1 ***Dolosigranulum pigrum* cooperation and competition in human nasal microbiota**

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Summary

Multiple epidemiological studies identify *Dolosigranulum pigrum* as a candidate beneficial bacterium based on its positive association with health, including negative associations with nasal/nasopharyngeal colonization by the pathogenic species *Staphylococcus aureus* and *Streptococcus pneumoniae*. To gain insight into *D. pigrum*'s functions, we used a multipronged strategy. We identified *in vivo* community-level and *in vitro* phenotypic cooperation by specific nasal *Corynebacterium* species. *D. pigrum* inhibited *S. aureus* growth *in vitro*. Whereas, *D. pigrum* plus a nasal *Corynebacterium* were needed to inhibit *S. pneumoniae* growth. Moreover, *D. pigrum* L-lactic-acid production was insufficient for this inhibition. Genomic analysis of 11 strains revealed that *D. pigrum* has a small genome (average 1.86 Mb) and multiple predicted auxotrophies, which indicate that *D. pigrum* relies on its human host and cocolonizing bacteria for key nutrients. This shift to genomic and phenotypic experimentation marks a significant advance in understanding *D. pigrum*'s role in human nasal microbiota.

37 **Introduction**

38 Colonization of the human nasal passages by *Staphylococcus aureus* or *Streptococcus*
39 *pneumoniae* is a risk factor for infection by the colonizing bacterium at a distant body site (Bogaert
40 et al., 2004; Kluytmans et al., 1997; von Eiff et al., 2001; Wertheim et al., 2004; Young et al.,
41 2017). Interventions that reduce the prevalence of colonization also reduce the risk of infection
42 and transmission, e.g., (Bode et al., 2010; Lexau et al., 2005). *S. aureus* and *S. pneumoniae* are
43 major human pathogens that cause significant morbidity and mortality worldwide (Collaborators,
44 2018; Tong et al., 2015; Turner et al., 2019; Wahl et al., 2018). There are also concerns regarding
45 rising rates of antimicrobial resistance (WHO, 2012) and the potential for long-term effects of
46 antibiotics early in life (Schulfer and Blaser, 2015). Thus, efforts have recently focused on the
47 identification of candidate bacteria that confer colonization resistance against *S. aureus* (Brugger
48 et al., 2016; Janek et al., 2016; Nakatsuji et al., 2017; O'Sullivan et al., 2019; Paharik et al., 2017;
49 Piewngam et al., 2018; Williams et al., 2019; Zipperer et al., 2016) and *S. pneumoniae* (Cardenas
50 et al., 2019; Coleman and Cervin, 2019; Manning et al., 2016; Tano et al., 2002), with particular
51 urgency for *S. aureus* in the absence of an effective vaccine.

52 Little is known about the biology of the Gram-positive, catalase-negative bacterium
53 *Dolosigranulum pigrum,* which was first described in 1993 (Aguirre et al., 1993). However, multiple
54 studies of the human upper respiratory tract microbiota have identified *D. pigrum* (Firmicute),
55 colonizing with or without *Corynebacterium* species (Actinobacteria), as potentially beneficial
56 and/or protective against colonization by *S. aureus* and *S. pneumoniae* (Biesbroek et al., 2014a;
57 Biesbroek et al., 2014b; Bomar et al., 2016; Bosch et al., 2017; Bosch et al., 2016; Camelo-
58 Castillo et al., 2019; Copeland et al., 2018; Escapa et al., 2018; Gan et al., 2019; Hasegawa et
59 al., 2017; Kelly et al., 2017; Langevin et al., 2017; Lappan et al., 2018; Laufer et al., 2011; Liu et
60 al., 2015; Man et al., 2019a; Man et al., 2019b; Perez-Losada et al., 2017; Pettigrew et al., 2012;
61 Sakwinska et al., 2014; Salter et al., 2017; Toivonen et al., 2019; Wen et al., 2018; Zhang et al.,

62 2016) (reviewed in (Bomar et al., 2018; Brugger et al., 2016; Esposito and Principi, 2018; Krismer
63 et al., 2017; Man et al., 2017)). Studies sampling either nostrils or nasopharynx show very similar
64 results; therefore, for simplicity, we use nasal or nasal passages henceforth to denote the area
65 inclusive of the nostrils through the nasopharynx. *D. pigrum* and *S. aureus* are inversely correlated
66 in adult nasal microbiota (Escapa et al., 2018; Liu et al., 2015; Yan et al., 2013). Whereas, in
67 pediatric nasal microbiota, *D. pigrum* and members of the genus *Corynebacterium* are
68 overrepresented when *S. pneumoniae* is absent (Bomar et al., 2016; Lappan et al., 2018; Laufer
69 et al., 2011). Moreover, children with *D. pigrum* colonization of the nasal passages are less likely
70 to have acute otitis media (Lappan et al., 2018; Pettigrew et al., 2012) and it has been speculated
71 that *D. pigrum*-dominated microbiota profiles might be more resistant to invasive pneumococcal
72 disease (Camelo-Castillo et al., 2019). Furthermore, *D. pigrum* abundance in the nasal passages
73 is inversely associated with wheezing and respiratory tract infections in infants (Biesbroek et al.,
74 2014a). The intriguing inferences from these studies that *D. pigrum* plays a beneficial role in
75 human nasal microbiota deserves further investigation.

76 In contrast to the above, there are very few reports of *D. pigrum* in association with human disease
77 (Hoedemaekers et al., 2006; LaClaire and Facklam, 2000; Lecuyer et al., 2007; Sampo et al.,
78 2013; Venkateswaran et al., 2014). Its frequent identification in human nasal microbiota (Bogaert
79 et al., 2011; Bosch et al., 2017; Bosch et al., 2016; Camarinha-Silva et al., 2012a; Camarinha-
80 Silva et al., 2012b; Camelo-Castillo et al., 2019; Caputo et al., 2019; Chonmaitree et al., 2017;
81 Copeland et al., 2018; De Boeck et al., 2017; Escapa et al., 2018; Gan et al., 2019; Kelly et al.,
82 2017; Langevin et al., 2017; Lappan et al., 2018; Laufer et al., 2011; Liu et al., 2015; Luna et al.,
83 2018; Man et al., 2019b; Peterson et al., 2016; Sakwinska et al., 2014; Salter et al., 2017; Song
84 et al., 2019; Teo et al., 2015; Toivonen et al., 2019; Walker et al., 2019; Wen et al., 2018; Wos-
85 Oxley et al., 2010; Zhang et al., 2016) coupled with its rare association with infection are
86 consistent with *D. pigrum* functioning as a commensal, and possibly as a mutualist, of humans–

87 –characteristics that support its potential for future use as a therapeutic. However, its metabolism
88 and its interplay with other nasal bacteria remain uncharted territory. Using a multipronged
89 approach, we have made significant advances in these areas. First, we identified, at the species-
90 level, candidate bacterial interactors with *D. pigrum* by analyzing nasal microbiota datasets from
91 adults and children. Second, we delineated key metabolic features of *D. pigrum*'s core genome
92 based on 11 distinct *D. pigrum* strains. Third, we used *in vitro* phenotypic assays to show that *D.*
93 *pigrum* exhibits distinct interaction phenotypes with nasal *Corynebacterium* species, *S. aureus*
94 and *S. pneumoniae*. These new insights into *D. pigrum*'s functional capacity advance the field
95 from compositional analysis to genomic and phenotypic experimentation on a potentially
96 beneficial bacterial resident of the human upper respiratory tract.

97

98 **Results**

99 **Individual species of *Corynebacterium* are positively associated with *D. pigrum* in the**
100 **nasal microbiota of both adults and children.** Multiple 16S rRNA gene-based microbiota
101 studies identify genus-level associations between *Dolosigranulum* and *Corynebacterium*, e.g.,
102 (Biesbroek et al., 2014a; Biesbroek et al., 2014b; Bosch et al., 2017; Copeland et al., 2018; de
103 Steenhuijsen Piters and Bogaert, 2016; Hasegawa et al., 2017; Lappan et al., 2018; Teo et al.,
104 2015). *D. pigrum* is the only member of its genus and data regarding species-level associations
105 with *Corynebacterium* and other taxa are lacking. Therefore, we identified bacterial species that
106 display differential relative abundance in the absence or presence of *D. pigrum* sequences. As a
107 prerequisite, we identified two nostril datasets with V1-V2/V1-V3 16S rRNA gene sequences,
108 regions that contain sufficient information for species-level identification of most nasal-associated
109 bacteria (Escapa et al., 2018; Laufer et al., 2011). After parsing sequences into species-level
110 phylotypes, we interrogated each dataset using Analysis of Composition of Microbiomes

111 (ANCOM) (Mandal et al., 2015). In the nostrils of 210 adult participants from the Human
112 Microbiome Project (HMP), four *Corynebacterium* species—*C. accolens, C. propinquum, C.*
113 *pseudodiphtheriticum* and an unresolved supraspecies of *C. accolens-macginleyi-*
114 *tuberculostearicum*—exhibited increased differential relative abundance in the presence of *D.*
115 *pigrum* (**Figure 1A, panels ii-v and Table S1**). In the nostrils of 99 children ages 6 and 78 months
116 (Laufer et al., 2011), *Corynebacterium pseudodiphtheriticum* exhibited increased differential
117 relative abundance in the presence of *D. pigrum*, as did *Moraxella nonliquefaciens* (**Figure 1B**).
118 Together, these results from children and adults identify a positive relationship between *D. pigrum*
119 and individual species of *Corynebacterium* in human nasal microbiota, with a positive association
120 of *D. pigrum* and *C. pseudodiphtheriticum* across age groups.

121 **Nasal *Corynebacterium* species enhance the growth of *D. pigrum in vitro*.** Various types of
122 microbe-microbe interactions could underlie the positive *in vivo* association between *D. pigrum*
123 and individual *Corynebacterium* species in human nasal microbiota (**Figure 1**). One being a direct
124 interaction between these species mediated by diffusible compounds. To test this possibility, we
125 quantified *D. pigrum* growth yields on unconditioned agar medium compared to on cell-free agar
126 medium conditioned by growth of *C. pseudodiphtheriticum, C. propinquum* or *C. accolens*.
127 Conditioning medium with any of these three nasal *Corynebacterium* species increased the yield
128 (measured as colony forming units, CFUs) of two *D. pigrum* strains (CDC 4709-98 and KPL1914)
129 by one to two orders of magnitude compared to growth on unconditioned agar medium (**Figures**
130 **2A** and **2B**). Additionally, one strain of *C. pseudodiphtheriticum* (**Figure 2A**) and the *C. accolens*
131 strain (**Figure 2B**) increased the growth yield of *D. pigrum* CDC 2949-98, a strain with a higher
132 baseline growth yield. Results of this phenotypic interaction assay are consistent with unilateral
133 cooperation of nasal *Corynebacterium* species—*C. pseudodiphtheriticum, C. propinquum* or *C.*
134 *accolens*—with *D. pigrum* in the nostril microbiota of both children and adults and support the
135 observed positive *in vivo* community-level relationships.

136 Although positive interactions between *C. accolens* and *D. pigrum* prevailed *in vivo* in adults
137 (**Figure 1A, panel ii**)*, in vitro* we observed both positive and negative interactions between *D.*
138 *pigrum* and *C. accolens* depending on the assay conditions. For example, we observed increased
139 *D. pigrum* growth yield on a semi-permeable membrane on *C. accolens* cell-free conditioned agar
140 medium (CFCAM) of Brain Heart Infusion (BHI) supplemented with triolein (**Figure 2B**). In
141 contrast, *D. pigrum* was inhibited when inoculated directly onto *C. accolens* CFCAM (**Table S2**).
142 Like *S. pneumoniae, D. pigrum* belongs to the order Lactobacillales; the inhibition was reminiscent
143 of our previous research demonstrating that *C. accolens*' triacylglycerol lipase, LipS1, releases
144 free fatty acids from model host epithelial-surface triacylglycerols, such as triolein, to inhibit *S.*
145 *pneumoniae in vitro* (Bomar et al., 2016). We observed that oleic acid also inhibited *D. pigrum*
146 (**Table S3**). Thus, *in vitro* data show *C. accolens* can both inhibit the growth of *D. pigrum* by
147 releasing antibacterial free fatty acids such as oleic acid from host triacylglycerols (**Tables S2**
148 **and S3**) and enhance the growth of *D. pigrum* by releasing as-yet unidentified factor(s) (**Figure**
149 **2B**). These results point to the existence of a complex set of the molecular interactions between
150 these two species.

151 ***D. pigrum* inhibits *S. aureus* growth.** Researchers have sought to identify commensal bacteria
152 that provide colonization resistance to pathobionts such as *S. aureus* (Janek et al., 2016;
153 Nakatsuji et al., 2017; O'Sullivan et al., 2019; Paharik et al., 2017; Piewngam et al., 2018; Williams
154 et al., 2019; Zipperer et al., 2016) (reviewed in (Brugger et al., 2016)). In contrast to the positive
155 association between *D. pigrum* and specific *Corynebacterium* species, ANCOM analysis of adult
156 nostril microbiota datasets revealed decreased differential relative abundance of *S. aureus* in the
157 presence of *D. pigrum* (**Figure 1A**). Direct antagonism could explain this observation. Therefore,
158 we assayed for the effect of *D. pigrum* on *S. aureus* and observed inhibition of *S. aureus* when it
159 was inoculated adjacent to a pregrown inoculum of *D. pigrum* on agar medium (**Figure 3**). (We
160 gave *D. pigrum* a head-start to compensate for its slower growth rate *in vitro.*) To assess whether

161 this *D. pigrum* inhibition of *S. aureus* was strain specific, we tested nine additional *D. pigrum*
162 strains. All nine also inhibited *S. aureus* (**Figure S1**). This, plus the observed cooperation from
163 nasal *Corynebacterium* species for *D. pigrum* growth yield (**Figure 2**), led us to explore the genetic
164 capacity of *D. pigrum.*

165 **The genomes of 11 *D. pigrum* strains reveal a small genome and high degree of sequence**
166 **conservation consistent with a highly host-adapted bacterium.** We analyzed one publicly
167 available genome of *D. pigrum* (ATCC51524) and sequenced 10 additional strains. The 10
168 sequenced strains were selected to ensure representation of distinct strains (see Methods). The
169 11 *D. pigrum* strain genomes had an average size of 1.86 Mb (median 1.88 Mb) with 1693
170 predicted coding sequences and a mean GC content of 39.6% (**Tables 1** and **S4**). This genome
171 size is consistent with *D. pigrum* being a highly host-adapted bacterium with reduced biosynthetic
172 capacities (Pfeiler and Klaenhammer, 2007) and is similar to or slightly smaller than other
173 Firmicutes commonly found in human oral, respiratory and skin environments, e.g., (Galac et al.,
174 2017; Hilty et al., 2014; Suzuki et al., 2012; Tettelin et al., 2001).

175 From the nucleotide sequences of these 11 strains, an estimate of the upper bound of a
176 conservative core genome for *D. pigrum* is 1200 coding sequencing (CDS) based on the overlap
177 of three ortholog prediction algorithms (**Figure S2**). Two of these algorithms together estimated
178 a lower bound of 1513 CDS for the *D. pigrum* accessory genome within a pangenome of 2729
179 CDS (based on an estimated core of 1216 CDS) (**Figure S3** and **Table S5**). Similarity matrices
180 of the core genes and proteins from the 11 *D. pigrum* strains revealed a high degree of nucleotide
181 (≥97%) and amino acid (≥96.8%) sequence conservation (**Figure S4A** and **S4B**, respectively).

182 To gain insight into the chromosomal structure of *D. pigrum*, we closed the genomes of two
183 strains, CDC 4709-98 (**Figure S5A**) and KPL1914 (**Figure S5B**) using SMRT sequencing.
184 MAUVE alignment revealed large blocks of synteny between these two closed genomes (**Figure**

185 **4**). Synteny analysis of the bidirectional best-hits core proteins from the RAST annotation revealed
186 1143 syntenic clusters (of 1206 total BDBH clusters). Ring plots using the closed genome of CDC
187 4709-98 as the reference (**Figure S5A**), because it is 5% (97 kb) larger than that of KPL1914,
188 showed discrete areas of sequence absence in KPL1914 (**Figure S5C**) along with large areas of
189 conservation. Similar areas of absence were visible when the other 9 strains were also compared
190 to CDC 4709-98 (**Figure S5D**).

191 **A core-genome phylogeny reveals relationships between the 11 *D. pigrum* strains.** In 16S
192 rRNA gene-based phylogenies, *D. pigrum* clades with other lactic acid bacteria and shares the
193 closest node with *Alloiococcus otitis* (aka *A. otitidis*); both species are within the family
194 *Carnobacteriaceae* (order *Lactobacillales*) (Yarza et al., 2008). Genomic content also identified
195 *Alloiococcus* as its closest genome-sequenced neighbor by RAST. Using *A. otitis* ATCC 51267
196 as an outgroup, a core-genome, maximum likelihood-based phylogeny revealed a monophyletic
197 *D. pigrum* clade (**Figure S6**). Although the 11 strains were isolated from different individuals in
198 different geographic regions, mostly in the late 1990s (**Table 1**), there was a relatively small
199 evolutionary distance. We next explored the metabolic capacity of *D. pigrum's* core genome to
200 gain insights into its functions in human nasal microbiota.

201 **Whole genome sequencing indicates that *D. pigrum* metabolizes carbohydrates via**
202 **homofermentation to lactic acid.** It is proposed that *D. pigrum* utilizes lactic acid production as
203 a mechanism for competition in the nasal passages (de Steenhuijsen Piters and Bogaert, 2016).
204 Lactic acid bacteria mainly perform either homo- or heterofermentation of carbohydrates (Kandler,
205 1983). Therefore, we examined the genomic capacity of *D. pigrum* for carbohydrate metabolism
206 and observed patterns of gene presence and absence that are consistent with homofermentation
207 of carbohydrates to lactic acid. *D. pigrum* genomes lacked genes required for a complete
208 tricarboxylic acid -cycle, which is consistent with fermentation. Moreover, we identified genes
209 encoding a complete glycolytic pathway in all 11 strains that are consistent with homofermentation

210 (supplemental text). All 11 strains harbored a predicted L-lactate-dehydrogenase (EC 1.1.1.27),
211 which catalyzes the reduction of pyruvate to lactate regenerating NAD+ for glycolysis (GAPDH
212 step), consistent with homofermentation to L-lactate as the main product of glycolysis. Supporting
213 this, we detected lactate production by *D. pigrum* during *in vitro* cultivation (**Figure 5A**).

214 ***D. pigrum* production of lactic acid is unlikely to be the primary mechanism for the negative**
215 **association with *S. pneumoniae* in pediatric nasal microbiota.** *D. pigrum* lactic acid
216 production has been proposed as a mechanism to explain epidemiologic observations of negative
217 associations between *D. pigrum* and *S. pneumoniae* (de Steenhuijsen Piters and Bogaert, 2016).
218 Under nutrient rich conditions *in vitro*, three tested strains of *D. pigrum* produced from 5.7 to 8.2
219 mM of L-lactic acid with strain KPL1914 producing the highest concentration (**Figure 5A**).
220 Therefore, we assayed for growth of *S. pneumoniae* in *D. pigrum* KPL1914 CFCM compared to
221 in BHI broth supplemented with varying concentrations of L-lactic acid. Three of the four *S.*
222 *pneumoniae* strains tested showed some growth in 22 mM lactic acid (**Figure 5B**), and all strains
223 displayed more growth in BHI supplemented with 11 mM L-lactic acid than in the *D. pigrum*
224 KPL1914 CFCM, which had 7.5 mM of *D. pigrum*-produced L-lactic acid (**Figure 5B**). Thus, the
225 restriction of *S. pneumoniae* growth in *D. pigrum* CFCM is unlikely to be due to *D. pigrum*
226 production of lactic acid. More likely, it reflects competition for nutrients since fresh medium was
227 not added to the CFCM, which, therefore, would have a lower concentration of sugars than BHI
228 broth. However, *D. pigrum* production of a toxin and/or an antipneumococcal compound in BHI
229 broth cannot be excluded. These results indicate that *D. pigrum* production of lactic acid in human
230 nasal passages is unlikely to be the primary molecular mechanism underlying the decreased
231 relative abundance of *S. pneumoniae* in children's nasal passages when *D. pigrum* is present.

232 *D. pigrum* is negatively associated with S. *aureus* in adult nostrils (Escapa et al., 2018; Liu et al.,
233 2015; Yan et al., 2013) and *D. pigrum* excreted a diffusible activity that inhibited *S. aureus* growth
234 on BHI agar (**Figures 3** and **S1**). Therefore, we also tested the *in vitro* effect of L-lactic acid on

235 two strains of *S. aureus*. Both grew in BHI supplemented with 22 mM L-lactic acid, with a
236 statistically significant decrease in growth in 33 mM L-lactic acid (**Figure 5C**). Thus, under the
237 tested conditions *D. pigrum* does not produce enough L-lactic acid to restrict *S. aureus* growth. In
238 contrast to *S. pneumoniae*, we would not expect depletion of sugars to impact *S. aureus* growth
239 in CFCM given its broader repertoire of energy source utilization options, e.g., amino acids, and
240 indeed both *S. aureus* strains showed no decrease in growth in *D. pigrum* CFCM. This also points
241 to a difference in *D. pigrum* production of the anti-*S. aureus* activity during growth on BHI agar
242 medium (**Figure 3; Fig S1**) versus in BHI broth. Excretion of metabolites may vary during growth
243 in liquid versus on agar medium and the mechanism of the *D. pigrum* anti-*S. aureus* activity is
244 yet-to-be identified.

245 ***D. pigrum* is a predicted auxotroph for some amino acids, polyamines and enzymatic**
246 **cofactors.** The nasal environment is low and/or lacking in key nutrients such as methionine
247 (Krismer et al., 2014). To gain insight into how *D. pigrum* functions in the nose, we examined all
248 11 genomes and found evidence of auxotrophy for some amino acids, polyamines and required
249 enzymatic cofactors (supplemental text). For example, all 11 strains lacked complete predicted
250 biosynthetic pathways for methionine and arginine but encoded putative degradation pathways
251 for these, suggesting these amino acids are acquired exogenously. Additionally, all 11 genomes
252 encoded predicted transporters for polyamines, such as putrescine and spermidine, but not the
253 genes for a complete polyamine biosynthetic pathway. In terms of enzyme cofactors, *D. pigrum*
254 genomes lacked genes for *de novo* synthesis of niacin/nicotinate/nicotinamide but encoded a
255 putative transporter. Similarly, all 11 strains lacked genes for the biosynthesis of cobalamin (B12),
256 thiamine (B1), pyridoxine (B6) and biotin (B7), with predicted biotin transport/uptake genes, and
257 10 of 11 lacked a riboflavin (B2) synthesis cluster (all except CDC 4199-99). Similarly, a complete
258 set of folate biosynthesis genes are lacking in all 11, which appear to encode folate salvage
259 pathways, along with folate to tetrahydrofolate conversion via dihydrofolate reductase (EC

260 1.5.1.3). Likely auxotrophy for the above points to nutrients that must be available either from the
261 host or from neighboring microbes in human nasal passages.

262 ***D. pigrum* encodes mechanisms for acquiring essential metal cofactors from the host**
263 **environment.** The nasal environment is low on essential metal ions such as iron, zinc and
264 manganese and host metal sequestration using lactoferrin and calprotectin is an important
265 defense mechanism against bacterial growth. Bacteria have acquired mechanisms to escape this
266 nutritional immunity (Krismer et al., 2014; Krismer et al., 2017). We therefore searched for genes
267 predicted to encode for siderophores and transporters for heme, manganese and zinc. All 11 *D.*
268 *pigrum* genomes harbored a predicted iron compound ABC uptake transporter ATP-binding
269 protein (hemin uptake system subsystem) and a manganese ABC-type transporter. Additionally,
270 six of the CDC strains (4294-98, 4420-98, 4545-98, 4199-99, 4791-99, 4792-99) had predicted
271 ferric iron ABC transporter and/or iron compound ABC uptake transporter genes.

272 Our analysis of the genomes of 11 distinct strains provided key insights into the biology and
273 environment of *D. pigrum*. It did not, however, identify a molecular mechanism for the negative
274 association between *D. pigrum* and *S. pneumoniae*. This led us to investigate whether a
275 cooperative interaction with nasal *Corynebacterium* species might be key to the negative
276 association of *D. pigrum* and *S. pneumoniae*, since many pediatric nasal microbiota composition
277 studies also detect a positive association between *D. pigrum* and *Corynebacterium*, e.g., (**Figure**
278 **1B**) and (Biesbroek et al., 2014a; Biesbroek et al., 2014b; Bosch et al., 2017; Copeland et al.,
279 2018; de Steenhuijsen Piters and Bogaert, 2016; Hasegawa et al., 2017; Lappan et al., 2018; Teo
280 et al., 2015).

281 **Together *D. pigrum* and *C. pseudodiphtheriticum* inhibit *S. pneumoniae* growth.** Since *C.*
282 *pseudodiphtheriticum* displayed increased differential relative abundance in the presence of *D.*
283 *pigrum* (**Figure 1**), we investigated the effect of a mixed *in vitro* population of *D. pigrum* and *C.*

284 *pseudodiphtheriticum* on *S. pneumoniae* growth. Agar medium conditioned with a coculture of *C.*
285 *pseudodiphtheriticum* strain KPL1989 and *D. pigrum* strain CDC4709-98 inhibited *S. pneumoniae*
286 growth, whereas agar medium conditioned with a monoculture of either species did not (**Figures**
287 **6** and **S7**). This is consistent with cocultivation resulting in either a greater level of nutrient
288 competition than monoculture of either commensal alone or in the production of diffusible
289 compound(s) toxic/inhibitory to *S. pneumoniae* by one or both of the commensal species when
290 grown together. Along with the *Corynebacterium* species enhancement of *D. pigrum* growth yield
291 (**Figure 2**) and the *D. pigrum* inhibition of *S. aureus* growth (**Figure 3**), these data indicate that
292 the negative associations of *D. pigrum* with *S. aureus* and *S. pneumoniae* are mediated by
293 different molecular mechanisms.

294 **The accessory genome of 11 *D. pigrum* strains contains a diversity of biosynthetic gene**
295 **clusters predicted to encode antibiotics**. Data presented above indicate that lactic acid alone
296 is unlikely to account for the negative associations of *D. pigrum* with *S. aureus* and with *S.*
297 *pneumoniae* in *in vivo* community composition data. Also, *D. pigrum*'s competitive interactions
298 with these two species may well be mediated by different mechanisms. Therefore, we delved into
299 the functional capacity of *D. pigrum*, including the accessory genome of the 11 sequenced strains.
300 What emerged was a diversity of biosynthetic gene clusters (BGCs) (**Table S6 and Figure S8**),
301 including a diversity of BGCs predicted to encode candidate antibiotics. Strikingly, although 10 of
302 10 strains tested displayed inhibition of *S. aureus* growth *in vitro* (**Figure S1**), there was no single
303 BGC common to all 10 strains that might encode a compound with antibiotic activity. Based on
304 this, we hypothesize that *D. pigrum* uses a diverse repertoire of BGCs to produce bioactive
305 molecules that play key roles in interspecies interactions with its microbial neighbors, e.g., for
306 niche competition, and potentially with its host. This points to a new direction for future research
307 on the functions that underlie the positive associations of *D. pigrum* in human nasal microbiota
308 with health.

309

310 **Discussion**

311 *D. pigrum* is associated with health in multiple genus-level compositional studies of human
312 URT/nasal passage microbiota. The above species-level genomic and phenotypic experimental
313 data mark a significant advance in the study of *D. pigrum* and set the stage for future mechanistic
314 research. Here, in nasal passage microbiota datasets, we identified positive associations of *D.*
315 *pigrum* with specific species of *Corynebacterium* in adults and children and a negative association
316 of *D. pigrum* with *S. aureus* in adults (**Figure 1**). We observed phenotypic support for these
317 associations during *in vitro* growth via cooperation from three common nasal *Corynebacterium*
318 species that increased *D. pigrum* growth yields (**Figure 2**) and by *D. pigrum* inhibition of *S. aureus*
319 (**Figure 3**). Our genomic analysis revealed auxotrophies consistent with reliance on cocolonizing
320 microbes and/or the human host for key nutrients. Genomic analysis also showed an aerotolerant
321 anaerobe that performs homofermentation to lactate. However, *D. pigrum* lactate production
322 (**Figure 5A**) was insufficient to inhibit either *S. aureus* (**Figure 5B**) or *S. pneumoniae* (**Figure 5C**),
323 and is, therefore, unlikely to be the sole contributor to negative associations with *S. pneumoniae*
324 and *S. aureus in vivo*. Consistent with the multiple reports of a negative association between *D.*
325 *pigrum*, usually in conjunction with the genus *Corynebacterium*, and *S. pneumoniae*, we observed
326 that cocultivation of *D. pigrum* and *C. pseudodiphtheriticum* produced a diffusible activity that
327 inhibited *S. pneumoniae* (**Figures 6** and **S7**). Finally, we identified a surprisingly diverse repertoire
328 of BGCs in 11 *D. pigrum* strains, revealing potential mechanisms for niche competition that were
329 previously unrecognized and opening up a new line of investigation in the field. The *in vitro*
330 interactions of *D. pigrum* with *S. aureus* and with *S. pneumoniae* support inferences from
331 composition-level microbiota data of competition between *D. pigrum* and each pathobiont.
332 However, these interactions differed *in vitro*. *D. pigrum* alone inhibited *S. aureus* but *D. pigrum*
333 plus *C. pseudodiphtheriticum*, together, inhibited *S. pneumoniae*. This points to a more complex

334 set of interactions among these specific bacterial members of the human nasal microbiota, which
335 likely exists in the context of a network of both microbe-microbe and microbe-host interactions.
336 To date, mechanisms for only a few such interactions are described. For example, a *C. accolens*
337 triacylglycerol lipase (LipS1) releases antipneumococcal free fatty acids from model host surface
338 triacyclglycerols *in vitro* pointing to habitat modification as a possible contributor to S*. pneumoniae*
339 colonization resistance (Bomar et al., 2016).

340 Multiple mechanisms could result in *D. pigrum* inhibition of *S. aureus in vitro* including nutrient
341 competition, excretion of a toxic primary metabolite or of an anti-*S. aureus* secondary metabolite
342 (i.e., an antibiotic). The latter is intriguing because the diverse repertoire of BGCs among the 11
343 *D. pigrum* strains includes predicted lanthipeptides and bacteriocins. For example, 4 of the 11
344 strains harbored putative type II lanthipeptide biosynthetic gene clusters. These clusters are
345 characterized by the presence of the LanM enzyme, containing both dehydration and cyclization
346 domains needed for lanthipeptide biosynthesis (Repka et al., 2017). Alignment of these enzymes
347 with the enterococcal cytolysin LanM revealed conserved catalytic residues in both domains
348 (Dong et al., 2015). Cleavage of the leader portion of the lanthipeptide is necessary to produce
349 an active compound and the presence of peptidases and transporters within these BGCs
350 suggests these *D. pigrum* strains might secrete an active lanthipeptide, which could play a role in
351 niche competition with other microbes. Additionally, 8 of the 11 *D. pigrum* genomes examined
352 contain putative bacteriocins, or bactericidal proteins and peptides. Intriguingly, the *D. pigrum*
353 strains (CDC4709-98, CDC39-95, KPL1914) exhibiting the strongest inhibition of *S. aureus*
354 (**Figure S1**), were the only strains that contained both a lanthipeptide BGC and a bacteriocin,
355 further indicating that *D. pigrum* may employ multiple mechanisms to inhibit *S. aureus* growth.

356 Mechanisms are coming to light for how other nasal bacteria interact with *S. aureus*. For example,
357 commensal *Corynebacterium* species excrete a to-be-identified substance that inhibits *S. aureus*
358 autoinducing peptides blocking *agr* quorum sensing (QS) and shifting *S. aureus* shifts towards a

359 commensal phenotype (Ramsey et al., 2016). Also, the to-be-identified mechanism of *C.*
360 *pseudodiphtheriticum* contact-dependent inhibition of *S. aureus* is mediated through phenol
361 soluble modulins (PSM), the expression of which increases during activation of *agr* QS (Hardy et
362 al., 2019). Within broader *Staphylococcus-Corynebacterium* interactions, *C. propinquum*
363 outcompetes coagulase-negative *Staphylococcus* (CoNS), but not *S. aureus*, for iron *in vitro* using
364 the siderophore dehydroxynocardamine, the genes for which are transcribed *in vivo* in human
365 nostrils (Stubbendieck et al., 2019). Interphylum Actinobacteria-Firmicutes interactions also occur
366 between *Cutibacterium acnes* and *Staphylococcus* species (reviewed in (Brugger et al., 2016)).
367 For example, some strains of *C. acnes* produce an anti-staphylococcal thiopeptide, cutimycin, *in*
368 *vivo* and the presence of the cutimycin BGC is correlated with microbiota composition at the level
369 of the individual human hair follicle (Claesen et al., 2019). Of note, Actinobacteria competition
370 with coagulase-negative *Staphylococcus* species could also have network-mediated (indirect)
371 effects on *S. aureus* via the well-known competition among *Staphylococcus* species (reviewed in
372 (Parlet et al., 2019)), which can be mediated by antibiotic production, e.g., (Janek et al., 2016;
373 Nakatsuji et al., 2017; O'Sullivan et al., 2019; Zipperer et al., 2016), interference with *S. aureus*
374 *agr* QS (Otto et al., 2001; Otto et al., 1999; Paharik et al., 2017; Williams et al., 2019) or
375 extracellular protease activity (Iwase et al., 2010), among other means (Brugger et al., 2016).
376 Further rounding out the emerging complexity of microbe-microbe interactions in nasal microbiota,
377 multiple strains of *Staphylococcus*, particularly *S. epidermidis*, inhibit the *in vitro* growth of other
378 nasal and skin bacteria, including *D. pigrum*, via to-be-identified mechanisms (Janek et al., 2016).
379 The above points to a wealth of opportunity to use human nasal microbiota as a model system to
380 learn how bacteria use competition to shape their community.

381 Direct cooperation could contribute to the observed positive associations between bacterial
382 species in epidemiological microbiome studies. Conditioning medium with any of the three nasal
383 *Corynebacterium* species positively associated with *D. pigrum in vivo* in human nasal microbiota

384 (**Figure 1**) enhanced the growth yield of some *D. pigrum* strains (**Figure 2**). This is possibly by
385 excretion of a limiting nutrient or by removal of a toxic medium component. The genomic
386 predictions of auxotrophy (above and supplemental text) might favor nasal *Corynebacterium*
387 species providing cooperation to *D. pigrum* by excretion of a limiting nutrient. Indeed, mass
388 spectrometry indicates a number of nutrients are limiting in the nose (Krismer et al., 2014).

389 Among the limitations of this study are these four. First, we analyzed the genomes of 11 strains,
390 and these were primarily isolated in the setting of disease. It is unclear whether these strains were
391 contaminants or pathogenic contributors (LaClaire and Facklam, 2000) but, with the exception of
392 keratitis, *D. pigrum* strains are infrequently associated with disease (Haas et al., 2012; Hall et al.,
393 2001; Hoedemaekers et al., 2006; Johnsen et al., 2011; Lecuyer et al., 2007; Lin et al., 2006;
394 Sampo et al., 2013; Venkateswaran et al., 2014). These 11 *D. pigrum* strains encoded only a few
395 potential virulence factors, which is consistent with *D. pigrum* acting primarily as a mutualistic
396 species of humans. Second, the ongoing search for a fully defined chemical medium permissive
397 for *D. pigrum* growth precluded experimental verification of predicted auxotrophies and further
398 investigation of how nasal *Corynebacterium* enhance *D. pigrum* growth yields. Third, our
399 approach assessed a limited set of possible interactions from among the complex multispecies
400 interplay discussed above that includes other common bacterial members of the nasal passages.
401 Fourth, to date, there is no animal model for nasal colonization with *D. pigrum* and
402 *Corynebacterium* species, which stymies directly *in vivo* testing the hypothesis of pathobiont
403 inhibition and points to another area of need within the nasal microbiome field.

404 In summary, we validated *in vivo* associations from human bacterial microbiota studies with
405 functional assays which support the hypothesis that *D. pigrum* is a mutualist, rather than a purely
406 commensal bacterium, with respect to its human host. The next step will be to identify the
407 molecular mechanisms of those interactions and to assess their role in the human host. Such
408 work could establish the premise for future studies to investigate the therapeutic potential of *D.*

409 *pigrum* as a topical nasal probiotic for use in patients with recurrent infections with *S. pneumoniae*,
410 possibly in conjunction with a nasal *Corynebacterium* species, or *S. aureus,* in conjunction with
411 established *S. aureus* decolonization techniques (Fritz et al., 2012).

412 **Materials and Methods.**

413 **Species-level reanalysis of a pediatric nostril microbiota dataset.** Laufer et al. analyzed
414 nostril swabs collected from 108 children ages 6 to 78 months (Laufer et al., 2011). Of these, 44%
415 were culture positive for *S. pneumoniae* and 23% were diagnosed with otitis media. 16S rRNA
416 gene V1-V2 sequences were generated using Roche/454 with primers 27F and 338R. We
417 obtained 184,685 sequences from the authors, of which 94% included sequence matching primer
418 338R and 1% included sequence matching primer 27F. We performed demultiplexing in QIIME
419 (Caporaso et al., 2010b) (split_libraries.py) filtering reads for those ≥250 bp in length, quality score
420 ≥30 and with barcode type hamming_8. Then, we eliminated sequences from samples for which
421 there was no metadata (n=108 for metadata) leaving 120,963 sequences on which we performed
422 *de novo* chimera removal in QIIME (USEARCH 6.1) (Edgar, 2010; Edgar et al., 2011), yielding
423 120,274 16S rRNA V1-V2 sequences. We then aligned the 120,274 chimera-cleaned reads in
424 QIIIME (PyNAST) (Caporaso et al., 2010a), using eHOMDv15.04 (Escapa et al., 2018) as a
425 reference database, and trimmed the reads using "o-trim-uninformative-columns-from-alignment"
426 and "o-smart-trim" scripts (Eren et al., 2015). 116,620 reads (97% of the chimera-cleaned) were
427 recovered after the alignment and trimming steps. After these initial cleaning steps, we retained
428 only the 99 samples with more than 250 reads. We analyzed this dataset of 99 samples with a
429 total of 114,909 reads using MED (Eren et al., 2015) with minimum substantive abundance of an
430 oligotype (-M) equal to 4 and maximum variation allowed in each node (-V) equal to 6 nt, which
431 equals 1.6% of the 379-nucleotide length of the trimmed alignment. Of the 114,909 sequences,
432 82.8% (95,164) passed the -M and -V filtering and are represented in the MED output. Oligotypes
433 were assigned taxonomy in R with the dada2::assignTaxonomy() function (an implementation of
434 the naïve Bayesian RDP classifier algorithm with a kmer size of 8 and a bootstrap of 100)
435 (Callahan et al., 2016; Wang et al., 2007) using the eHOMDv15.1 V1-V3 Training Set (version 1)

436 (Escapa et al., 2018) and a bootstrap of 70. We then collapsed oligotypes within the same
437 species/supraspecies yielding the data shown in **Table S7**.

438 **Microbiota community comparison (Figure 1).** The pediatric 16S rRNA gene V1-V2 dataset
439 analyzed at species level here (**Table S7**), as well as the HMP adult 16S rRNA gene V1-V3
440 dataset previously analyzed at species level (Table S7 in (Escapa et al., 2018)) were used as
441 input for the ANCOM analysis, including all identified taxa (i.e., we did not remove taxa with low
442 relative abundance). ANCOM (version 1.1.3) was performed using the presence or absence of *D.*
443 *pigrum* as group definer. ANCOM default parameters were used (sig = 0.05, tau = 0.02, theta =
444 0.1, repeated = FALSE) except that we performed a correction for multiple comparisons (multcorr
445 = 2), instead of using the default no correction (multcorr = 3) (Mandal et al., 2015).

446 **Ethics statement.** We isolated *D. pigrum* KPL1914 and *C. pseudodiphtheriticum* KPL1989 from
447 the nostril of an adult as part of a protocol to study the bacterial microbiota of the nostrils of healthy
448 adults that was initially approved by the Harvard Medical School Committee on Human Studies
449 (Lemon et al., 2010), and subsequently approved by the Forsyth Institute Institutional Review
450 Board.

451 **Cultivation from frozen stocks.** Bacterial strains were cultivated as described here unless
452 stated otherwise. Across the various methods, strains were grown at 37°C with 5% CO_2 unless
453 otherwise noted. *D. pigrum* strains were cultivated from frozen stocks on BBL Columbia Colistin-
454 Nalidixic Acid (CNA) agar with 5% sheep blood (BD Diagnostics) for 2 days. *Corynebacterium*
455 species were cultivated from frozen stocks on BHI agar (*C. pseudodiphtheriticum* and *C.*
456 *propinquum*) or BHI agar supplemented with 1% Tween80 (*C. accolens*) for 1 day.
457 Resuspensions described below were made by harvesting colonies from agar medium and
458 resuspending in 1X phosphate buffered saline (PBS).

459 **Growth yield of *D. pigrum* on a membrane atop media conditioned by growth of**
460 ***Corynebacterium* species (Figure 2).** Each *Corynebacterium* strain was resuspended from
461 growth on agar medium to an optical density at 600 nm (OD_{600}) of 0.50 in 1x PBS. Then 100 μL
462 of each resuspension was individually spread onto a 0.2-μm, 47-mm polycarbonate membrane
463 (EMD Millipore, Billerica, MA) atop 20 mL of either BHI agar for *C. pseudodiphtheriticum* and *C.*
464 *propinquum* or BHI agar supplemented with Triolein (CAS # 122-32-7, Acros) spread atop the
465 agar medium, as previously described (Bomar et al., 2016), for *C. accolens*. After 2 days,
466 membranes with *Corynebacterium* cells were removed, leaving cell-free conditioned agar medium
467 (CFCAM). On each plate of CFCAM, we placed a new membrane onto which we spread 100 μL
468 of *D. pigrum* cells that had been resuspended to an OD_{600} of 0.50 in 1x PBS. After 2 days,
469 membranes with *D. pigrum* were removed, placed in 3 mL 1x PBS, and vortexed for 1 min. to
470 resuspend cells. Resuspensions were diluted 1:10 six times, dilutions were inoculated onto BBL
471 CNA agar with 5% sheep blood and colony forming units (CFUs) were enumerated after 2-3 days
472 of growth.

473 **Growth of *D. pigrum* directly on BHI agar medium supplemented with triolein and**
474 **conditioned by growth of nasal *Corynebacterium* species (Table S2).** Onto BHI agar
475 supplemented with 200 U/mL of bovine liver catalase (C40-500MG, Sigma) (BHIC), we spread
476 50 μL of 100 mg/mL of Triolein (BHICT). We then spread 50 μL of a resuspension (OD_{600} of 0.50)
477 of each *Corynebacterium* strain onto a 0.2-μm, 47-mm polycarbonate membrane placed atop 10
478 mL of BHICT agar in a 100-mm-by-15-mm petri dish. After 2 days, we removed each membrane
479 with *Corynebacterium* cells leaving CFCAM. Using a sterile cotton swab, we then spread either a
480 lawn of *D. pigrum* (from cells resuspended to an OD_{600} of 0.50 in 1x PBS) or *S. pneumoniae*
481 (taken directly from agar medium) onto the CFCAM. Each lawn then grew for 1-2 days before
482 documenting growth or inhibition of growth with digital photography.

483 **Oleic acid disc diffusion assay (Table S3).** A lawn of *D. pigrum* or *S. pneumoniae* was spread
484 onto 10 mL of BHIC agar using a sterile cotton swab as described above. Oleic acid (Sigma-
485 Aldrich) was dissolved to a final concentration of 2 mg/mL, 5 mg/mL and 10 mg/mL in ethanol and
486 then we added 10 μl of each to separate, sterile 0.2-μm, 6-mm filter discs (Whatman), with 10 μL
487 of ethanol alone added to a disc as a control. After allowing the solvent to evaporate, filter discs
488 were placed onto the bacterial lawns which were then allowed to grow for 1 day before measuring
489 zones of inhibition and photographing.

490 ***D. pigrum*–*S. aureus* side-by-side coculture assay (Figures 1 and S1).** *D. pigrum* cells were
491 harvested with sterile cotton swabs and resuspended in sterile 1x PBS to a minimal OD_{600} of 0.3
492 then 5 µl drops were individually inoculated on BHI agar medium and incubated for 2 days. *S.*
493 *aureus* JE2 was grown overnight on BBL Columbia CNA agar with 5% sheep blood and
494 resuspended in PBS to an OD_{600} of 0.1. Then 5 µl drops of *S. aureus* were inoculated at different
495 distances from the pregrown *D. pigrum*. Inhibition was assessed daily and photographically
496 documented.

497 **Selection of strains and preparation of DNA for whole genome sequencing.** *D. pigrum*
498 KPL1914 was isolated from the nostril of a healthy adult (above). In addition, we selected 9 of 27
499 *D. pigrum* strains from a CDC collection (LaClaire and Facklam, 2000) using an *rpoB*-based typing
500 system with a preference for strains isolated from the nasal passages and/or from children (**Table**
501 **1**). Primers Strepto F MOD (AAACTTGGACCAGAAGAAAT) and R MOD
502 (TGTAGCTTATCATCAACCATGTG) were generated *in silico* by mapping primers Strepto F and
503 R (Drancourt et al., 2004) to the *rpoB* sequence of *D. pigrum* ATCC 51524 (genome obtained
504 from NCBI; RefSeq: NZ_AGEF00000000.1) with BLAST (Altschul et al., 1990) and manually
505 correcting misalignments in SnapGene viewer 2.8.2 (GSL Biotech, Chicago, IL). PCR were
506 performed using extracted genomic DNA of *D. pigrum*. PCR conditions were as follows: initial
507 denaturation 95°C for 2 minutes, then 30 cycles of denaturation for 30 seconds at 98°C, annealing

508 at 50°C for 30 seconds, elongation 72°C for minutes and a final extension step at 72°C for 10
509 minutes. PCR products were cleaned using QIAquick PCR purification kit (Qiagen, Germantown,
510 MD) and sequence determined by Sanger sequencing (Macrogen USA, Boston, MA, USA). In the
511 genomic analysis, we also included the publicly available genome for *D. pigrum* ATCC 51524,
512 which was sequenced by the BROAD institute as part of the HMP (RefSeq
513 NZ_AGEF00000000.1).

514 *D. pigrum* strains were grown atop membranes for 48 hrs as described above. Cells were
515 harvested with a sterile tip, resuspended in 50 µl of sterile PBS and frozen at -80°C. Genomic
516 DNA was extracted using the Epicentre MasterPure nucleic acid extraction kit (Epicentre,
517 Madison, WI) per the manufacturer's instructions. We assessed DNA purity using a Nanodrop
518 spectrophotometer (Nanodrop, Wilmington, DE), concentration using Qubit fluorometer
519 (Invitrogen, Carlsbad, CA) and fragment size/quality via agarose gel electrophoresis.

520 **Whole genome sequencing, read assembly and annotation.** Genomic DNA was sequenced
521 at the Yale Center for Genome Analysis (YCGA), New Haven, CT, on an Illumina MiSeq platform
522 using mated paired-end (2 x 250 bp) technology, assembled using de Bruijn graph algorithms
523 with Velvet (Zerbino and Birney, 2008) with a kmer size of 139 bp and annotated with RAST with
524 FIGfam release 70 (Aziz et al., 2008) and Prokka (Seemann, 2014). In addition, *D. pigrum* strains
525 KPL1914 and CDC#4709-98 (LaClaire and Facklam, 2000) were sequenced on a PacBio RS II
526 (Pacific Biosystems, Menlo Park, CA) and sequences were assembled using HGAP version 3.0
527 (Chin et al., 2013). We used an iterative procedure to error correct the PacBio genomes, which
528 involved mapping Illumina reads to the PacBio genomes until there were no differences detected
529 between the Illumina reads and the PacBio assembly (Pettigrew et al., 2018). To estimate the
530 degree of assembly errors and missing content that might contribute to the variation in gene
531 content, we compared the Illumina assembly of KPL1914 with the Illumina-corrected PacBio
532 assembly of KPL1914 to estimate the possible divergence (Hilty et al., 2014). Within Illumina

533 assemblies, we identified 139 (1566 vs. 1705) predicted coding sequences as determined by
534 RAST annotation absent in the assembly received by PacBio sequencing. Genomes were
535 deposited at NCBI (GenBank: NAJJ00000000, NAQW00000000, NAQX00000000,
536 NAQV00000000, NAQU00000000, NAQT00000000, NAQS00000000, NAQR00000000,
537 NAQQ00000000 and NAQP00000000 in BioProjects PRJNA379818 and PRJNA379966).

538 **Identification of the *D. pigrum* core, shell and cloud genome based on Illumina-sequenced**
539 **genomes from 11 strains.** Core proteins from RAST-annotated GenBank-files were determined
540 using the intersection of bidirectional best-hits (BDBH), cluster of orthologous (COG) triangles
541 and Markov Cluster Algorithm (OrthoMCL) clustering algorithms using GET_HOMOLOGUES
542 package version 02012019 on Ubuntu-Linux (Contreras-Moreira and Vinuesa, 2013) excluding
543 proteins with more than one copy in an input species (as single-copy proteins are safer
544 orthologues, i.e., using flag t-11). GenBank files derived from RAST annotation (see above) were
545 renamed with KPL strain names except for strain ATCC51524. As an initial control, amino acid
546 fasta files (*.faa) were used for the determination of core proteins. We determined the cloud, shell
547 and core genome of each of the 11 sequenced *D. pigrum* strains using the
548 parse_pangenome_matrix.pl script (./parse_pangenome_matrix.pl -m
549 sample_intersection/pangenome_matrix_t0.tab -s) of the GET_HOMOLOGUES package version
550 30062017 (Contreras-Moreira and Vinuesa, 2013). Definition of cloud, shell and core genome
551 were based on (Kaas et al., 2012). In brief, cloud is defined as genes only present in a 1 or 2
552 genomes (cut-off is defined as the class next to the most populated non-core cluster class). The
553 core genome is composed of clusters present in all 11 strains, soft core contains clusters present
554 in 10 genomes and shell includes clusters present in 3 to 9 genomes. Synteny analysis on BDBH
555 core (with flag t11) was performed using the compare_clusters script (-s) and synteny
556 visualization was done in MAUVE using standard settings (Darling et al., 2010) after the KPL1914

557 genome was reverse complemented and both genomes had the origin set at the beginning of
558 *dnaA*.

559 **Phylogenetic reconstruction, sequence and protein similarities.** A monophyletic (clade) core
560 genome phylogenic tree was constructed by including *A. otitis* (closest neighbor based on the
561 Living Tree Project (Yarza et al., 2008)) an outgroup (**Figure S6B**). A phylogenic tree without an
562 outgroup was also constructed similarly (**Figure S6A**). *A. otitis* ATCC 51267 contigs were
563 downloaded from NCBI (NZ_AGXA00000000.1) and annotated using RAST (see above).
564 Predicted core proteins common to *A. otitis* and *D. pigrum* genomes were identified as described
565 above using GET_HOMOLOGUES package. Alignments were done using a loop with Clustal
566 Omega V. 1.2.4 ($ for filename in *.faa; do clustalo -i "$filename" -o
567 clustalo_out/${filename%coral} -v; done) and resulting alignments were concatenated using
568 catfasta2phyml perl script (https://github.com/nylander/catfasta2phyml) $./catfasta2phyml.pl *.faa
569 --verbose > outv.phy. PhyML 3.0 (Guindon et al., 2010) with smart model selection (Lefort et al.,
570 2017) using Akaike information criterion was used for phylogenetic analysis (maximum-likelihood)
571 with 100 regular bootstrap replicates and FigTree (http://tree.bio.ed.ac.uk/software/figtree/) for
572 tree visualization.

573 BLAST Ring Image Generator (BRIG) was used for visualization of the other sequenced genomes
574 compared to the closed CDC 4709-98 genome (**Figure S5**) (Alikhan et al., 2011). Average amino
575 acid and nucleic acid identity (**Figure S4**) was calculated using GET_HOMOLOGUES package
576 version 30062017 (Contreras-Moreira and Vinuesa, 2013). In brief, a pangenome matrix was
577 generated using the OMCL algorithm (./get_homologues.pl -d dpig_folder -t 0 -M (OMCL)) for
578 homologues identification. Both, ANI and AAI were calculated with all available clusters (t 0).
579 Commands used: Generation of an AA identity matrix: $ ./get_homologues.pl -d “gbk-files” -A -t
580 0 -M and CDS identity matrix with the command $./get_homologues.pl -d “gbk files” -a 'CDS' -A -
581 t 0 -M.

582 **Measurement of L-Lactic Acid Concentration (Figure 5A).** *D. pigrum* cells were grown from
583 frozen stocks as above. Cells were then harvested with a sterile cotton swab, resuspended to an
584 OD_{600} of 0.50 in 1x PBS and inoculated at 1:25 in BHI broth for overnight growth gently shaking
585 (~50-60 rpm) at 37°C under atmospheric conditions. The overnight culture was then inoculated
586 at 1:25 into fresh BHI broth and grown for 24 hrs at 37°C prior to measuring the lactic acid
587 concentration (mmol/L) using a D-lactic acid/L-lactic acid kit per the manufacturer's instructions
588 (Cat. no. 11112821035, R-Biopharm AG).

589 **Growth of *S. aureus* and *S. pneumoniae* in *D. pigrum* cell-free conditioned liquid medium.**
590 **(**CFCM in **Figures 5B** and **5C**) After growth in BHI, as described for L-lactic acid measurement,
591 *D. pigrum* KPL1914 cells were removed with a 0.22-μM sterile filter yielding cell-free conditioned
592 medium (CFCM). We adjusted the pH of the *D. pigrum* CFCM using 2N H_2SO_4 and 10M KOH to
593 match that of BHI broth alone within 0.02 pH units. *S. aureus* strains Newman and JE2 and *S.*
594 *pneumoniae* strains TIGR4, DBL5, 603, WU2 were each grown on BBL Columbia CNA agar with
595 5% sheep blood for 1 day, harvested with a sterile cotton swab, resuspended to an OD_{600} of 0.30
596 in 1x PBS, inoculated at 1:100 into both *D. pigrum* CFCM and BHI broth and grown for 19-20 hrs
597 at 37°C in shaking (*S. aureus*; 50 rpm) or static (*S. pneumoniae*) culture under atmospheric
598 conditions. Growth yield was quantified as OD_{600} absorbance.

599 **Growth of *S. aureus* and *S. pneumoniae* in BHI broth supplemented with L-lactic acid.**
600 (Lactic Acid in **Figures 5B** and **5C**) Strains of *S. aureus* and *S. pneumoniae* were grown and
601 harvested as described above for inoculation. BHI broth, supplemented with L-lactic acid (CAS
602 no. 79-33-4; Fisher BioReagents) at varying concentrations from 11mM – 55 mM, was sterilized
603 through a 0.22-μM filter. After inoculating each strain separately into BHI broth with L-lactic acid,
604 cultures were grown as described above for growth in CFCM. Growth yield was quantified as
605 OD_{600} absorbance.

606 **Growth assay for *S. pneumoniae* on BHI agar medium conditioned by mono- vs. coculture**
607 **of *D. pigrum* and/or *C. pseudodiphtheriticum* (Figures 6 and S7).** *D. pigrum* and *C.*
608 *pseudodiphtheriticum* strains were grown from freezer stocks as described above. Cells were
609 harvested with sterile cotton swabs and resuspended in sterile PBS to an OD_{600nm} of 0.5. We then
610 spotted 100 µl of 1:1 mixed resuspension on a polycarbonate membrane (see above) on BHI agar
611 medium containing 400U/mL bovine liver catalase. After 2 days of growth, the polycarbonate
612 membrane with *D. pigrum* and/or *C. pseudodiphtheriticum* was removed from each plate leaving
613 CFCAM. *S. pneumoniae* 603 (Malley et al., 2001) was grown overnight on BBL Columbia CNA
614 agar with 5% sheep blood as described above and, using a sterile cotton swab, a lawn was
615 streaked onto the CFCAM and allowed to grow for 24 hours. Growth/inhibition was assessed daily
616 and photographically recorded. Imaging was difficult due to the transparency of *S. pneumoniae*
617 lawns.

618 **Biosynthetic gene clusters and antibiotic resistance genes (Table S6 and Figure S8).**
619 AntiSMASH (antibiotics & Secondary Metabolite Analysis SHell) and ClusterFinder (Medema et
620 al., 2011; Weber et al., 2015) were accessed at https://antismash.secondarymetabolites.org/
621 using default setpoints. Putative antibiotic resistance genes or mutations in genes conferring
622 antibiotic resistance were predicted using Resistance Gene Identifier (RGI) on the
623 Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013). Assembly
624 contigs were submitted at RGI (https://card.mcmaster.ca/analyze/rgi) and only perfect and strict
625 hits were allowed. ResFinder version 2.1. (https://cge.cbs.dtu.dk/services/ResFinder/) with 90%
626 threshold for %ID and 60% minimum length (Zankari et al., 2012).

627 **Statistical analyses.** Stata version 14.2 (College Station, TX) and R version 3.3.1 using RStudio
628 version 0.99.896 were used for statistical analysis and figure design. A two-tailed p value of $<$
629 0.05 was considered statistically significant and effect sizes are reported.

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641 Investigation: SDB, SME, IFE, YK. Interpretation of data: SDB, SME, MMP, IFE, MH, YK, KPL.
642 Visualization: SDB, SME, IFE. Wrote Original Draft: SDB, SME, KPL. Editing and review: SDB,
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644 **Declaration of interests.** The authors declare no competing interests.

645 **Table 1. Characteristics of *D. pigrum* strains and Illumina genomes in this study.**

Strain name	Internal reference	Source (citation)	Geography / body site / age	Median fold coverage	CDS[+]	RNAs[+]	GC content (%)[+]	Size (bp)
KPL1914	KPL1914	This study	Massachusetts / nostril / adult	83	1566	72	40	1,726,398
CDC 39-95	KPL1922	CDC (LaClaire and Facklam, 2000)	Canada / sinus / 3 years	62	1666	80	39.7	1,859,258
CDC 2949-98	KPL1930	CDC (LaClaire and Facklam, 2000)	AZ / nasopharyngeal / NA	60	1644	73	39.6	1,886,398
CDC 4294-98	KPL1931	CDC (LaClaire and Facklam, 2000)	SC / blood / 2 months	73	1841	78	39.5	2,014,679
CDC 4420-98	KPL1932	CDC (LaClaire and Facklam, 2000)	TN / blood / 11 years	63	1745	73	39.7	1,934,436
CDC 4545-98	KPL1933	CDC (LaClaire and Facklam, 2000)	AZ / nasopharyngeal / NA	128	1680	61	39.6	1,861,299
CDC 4709-98	KPL1934	CDC (LaClaire and Facklam, 2000)	GA / eye / 2 months	81	1686	80	39.6	1,912,682
CDC 4199-99	KPL1937	CDC (LaClaire and Facklam, 2000)	GA / blood / 1.8 years	107	1746	85	39.6	1,976,602
CDC 4791-99	KPL1938	CDC (LaClaire and Facklam, 2000)	AZ / nasopharyngeal / NA	61	1651	80	39.6	1,873,869
CDC 4792-99	KPL1939	CDC (LaClaire and Facklam, 2000)	AZ / nasopharyngeal / NA	92	1704	80	39.4	1,893,917
SS-1342 (NCFB2967) (ATCC 51524)	NA	BROAD / HMP R91/1468 (Aguirre et al., 1993)	England / spinal cord (autopsy)	200*	1651	31	39.6	1,846,028

646 ***sequenced by the BROAD institute for the Human Microbiota Project**

647 **+ as determined by RAST annotation (see text)**

648

649 **Table 2: Non-*D. pigrum* bacterial strains used in this stud**

Species	**Strain**	**Internal Reference**	**Reference**	**Characteristics**
C. accolens	KPL1818	KPL1818	(Bomar et al., 2016)	primary adult human nostril isolate; lipid dependent
C. pseudodiphtheriticum	KPL1989	KPL1989	This study	primary adult human nostril isolate; lipid independent
C. pseudodiphtheriticum	DSM44287^T	KPL2589	(Lehmann and Neumann, 1896)	type strain; lipid independent
C. propinquum	DSM44285^T	KPL1955	(Riegel et al., 1993)	type strain; lipid independent
S. aureus	Newman	KPL2023	(Miller et al., 1977)	lab-adapted strain
S. aureus	JE2	KPL2115	(Fey et al., 2013)	plasmid-free derivative of USA300 LAC
S. pneumoniae	TIGR4	KPL1904	(Tettelin et al., 2001)	Clinical isolate
S. pneumoniae	DBL5	KPL1905	(Lu et al., 2010)	Clinical isolate
S. pneumoniae	603	KPL1906	(Malley et al., 2001)	Clinical isolate
S. pneumoniae	WU2	KPL1907	(Briles et al., 1981)	Clinical isolate

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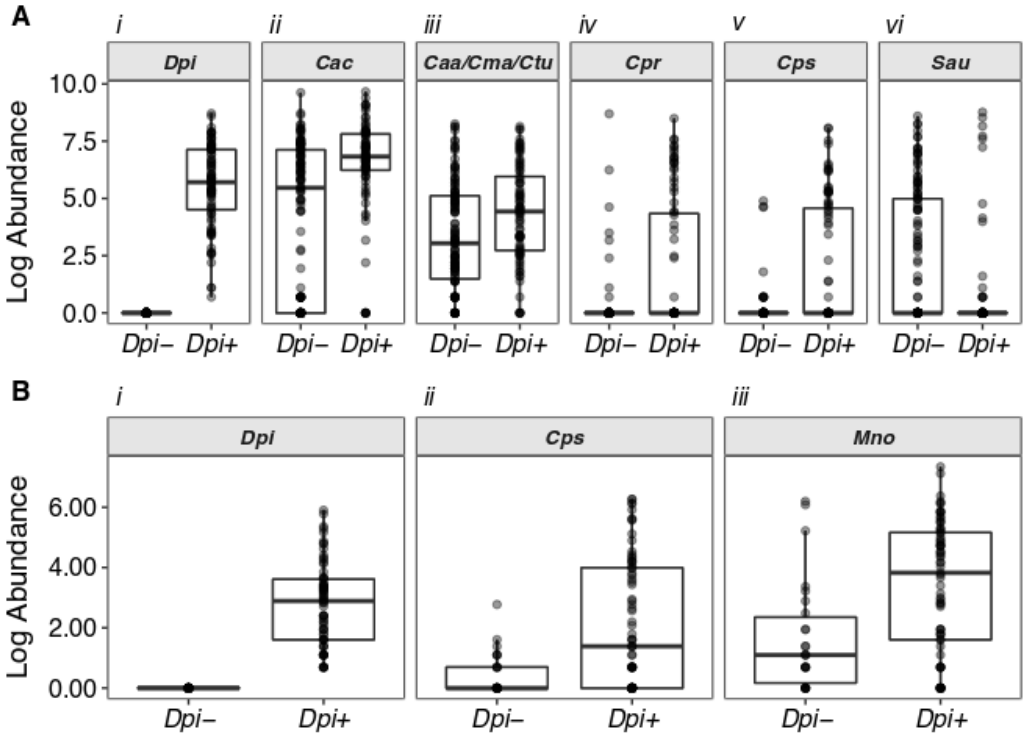
1 **Figures**



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3 **Figure 1. Individual nasal *Corynebacterium* species exhibit increased differential relative**
4 **abundance in the presence of *D. pigrum* in human nostril microbiota.** We used ANCOM to
5 compare species/supraspecies-level composition of 16S rRNA gene nostril datasets from (**A**) 210
6 adults and (**B**) 99 children ages 6 and 78 months when *D. pigrum* was either absent (Dpi-) or
7 present (Dpi+). Plots show statistically significant findings after correction for multiple testing
8 within ANCOM. The dark bar represents the median; lower and upper hinges correspond to the
9 first and third quartiles. Each gray dot represents the value for a sample, and multiple overlapping
10 dots appear black. Dpi = *Dolosigranulum pigrum*, Cac = *Corynebacterium accolens*,
11 Caa/Cma/Ctu= supraspecies *Corynebacterium accolens_macginleyi_tuberculostearicum*, Cpr =
12 *Corynebacterium propinquum*, Cps = *Corynebacterium pseudodiphtheriticum*, Mno = *Moraxella*
13 *nonliquefaciens.*

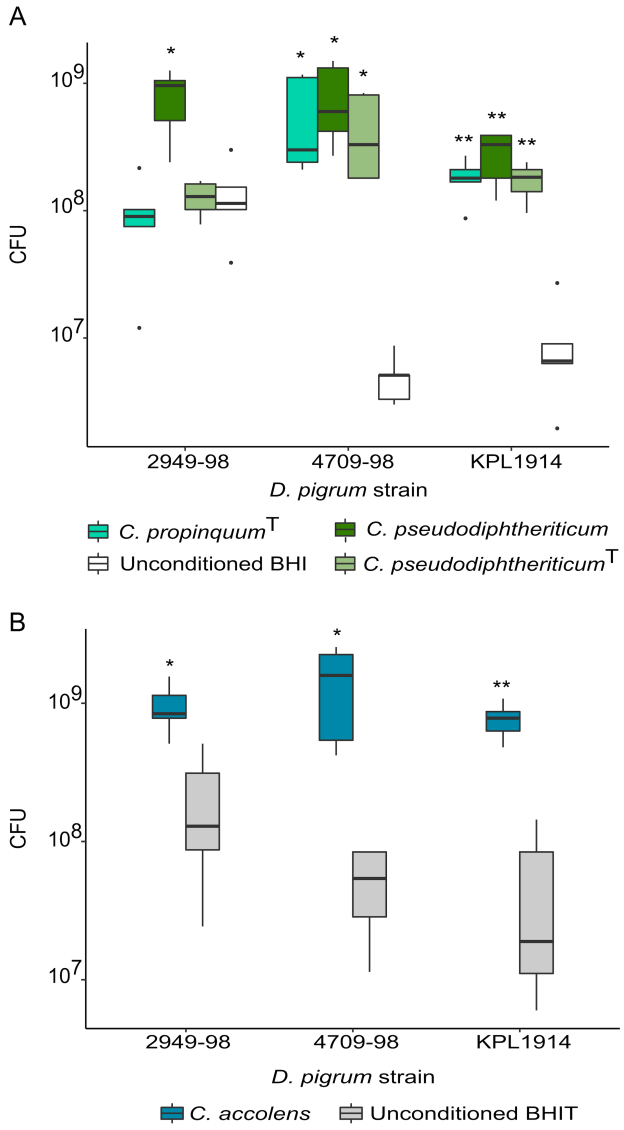
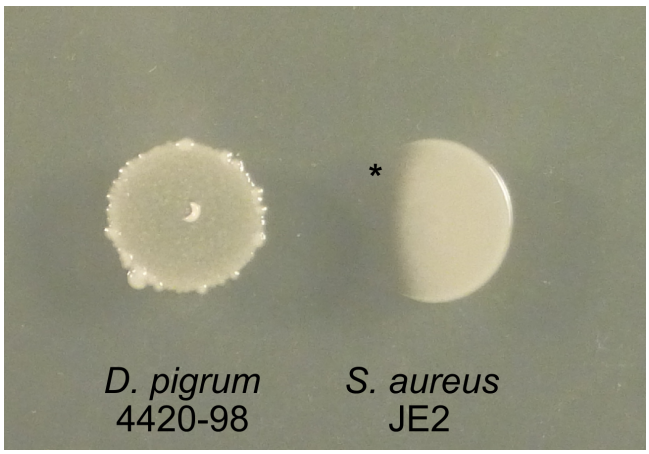


Figure 2. *D. pigrum* growth yields increase on cell-free conditioned agar medium from nasal *Corynebacterium* species. Growth yield of *D. pigrum* strains CDC 2949-98, CDC 4709-98 and KPL1914 was quantified as the number of CFUs grown on a polycarbonate membrane placed onto (**A**) cell-free conditioned BHI agar from *C. propinquum* (aqua green) or *C. pseudodiphtheriticum* (light and dark green) or (**B**) cell-free conditioned BHI-Triolein (BHIT) agar from *C. accolens* (blue) and compared to growth on unconditioned BHI agar (white) or unconditioned BHIT agar (grey), respectively. Data were analyzed separately for each *D. pigrum*

22 strain. CFU counts for each condition were compared to the unconditioned medium (n=5) using

23 a pairwise t test with Bonferroni correction for multiple comparisons. *, $p \leq 0.05$; **, $p \leq 0.001$

24 

25 **Figure 3. *D. pigrum* inhibits methicillin-resistant *S. aureus* USA300.** A pregrown *D. pigrum*
26 CDC 4420-98 inoculum produced a diffusible activity that inhibited the growth of *S. aureus* strain
27 JE2 on BHI agar (*n*=3). A representative image of CDC 4420-98 inhibiting (asterisk) *S. aureus*
28 JE2 is shown (48 hrs). Images of eight additional *D. pigrum* strains inhibiting *S. aureus* are in
29 Figure S1.

30

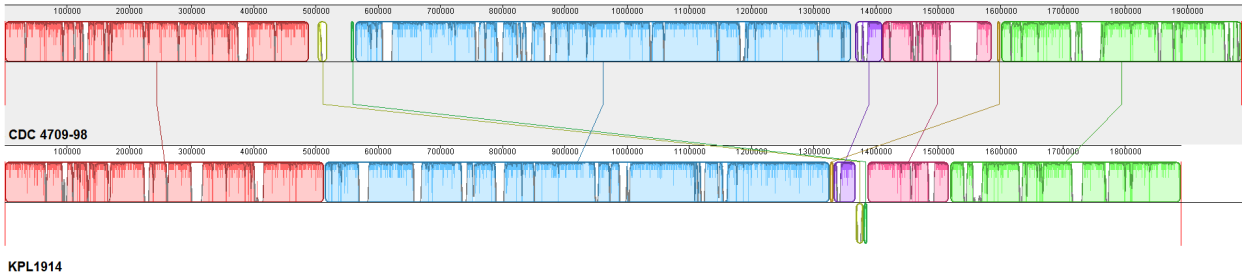


Figure 4. Comparative analysis of two *D. pigrum* genomes reveals a high degree of synteny. Multiple genome alignment for synteny analysis of the two closed *D. pigrum* genomes CDC 4709-98 and KPL1914 was performed using MAUVE (Darling et al., 2010). Locally collinear blocks and similarity profiles are presented, and genome boundaries are indicated (red bar after non-aligned sequences, i.e., sequences that are unique for the corresponding genome).

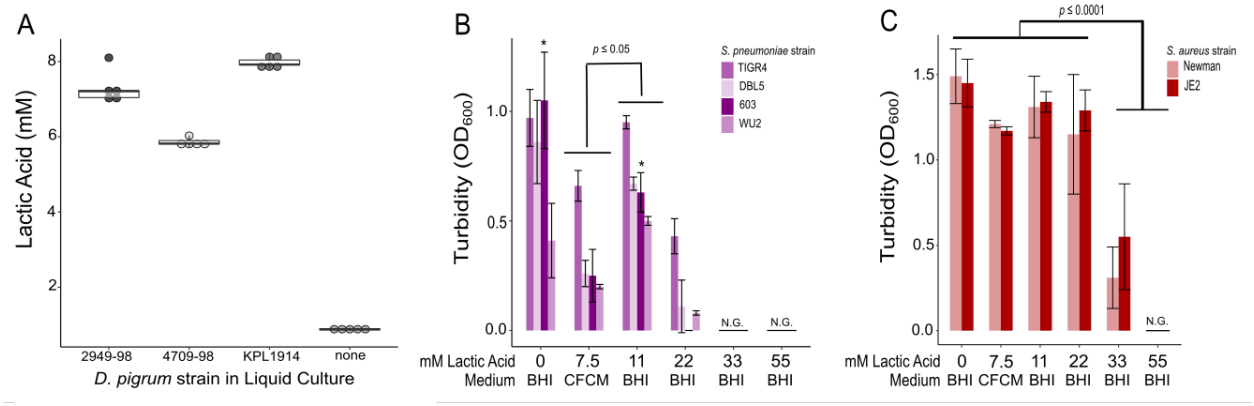


Figure 5. Lactate production by *D. pigrum* is insufficient to inhibit pathobiont growth. Strains of *S. pneumoniae* and *S. aureus* grew in the presence of higher levels of L-lactic acid than those produced by *D. pigrum in vitro*. (**A**) The concentration of L-lactic acid (mM) produced by three *D. pigrum* strains was measured after 24 hrs of gentle shaken aerobic growth in BHI broth at 37°C (*n*=5) as compared to the basal concentration of L-lactic acid in BHI alone (none). (**B**) The average growth (OD_{600}) of 4 *S. pneumoniae* strains in *D. pigrum* KPL1914 CFCM or in unconditioned BHI broth supplemented with different concentrations of L-lactic acid measured after 19–20 hrs of static aerobic growth at 37°C (*n*=4). (C) The average growth (OD_{600}) of 2 *S. aureus* strains in *D. pigrum* KPL1914 CFCM or in unconditioned BHI broth supplemented with different concentrations of L-lactic acid measured after 19–20 hrs of shaken aerobic growth at 37°C (*n*=4). Average growth of *S. pneumoniae* in BHI, CFCM and 11 mM L-lactic acid and of *S. aureus* in all conditions were analyzed for each individual strain using an ANOVA and Tukey's HSD test for multiple comparisons. * indicates $p \leq 0.05$ for *S. pneumoniae* 603 growth in BHI vs. 11mM L-lactic acid. Error bars represent standard deviation. N.G. is no growth.

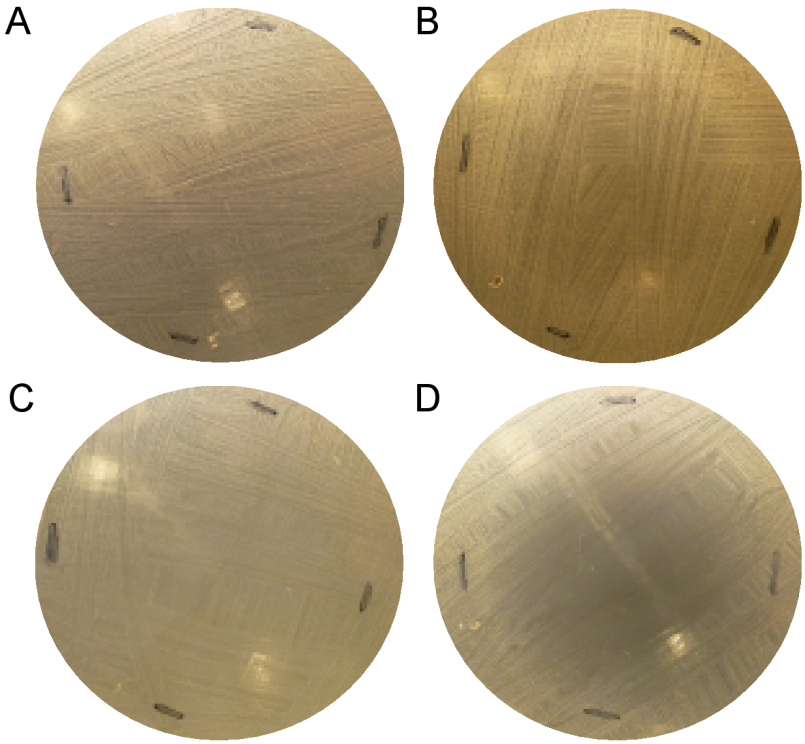


Figure 6. *D. pigrum* and *C. pseudodiphtheriticum* grown together but not *D. pigrum* alone inhibit *S. pneumoniae* in an *in vitro* agar medium-based assay. Representative images of *S. pneumoniae* 603 growth on (**A**) BHI alone or on CFCAM from (**B**) *C. pseudodiphtheriticum* KPL1989, (**C**) *D. pigrum* KPL1914 or (**D**) both *D. pigrum* and *C. pseudodiphtheriticum* grown in a mixed inoculum (*n*=4). To condition the medium, we cultivated *D. pigrum* and/or *C. pseudodiphtheriticum* on a membrane, which was then removed prior to spreading a lawn of *S. pneumoniae*. Images were cropped. Black marks indicate edges of where the membrane had been.

Table S1A. Adult nostril dataset ANCOM results. These Log abundance values are plotted in Figure 1A. (See excel file)

Table S1B. Pediatric nostril dataset ANCOM results. These Log abundance values are plotted in Figure 1B. (See excel file)

Table S2. *D. pigrum* is inhibited when grown directly on cell-free *C. accolens*-conditioned BHI agar supplemented with triolein. Related to Figure S2B.

	Growth of Target Strains[a]		
Conditioning Strain	*S. pneumoniae* 603 (6B)	*D. pigrum* CDC 4709-98	*D. pigrum* KPL1914
C. accolens KPL1818	0	0	0
C. propinquum[1] DSM44285	0	+	+
C. pseudodiphtheriticum KPL1989	+	+	+

[a]0, no growth; +, growth detected, $n \geq 3$

Table S3. Oleic acid inhibits *D. pigrum* growth. Related to section "Nasal *Corynebacterium* species enhance the growth of *D. pigrum in vitro*."

	ZOI (mm)[a]		
Oleic Acid (μg/disc)	*S. pneumoniae* 603 (6B)	*D. pigrum* CDC 4709-98	*D. pigrum* KPL1914
20	10.3 ± 4.7	12.0 ± 2.9	17.0 ± 2.1
50	22.0 ± 5.4	26.8 ± 4.4	28.4 ± 7.0
100	26.3 ± 6.7	35.8 ± 4.5	39.4 ± 5.0

[a]Mean ZOI ± SD produced in a disc-diffusion assay. ZOIs were measured as the smallest diameter of inhibited growth and measurements include disc diameter (6 mm). Biological replicates (n=4 for *S. pneumoniae*, n=5 for *D. pigrum*) were averaged.

Figure S1. Ten different *D. pigrum* strains inhibit methicillin-resistant *S. aureus* USA300 strain JE2. Related to Figure 3. *D. pigrum* isolates inhibited *S. aureus* strain JE2 on BHI agar ($n \geq 3$). Representative images are shown for each strain. *D. pigrum* was resuspended in PBS then a 5µl drop was placed onto BHI agar and pregrown for 48 hrs. After that, *S. aureus* was inoculated adjacent to the *D. pigrum*. Inhibition was assessed after 24 and 48 hrs (48 hrs shown here).

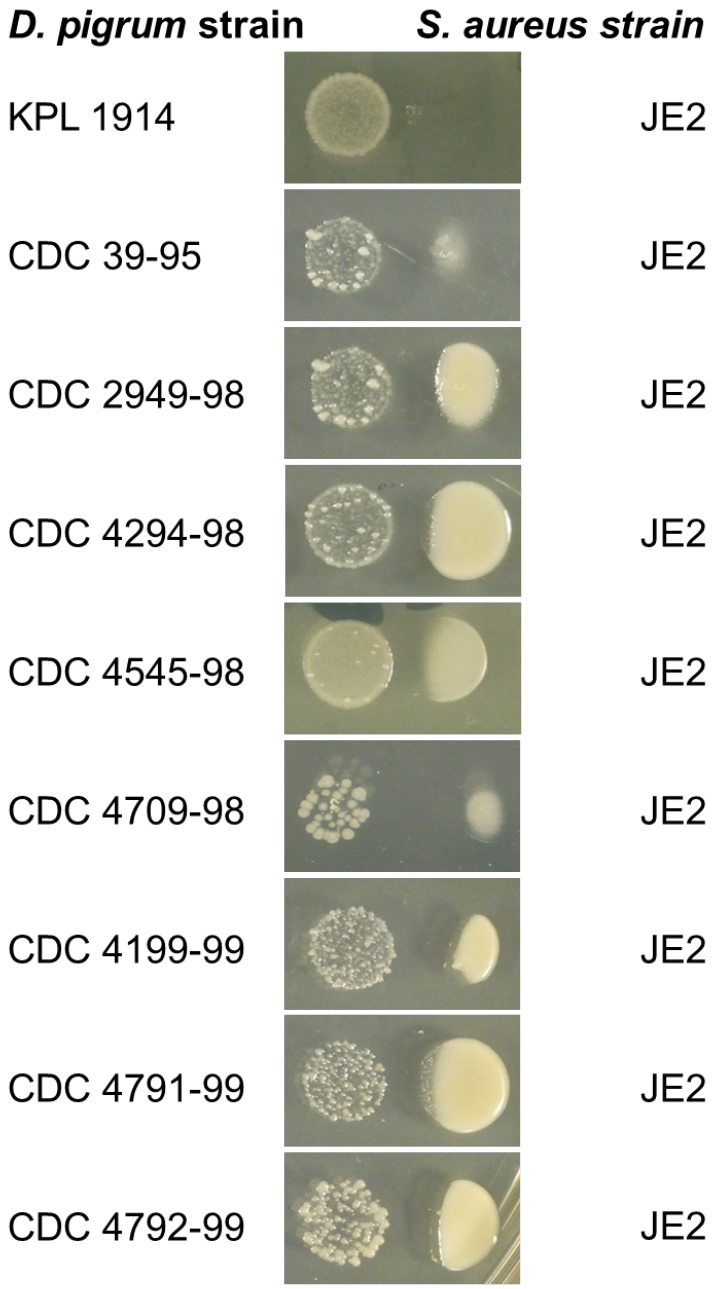


Table S4. Assembly characteristics of *D. pigrum* Illumina genomes in this study. Related to Table 1.

CDC / internal strain number		Nodes [N]	N50 [bp]	Max [bp]	Total [bp]	Reads [N]
	KPL1914	107	87,900	210,575	1,726,398	1,640,818
39-95	KPL1922	138	153,015	273,357	1,859,258	1,819,350
2949-98	KPL1930	179	127,744	456,484	1,886,398	1,699,318
4294-98	KPL1931	139	88,498	198,469	2,014,679	2,357,776
4420-98	KPL1932	134	209,743	328,871	1,934,436	2,098,746
4545-98	KPL1933	50	283,724	492,087	1,861,299	2,738,030
4709-98	KPL1934	142	110,284	268,980	1,912,682	2,198,156
4199-99	KPL1937	109	128,019	379,812	1,976,602	2,528,652
4791-99	KPL1938	129	132,767	316,186	1,873,869	1,831,854
4792-99	KPL1939	86	253,067	460,850	1,893,917	2,039,094

Figure S2. The conservative core genome of 11 *D. pigrum* strains encodes 1200 orthologs. Related to section "The genomes of 11 *D. pigrum* strains reveal a small genome and high degree of sequence conservation consistent with a highly host-adapted bacterium." A Venn diagram generated using the bidirectional best-hits (BDBH), cluster of orthologous groups (COG) triangle and OrthoMCL (OMCL) algorithms identified predicted protein orthologs (RAST annotation) shared between the 11 *D. pigrum* genomes (GET_HOMOLOGUES package version 02012019) (Contreras-Moreira and Vinuesa, 2013). Flag t=11 was used to only include clusters containing single-copy orthologs from all input species since these are likely the most reliable ortholog predictions.

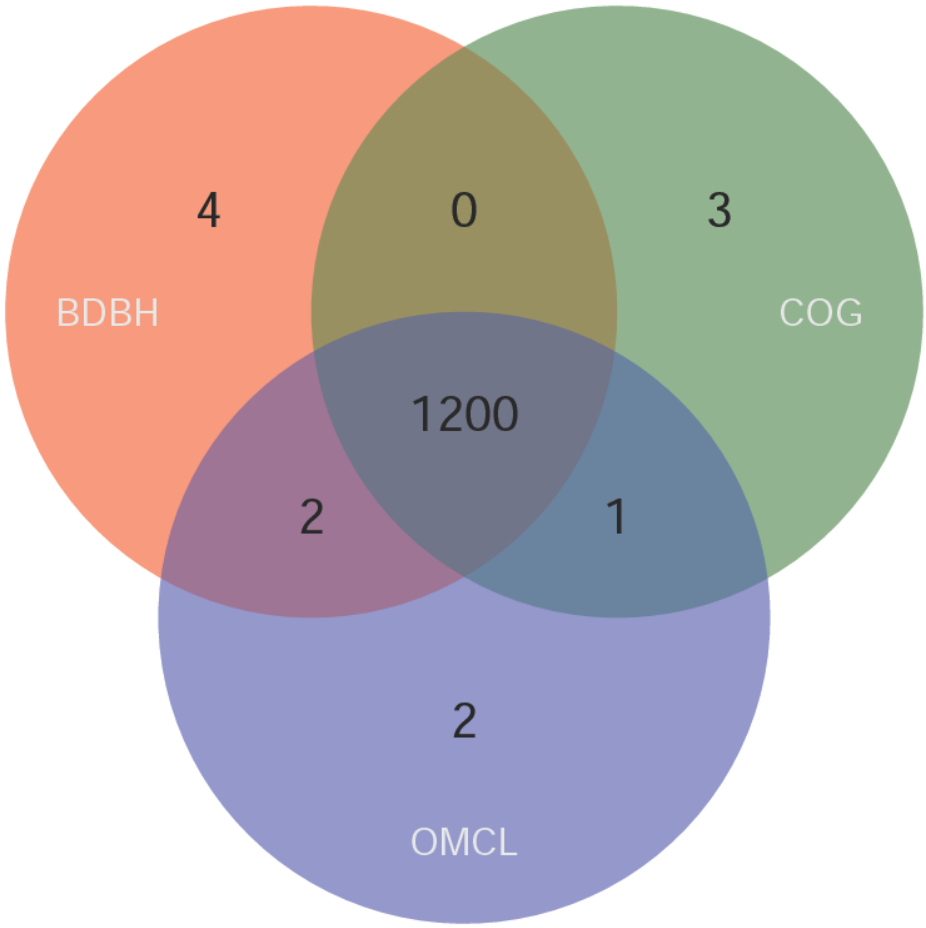


Figure S3. Core, shell and cloud pangenome of 11 *Dolosigranulum pigrum* strains. Related to section "The genomes of 11 *D. pigrum* strains reveal a small genome and high degree of sequence conservation consistent with a highly host-adapted bacterium." The pangenome of the 11 *D. pigrum* strains includes an estimated core of 1216, soft core of 116, shell of 373 and cloud of 1024 genes (CDS), as determined by the parse_pangenome_matrix.pl script (using the OMCL / COG intersection) of the GET_HOMOLOGUES package version 02012019 (Contreras-Moreira and Vinuesa, 2013). The core genome is composed of genes that are present in all strains and soft core contains clusters present in 10 genomes but not the core as defined in (Kaas et al., 2012). Cloud is defined as genes only present in a few genomes (cut-off is defined as the class next to the most populated non-core cluster class). Shell genes are the remaining genes and displayed sorted to the number of genomes in which these are present (*n*=3-9).

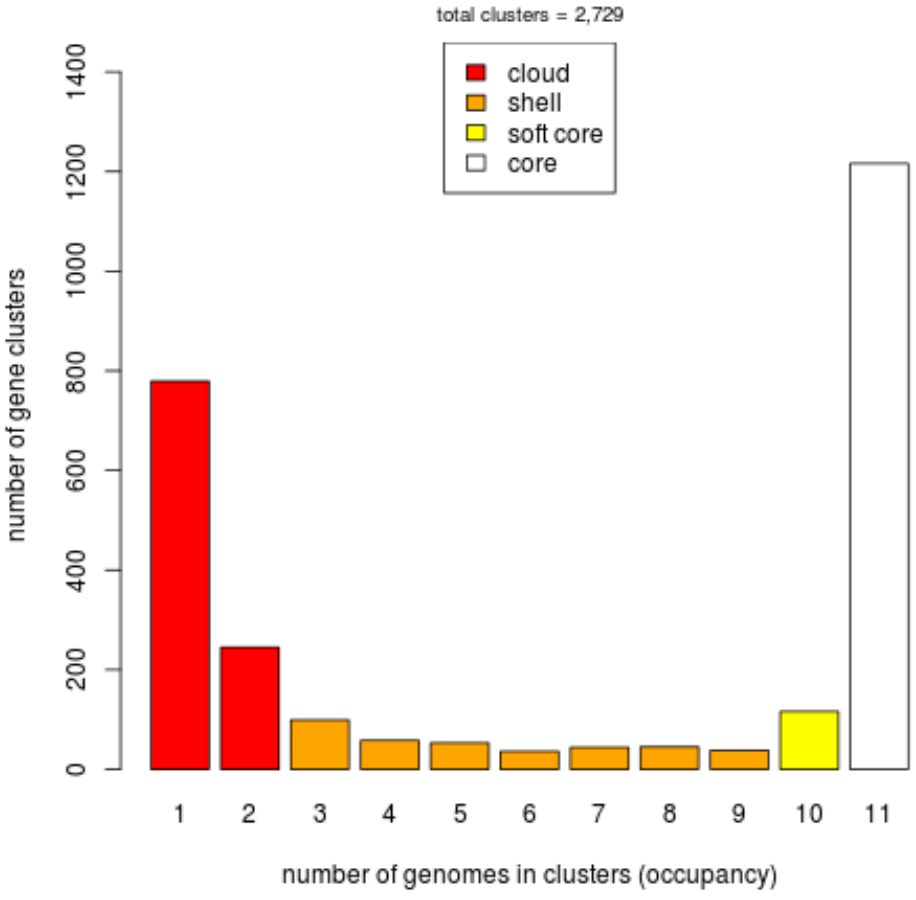


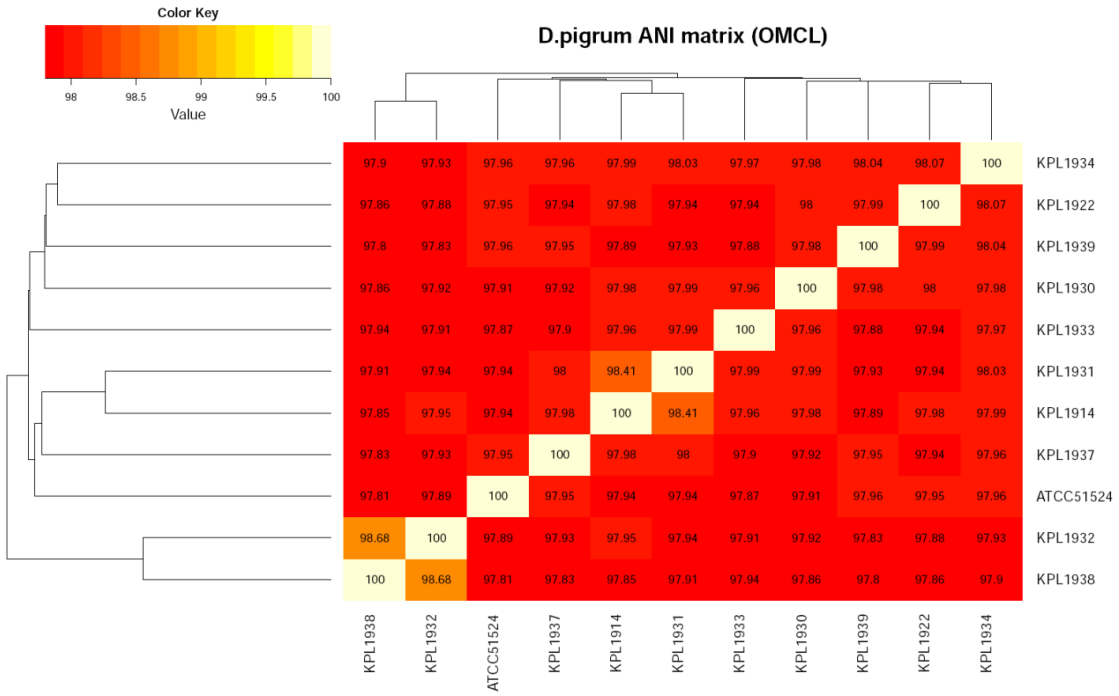
Table S5. Distribution by strain of genes in the core, soft core, shell and cloud *D. pigrum* genome.* Related to section "The genomes of 11 *D. pigrum* strains reveal a small genome and high degree of sequence conservation consistent with a highly host-adapted bacterium."

CDC or other strain #	Internal reference	Cloud [N]	Shell [N]	Soft core [N]	Core [N]
KPL1914	KPL1914	79	135	89	1216
39-95	KPL1922	81	189	109	1216
2949-98	KPL1930	88	167	113	1216
4294-98	KPL1931	243	183	110	1216
4420-98	KPL1932	134	205	114	1216
4545-98	KPL1933	102	202	96	1216
4709-98	KPL1934	105	185	111	1216
4199-99	KPL1937	186	174	94	1216
4791-99	KPL1938	73	192	108	1216
4792-99	KPL1939	110	194	108	1216
ATCC51524		68	194	108	1216

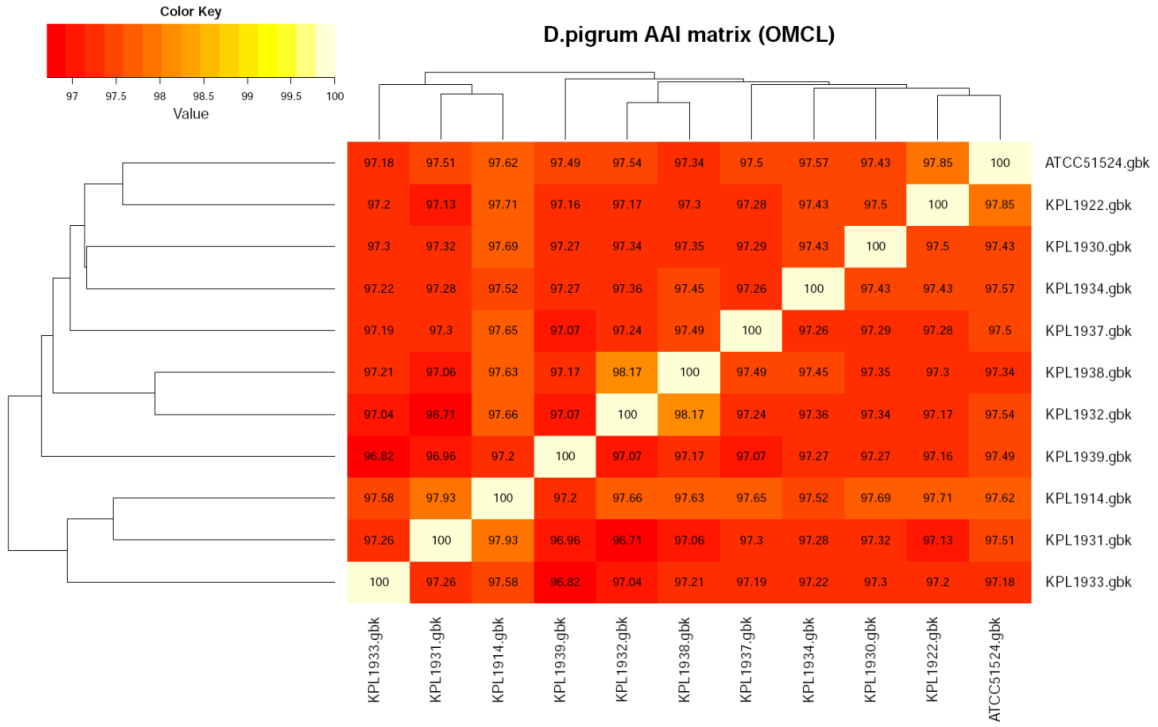
* These results were determined as described for Figure S3.

Figure S4. The (A) average nucleotide identities (ANI) and (B) amino acid identities (AAI) show a high degree of conservation across all 11 *D. pigrum* genomes. Related to section "Phylogenetic reconstruction, sequence and protein similarities." Average identity matrices of clustered coding sequences were calculated using GET_HOMOLOGUES with the OrthoMCL algorithm. Both ANI and AAI were calculated with all available clusters (t 0). Commands used: Generation of an AA identity matrix: $ ./get_homologues.pl -d "gbk-files" -A -t 0 -M and CDS identity matrix with the command $./get_homologues.pl -d "gbk files" -a 'CDS' -A -t 0 -M.

A



B


Color Key
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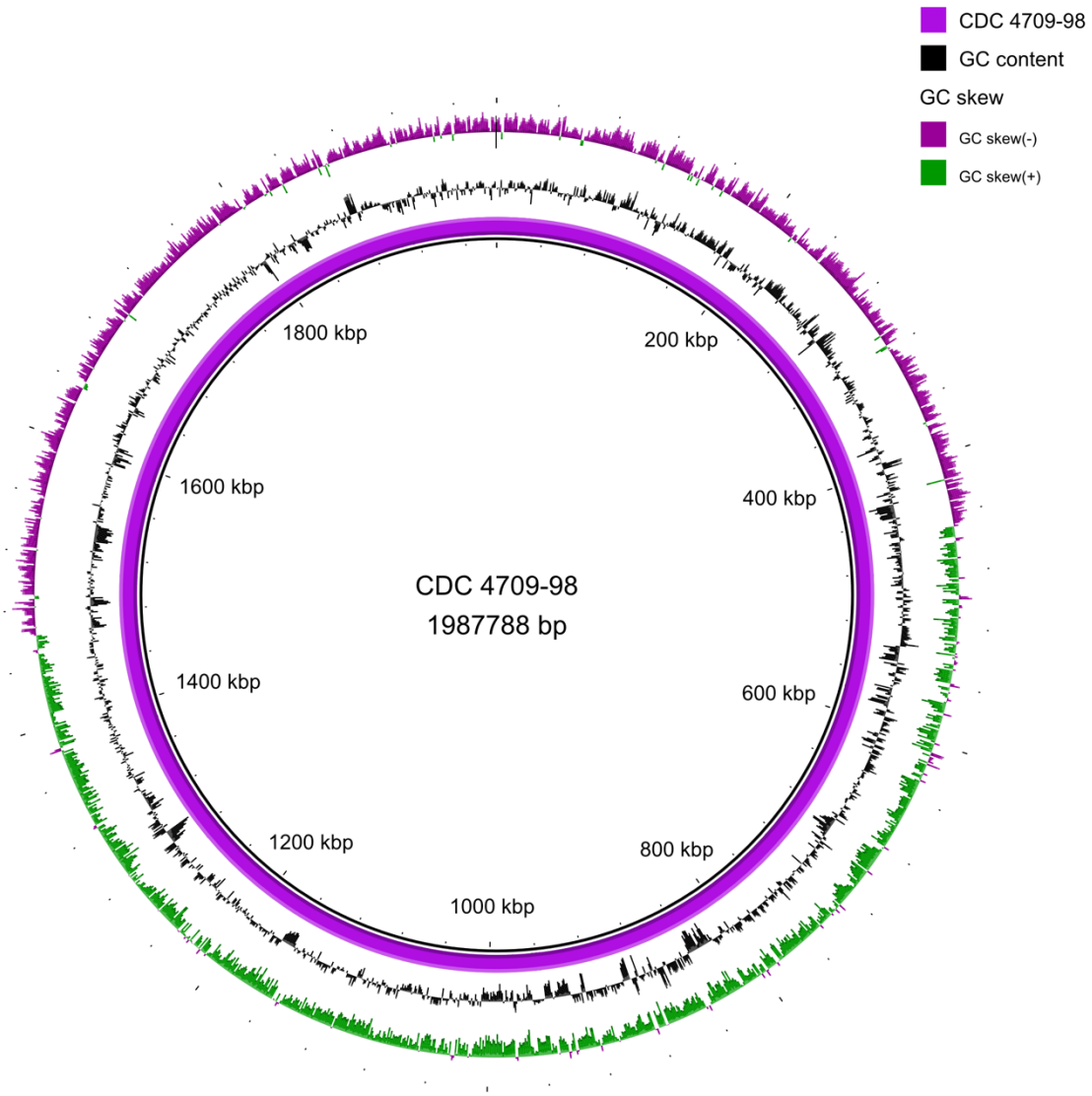
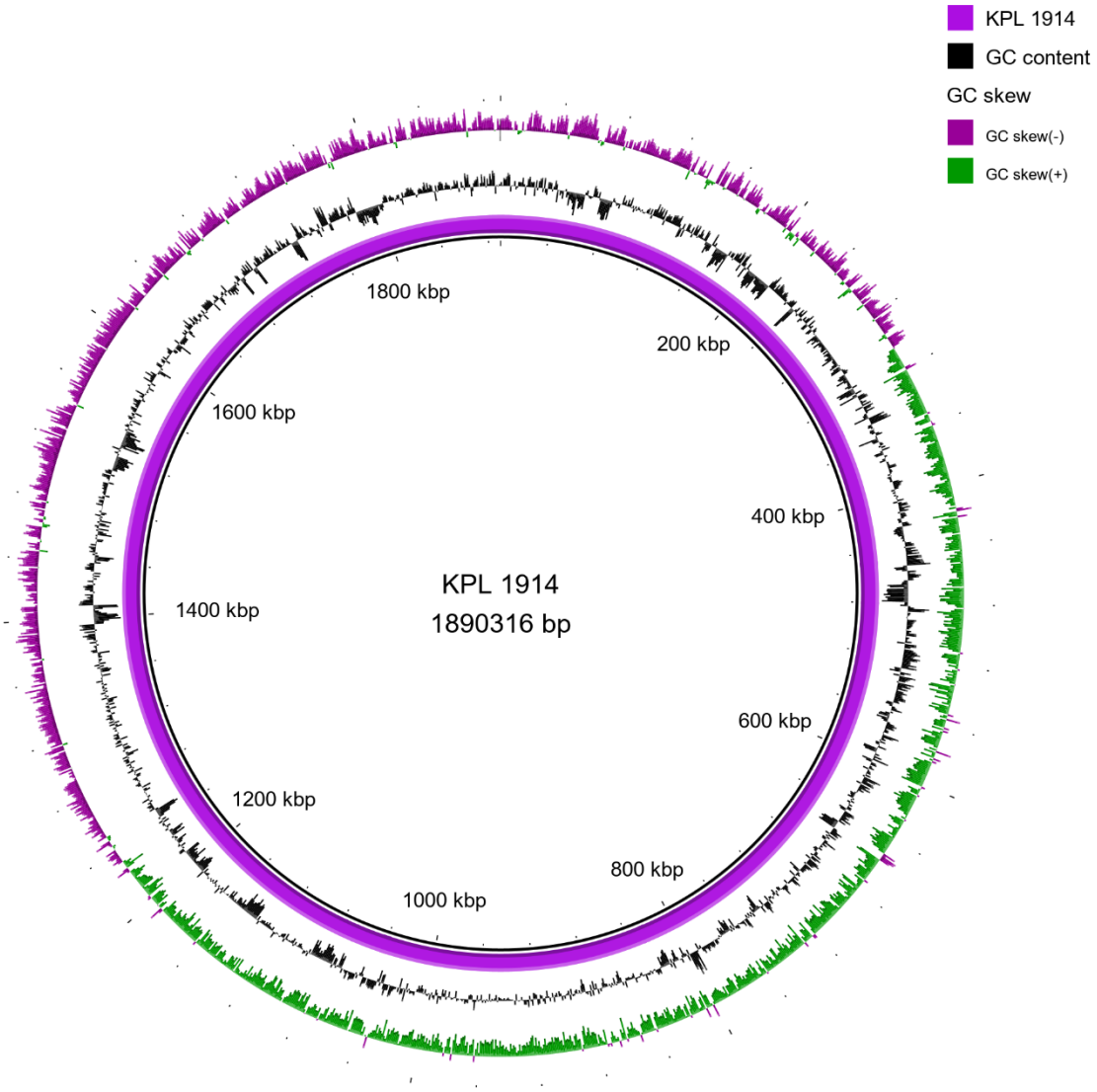
97.5
98
98.5
99
99.5
100
Value
D.pigrum AAI matrix (OMCL)
ATCC51524.gbk 97.18 97.51 97.62 97.49 97.54 97.34 97.5 97.57 97.43 97.85 100
KPL1922.gbk 97.2 97.13 97.71 97.16 97.17 97.3 97.28 97.43 97.5 100 97.85
KPL1930.gbk 97.3 97.32 97.69 97.27 97.34 97.35 97.29 97.43 100 97.5 97.43
KPL1934.gbk 97.22 97.28 97.52 97.27 97.36 97.45 97.26 100 97.43 97.43 97.57
KPL1937.gbk 97.19 97.3 97.65 97.07 97.24 97.49 100 97.26 97.29 97.28 97.5
KPL1938.gbk 97.21 97.06 97.63 97.17 98.17 100 97.49 97.45 97.35 97.3 97.34
KPL1932.gbk 97.04 96.71 97.66 97.07 100 98.17 97.24 97.36 97.34 97.17 97.54
KPL1939.gbk 96.82 96.96 97.2 100 97.07 97.17 97.07 97.27 97.27 97.16 97.49
KPL1914.gbk 97.58 97.93 100 97.2 97.66 97.63 97.65 97.52 97.69 97.71 97.62
KPL1931.gbk 97.26 100 97.93 96.96 96.71 97.06 97.3 97.28 97.32 97.13 97.51
KPL1933.gbk 100 97.26 97.58 96.82 97.04 97.21 97.19 97.22 97.3 97.2 97.18
KPL1933.gbk
KPL1931.gbk
KPL1914.gbk
KPL1939.gbk
KPL1932.gbk
KPL1938.gbk
KPL1937.gbk
KPL1934.gbk
KPL1930.gbk
KPL1922.gbk
ATCC51524.gbk

Figure S5. *D. pigrum* genomes BLAST ring comparisons. Related to Figure 4. BLAST Ring Image Generator (BRIG) version 0.95 (Alikhan et al., 2011) was used for visualization of the sequenced genomes with the closed genome of CDC 4709-98 as a reference. (**A**) Closed genome of CDC 4709-98 with GC plots (**B**) Closed genome of KPL 1914 with GC plots (**C**) BLASTN based comparison of the closed genomes of CDC 4709-98 and KPL 1914 (**D**) BLASTN based comparison of CDC 4709-98 and the remaining 10 Illumina contig-based genomes as well as ATCC 51524

5A



5B


KPL 1914
GC content
GC skew
GC skew(-)
GC skew(+)
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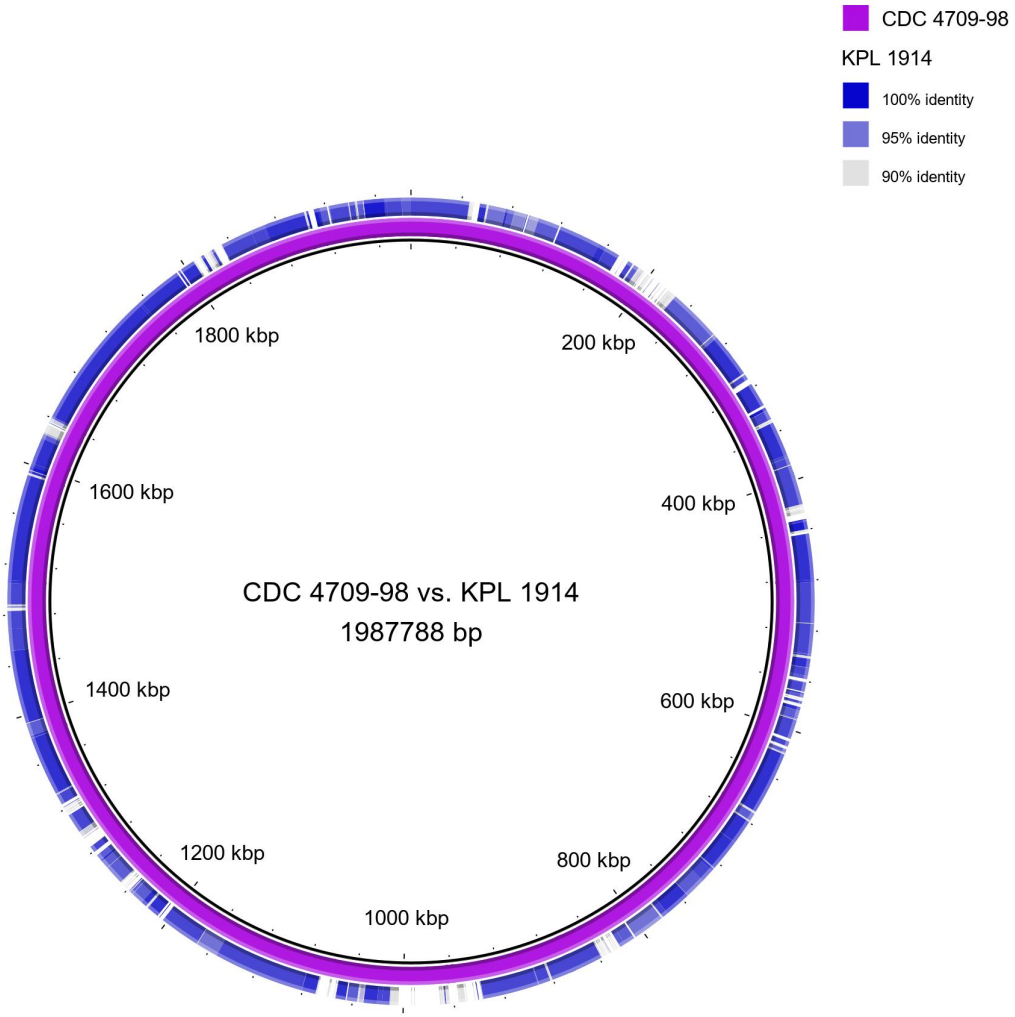
1600 kbp
400 kbp
KPL 1914
1890316 bp
1400 kbp
600 kbp
1200 kbp
800 kbp
1000 kbp

5C


CDC 4709-98
KPL 1914
100% identity
95% identity
90% identity
1800 kbp
200 kbp
1600 kbp
400 kbp
CDC 4709-98 vs. KPL 1914
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1400 kbp
600 kbp
1200 kbp
800 kbp
1000 kbp

5D

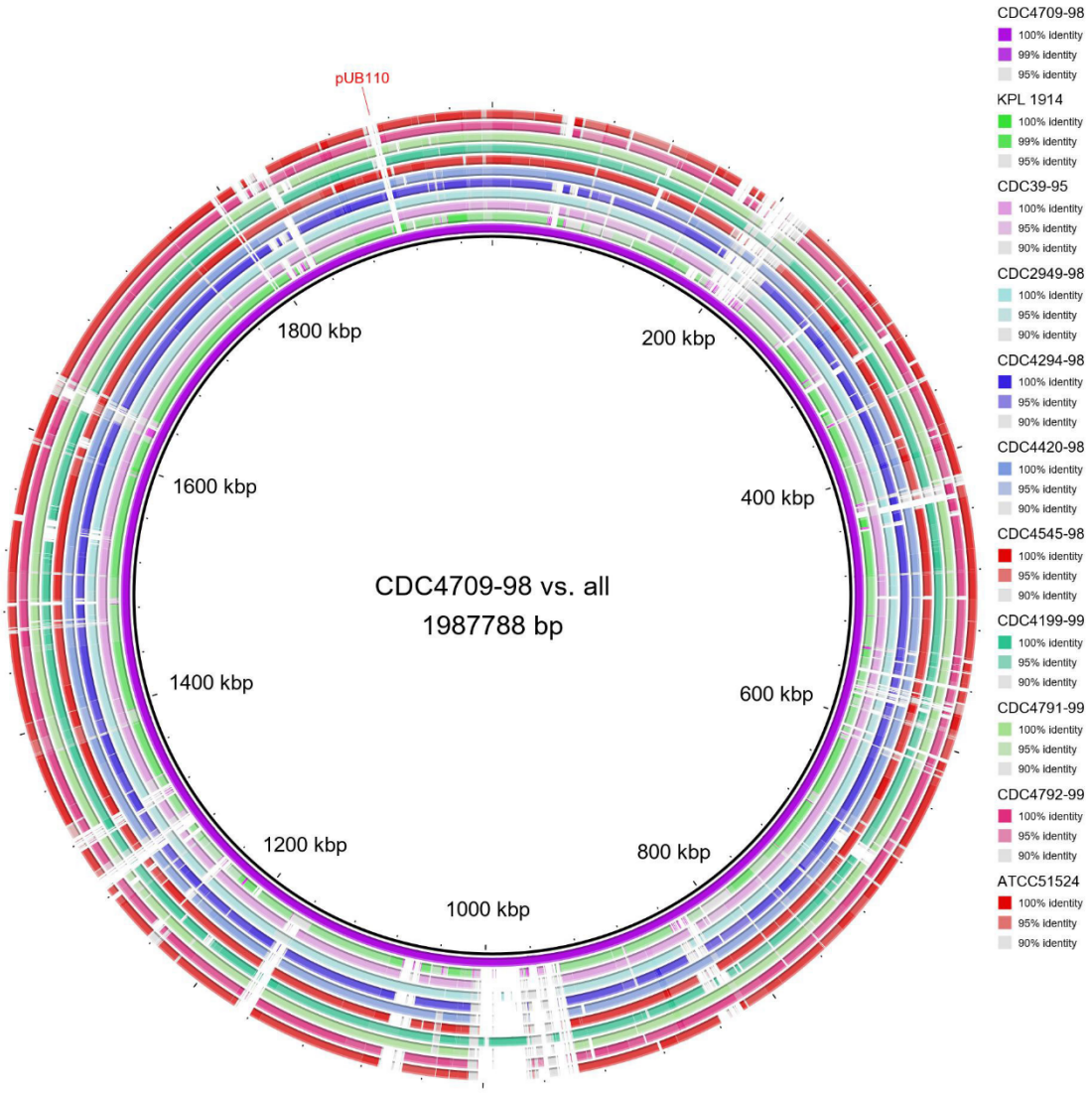
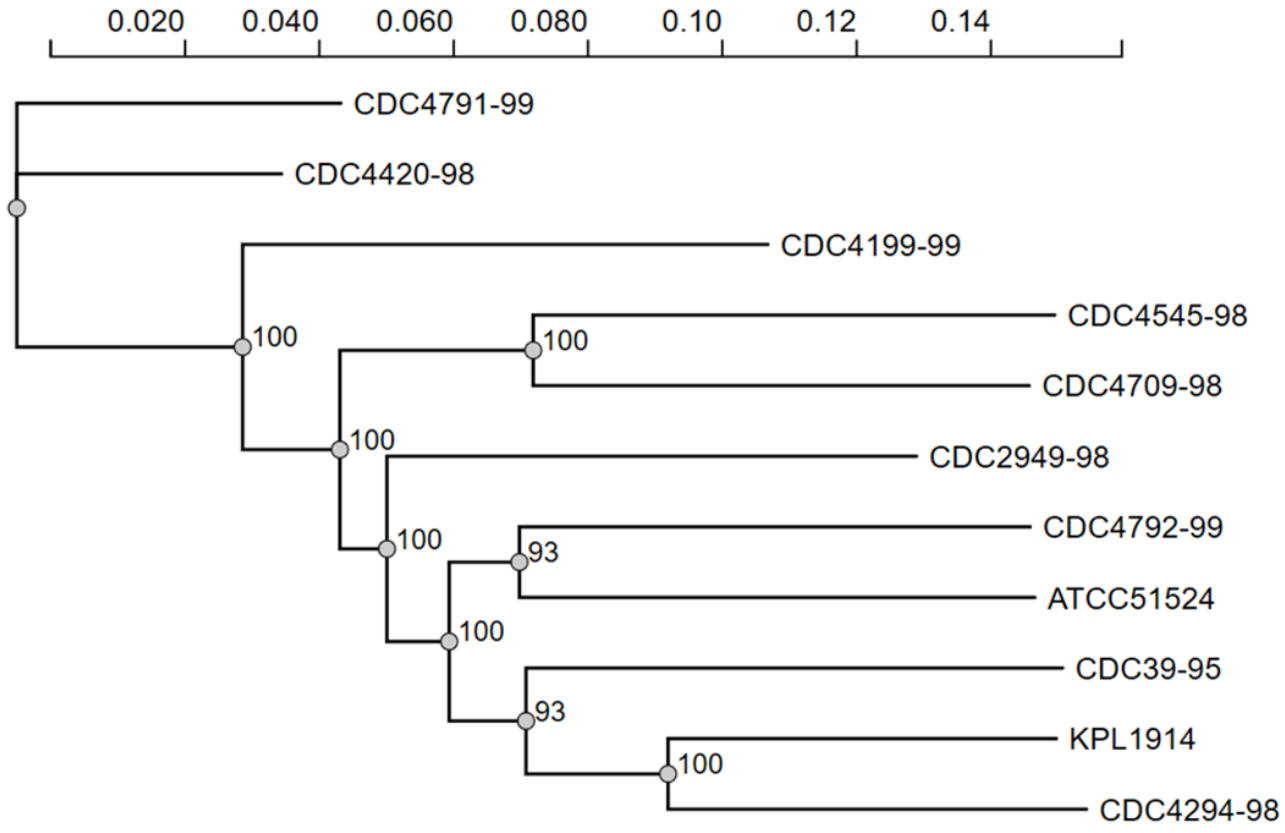

pUB110
CDC4709-98 vs. all
1987788 bp
200 kbp
400 kbp
600 kbp
800 kbp
1000 kbp
1200 kbp
1400 kbp
1600 kbp
1800 kbp
CDC4709-98
100% identity
99% identity
95% identity
KPL 1914
100% identity
99% identity
95% identity
CDC39-95
100% identity
95% identity
90% identity
CDC2949-98
100% identity
95% identity
90% identity
CDC4294-98
100% identity
95% identity
90% identity
CDC4420-98
100% identity
95% identity
90% identity
CDC4545-98
100% identity
95% identity
90% identity
CDC4199-99
100% identity
95% identity
90% identity
CDC4791-99
100% identity
95% identity
90% identity
CDC4792-99
100% identity
95% identity
90% identity
ATCC51524
100% identity
95% identity
90% identity

Figure S6. Phylogenetic relationship between the 11 *D. pigrum* strains. Related to section "Phylogenetic reconstruction, sequence and protein similarities." *D. pigrum* phylogenetic trees were constructed by using concatenated alignments of the intersection of predicted core genes (BDBH, COG, and OMCL, see above). Amino acids were aligned using Clustal Omega V.1.2.4 (Sievers et al., 2011) and a maximum likelihood phylogenetic tree was generated using the LG model of amino-acid replacement matrix (Le and Gascuel, 2008) as selected by smart model selection with Akaike information criterion (Lefort et al., 2017) and 100 bootstrap replicates for branch support with PhyML (phylogenetic maximal likelihood) V. 3.0 (Guindon et al., 2010) and visualized using FigTree V.1.4.4. A BIONJ distance-based tree was used as a starting tree to be redefined by the maximum likelihood algorithm. Bootstrap support values from 100 replicates are indicated above the branches. (**A**) Core genome AA tree of the 11 *D. pigrum* genomes. (**B**) Core genome AA tree with *Alloiococcus otitis* ATCC 51267 as an outgroup. Core genome size decreased to 866 clusters when including *A. otitis*.

6A


0.020
0.040
0.060
0.080
0.10
0.12
0.14
CDC4791-99
CDC4420-98
CDC4199-99
CDC4545-98
CDC4709-98
CDC2949-98
CDC4792-99
ATCC51524
CDC39-95
KPL1914
CDC4294-98
100
100
100
100
93
100
93
100

ATCC51524
69
KPL1914
100
82
CDC4294-98
CDC39-95
97
CDC4792-99
CDC4199-99
23
CDC4545-98
73
CDC4709-98
24
CDC2949-98
14
CDC4791-99
100
CDC4420-98
ATCC51267
0.2

6B

Figure S7. When grown together *D. pigrum* and *C. pseudodiphtheriticum* inhibit *S. pneumoniae*. Related to Figure 6. A second set of representative images of *S. pneumoniae* 603 growth on (**A**) BHI alone or CFCAM from (**B**) *C. pseudodiphtheriticum* KPL1989, (**C**) *D. pigrum* KPL1914 or (**D**) both *D. pigrum* and *C. pseudodiphtheriticum* grown in a mixed inoculum (*n*=4). To condition the medium, we cultivated *D. pigrum* and/or *C. pseudodiphtheriticum* on a membrane, which was then removed prior to spreading a lawn of *S. pneumoniae*. Images were cropped. Black marks indicate edges of where the membrane had been.

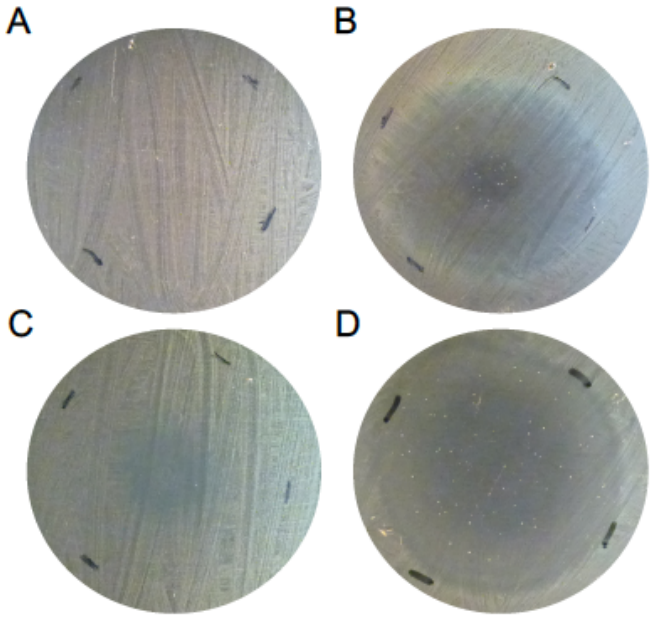


Table S6. Predicted biosynthetic gene clusters / secondary metabolites regions (antiSMASH version 5.0.0beta1-4e548fe) (Blin et al., 2019)**. Related to section "The accessory genome of 11 *D. pigrum* strains contains a diversity of biosynthetic gene clusters predicted to encode antibiotics."**

Strain	Bacteriocins	Lanthipeptides
KPL1914	2	1
39-95	1	1
2949-98	1	0
4294-98	2	0
4420-98	0	0
4545-98	1	0
4709-98	2	1
4199-99	3	0
4791-99	0	0
4792-99	2	0
ATCC51524	0	2

Table S7. Species-level reanalysis of a pediatric nostril microbiota dataset. Related to Fig. 1. (see excel file)

Figure S8. Predicted biosynthetic gene clusters / secondary metabolites regions (antiSMASH version 5.0.0beta1-4e548fe) (Blin et al., 2019)**. Related to section "The accessory genome of 11 *D. pigrum* strains contains a diversity of biosynthetic gene clusters predicted to encode antibiotics."**

KPL1914

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record 1:						
Region 1	Bacteriocin	166955	178641			
Region 2	Lanthipeptide	459577	487064			
Region 3	Bacteriocin	1238792	1250948			

CDC4709-98

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record 1:						
Region 1	Lanthipeptide	418481	441708			
Region 2	Bacteriocin	536284	545549			
Region 3	Bacteriocin	1239084	1249380			

CDC39-95

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00009_NODE_10.. (original name was: NODE_10_length_97139_cov_86.161758):						
Region 9.1	Bacteriocin	83002	95155	Non-acetylated open-chain sophorolipid / diacetylated lactonic other	100%	BGC0001274
The following regions are from record c00017_NODE_75.. (original name was: NODE_75_length_77128_cov_66.578545):						
Region 17.1	Lanthipeptide	41076	63532			

CDC2949-98

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00036_NODE_16.. (original name was: NODE_165_length_169774_cov_66.562698):						
Region 36.1	Bacteriocin	132959	145112			

CDC4294-98

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00014_NODE_14.. (original name was: NODE_14_length_25558_cov_121.382584):						
Region 14.1	Bacteriocin	14627	25696			
The following regions are from record c00028_NODE_36.. (original name was: NODE_36_length_49203_cov_111.674515):						
Region 28.1	Bacteriocin	38167	49341			

CDC4545-98

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00010_NODE_30.. (original name was: NODE_30_length_491949_cov_138.342133):						
Region 10.1	Bacteriocin	333003	345156			

CDC4199-99

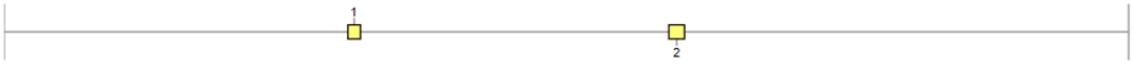
Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00016_NODE_52.. (original name was: NODE_52_length_379674_cov_119.267921):						
Region 16.1	Bacteriocin	254448	265629			
Region 16.2	Bacteriocin	283318	295471			
The following regions are from record c00030_NODE_10.. (original name was: NODE_100_length_155311_cov_111.723755):						
Region 30.1	Bacteriocin	23742	34038			

CDC4792-99

Region	Type	From	To	Most similar known cluster	Similarity	MIBiG BGC-ID
The following regions are from record c00013_NODE_48.. (original name was: NODE_48_length_252929_cov_101.571663):						

Region 13.1	Bacteriocin	141759	153571			
Region 13.2	Bacteriocin	172804	184957			

ATCC51524



Region	Type	From	To	Most similar known cluster	Similarity
Region 1	lanthipeptide	564,388	585,116		
Region 2	lanthipeptide	1,091,260	1,117,015		

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Supplemental text

Antibiotic resistance prediction. We analyzed all 11 *D. pigrum* genomes for putative antibiotic resistance genes and mutations that confer antibiotic resistance using the Resistance Gene Identifier (RGI) on the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) allowing only perfect and strict results. This identified a candidate in only one strain. These data are consistent with the report from LaClaire and Facklam of *D. pigrum* susceptibility to amoxicillin, cefotaxime, cefuroxime, clindamycin, levofloxacin, meropenem, penicillin, quinupristin-dalfopristin, rifampin, tetracycline, and vancomycin for all tested *D. pigrum* strains (LaClaire and Facklam, 2000). *D. pigrum* strain CDC 4709-98 alone encoded a predicted bleomycin resistance protein (BRP), which was predicted with 100% amino acid in a protein homolog model. In agreement, ResFinder (Zankari et al., 2012) identified kanamycin nucleotidyltransferase (*aadD*, aka ANT(4')-Ia, aminoglycoside adenyltransferase AAD, spectinomycin resistance; streptomycin resistance; transferase) in this strain with 99.74 % sequence identity. Detailed analysis of this region with PlasmidFinder (Carattoli et al., 2014) and BLAST (Altschul et al., 1990) revealed loci with sequence identity to portions of plasmid pUB110 from *S. aureus* (McKenzie et al., 1986) suggesting integration of, or at least part of, this plasmid into the genome of strain CDC 4709-98.

Glycolysis (Embden-Meyerhof-Parnas pathway, EMP). Enzymes present in all 11 isolates included glucokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase class II-1,6-bisphosphate-aldolase, triose phosphate isomerase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, enolase and pyruvate kinase. Three strains (KPL1914, CDC 39-95, CDC 4792-99) also encoded a predicted fructose-bisphosphate aldolase class I (EC 4.1.2.13), whereas all strains harbored a predicted triosephosphate isomerase.

As noted in the main text, all 11 strains encoded a predicted L-lactate-dehydrogenase (EC 1.1.1.27), which catalyzes the reduction of pyruvate to lactate regenerating NAD+ for glycolysis (GAPDH step), consistent with homofermentation to L-lactate as the primary product of glycolysis. Moreover, the absence of xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) is inconsistent with (obligate) heterofermentation, since bacteria that heteroferment lack aldolase and have to shunt through the pentose phosphate or phosphoketolase pathway (Buyze et al., 1957). In addition to homofermentation to lactate, some end product flexibility, which is probably produced under differing environmental/nutritional conditions, is predicted by the presence in all 11 genomes of genes needed to accomplish mixed-acid fermentation with potential production of formate, acetate and ethanol (i.e., enzymes pyruvate formate-lyase (EC 2.3.1.54), phosphate acetyltransferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1), as well as acetaldehyde dehydrogenase (1.2.1.10) and alcohol dehydrogenase (EC 1.1.1.1)).

Identification of a V-type ATPase in all 11 isolates. V-ATPases hydrolyse ATP to drive a proton pump but cannot work in reverse to synthesize ATP.

There is no tricarboxylic acid (TCA) cycle present in *D. pigrum*. Only fumarate-hydratase (EC 4.2.1.2) and TCA associated dihydrolipoyl dehydrogenase (EC 1.8.1.4) were predicted in all *D. pigrum* genomes.

Anaerobic respiratory reductases. We did not identify butyryl-CoA-reductase (EC 1.3.8.1) or any other predicted anaerobic reductases in all *D. pigrum* strains. However, *D. pigrum* CDC 4545-98 encoded an arsenate reductase (EC 1.20.4.1).

Sialidases. The original species description of *D. pigrum* reports production of acid from D-glucose, galactose, D-fructose, D-mannose, maltose and L-fucose in two isolates with strain-level variation in producing acid from D-arabinose, mannitol, sucrose and N-acetyl-glucosamine (Aguirre et al., 1993). Glucose is the main monosaccharide detected in the nasal environment

(Krismer et al., 2014). Host-cell-surface- and host-mucin-derived sialic acids are another important potential source of monosaccharides and all 11 *D. pigrum* genomes harbor a putative sialidase as well as a predicted transporter (sodium solute symporter) and catabolic enzymes suggesting it utilizes sialic acid in the nasal passages.

Polyamine auxotrophy and transport. The absence of predicted genes required for *de novo* synthesis of the essential polyamines putrescine and spermidine indicates that *D. pigrum* likely relies on extracellular polyamines. Consistent with this, all strains harbor putative ABC type spermidine transporter components (SPERta, SPERtb, SPERtc, SPERtd) adjacent to each other as well as putative putrescine transporter genes (*potA*, *potB*, *potC*, and *potD*).

Nicotinic acid (niacin) auxotrophy and transport. NAD and NADP are critical factors for cellular metabolism**.** However, *D. pigrum* lacked known genes for *de novo* synthesis and for salvage of nicotinate or nicotinamide. However, the presence of *niaP* predicts uptake of nicotinic acid (niacin) by all 11 strains. All strains also encoded genes needed to convert niacin or nicotinamide to NAD+ and NADP: nicotinamidase (EC 3.5.1.19), nicotinate phosphoribosyltransferase (EC 2.4.2.11), nicotinate-nucleotide adenylyltransferase (EC 2.7.7.18), NAD synthetase (EC 6.3.1.5), NAD kinase (EC 2.7.1.23) and NadR transcriptional regulator.

Biotin auxotrophy and transport. A biotin uptake system was predicted in all 11 *D. pigrum* strains (Substrate-specific component BioY of biotin ECF transporter) and no biosynthesis was predicted. Biotin-protein ligase (EC 6.3.4.15) was predicted only in KPL1914 and ATCC51524.

Methionine auxotrophy and degradation. All 11 *D. pigrum* genomes lacked a complete known pathway for methionine biosynthesis. Methionine has been reported to be a limiting nutrient in the nasal passages of humans and its synthesis is upregulated in *S. aureus* growing in synthetic nasal medium (Krismer et al., 2014). In contrast, all 11 encoded two full sets of the genes required for

methionine degradation in different regions on their chromosome suggesting external dependence. Each set includes *metN* (methionine ABC transporter ATP-binding protein), *metP* (methionine ABC transporter permease protein), *metQ* (methionine ABC transporter substrate-binding protein) and *metT* (methionine transporter).

Glutamine. Glutamine synthetase I (EC 6.3.1.2) was predicted in all 11 strains and catalyzes the reaction of L-glutamine from L-glutamate and NH_4^+ using ATP. Glutamate racemase (EC 5.1.1.3) was predicted in all 11 and catalyzes D- / L-glutamate interconversion. NAD(P)-specific glutamate dehydrogenase (EC 1.1.1.4) GdhA was predicted in all 11 and catalyzes the formation of L-glutamate and H_2O from 2-oxoglutarate, ammonia and $NADH+H^+$ and vice versa.

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