1   TITLE: A prospective role for the rumen in generating antibiotic resistance

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28

29   ABSTRACT

30

31   Antibiotics were a revolutionary discovery of the 20th century, but the ability of bacteria to

32   spread the genetic determinants of resistance via horizontal gene transfer (HGT) has quickly

33   endangered their use[1]. Indeed, there is a global network of microbial gene exchange, the analysis

34   of which has revealed particularly frequent transfer of resistance determinants between farm

35   animals and human-associated bacteria[2]. Here, we leverage the recent release of a rumen

36   microbial genome reference set and show that the wide-spread resistance gene cluster *aadE-sat4-*

37   *aphA-3* is harboured in ruminal Bacteroidetes. While this cluster appears to have been recently

38   transferred between commensal bacteria in the rumen and many diverse animal and human

39   pathogens, comparative analysis suggests that the cluster stabilized in the pathogens. Then,

40   focusing on streptomycin resistance, it was found that homologues from the rumen span much of

41   the known diversity of aminoglycoside O-nucleotidyltransferases (AadEs) and that distinct

42   variants of the enzyme are present in a single rumen bacterial genome. Notably, a second variant

43   of AadE has also been recently transferred, albeit more often as a single gene, throughout a

44   different set of animal and human associated bacteria. By examining the synteny of AadE

45   orthologues in various bacterial genomes and analyzing corresponding gene trees in an

46 environmental context, we speculate that the ruminant associated microbiome has a salient role
47 in the emergence of specific resistance variants and clusters. In light of the recent literature on
48 the evolutionary origin of antibiotic resistance, we further suggest that the rumen provides a
49 possible route of dissemination of resistance genes from soil resistomes, throughout the farm,
50 and to human pathogens[3].
51
52 MAIN TEXT
53
54 Since the introduction of antibiotics in 1937, the emergence and spread of antibiotic resistance
55 determinants (ARDs) has become one of the largest threats to human health[1,4]. In fact, the history
56 of antibiotic use is concurrent with the history of increasing antibiotic resistance (AR), which
57 now vastly outpaces new antibiotic discovery[5]. Considering the lack of new antimicrobial
58 compounds entering the clinic, there have been many renewed calls for efforts to discover new
59 compounds or to return to modifying well-understood classes of antibiotics, such as the
60 aminoglycosides[5–8]. In addition to restricting the use of current and future antibiotics, there is a
61 need to better understand the extensive evolutionary history of specific ARDs and their routes of
62 dissemination [3,9–11]. In doing so, attempting to re-trace evolutionary events involving ARDs and
63 resistance clusters will be essential to move from the metagenomic description of AR reservoirs
64 to identifying particular sources where AR variants emerge, assemble into clusters, and
65 subsequently transfer to human pathogens[12,13]. Fortunately, the ability to carry out such analysis
66 is constantly improving with the number of publicly available genome sequences[14]. Recently,
67 several high-quality datasets containing hundreds of bacterial and archaeal genomes from the
68 rumen microbiome have been published, such as the Hungate1000 collection[15,16].
69
70 In order to search for ARDs that may form clusters in commensal rumen bacteria, we first
71 collected prokaryotic genome sequences from cultured organisms and combined them with a set
72 of metagenome assembled genomes (MAGs), all sourced from the rumen[15,16]. This led to a total
73 of 1585 genomes (453 genomes from cultured organisms and 1133 MAGs). Predicted open
74 reading frames (ORFs) from this dataset were then compared to the comprehensive antibiotic
75 resistance database (CARD) and it was noticed that 2 characterized ARDs from the antibiotic
76 inactivation category, AadE and AphA-3 from *Streptococcus oralis* and *Campylobacter coli*,
77 respectively, each shared 100% amino acid identity with an ORF from three different genomes in
78 the rumen dataset[17]. In all three genomes, these two ORFs were proximal on the same contig,
79 indicating that they may be organized in a cluster (Table S1). Two of the genomes derive from
80 different species of *Bacteroides* from cows in the US, while the third came from a MAG
81 classified as *Prevotella* sampled from a cow in Scotland. When compared at the nucleotide level,
82 the three contigs identified from the rumen bacterial genomes shared a region of approximately
83 ~10kB at 100% nucleotide identity, which upon further annotation, was found to contain the
84 well-known aminoglycoside-streptothricin AR cluster *aadE-sat4-aphA-3*[18]. This cluster was
85 originally identified as the transposon Tn*5405* in *Staphylococcus aureus* and the genes *aadE*,
86 *sat4*, and *aphA-3* encode for an aminoglycoside O-nucleotidyltransferase, a streptothricin N-
87 acetyltransferase and a aminoglycoside O-phophotransferase, respectively (Figure 1)[19,20]. The
88 Tn*5405* sequence itself is also among those conserved at 100% nucleotide identity and to date,
89 the cluster has been observed across a wide range of human and animal pathogens[18–27]. When
90 compared to the NCBI non-redundant nucleotide database, it was found that a highly-conserved
91 region that spanned ~6kB of the ~10kB region was present in a diverse set of pathogens (Figure

92 1A, Table S2). The segment of this ~6kB which contained the *aadE-sat4-aphA-3* cluster ranged
93 from 99.8-100% nucleotide identity in 32 unique sequences as compared to the rumen sourced
94 contigs, while the flanking regions ranged from 89.9-100% (Figure 1A). Interestingly, the
95 regions that were missing in the pathogens as compared to the rumen bacterial genomes
96 contained only annotated transposases, including a transposase located between *aphA-3* and *sat4*
97 in the cluster, indicating that the cluster has stabilized in the pathogens (Figure 1B, Table S3)[28].
98 It is worth noting that the only example found where the cluster was not shared as a whole was in
99 *Bacteroides fragilis*, a common reservoir of AR and an opportunistic pathogen, where *aphA-3*
100 appears to have recombined into a different multi-drug resistance cluster, CTnHyb[29,30]. Further,
101 *B. fragilis* was the only non-rumen sequence found with an additional highly conserved region
102 and is the most closely related organism phylogenetically to the three genomes sourced from the
103 rumen. Taken together, the version of *aadE-sat4-aphA-3* identified in rumen *Bacteroides* is
104 highly-conserved in diverse human pathogens, was therefore likely recently horizontally
105 transferred and the loss of transposases, only observed in the pathogenic isolates, implies
106 stabilization of the cluster outside of the rumen. We then sought to gain more evolutionary
107 insight into the individual ARDs within the cluster.
108
109 Since genes are the units of evolution and proliferation for mobile traits, we attempted to analyze
110 the evolutionary history of a single enzyme within the cluster. We focused on AadE (also known
111 as ANT(6)), an enzyme characterized to be involved in streptomycin resistance, as it is known to
112 have diverse homologues with the same activity and streptomycin resistance has been long
113 observed in the rumen[31,32]. For instance, in 1966, a range of rumen isolates were screened against
114 various antibiotics and the only compound that demonstrated resistance in all cases was
115 streptomycin[32]. We used 1354 homologues of AadE from the NCBI non-redundant (nr) protein
116 sequences database to build a gene tree (Figure 2)[33,34]. The majority of the homologues (78%)
117 came from Firmicutes, where AadEs likely originated, followed by the Bacteroidetes (13%)[31].
118 The taxonomic origin of the remaining sequences was diverse and interestingly, despite the fact
119 that only 7% of the sequences derive from the rumen microbiome, they span much of the
120 diversity represented in the NCBI nr database (Figure 2). This indicates that the rumen has been
121 exposed to a large and diverse gene pool with respect to sequences homologous to AadE. Then,
122 we noticed that a sub-clade (clade 7) contained both the AadE from the *aadE-sat4-aphA-3*
123 cluster, as well as a homologous variant from the same rumen *Bacteroides* genome (*Bacteroides*
124 *thetaiotaomicron* nale-zl-c202 (Hungate collection 4309680)) (Figure 2). These two variants
125 were annotated as ANT(6)-Ia (AadE-Ia) and ANT(6)-Ib (AadE-Ib), respectively. As these two
126 enzymes are thought to have the same activity, we were interested to see how the horizontal
127 transfer of *aadE-Ib* compared with that of *aadE-Ia*[31]. To do so, we carried out the same type of
128 analysis as shown in Figure 1, but instead analyzed the *aadE-Ib* containing contig from the *B.*
129 *thetaiotaomicron* nale-zl-c202 (Figure 3, Table S2). In this case, *aadE-Ib* was widely distributed
130 in pathogens and commensal bacteria, albeit with lower nucleotide identities as compared to
131 *aadE-Ia* (81.3-100%) and seems to be transferred alone or with a different aminoglycoside O-
132 nucleotidyltransferase (*aad9* or ANT(9)) (Figure 3, Table S3). Considering that the most closely
133 related sequences to *aadE-Ib* are not as conserved and not exclusively found in pathogens, this
134 gene is likely not under as strong of selection as *aadE-Ia*. It is however recombining in context
135 with other ARDs. For example, it was found to recombine near Tet(O) in *C. coli* SX8, a gene
136 which is also highly conserved in several ruminal bacteria at the nucleotide level (Figure 3B,
137 Figure S1A). When looking at further syntenic regions, AadE-Ib was often found in context of

138   AadE-Ia and the *aadE-sat4-aphA-3* cluster. We therefore were interested to further compare
139   AadE-Ia and AadE-Ib across many environments and bacterial genomes and better understand
140   how these two variants may have emerged.
141
142   By building a gene tree with all protein sequences within clade 7, shown in Figure 2, we
143   observed four clear sub groups that each corresponded to a different annotated version of AadE
144   (Figure 4A). Outside of bacteria from the rumen or pathogens, the two groups representing
145   AadE-Ia and AadE-Ib contained sequences that were mostly sourced from various animal or
146   human intestinal samples (Figure 4A). Moreover, the sequences from the rumen tended to span
147   these two groups, whereas the sequences from the two more deeply branching sub groups,
148   containing ANT(6)-Id (AadE-Id) and ANT(6)-Ic (AadE-Ic), were mostly sourced from diverse
149   environmental samples, such as plants and soil (Figure 4A, Table S4). This may not be surprising
150   in light of several genomic analyses of the transfer of horizontal resistance, which have pointed
151   to the gut as an interconnection between soil and clinical pathogens or found that farm animal
152   microbiomes are enriched for transfer events with human-associated bacteria[2,11]. This does
153   however point more specifically to the rumen as a link between the environment and the human
154   or animal intestinal tract. Two questions that then arise are: why did these two variants, AadE-Ia
155   and AadE-Ib, emerge evolutionarily and why are they both often present in a single genome (e.g.
156   *B. thetaiotaomicron* nale-zl-c202)? Especially considering that the characterized versions have
157   the same activity[31]. When looking at those genomes that were selected for sharing high
158   nucleotide identity with the *aadE-Ia* or *aadE-Ib* from *B. thetaiotaomicron* nale-zl-c202, several
159   of them were also found to have both or multiple copies of AadE (Figure 4B and Figure 4C). By
160   comparing their identity and synteny, it is clear that several AadEs have arisen via gene
161   duplication events and often remained in context of each other (Figure 4B and Figure C).
162   Interestingly, outside of the *aadE-sat4-aphA-3,* the genes found in context of the two AadEs are
163   mostly streptomycin or aminoglycoside modifying enzymes (Figure 4B and Figure C). Other
164   ARDs in context include tetracycline and lincosidamide resistance genes, which are also heavily
165   represented in the rumen and act on compounds produced by *Streptomyces* (Table S1, Table S5,
166   Figure S1). It is interesting to note, although often observed with other ARDs, that AadE-Ia and
167   AadE-Ib further recombine into clusters with genes which would theoretically yield the same
168   resistance phenotype. A logical suggestion is that aminoglycoside producing bacteria from soil
169   are also the sources of AR, and that these genes may have served modifying roles outside of
170   resistance to the toxicity of the compounds[35–37]. Altogether, it is possible that recombining
171   variants of *aadE* from the environment further duplicated, potentially including the events that
172   spawned AadE-Ia and AadE-Ib, adapted, and refined their syntenic context in the rumen. During
173   the process, there were likely many subsequent transfer events, often with commensal bacteria of
174   the intestinal tract of humans and other animals.
175
176   In terms of food-producing animals, aminoglycosides accounted for 3.5% of the total sales of
177   antimicrobials in 2015 and are most frequently used to treat infections[38]. Considering the
178   diversity of homologues of aminoglycoside inactivating or modifying enzymes and that cattle are
179   not directly fed aminoglycosides, it is worth considering that the rumen is also exposed to the
180   compounds and an ARD gene pool via natural sources. Soil, for example, is a well characterized
181   reservoir of antibiotic producing organisms and ARDs, which long predate the use of antibiotics,
182   and aminoglycosides have particularly high sorption in soils[8,9,39–41]. Additionally, *Streptomyces*
183   are often isolated from agricultural soils, including in the case of the discovery to streptomycin[42],

184 as well as from feed sources such as hay directly[43]. The rumen takes in enormous amount of feed
185 and in various ways, it has been shown to provide favourable conditions for genetic exchange[44–
186 46]. Considering that ecology shapes gene exchange, it is reasonable to assume that the rumen, a
187 100-200L anaerobic bioreactor constantly interfacing with the feed containing a diversity of
188 antibiotic related compounds and the microorganisms producing them, provides an opportunity
189 for a ARD gene pool to exchange and adapt within an animal associated microbiome and
190 environment. While streptomycin is not regularly detected in feed, other compounds produced by
191 *Streptomyces*, which are easier to detect, such as chloramphenicol, are found regularly[47].
192 Ultimately, a wide range of aminoglycoside modifying enzymes sourced from soils or sediments
193 may be transferred to and refined the rumen, especially in terms of genetic synteny, before being
194 spread throughout the farm and potentially strongly selected or co-selected for when treating an
195 animal infection or when a field is contaminated with antibiotics (Figure S2)[48]. In terms of
196 spreading throughout the farm, the humans, whose associated microbes show 25 fold more HGT
197 as compared to non-human isolates, in contact with the animals are the most obvious conduit[2]. It
198 was however also interesting to find a common dog pathogen (*Staphylococcus*
199 *pseudointermedius*) in the analysis which contained the highly conserved *aadE-sat4-aphA-3*
200 cluster (Figure 1A). Overall, we observed recent horizontal transfer events of ARDs between
201 ruminal bacteria, farm animals, pets and pathogens infecting humans, whose history of assembly
202 points towards the rumen as the source. Therefore, while only one of many sources of AR, the
203 rumen should be considered an environment with high potential for generating clusters of ARDs
204 and providing a central link to other reservoirs, especially on the farm, before going on to create
205 problems in the clinic. If further evidence corroborates this suggestion, antibiotic discovery
206 efforts could focus on antibiotic compounds from organisms that evolved in environments with
207 little or no connection to agricultural feed.
208
209 FIGURE LEGENDS
210
211 Figure 1. A. Aligned regions from nucleotide blast comparisons of the most similar sequences
212 from NCBI to three rumen contigs (see Table S1) compared to a *Prevotella* sp. metagenome
213 assembled genome (MAG)(RUG782) contig. B. Gene diagram comparison between 2 rumen
214 sourced and 2 pathogen bacterial genomes to show conservation of genetic synteny from a few
215 selected examples. Gene numbering maps to annotations in Table S3 and grey connections
216 between genes represent homologues.
217
218 Figure 2. A. Maximum likelihood phylogenetic tree using the top 1354 most similar sequences to
219 AadE-V1 from both the rumen database and NCBI nr with lengths between 250 and 350 amino
220 acids. Clades are numbered for reference. Pie charts show the distribution of phyla from which
221 the sequences were obtained. Numbers within the pie charts indicate how many sequences make
222 up the clade. Clade 7 contains ANT(6)-Ia (AadE-Ia) and ANT(6)-Ib (AadE-Ib) from *B.*
223 *thetaiotaomicron* nale-zl-c202.
224
225 Figure 3. A. Aligned regions from nucleotide blast comparisons of the most similar sequences
226 from NCBI to a single rumen contig (see Table S1) compared to a *B. thetaiotaomicron* nale-zl-
227 c202 genome (Hungate collection 4309680) contig. B. Gene diagram comparison between a
228 rumen sourced and 2 pathogenic organism genomes to show conservation of genetic synteny

229 from a few selected examples. Gene numbering maps to annotations in Table S3 and grey
230 connections between genes represent homologues.
231
232 Figure 4. A. A maximum likelihood tree using all sequences falling within clade 7 (Figure 2).
233 Ultrafast bootstrap values are shown and sequences in bold are from the Hungate 1000
234 collection. Clades are labelled based on containing specific variants of AadE. The outgroup used
235 was a randomly selected sequence taken from clade 22 in Figure 2. B and C. Each point around
236 the circle is an antibiotic resistance determined (ARD) coloured by the contig containing it. A
237 contig is represented if the genome was used in Figure 1 or Figure 3 and contained two or more
238 AadE. An ARD is shown if it is annotated as AadE-Ia or AadE-Ib or another annotated ARD that
239 is syntenic with one of the AadE variants (within a resistance cluster). B. Connections show
240 amino acid identity with AadE-Ib from *B. thetaiotaomicron* nale-zl-c202 (Hungate collection
241 4309680). C. Connections show amino acid identity with AadE-Ia from *B. thetaiotaomicron*
242 nale-zl-c202 (Hungate collection 4309680). Genes are labelled if they are annotated as AadE-Ib*,*
243 AadE-Ia*,* part of the *aadE-sat4-aphA-3* cluster or annotated to act on aminoglycosides. Other
244 ARDs present in the resistance cassettes are shown in Table S5.
245
246 Figure S1. A. Aligned regions from nucleotide blast comparisons of rumen bacterial genomes to
247 a *C. coli* (JQ655275)(A) and *Erysipelothrix rhusiopathiae* (KP339868)(B) genome.
248
249 Figure S2. Graphical overview of a scenario where the rumen plays a predominant role in
250 connecting soils and crops, harboring the organisms who produce antibiotics and have evolved
251 ARDs, to the rest of the farm. It is suggested that the rumen provides a significant opportunity
252 for ARDs to transfer into an animal associated microbiome, recombine and adapt before being
253 spread throughout the farm, where the resulting antibiotic resistance cassettes are (co-) selected
254 for in treating human or animal pathogens.
255
256 METHODS
257
258 Comparative analysis of *aadE-sat4-aphA-3* and *aadE-Ib*
259
260 Genome sequences from the Hungate1000 project, including those listed as previously
261 published, were combined with MAGs from Steward *et al.*[15,16]. Using a concatenated fasta file
262 containing all genome and MAG nucleotide sequences, ORFs were predicted using prodigal
263 v2.6.3[49]. The resulting ORFs were then blasted against the CARD using local blastp v2.9.0+[50].
264 The 3 contigs (Hungate collection 4309689_79 and 43809680_52, MAG RUG782_1) which
265 coded for the top 6 blast hits, in terms of bitscore, were then blasted against the NCBI nucleotide
266 collection (nr/nt) using web-based blastn and the full-length sequence for each of the top 50 hits
267 was downloaded[33,34]. After removing identical sequences, a total of 54 sequences were used in
268 the downstream analysis, the accession numbers and descriptions for which are listed in Table
269 S2. Each of the downloaded sequences was then blasted against the rumen sourced *Prevotella* sp.
270 contig (RUG782_1) using local blastn 2.9.0+[50]. A sequence was displayed in Figure 1A if the
271 total combined length of alignments was over 4000 bp for each query and the percent identity of
272 the alignment was over 80%. For the gene diagrams displayed in Figure 1B, annotations from up
273 to 10 of the top blastp hits from the NCBI non-redundant protein sequences database are listed in
274 Table S3[33,34]. The same was done for Figure 3A, except starting with the contig from *B.*

275   *thetaiotoamicron* nale-zl-c202 genome (43809680_59) containing *aadE-Ib*. Again, top 50 hits
276   from the NCBI nucleotide collection (nr/nt) were downloaded (Table S2) and subsequently
277   blasted against the rumen *B. thetaiotoamicron* contig (43809680_59). Here, a sequence was
278   displayed in Figure 3A if the total combined length of alignments was over 1000 bp and the
279   percent identity of the alignments were over 80%.
280
281   Phylogenetic and syntenic analysis of AadE
282
283   The predicted ORF for AadE from the 3 selected rumen contigs (Hungate collection
284   4309689_79 and 43809680_52, MAG RUG782_1), being identical ORFs, was blasted against
285   the NCBI non-redundant protein sequences database[33,34]. All hits with an e-value below 1e-4
286   were downloaded. Sequences were further eliminated if the length was below 250 bp or above
287   350 bp and an initial alignment was then made using MUSCLE (including the following flags: -
288   maxiters 3 -diags -sv -distance1 kbit20_3)[51]. This alignment was inspected using Geneious
289   v9.1.8, trimmed to between position 64 and 609, and further refined using the default setting
290   from MUSCLE, while allowing for up to 50 iterations[51,52]. The phylogenetic tree shown in
291   Figure 2 was subsequently constructed FastTree on the default settings[53]. In terms of
292   visualization, clades were collapsed whose average branch length to the leaves was below 1.5
293   using the interactive tree of life (iTOL) online tool[54]. The resulting tree is down in Figure 2.
294
295   The tree shown in Figure 4A was constructed using the sequences extracted from clade 7 in
296   Figure 2, with the addition of any homologues of AadE-Ia or AadE-Ib (>200 amino acids and
297   >60% identity to the two versions from *B. thetaiotoamicron* nale-zl-c202 when compared using
298   local blastp 2.9.0+) from the genomes used in Figure 1 and 3, if they contained multiple copies
299   of the homologues[50]. The sequences were aligned using MUSCLE with 50 iterations, inspected
300   using Geneious v9.1.8, and trimmed to between positions 25 and 305. Moreover, truncated
301   proteins were removed, resulting in an alignment of 156 sequences, which was again refined
302   using MUSCLE. This was then used as the input file for IQ-TREE using the standard settings
303   with the following flags: -m TEST -bb 1000 -alrt 1000. An Le Gascuel (LG) model was selected
304   using Gamma with 4 categories for the rate of heterogeneity[55–57]. The resulting tree, along with
305   ultrafast bootstrap values, was visualized using iTOL.
306
307   To analyze synteny, any ORFs annotated as ARDs surrounding the AadE-Ia or AadE-Ib
308   homologues (within maximum ~50kB) that were taken from the genomes used in Figure 1 and 3
309   are shown in Figure 4B and C. These were compared to AadE-Ib (Figure 4B) AadE-Ia (Figure
310   4C) or using local blastp 2.9.0+[50]. The annotation based on the top blastp hits from the NCBI
311   non-redundant protein sequences database are listed in Table S5[33,34].
312
313
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315

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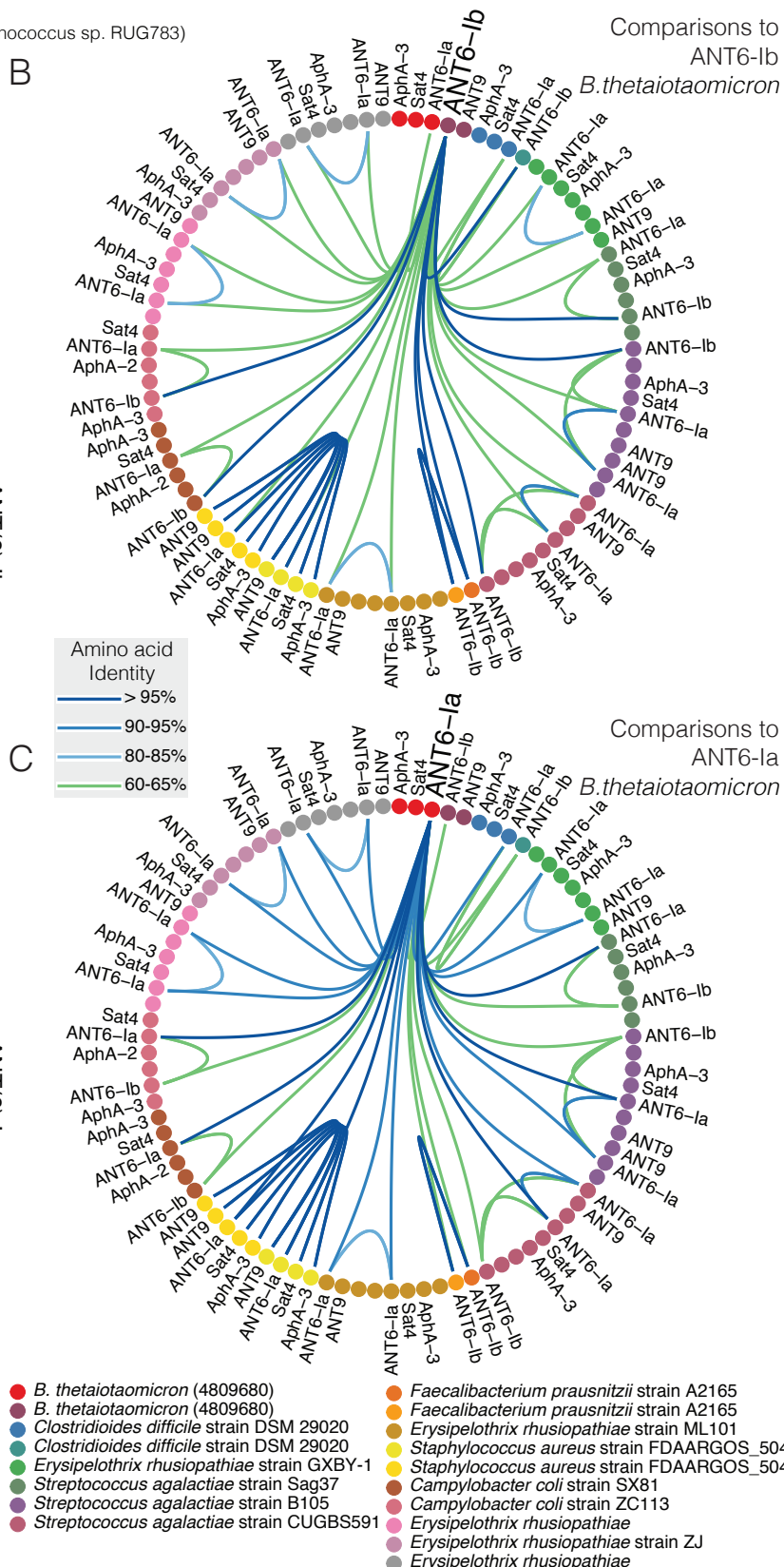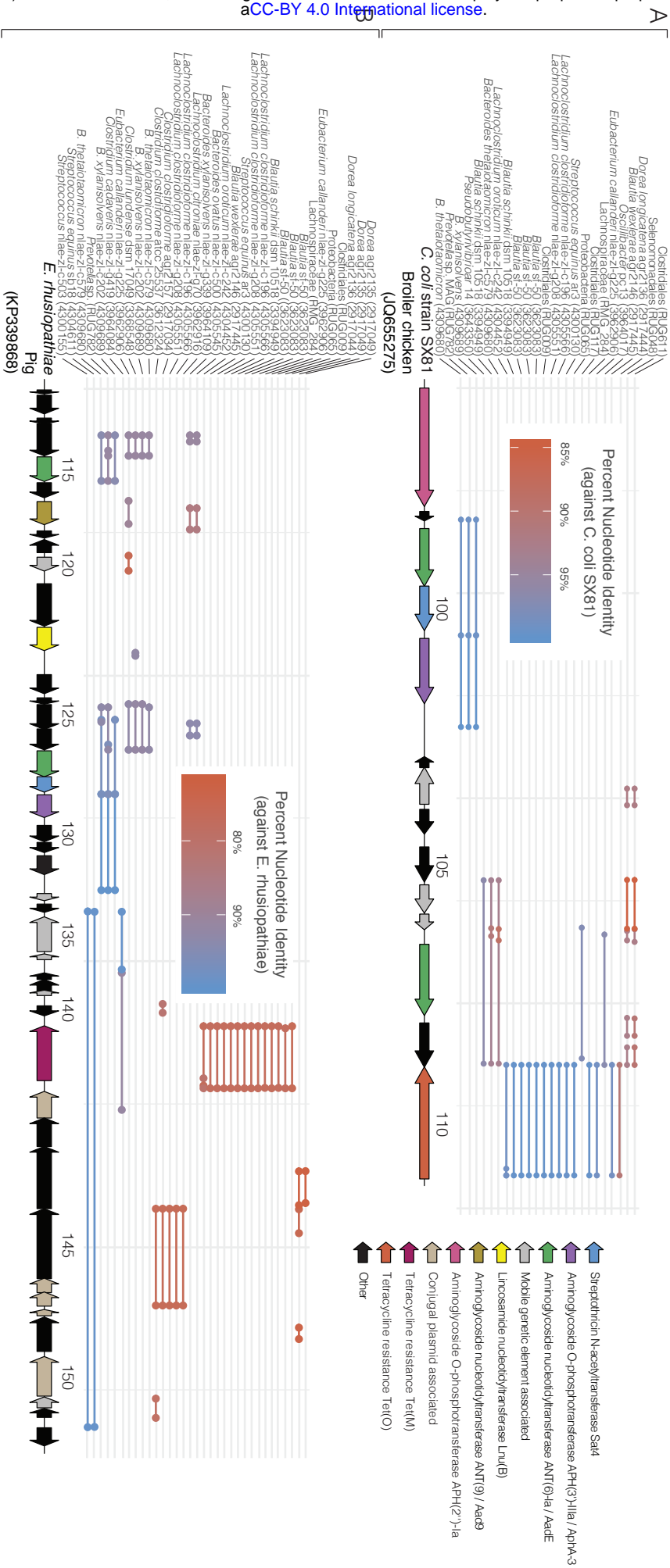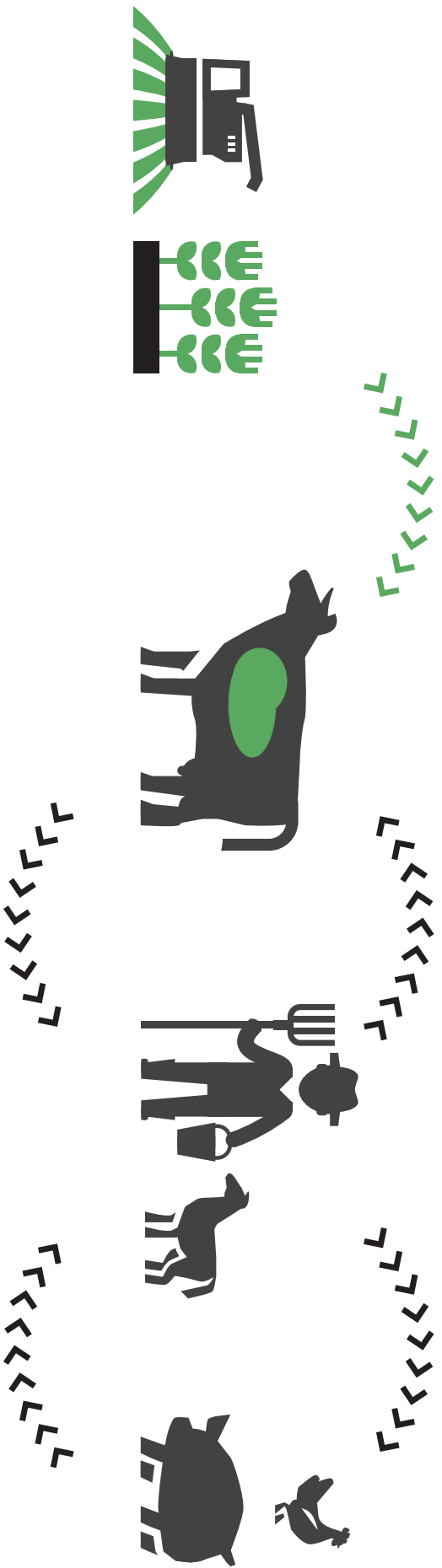467

Figure 1
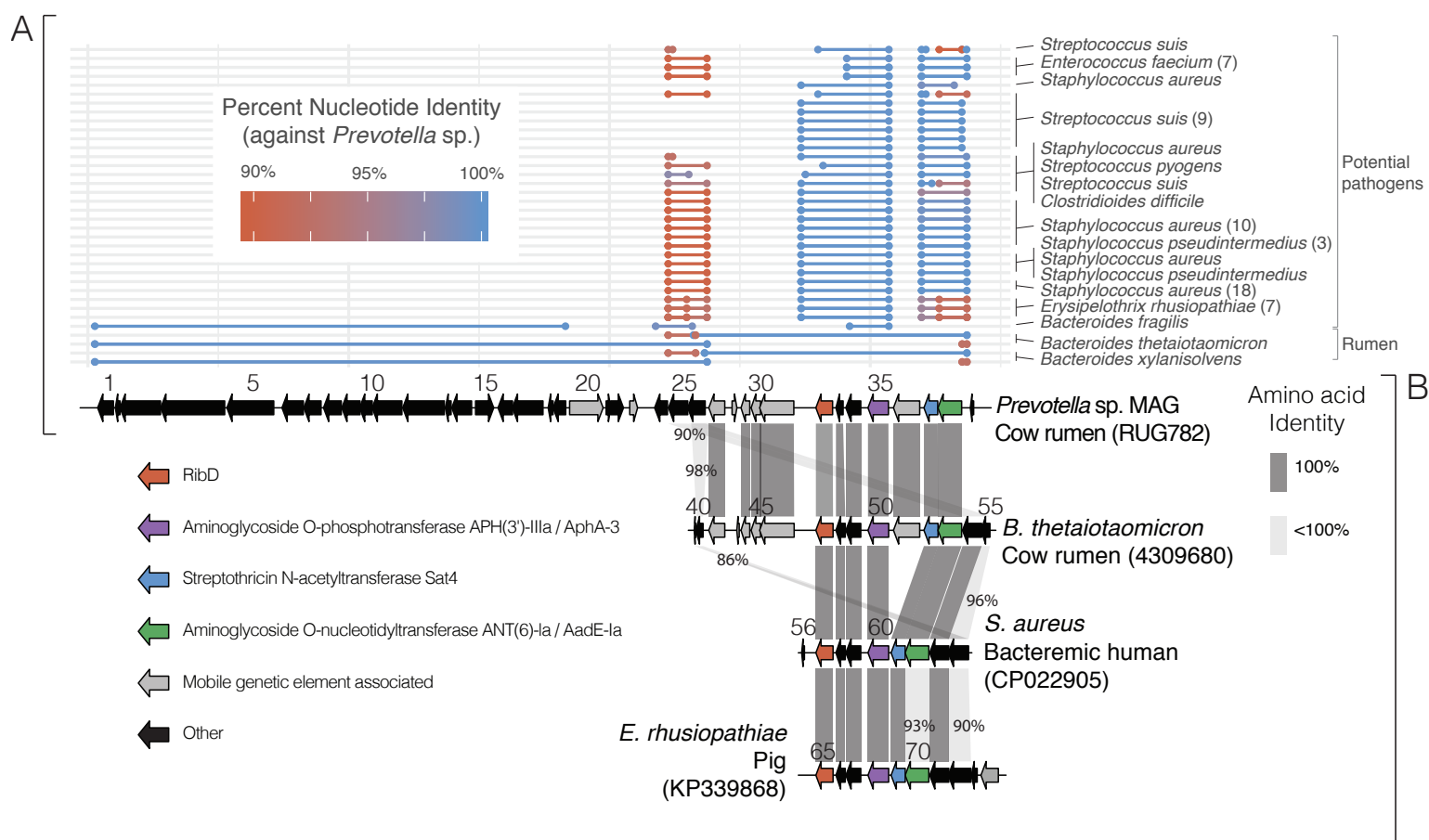
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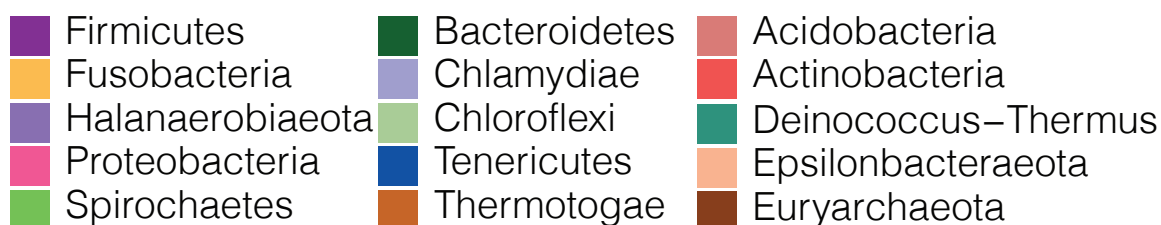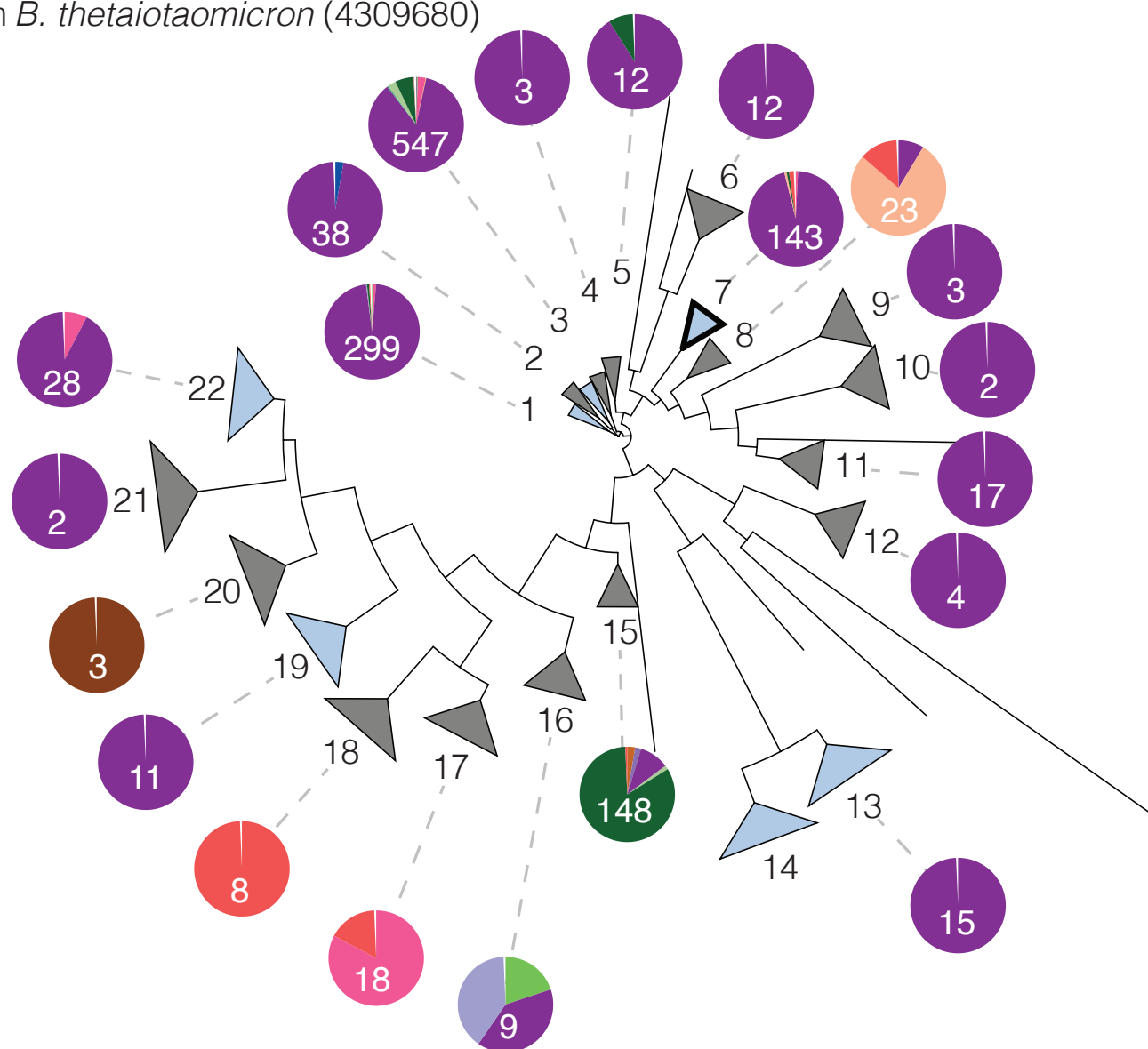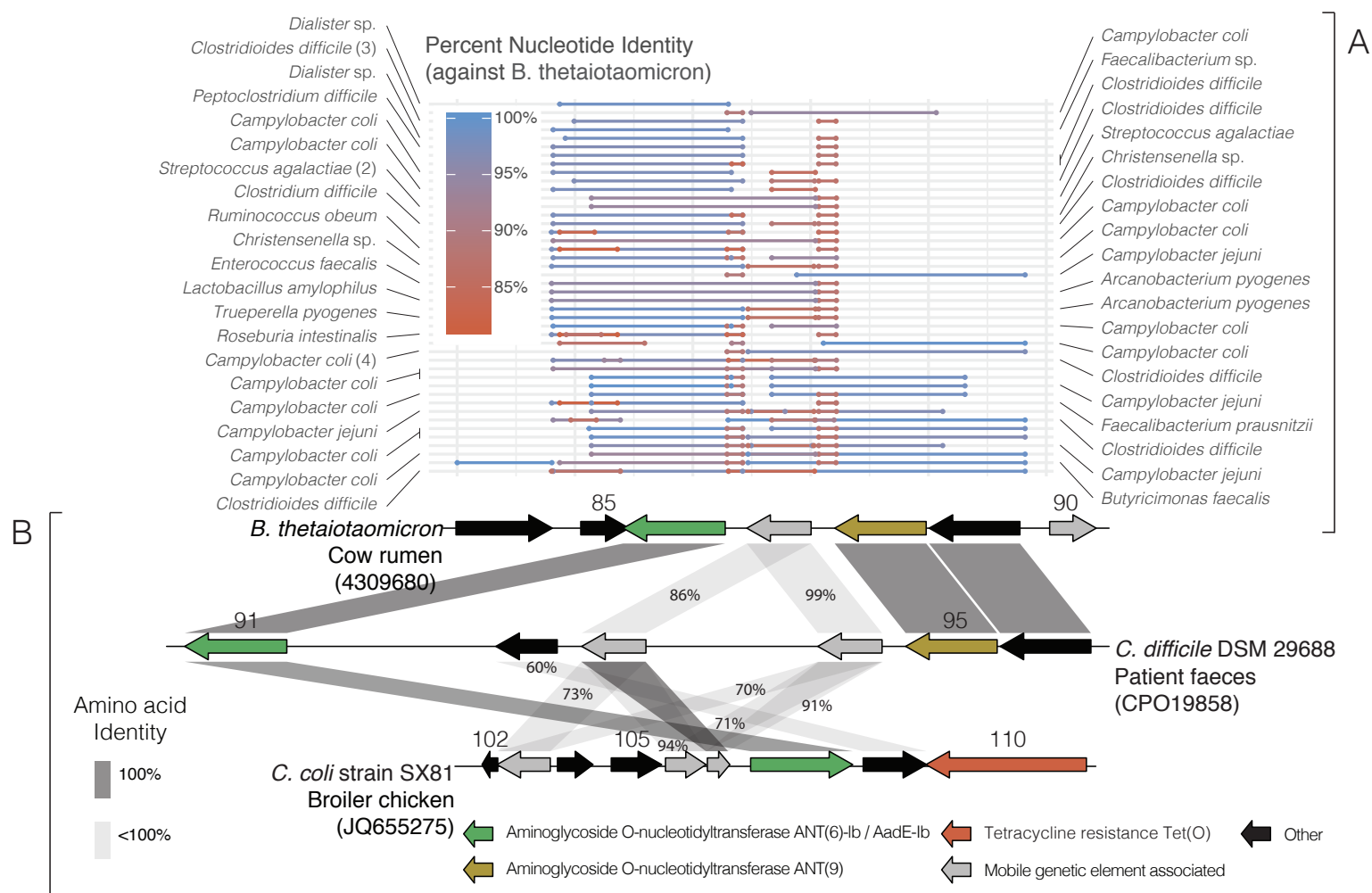
Figure 3

Figure 4

Figure S1

Figure S2

B. ARDs diversify, recombine and adapt predominantly in the rumen - an animal associated microbiome constantly exposed to soils and crops.

A. Soils and crops harbour organisms containing the original ARDs.

C. ARDs transfer throughout the farm via humans and pets. Here, the treatment of disease can result in the strong (co-) selection and stabilization (ex. gene loss and/or further recruitment) of antibiotic resistance clusters originally sourced from the rumen.