Core Sequencing Core Facility.

To quantify gene expression and determine if genes within RUSTI were differentially expressed when grown with the competitor *Penicillium*, we used Rockhopper (McClure et al. 2013). Only forward reads were used for this analysis. The assembled and annotated *Glutamicibacter*

arilaitensis strain JB182 or *Brevibacterium linens* JB5 (described above) genomes were used as reference genomes for mapping. We considered genes that had a greater than 4-fold difference in expression when grown with *Penicillium* or *Staphylococcus* and were significantly different (based on Rockhopper's *q*-values, which control for false discovery rate using the Benjamini-Hochberg procedure) to be differentially expressed genes.

Additional Software

Annotated genomes were compared using blastn from BLAST+ (v2.3.0) (Camacho et al. 2009). Protein coding genes were considered potential HGT if their sequence was at least 99% identical for at least 500 nucleotides. Neighboring candidate HGT were identified as part of the same island if they were separated by no more than 5000 nucleotides. Scripts to import and store genome information and blast results and to analyze results are available on github (Bonham 2016).

Genomic average nucleotide identity (ANI) was calculated using the "ani.rb" script from the enveomics collection (commit "e8faed01ff848222afb5820595cccc4e50c89992") with default settings (Rodriguez-R and Konstantinidis 2016).

Metagenomes

Shotgun metagenomic data from (Wolfe et al. 2014) and (Kastman EK, Kamelamela N, Norville JW, Cosetta CM, Dutton RJ, Wolfe BE. 2016) were assembled with CLC Genomic Workbench 8.0. Representative sequences for ActinoRUSTI or ProteoRUSTI were compared to assembled metagenomes by BLAST. Hits with >97% similarity were considered positive hits for target regions.

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Figures

Figure 1: Extensive Horizontal Gene Transfer in Cheese Microbiome

(A) All HGT events in analyzed cheese-associated bacteria. Connection thickness is scaled to # of shared protein coding sequences. Phylogram based on 16S RNA alignment using Ribosomal Database Project (RDP). (B) Mean number of genes per species in HGT groups. Diameter of each circle is proportional to the total number of species in the group. Groups highlighted in red

are described further in the text. (C) Quantification of KEGG modules and submodules for protein coding genes (CDS) identified as horizontally transferred. Annotations were generated by BLAST-Koala. Genes without function prediction are not depicted.

Figure 2: HGT Groups in Actinobacteria, Firmicutes, and γ-Proteobacteria Groups

(A) The 31 largest HGT groups that contain predominantly Actinobacteria. The areas of colored circles are scaled to log2(n), where n is the total number of nucleotides in that group for each species. The largest circle size represents the largest HGT group in that phylum. Protein function matrix (top) is shaded if at least one protein coding sequence in the group has that function. Phylogenies (left) are based on small subunit ribosomal RNA alignment. (B) The 25 largest HGT groups that contain predominantly Firmicutes. (C) The 28 Largest groups that contain predominantly γ-Proteobacteria.

Figure 3: Structure of RUSTI Islands

(A) At-scale schematics for genomic context of HGT Group 1 (top) for *B. linens* JB5 and *G. arilaitensis* JB182 and alignment of RUSTI operon (bottom). Nucleotide position values (top) refer to contigs Ga0099665_11 and Ga0099663_102 respectively. Dotted line for JB5 depicts regions of the contig that are not shown. Nucleotide position values (bottom) refer to operon starting from stop codon of leading AraC coding sequence. (B) At scale schematics for genomic context of HGT Group 7 for *Halomonas sp.* JB37 and *V. casei* JB196 (top) and alignment of iron and phosphonate metabolism genes (bottom). Nucleotide position values (top) refer to contigs Ga0099667_11 and Ga0099672_104 respectively. Grey lines for JB196 depicts gaps in the alignment due to insertions in JB37. Nucleotide position values (bottom) refer to operon starting from stop codon of leading protein coding sequence. (C) At-scale schematics for genomic context of HGT Group 31 for *S. fleuretti.* CIP106114 and *S. vitulinus* Ma1 (top). For both species, the group is split across 2 different contigs and nucleotide position values (top) refer to the relative position for that contig. Alignment of iron and phosphonate metabolism genes from Group 31 (bottom).

Figure 4: Presence of RUSTI in Cheese Metagenomes

Genes in ActinoRUSTI (*G. arilaitensis* JB182) and ProteoRUSTI (*V. casei* JB196) regions were compared to 32 assembled metagenomes from the US and Europe. Filled CDS represents positive (>97% identical nucleotides) hit in that metagenome.

Figure 5: Mobility of RUSTI

(A) Schematic for PCR primer design - see materials and methods for details. (B) PCR testing for the presence of RUSTI and for the excision of the ICE in an overnight culture of *G. arilaitensis* JB182. (C) DNA was extracted from 5 commercially available starter cultures and tested for the presence of RUSTI using PCR with primers specific for the HGT region (Materials

and Methods). Starter culture 3 was plated on PCAMS media, and 4 isolates selected based on colony morphology were also tested. The expected size for the amplicon is ~1.4kb. Sequencing of the 16S ribosomal RNA genes for these isolates suggested that two isolates are *Glutamicibacter arilaitensis* and two are *Brevibacterium linens*. *G. arilaitensis.* JB182 was used as a positive control.

Supplementary Table 1: Genome Information

Genome statistics for newly sequenced genomes, determined by IMG/ER. Gene IDs refer to IMG bioproject or RefSeq Accession. Genomes from Almeida et. al. do not yet have Accession numbers.

Supplementary Table 2: Pairwise Species Comparison Summary

Total protein coding sequences (CDS) and nucleotides determined to be horizontally transferred For every pair of species that were compared by the HGT detection pipeline. Also shows calculated ANI and 16S distance (see materials and methods). Species pairs that have ANI > 0.89 are not shown.

Supplementary Table 3: Full Group Annotations

All protein coding sequences identified as HGT, sorted by group # (ranked by total nucleotide content), species and genome location within species. Certain functional annotations are identified by color (eg orange for iron) based on text in annotation. Locus tags and contig IDs beginning with lower-case letters were assigned by kvasir, and do not correspond to any published database.

Supplementary Table 4: Group Summary Statistics

Summary statistics for each HGT group.

Supplementary Table 5: Highly Conserved Genes in *Brevibacterium* species

Protein coding sequences from Group 27, as well as selected highly conserved genes from *Brevibacterium antiquum* CNRZ918 were compared to other *Brevibacterium* strains by BLAST. *B. linens* 947.7 has substantially lower nucleotide identity for the 4 genes found in Group 27 than other *B. linens* strains, despite similar nt distance for other highly conserved genes. This suggests Group 27 is a true example of HGT between CNRZ918 and other *B. linens* strains, rather than a false-positive.

Supplementary Table 6: TCBD Hits for Transporters in RUSTI

Representative CDS of Actino- and ProteoRUSTI from *G. arilaitensis* JB182 and *V. casei* JB196 respectively were compared to the Transporter Classification Database (TCDB). Results colored according to type of siderophore transported according to annotation.

Supplementary Table 7: RefSeq BLAST

Actino- and ProteoRUSTI from *G. arilaitensis* JB182 and *V. casei* JB196 respectively, as well as the consensus sequence for StaphRUSTI (see figures 3 and 4) were compared to the NCBI RefSeq database using BLAST.

Supplementary Figure 1: Kvasir Schematic

Schematic of software pipeline to identify HGT. (1) Sequenced genomes are annotated with IMG/ER and downloaded in Genbank format. (2) All annotated genes in all genomes are used to assemble a BLAST database using BLAST+ command-line tools. (3) All protein-coding genes (CDS) from all species are queried against the BLAST database. Hits from the same species are discarded; other hits are saved. (4) For each species, coding sequences that have at least one BLAST hit are grouped into islands based on proximity. Genes that are within 5kb of each other on the same contig are considered part of the same island. (5) Islands in each species are compared to islands in each other species to form groups. Islands that share at least 1 gene in common according to BLAST parameters in step 3 are placed in the same group.

Supplementary Figure 2: HGT Identification Parameters

Different parameters for minimum length of gene match for HGT, maximum % ANI identity for related species, and maximum distance between genes in an island were compared. (A) Number of positive HGT hits identified when varying the minimum protein coding gene length. (B) Number of HGT groups constructed when varying the maximum separation between hits that are classified as belonging to the same group. (C) Number of nucleotides or (D) number of protein coding sequences in HGT regions by 16S similarity. Note - since 500bp is the minimum length for protein coding sequences in this analysis.

Supplementary Figure 3: Group Clustering

(A) Expected clustering: contiguous genes in multiple species are in a single group. Though island 6 (i6) lacks one gene present in i1 and i4, (possibly because of a transposon insertion), it is still correctly considered related. (B) Ambiguous grouping: Islands 2 and 3 from species 1 are found on different contigs, but are grouped together. They may be found in close proximity in the genome, but on different sides of a gap in the assembly, or they may be quite distant from each other. The grouping of related genes in species 2 into a single island suggests that they may have been transferred in a single event, but the possibility of two unrelated HGT events landing in the same spot cannot be excluded. (C) Possible mis-grouping of two HGT events in a

single group: Though species 4 does not share any genes with species 1 and 2, these islands are nevertheless clustered due to the proximity of coding sequences in species 3. This may correctly represent a single gene cluster that subsequently diverged in each species, or unrelated HGT that happened to insert in close proximity. (D) Mis-grouping due to mobile element: Mobile elements (outlined in red) found in multiple locations in multiple genomes may insert next to unrelated HGT islands, causing spurious grouping by the algorithm.

Supplementary Figure 4: ICE integrate and Excision Sequencing

The ~2000bp band from the PCR amplification using primers 1 and 6 (A) and the ~500bp band from amplification using primers 2 and 5 (B) were extracted and sequenced. Alignment with the JB182 genome reveals 100% alignment with expected and the spliced chromosomal region containing the 3' repeat and the excision circle containing the 3' repeat respectively.

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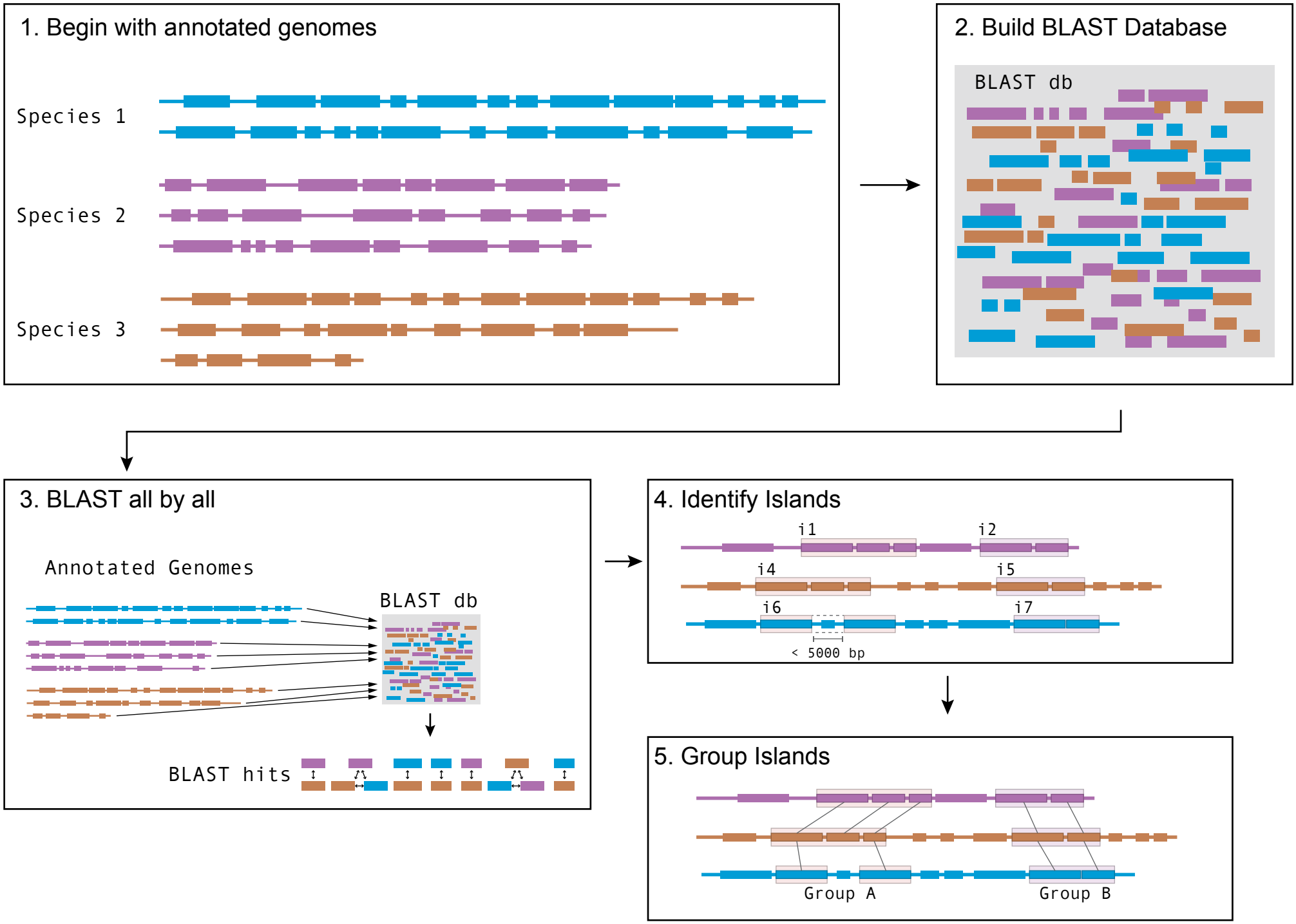
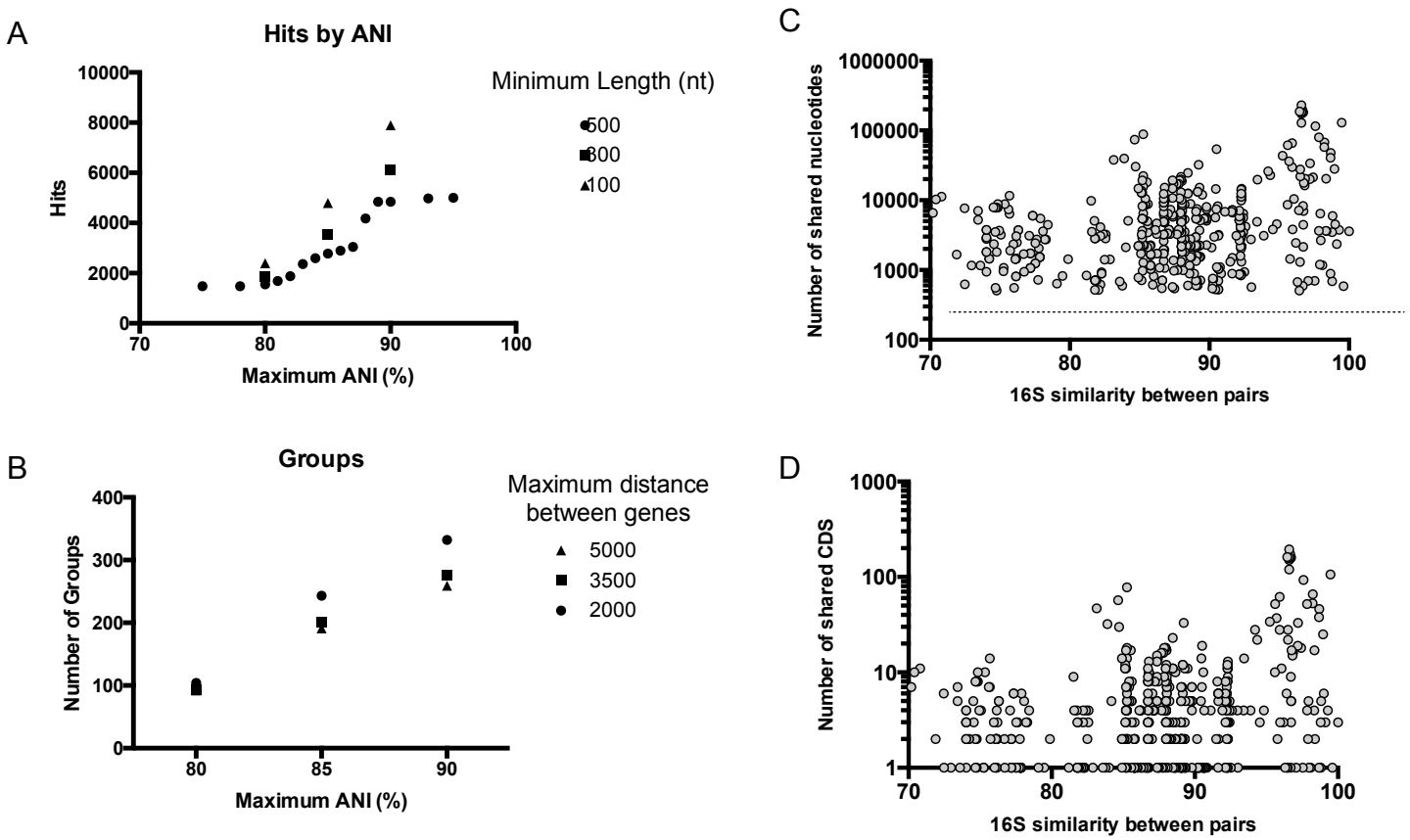
Supplementary Figure 1



Supplementary Figure 2



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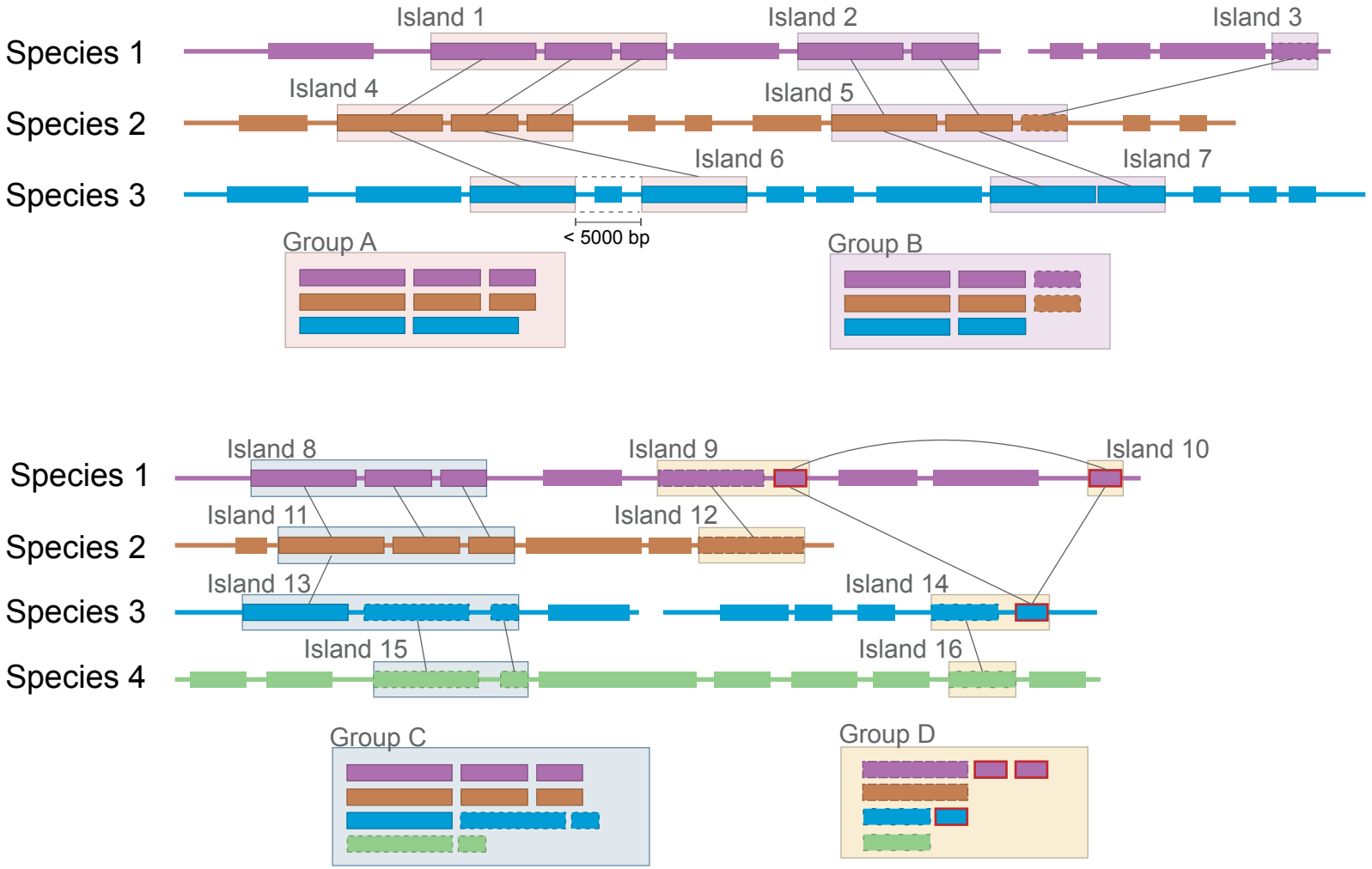
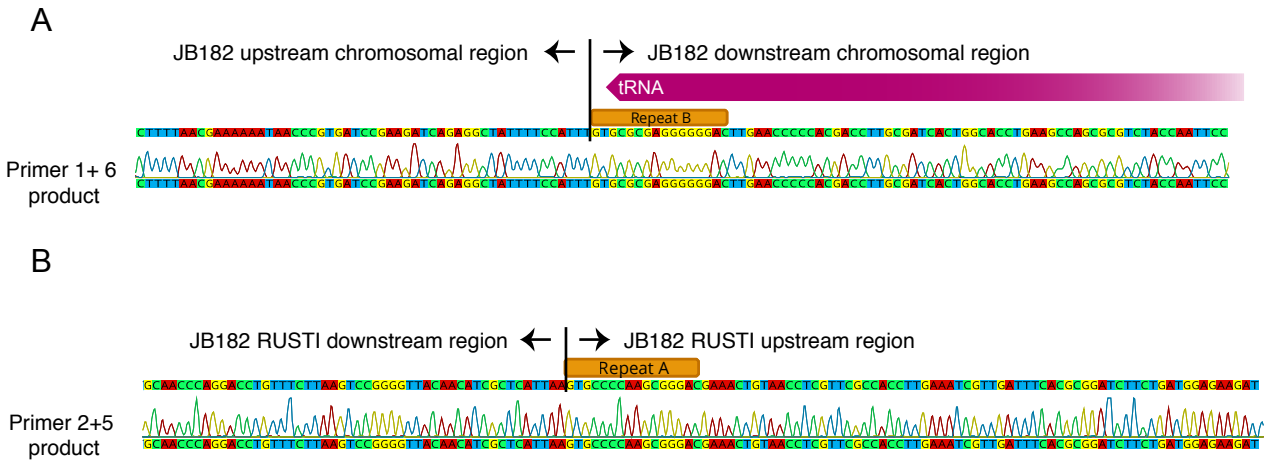


Supplementary Figure 4



Supplementary Figure 5