Corynebacterium accolens Releases Antipneumococcal Free Fatty Acids from Human Nostril and Skin Surface Triacylglycerols

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ABSTRACT Bacterial interspecies interactions play clinically important roles in shaping microbial community composition. We observed that *Corynebacterium* spp. are overrepresented in children free of *Streptococcus pneumoniae* (pneumococcus), a common pediatric nasal colonizer and an important infectious agent. *Corynebacterium accolens*, a benign lipid-requiring species, inhibits pneumococcal growth during *in vitro* cocultivation on medium supplemented with human skin surface triacylglycerols (TAGs) that are likely present in the nostrils. This inhibition depends on LipS1, a TAG lipase necessary for *C. accolens* growth on TAGs such as triolein. We determined that *C. accolens* hydrolysis of triolein releases oleic acid, which inhibits pneumococcus, as do other free fatty acids (FFAs) that might be released by LipS1 from human skin surface TAGs. Our results support a model in which *C. accolens* hydrolyzes skin surface TAGS *in vivo* releasing antipneumococcal FFAs. These data indicate that *C. accolens* may play a beneficial role in sculpting the human microbiome.

IMPORTANCE Little is known about how harmless *Corynebacterium* species that colonize the human nose and skin might impact pathogen colonization and proliferation at these sites. We show that *Corynebacterium accolens*, a common benign nasal bacterium, modifies its local habitat *in vitro* as it inhibits growth of *Streptococcus pneumoniae* by releasing antibacterial free fatty acids from host skin surface triacylglycerols. We further identify the primary *C. accolens* lipase required for this activity. We postulate a model in which higher numbers of *C. accolens* cells deter/limit *S. pneumoniae* nostril colonization, which might partly explain why children without *S. pneumoniae* colonization have higher levels of nasal *Corynebacterium*. This work narrows the gap between descriptive studies and the needed in-depth understanding of the molecular mechanisms of microbe-microbe interactions that help shape the human microbiome. It also lays the foundation for future *in vivo* studies to determine whether habitat modification by *C. accolens* could be promoted to control pathogen colonization.

*S*treptococcus pneumoniae (pneumococcus) is a major cause of pneumonia, septicemia, meningitis, otitis media, and sinusitis in children and adults worldwide. The World Health Organization estimates that each year *S. pneumoniae* leads to more than 1 million deaths, the majority of which occur in developing countries and are of children under the age of 5 years (1, 2). More than 20,000 deaths annually have been estimated in the United States (3–5). Although most individuals colonized with *S. pneumoniae* do not develop pneumococcal infection, colonization greatly increases the risk of, and is a prerequisite for, both infection and transmission. Children are the primary reservoir, with those between 6 months and 7 years of age having the highest rate of nasal colonization (40 to 60% on average in developed countries), and this rate decreases in adults. Colonization rates show extensive variation depending on multiple factors, such as geographical strain epidemiology, health status, socioeconomic environment, day care use, etc. (1, 6). Because *S. pneumoniae* can be part of the healthy microbiota without causing disease, it is considered a pathobiont—a commensal bacterium that is also a pathogen (7).

A challenge to controlling pneumococcus in humans is that there are over 92 known capsular serotypes of *S. pneumoniae* and these display different antigenic properties and biological behaviors (8–12). Introduction of conjugated vaccines, which are effective in infants, against the most commonly invasive serotypes has led to a significant reduction of severe infections, i.e., invasive pneumococcal disease (13, 14). In contrast, conjugate vaccines have been less effective in controlling milder, noninvasive pneumococcal disease, such as middle ear infections (otitis media). In addition, while vaccinated individuals are uncommonly colonized by vaccine serotypes, the ability of nonvaccine pneumococcal serotypes to occupy the niche vacated by vaccine serotypes (serotype replacement) means that overall pneumococcal colonization rates remain unchanged (15–17). Thus, an increased understanding of *S. pneumoniae* colonization dynamics, especially in children, is important for developing new approaches to prevent pneumococcal colonization.

The pediatric nasal passages are the primary reservoir for *S. pneumoniae*. Pneumococcus is present from where the nostrils

(anterior nares) open onto the skin of the nasal vestibules (18, 19), a habitat complete with sweat and sebaceous glands, through the mucosal surfaces of more posterior sections of the nasal cavity to the nasopharynx (see Fig. S1 in the supplemental material). (Henceforth, we refer to the nostrils and nasal vestibules simply as the nostrils.) In prepubertal children, many other bacteria cocolonize these nasal surfaces with pneumococcus, including other members of the phylum *Firmicutes*, along with members of the phyla *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (18, 20, 21). We and others (18, 19) have hypothesized that pneumococcal interactions with commensal bacteria impact the ability of pneumococcus to colonize, proliferate, and persist. If so, then defining pediatric nasal microbiota in the presence and absence of pneumococcal colonization might reveal potential clinically relevant interactions that affect pneumococcal colonization dynamics. For example, Pettigrew and colleagues observed that children free of nostril pneumococcal colonization had a greater relative abundance of *Corynebacterium* spp. than did children with pneumococcal colonization (18). In a separate cohort, Pettigrew et al. found that children harboring low levels of *Streptococcus* spp. had high levels of *Corynebacterium* spp. (19). These observations led the authors to speculate that commensal *Corynebacterium* spp. may play a protective role against pneumococcus. Determining the mechanism(s) that underlies such antagonism is of particular interest. Because there is no established animal model for commensal *Corynebacterium* spp. colonization, we chose to approach identification of a potential mechanism(s) by using *in vitro* cocultivation.

Although non-*diphtheriae* *Corynebacterium* spp., along with pneumococcus, commonly colonize the skin and nasal passages (see Fig. S1 in the supplemental material), their function(s) in these complex microbial communities is poorly understood (21–31). Among the *Corynebacterium* spp., a subset require an exogenous source of free fatty acids (FFAs) for growth because they lack a fatty acid synthase (32). Culture media for these fatty acid synthase-deficient *Corynebacterium* spp. commonly incorporate Tween 80 (Tw80; polyoxyethylenesorbitan mono-oleate), a synthetic lipid that contains an ester of oleic acid, a monounsaturated 18-carbon fatty acid (32). Many bacterial lipases and esterases can hydrolyze the ester bond in Tw80, releasing free oleic acid (33). Growth of *C. accolens* on Tw80 suggests that lipids containing oleic acid might serve as a source of required FFAs *in vivo*. However, to our knowledge, the host lipids from which fatty acid-requiring *Corynebacterium* spp. harvest FFAs *in vivo* have not been thoroughly investigated, nor have the genes required for such harvest.

In humans, unlike other animals tested to date, e.g., mice and rats (34, 35), triacylglycerols (TAGs) constitute a large percentage (40 to 60%) of the total lipid content of sebum and a slightly lower percentage of skin surface lipids (SSLs). Thus, TAGs are a major source of FFAs on skin (36, 37). TAGs consist of three fatty acid moieties, each linked via an ester bond to a glycerol backbone. Recent analyses using high-resolution chromatography coupled to mass spectrometry reveal an amazing diversity of TAGs on human skin (37). This diversity arises from variations in both the combination and the composition of the fatty acid moieties, which on human skin reportedly range in length from 12 to 18 carbon atoms with differing degrees of saturation (36, 37). Lipases act primarily on esters of water-insoluble fatty acids with chain lengths greater than nine carbon atoms. Microbiota- and host-
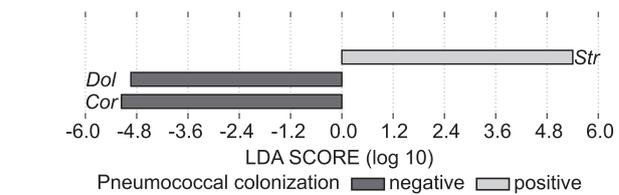
FIG 1 Nasopharyngeal *Corynebacterium* spp. are overrepresented in children without *S. pneumoniae* nasal colonization. Shown is a linear discriminant analysis (LDA) plot of the nasopharyngeal bacterial genera overrepresented when *S. pneumoniae* was absent (dark gray) or present (light gray) by cultivation. Abbreviations indicate the following genera: *Str*, *Streptococcus*; *Dol*, *Dolosigranulum*; *Cor*, *Corynebacterium*. Data were obtained from 27 nasopharyngeal swabs collected from healthy children aged 6 months to 7 years. Swabs used to inoculate medium for *S. pneumoniae* cultivation were frozen and later used to harvest nucleic acids, which were analyzed by 16S rRNA V1-3 tag pyrosequencing. Sequences were analyzed as described in Materials and Methods.

produced lipases are expected to hydrolyze sebum and skin surface TAGs, including those coating the human nostrils, releasing FFAs. These FFAs are likely used by lipid-requiring *Corynebacterium* spp. such as *C. accolens* that colonize human nasal and skin surfaces.

In this study, we present data for pediatric nasopharyngeal bacterial microbiota that corroborate the finding that *Corynebacterium* spp. are overrepresented in children free of pneumococcal nasal colonization (18). Based on these data, we hypothesized that *Corynebacterium* spp. might directly antagonize pneumococcus during *in vitro* cocultivation. We found that, *in vitro*, *C. accolens*, a lipid-requiring species, uses representative human skin surface TAGs to satisfy this nutritional requirement and, in doing so, releases FFAs that inhibit pneumococcal growth. Our genetic analyses indicate that hydrolysis of a representative skin surface TAG depends on the *C. accolens* lipase LipS1. In addition, we have shown that many of the FFAs that would likely be released by *C. accolens* LipS1 from human skin and nostril surface TAGs inhibit pneumococcus *in vitro*. Thus, based on our data, we hypothesize that this release of antipneumococcal FFAs from human TAGs represents a mechanism by which *C. accolens* antagonizes pneumococcus *in vivo* and thus shapes human nasal microbiota in a manner that contributes to pneumococcal colonization resistance.

RESULTS

***Corynebacterium* species are overrepresented in nasopharyngeal samples from children who are free of pneumococcal colonization compared to samples from those with pneumococcal colonization.** To test the hypothesis that nasopharyngeal microbiota differ in children with and without pneumococcal nasopharyngeal colonization, we performed 16S rRNA gene sequencing on a small subset of samples collected as part of a large epidemiological study of pneumococcal colonization in eastern Massachusetts (16, 17, 38, 39). Data were analyzed using linear discriminant analysis effect size (LEfSe) (40) to compare the microbiota composition of children positive and negative for culture-proven *S. pneumoniae* nasopharyngeal colonization. This revealed that the genera *Corynebacterium* and *Dolosigranulum* were overrepresented in children negative for pneumococcal colonization by cultivation (Fig. 1, dark gray bars; see also Fig. S2 in the supplemental material), whereas *Streptococcus* was overrepresented in children positive for pneumococcal colonization (Fig. 1, light gray bar; see

also Fig. S2). Our results from nasopharyngeal swabs complement published data from 16S rRNA gene studies of bacterial microbiota of nostril swabs from a different cohort of children that were analyzed using a different technique (18, 19), suggesting these data are robust. One potential explanation for these data is that there is direct antagonism between *Corynebacterium* spp. and *S. pneumoniae* in the human nose. However, these data do not indicate a mechanism for this hypothetical microbiological antagonism.

Interestingly, at 6 weeks of age, *Corynebacterium* spp. and *Dolosigranulum* spp. are also overrepresented in the nasopharyngeal microbiota of breastfed infants compared to formula-fed infants (41). In these breast-fed infants, the *Corynebacterium* spp. operational taxonomic units (OTUs) (identified by sequencing the V5-V7 region of the 16S rRNA gene) are reportedly most homologous with *C. pseudodiphtheriticum* and *C. propinquum* or with *C. accolens*. Metagenomic or cultivation-dependent studies are needed to conclusively determine which species of *Corynebacterium* are most common in the nasal microbiota of children younger than 7 years. However, given the available data, we focused our analyses on *C. accolens*, since this species is commonly isolated from/detected in adult noses (nostrils, nasal mucosa, and nasopharynx) (28–30), is present in the nasopharynx of young infants (41), and is rarely reported in association with infection (e.g., a PubMed search for "*accolens* AND infection" yielded four reports clearly implicating *C. accolens* in human infection, which is fewer than for *C. pseudodiphtheriticum* or *C. propinquum*). All of these are desirable characteristics for any potential nasal probiotic (42).

Representative human skin surface TAGs support the growth of *C. accolens*, a fatty acid-requiring species. To test the hypothesis that there is direct antagonism between *C. accolens* and pneumococcus, we first established conditions under which both species will grow. To satisfy the fatty acid requirement of *C. accolens*, we opted to identify physiologically relevant lipids, i.e., SSLs that support cultivation of *C. accolens*. Oleic and linoleic acids are detected in nasal fluid (43) as well as on human skin (36, 37, 44). Triolein (Fig. 2A) and trilinolein (Fig. 2B) contain esters of oleic acid (18:1 *cis*-9) and linoleic acid (18:2 *cis*-9,12), respectively, and serve as models for human sebum and skin surface TAGs (34, 45–47). Therefore, we tested these two commercially available, representative human skin surface TAGs for the ability to support the growth of *Corynebacterium* sp. KPL1818, a primary nostril isolate from a healthy adult that we identified as *C. accolens* (see Fig. S3 in the supplemental material). (Henceforth, *Corynebacterium* sp. KPL1818 is referred to as *C. accolens*.) *C. accolens* grew robustly in brain heart infusion (BHI) broth supplemented with 100 μg/ml of either triolein or trilinolein but poorly, if at all, in BHI broth supplemented with solvent alone (Fig. 2C). Having identified these TAGs as physiologically relevant sources of fatty acids for *C. accolens*, we used one as a medium supplement for all subsequent experiments (in place of Tw80).

***C. accolens* inhibits pneumococcal growth during cocultivation.** To test the hypothesis that *C. accolens* produces a diffusible activity that antagonizes *S. pneumoniae*, we developed a solid agar medium-based assay that permits side-by-side cocultivation of *C. accolens* and *S. pneumoniae*, where growth antagonism is detected as zones of inhibition (ZOI). For this assay, 5 mg of either triolein or trilinolein was thinly spread on the surface of 10 ml of BHI agar, since neither one is readily soluble in aqueous-based agar medium, and served as the source of FFA for *C. accolens* (oleic
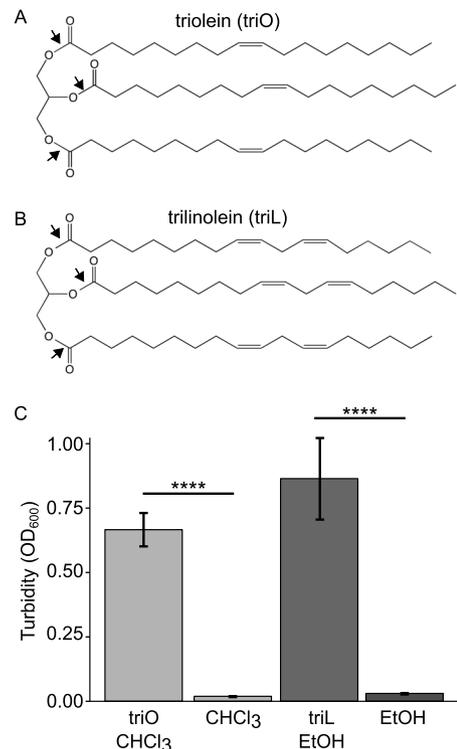
FIG 2 Model human skin surface TAGs triolein (triO) and trilinolein (triL) support the growth of the fatty acid-requiring species *C. accolens*. Black arrows indicate the oleic acid esters in (A) triolein and the linoleic esters in (B) trilinolein. (C) The mean turbidity (OD_{600}) after 48 h of shaken aerobic growth at 37°C demonstrated increased *C. accolens* growth in BHI broth supplemented with one of the TAGs compared to solvent-only controls ($n = 9$). Data were analyzed using an ANOVA and Tukey's multiple-comparisons test. Error bars represent standard errors of the means. ****, $P \leq 0.0001$.

or linoleic acid, respectively). In addition, the medium was supplemented with catalase to mitigate potential *S. pneumoniae*-produced hydrogen peroxide (H_2O_2) inhibition of *Corynebacterium* for two reasons. First, *in vivo*, other human and bacterial catalases present on the surface of the nasal passages likely contribute to detoxification of pneumococcus-produced H_2O_2, as occurs in saliva (48–50). Second, data indicate that H_2O_2-mediated killing *in vitro* does not necessarily reflect interactions *in vivo*. For example, although *S. pneumoniae*-produced H_2O_2 kills *Staphylococcus aureus in vitro*, data from animal models and pediatric upper respiratory tract epidemiological studies that show a negative association between *S. pneumoniae* and *S. aureus* cannot be explained by H_2O_2-mediated killing of cooccurring bacteria (51–54). Similarly, although pneumococcus-produced H_2O_2 kills *Haemophilus influenzae in vitro*, this effect does not explain *in vivo* animal model observations, which show *H. influenzae*-induced clearance of *S. pneumoniae* in a nasopharyngeal colonization mouse model (55, 56). Finally, to compensate for the differential growth rate of the two species, *C. accolens* was grown for 42 h prior to adjacent inoculation of *S. pneumoniae* 603.

On agar medium with either triolein or trilinolein as the FFA source, *C. accolens* inhibited *S. pneumoniae* (Fig. 3A and C). In contrast, in the absence of exogenous TAG (solvent alone), *C. accolens* grew poorly and did not inhibit *S. pneumoniae* (Fig. 3B and D). These results indicate that *C. accolens* produces an extracellu-
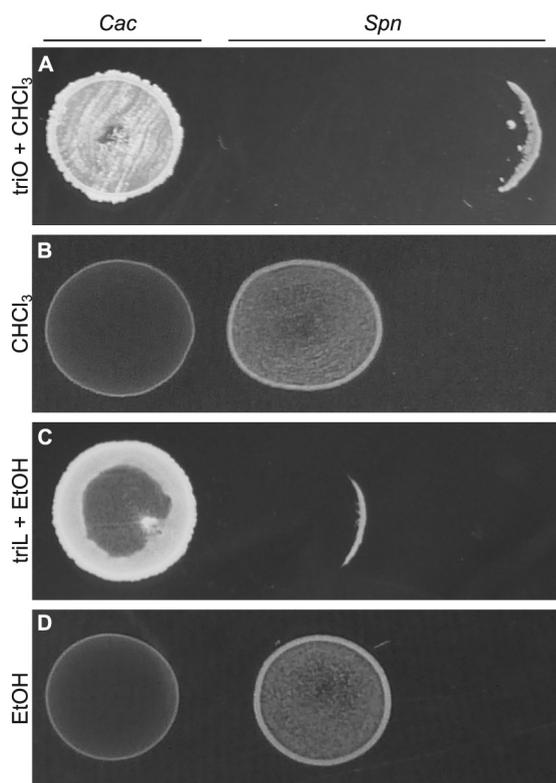
FIG 3 *C. accolens* inhibits *S. pneumoniae*. *C. accolens* KPL1818 (*Cac*) inhibited *S. pneumoniae* 603 (*Spn*) growth on BHIC agar spread with 50 μl of a 100-mg/ml solution of (A) triolein solubilized in chloroform (triO + CHCl₃) or (C) trilinolein emulsified in ethanol (triL + EtOH), but not on medium spread with 50 μl of solvent alone: (B) chloroform (CHCl₃) or (D) EtOH. Representative images are shown (*n* = 3). To permit visualization of partial inhibition of the pneumococcal spot, *S. pneumoniae* was inoculated at various distances from *C. accolens*, as shown by the varied sizes of ZOIs, which are likely due to differences in diffusion of the antipneumococcal molecule(s).

TABLE 1 Methanol extracts of *C. accolens* KPL1818 CFCM inhibit *S. pneumoniae* 603 and contain free oleic acid

Methanol extract source (growth medium)	ZOI (mm)[a]	Free oleic acid concn (μM)[b]
C. accolens CFCM (LB agarose triolein)	11.7 ± 3.8	1,099.40 ± 422.98
Sterile medium (LB agarose triolein)	0.00 ± 0.00	6.07 ± 12.15

[a] The mean ZOI ± SD produced in a disk-diffusion assay versus *S. pneumoniae* 603. The ZOI includes the disk diameter (6 mm). Extracts from each biological replicate (*n* = 4) were assayed in three technical replicates and results were then averaged.
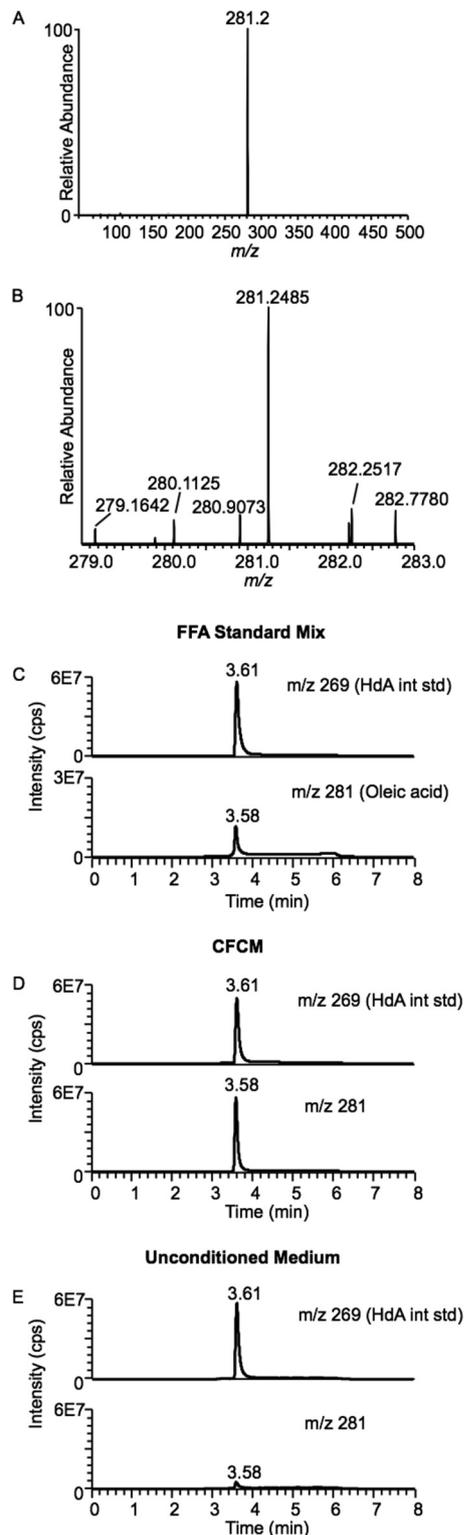
[b] The mean ± SD concentration of oleic acid found in methanol extracts. For samples in which no free oleic acid was detected, a value of 0 was used for calculating the mean and SD and for statistical analysis.

lar, diffusible activity that inhibits *S. pneumoniae* growth. *C. accolens* strains DSM 44278[T] and DSM 44279 also inhibited pneumococcal growth in either this assay or a simpler membrane-based assay (see Table S1 in the supplemental material). Having established that this inhibitory phenotype is not strain specific, we used *C. accolens* KPL1818 for all further experiments, as it was isolated directly from a human nostril.

Methanol extracts of *C. accolens* cell-free conditioned medium exhibit antipneumococcal activity. Because many antibacterial molecules are soluble in methanol, we hypothesized that the diffusible antipneumococcal activity produced by *C. accolens* (Fig. 3A and C; see also Table S1 in the supplemental material) might be extractable with methanol from medium conditioned by growth of *C. accolens* in the absence of pneumococcus. *C. accolens* produced antipneumococcal activity with both triolein and trilinolein supplementation (Fig. 3A and C), and we selected triolein as the medium supplement for further experimentation. We prepared crude methanol extracts of cell-free conditioned LB agarose medium spread with triolein (CFCM). For ease of harvesting CFCM, *C. accolens* was grown atop 0.2-μm polycarbonate membranes that were removed prior to extraction. Concentrated aliquots of crude CFCM methanol extracts killed pneumococcus, whereas equivalent amounts of control extracts of unconditioned

sterile medium did not (Table 1). These data indicate that *C. accolens* produces an antipneumococcal activity that is extractable in methanol and does so in the absence of pneumococcus under the growth conditions of this assay.

Extracts of *C. accolens* CFCM are enriched in free oleic acid. To identify the compound(s) with antipneumococcal activity, we compared qualitative mass spectrometry (MS) analysis results for methanol extracts from CFCM to those for unconditioned medium (LB agarose triolein) in search of compounds uniquely present or enriched in *C. accolens* CFCM. Mass spectrometric analysis of methanol extracts of CFCM revealed that oleic acid was the most abundant compound uniquely enriched in the CFCM extracts (Fig. 4). Compounds enriched in CFCM versus unconditioned medium were identified by performing limited mass scanning in negative ion mode and subtracting the spectrum for unconditioned medium from that for CFCM. One large novel peak with *m/z* 281 was identified in CFCM (Fig. 4A). Using high-resolution mass spectrometric analysis, the measured mass-to-charge ratio of this compound was 281.2485 (Fig. 4B), differing from the calculated mass of the [M-H]⁻ ion of oleic acid of *m/z* 281.2486 by less than 5 ppm, consistent with the compound being oleic acid. In gradient reversed-phase ultraperformance liquid chromatography, the CFCM *m/z* 281 compound perfectly coeluted with authentic free oleic acid (Fig. 4C and D), further confirming that the compound present in CFCM is oleic acid. The concentrations of free oleic acid in CFCM extract were consistently greater than those observed in extracts of unconditioned medium (CFCM versus LB agarose triolein, two-tailed paired *t* test, *P* < 0.05) (Table 1; Fig. 4D versus E). These data clearly indicate that *C. accolens* releases free oleic acid from triolein, likely via lipase activity, and raise the possibility that free oleic acid might be responsible for the antipneumococcal activity produced by *C. accolens*.

Authentic FFAs that can be released from human skin/nasal TAGs inhibit pneumococcal growth. Based on the combination of our observation that *C. accolens* released free oleic acid from triolein (Fig. 4; Table 1) and previous reports that some FFAs inhibit bacterial growth (57–60), we hypothesized that *C. accolens*-liberated FFAs inhibit pneumococcal growth during both cocultivation (Fig. 3A and C) and exposure to CFCM extracts (Table 1). We tested this in a disk diffusion assay and observed that both authentic oleic and linoleic acids, which would be liberated from triolein and trilinolein, respectively, inhibited the growth of *S. pneumoniae* 603 (serotype 6B) and five other tested *S. pneumoniae* strains (TIGR4, DBL5, WU2, DSM 24048, and DSM

11868, which represent capsular serotypes 4, 5, 3, 19F, and 23F, respectively) (Table 2). These strains were selected, in part, because they represent a range of encapsulation capacities, from low encapsulation to heavy encapsulation. Next, we expanded the panel of FFAs tested to include other commercially available FFAs that are detected on human skin or in human nasal secretions (36, 37, 43, 44, 61). This included sapienic acid, which is the most abundantly detected FFA in sebum and SSLs (36, 37). We based these experiments on the hypothesis that *C. accolens* also liberates these other FFAs from the diversity of human skin surface TAGs (36, 37) via a lipase(s). Sapienic, palmitioleic, lauric, and myristic acids also inhibited pneumococcus, whereas stearic and palmitic acids did not inhibit pneumococcus under the assay conditions used (Table 2). The only difference between stearic and oleic acids is that stearic acid is a fully saturated 18-carbon FFA and thus is more difficult to emulsify in solvent at room temperature. Palmitic acid is similarly a saturated 16-carbon FFA. It is possible these two saturated 16- and 18-carbon FFAs do not diffuse from the filter disk and/or that they do not kill encapsulated pneumococcus at the quantities tested. (Both are reported to kill the unencapsulated mutant strain R6 [59].) Arsic and colleagues report that palmitic and stearic acids do not inhibit *S. aureus*, which leads us to speculate that these might not inhibit encapsulated pneumococcus (62). Several of the observed antipneumococcal FFAs (Table 2) have been reported to inhibit other microbes (58, 61), including *S. pneumoniae* (57, 59, 63). Here, we demonstrated that six serotypes are inhibited by a range of FFAs which can be released from human skin/nasal TAGs, and we predict that these FFAs inhibit additional pneumococcal serotypes.

Identification of a putative *C. accolens* TAG lipase. TAG lipases preferentially hydrolyze ester bonds to release fatty acids with chain lengths longer than nine carbon atoms from TAGs (64). Based on this, we hypothesized that the enzyme required for *C. accolens* to hydrolyze triolein and trilinolein, releasing free oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids, respectively, might be an extracellular TAG lipase. To identify candidate TAG lipases, we queried the draft genome of *C. accolens* KPL1818 (RefSeq accession NZ_AXMA00000000) against the Conserved Domain Database (CDD) of NCBI (65). We searched for *C. accolens* proteins that aligned to the LIP family (pfam03583), the Lipase_2 family (pfam01674), or the LipA/EstA cluster of orthologous groups (COG1075). We focused on these groups because characterized TAG lipases from other common human skin- and nostril-associated bacteria, i.e., *S. aureus*, *S. epidermidis*, and *Proprionibacterium acnes* (45, 66–68) and those putatively identified in other *Corynebacterium* spp. (69, 70) align to at least one of these groups. This search revealed two candidate TAG lipases in *C. ac-*

FIG 4 Oleic acid is enriched in methanolic extracts of *C. accolens* CFCM. We used mass spectrometric analysis to identify compounds uniquely enriched in *C. accolens* CFCM. Methanolic extracts were analyzed by direct line infusion into a triple-quadrupole mass spectrometer operating in negative-ion scanning mode from *m/z* 50 to *m/z* 500. (A) To identify novel compounds present in the CFCM, the limited mass spectrum of the methanolic extract from unconditioned medium was subtracted from the spectrum for *C. accolens* CFCM. (B) Limited mass scanning of the novel *m/z* 281 peak present in CFCM, deter-

(Continued)

Figure Legend Continued

mined via high-resolution mass spectrometry, which gave a measured mass of *m/z* 281.2485, consistent with the calculated mass of oleic acid (*m/z* 281.2486). (C) An FFA standard mixture containing authentic oleic acid and heptadecanoic acid (HdA), as an internal standard, was analyzed by reversed-phase ultraperformance liquid chromatography coupled to tandem mass spectrometry, operating in multiple reaction mode monitoring transition of *m/z* 281 to *m/z* 281 at 10 eV for HdA and *m/z* 269 to *m/z* 269 at 10 eV for oleic acid, respectively. (D) CFCM with HdA added as an internal standard, analyzed under the same chromatographic conditions. (E) Unconditioned medium with HdA added as an internal standard, analyzed under the same chromatographic conditions. Representative spectra (A and B) or chromatographs (C, D, and E) are shown.

TABLE 2 Antipneumococcal activity of FFAs reported on human skin or in human nasal fluid

Test FFA[a]	FFA description	ZOI[b] (mm) for *S. pneumoniae* strain (serotype)					
		603 (6B)	TIGR4 (4)	DBL5 (5)	WU2 (3)	DSM 24048 (19F)	DSM 11868 (23F)
Oleic	18:1 *cis*-9	33.0 ± 3.0	24.0 ± 3.6	24.3 ± 4.0	35.7 ± 3.8	20.3 ± 4.0	30.0 ± 4.4
Linoleic	18:2 *cis*-9,12	30.3 ± 1.5	28.7 ± 3.5	25.0 ± 3.0	30.0 ± 6.6	20.3 ± 3.2	26.0 ± 6.1
Stearic	18:0	ND	ND	ND	ND	ND	ND
Sapienic	16:1 *cis*-6	29.3 ± 5.8	28.7 ± 4.2	24.7 ± 3.5	33.7 ± 5.7	25.3 ± 6.7	28.7 ± 5.7
Palmitoleic	16:1 *cis*-9	19.0 ± 1.7	20.3 ± 2.9	19.0 ± 3.0	24.3 ± 0.6	17.7 ± 0.6	18.0 ± 3.6
Palmitic	16:0	ND	ND	ND	ND	ND	ND
Myristic	14:0	11.3 ± 0.6	10.0 ± 0.0	10.0 ± 0.0	9.7 ± 0.6	10.0 ± 0.0	10.3 ± 0.6
Lauric	12:0	20.7 ± 0.6	14.3 ± 6.7	8.3 ± 0.6	15.3 ± 1.2	11.3 ± 1.5	16.0 ± 2.6
Ethanol	Negative control	ND	ND	ND	ND	ND	ND

[a] One hundred micrograms of each FFA in 10 μl of 200-proof ethanol was pipetted onto a 6-mm filter disk that was placed on a lawn of *S. pneumoniae*.
[b] The diameter of the ZOI (mean ± standard deviation; $n = 3$) was measured after overnight incubation at 37°C with 5% CO_2. ND, ZOI not detected.

colens KPL1818: WP_034657805.1 and WP_023029886.1, here abbreviated WP_03 and WP_02.

The CDD analysis predicted that WP_03 belongs to the LIP protein family (E value, $3.39e^{-36}$), whereas WP_02 aligned to both the LipA/EstA COG (E value, $4.88e^{-40}$) and the Lipase_2 family (E value, $1.08e^{-08}$). Both proteins contain the canonical lipase motif G-X-S-X-G, complete with the predicted active site serine residue (64). SignalP analysis (version 4.1) (71) of the two protein sequences predicted that only WP_03 is secreted via the general secretion pathway, similar to other skin-associated bacterial TAG lipases (66–68). It is possible that WP_02 is secreted by mechanisms that are not dependent on the general secretion pathway. However, based on the CDD and SignalP analyses, we hypothesized that WP_03 is the primary extracellular *C. accolens* TAG lipase, and we proceeded to test this by using a genetic approach.

***C. accolens lipS1* (WP_034657805.1) is necessary for growth when triolein is the only source of fatty acids.** A *C. accolens* KPL1818 mutant strain with an in-frame deletion of WP_03 did not grow in BHI supplemented with triolein, whereas the wild type did (Fig. 5A). These results indicated that WP_03 is required for hydrolysis of the TAG triolein; therefore, we named this gene *lipS1*, in adherence with existing nomenclature for *Corynebacterium* TAG lipases predicted to be members of the LIP family (69, 70). In contrast to its inability to grow in medium supplemented with triolein, the *lipS1*-deficient mutant grew comparably to the wild type in medium supplemented with Tw80 (see Fig. S4A in the supplemental material). This growth on Tw80 is likely due to esterase activity, since some esterases can hydrolyze the ester of oleic acid ($C_{18:1}$) in Tw80 but not in a TAG (33); in TAGs, esterases preferentially hydrolyze ester bonds linking water-soluble fatty acids of chain lengths less than 10 carbon atoms ($<C_{10}$) to the glycerol backbone (64).

Complementation of the *lipS1* deletion with *lipS1* (with a His6 tag) expressed from its native promoter on a multicopy plasmid (see Fig. S5 in the supplemental material) restored the ability of *C. accolens* to grow to wild-type levels with triolein as the sole source of fatty acids (Fig. 5B). In contrast, there were no growth differences among these strains in medium supplemented with Tw80 (see Fig. S4B in the supplemental material). Again, this is likely due to esterase activity encoded by another gene(s). In addition to identifying the primary *C. accolens* lipase, this work demonstrates that *C. accolens* is genetically tractable.

Among *Corynebacterium* strains with an assigned species, LipS1 homologs are detected only in *C. accolens*. To determine whether homologs of LipS1 are present in other *Corynebacterium* spp., we queried the nonredundant database at NCBI with the predicted 464-amino-acid sequence of LipS1 via BLASTP (72). We restricted our search to the *Corynebacterium* genus (taxid 1716) using a ≥96% protein identity to LipS1 as the cutoff for homology. LipS1 was detected in the *C. accolens* type strain (98% identity) which inhibited pneumococcal growth (see Table S1 in the supplemental material). LipS1 was also detected in the *C. accolens* primary nostril isolate KPL1855 (73) and in ATCC 49726, suggesting that these strains likely possess the ability to hydrolyze TAGs to release antipneumococcal FFAs. Based on this bioinformatic analysis and the genetic data described above, we conclude that LipS1 is a primary TAG lipase that *C. accolens* uses to harvest FFAs from host skin surface TAGs.

DISCUSSION

To our knowledge, this work provides the first evidence that *C. accolens*, a commensal *Corynebacterium* species that colonizes the nose, can inhibit *S. pneumoniae*. The goal of identifying antago-
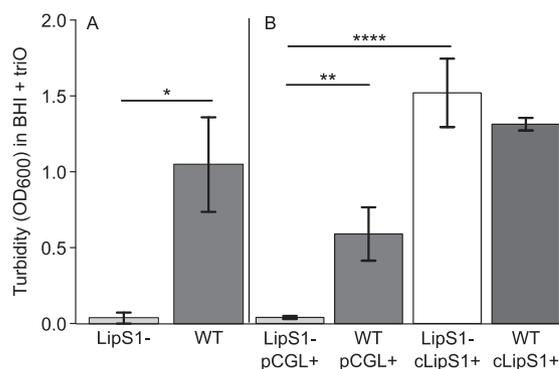
FIG 5 *C. accolens* LipS1 is necessary for growth on triolein (triO) as the sole source of fatty acids. (A) A *C. accolens* mutant strain (LipS1⁻) harboring an in-frame deletion in *lipS1* did not grow in BHI supplemented with triolein (0.1 mg/ml), whereas the wild-type (WT) did. Growth was measured as the mean OD_{600} following 48 h of aerobic growth with shaking at 37°C. Data were analyzed using a Welch two-sample *t* test. $n = 3$. Error bars represent standard deviations. *, $P \leq 0.05$. (B) The lipase-deficient mutant carrying the empty vector (LipS1⁻ pCGL⁺) did not grow in BHI supplemented with triolein; however, the complemented mutant (LipS1⁻ cLipS1⁺) did. For comparison, the empty vector and complemented vector controls for the parental strain (WT pCGL⁺ and WT cLipS1⁺, respectively) are included. Data ($n = 3$) were analyzed using an ANOVA and Tukey's multiple-comparisons test. Error bars represent standard deviations. **, $P \leq 0.01$; ****, $P \leq 0.0001$.

nistic interactions between commensal nasal bacteria and *S. pneumoniae* is to lay the foundation for future translational research aimed at preventing pneumococcal colonization and subsequent infection. As illustrated here, we approached this by starting with data on the community composition of the pediatric nasal microbiota (Fig. 1) (18, 19). We then used a reductionist approach to test our hypothesis that growth of the medically important pathobiont *S. pneumoniae* is antagonized by *C. accolens*, a commensal bacterium that is rarely reported in conjunction with infection and, thus, might have potential as a probiotic. Liquid chromatography-mass spectroscopy (LC/MS) of *C. accolens* CFCM and disk-diffusion assays with both CFCM extracts and authentic FFAs demonstrated that FFAs released from skin surface TAGs by *C. accolens* inhibit pneumococcal growth. We also showed that FFAs present on skin and in nasal secretions (36, 37, 43, 44, 61) inhibit pneumococcus (Table 2). The antibacterial activity of FFAs is well documented, and oleic acid inhibits pneumococcal growth through membrane depolarization and cell rupture (57). Overall, our results lay the foundation for future testing of *C. accolens* as a potential skin and nasal probiotic. There is already a precedent for testing *Corynebacterium* spp. as probiotics for the eradication of nasal colonization by the pathobiont *S. aureus* (31, 74). Our results also pave the way for future testing of representative human skin surface TAGs as potential prebiotics, the addition of which is predicted to result in decreased levels of FFA-susceptible pathogenic streptococcal species, such as *S. pneumoniae* and *Streptococcus pyogenes* (60). Since colonization is a prerequisite for infection with these pathobionts, eradication or a decrease in the level of colonization could prevent infection.

The discovery that *C. accolens* releases antibacterial FFAs from human TAGs provides an impetus for further investigations. For example, because *C. accolens* grows poorly in the absence of an exogenous FFA source (Fig. 3), we cannot exclude the possibility that in the presence of a fatty acid source, *C. accolens* produces another compound(s) that also contributes to pneumococcal growth inhibition. It remains unknown whether other *Corynebacterium* spp. inhibit pneumococcal growth via the release of antibacterial FFAs and/or via an as-yet-undiscovered alternative mechanism. Further, future investigations will determine what species of *Corynebacterium* are most commonly found in the nasal microbiota of children 6 months to 7 years of age, as it is not yet known how common natural *C. accolens* nasal colonization is in this age group. Finally, the absence, or low levels, of skin surface TAGs in other mammals tested to date (34, 35), including mice and rats, limits the testing of our model (Fig. 6) in animals. In addition, although animal models exist for *S. pneumoniae* nasal colonization, there is not yet an established model for nasal colonization by commensal *Corynebacterium* spp. However, the use of some TAGs, including both trilinolein and triolein (i.e., triglycerides) in some cosmetics (75) opens up the possibility for future testing directly on human skin.

Based on the *in vitro* mechanistic data presented, we developed a model that addresses the potential relevance of our findings to the *in vivo* ecology of *C. accolens* and *S. pneumoniae* and generates testable hypotheses for future research. In this model (Fig. 6), the *C. accolens* TAG lipase LipS1, which is likely to be extracellular, hydrolyzes skin surface TAGs, releasing antibacterial FFAs, many of which inhibit pneumococcus. This current model might partly explain the greater relative abundance of nasal *Corynebacterium* spp. in children who lack pneumococcal nasal colonization
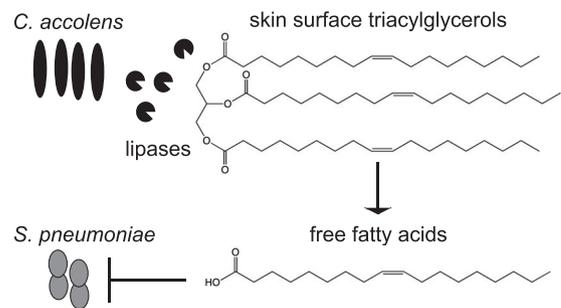

FIG 6 One model is that *C. accolens* modifies the nasal and/or skin habitat, making it inhospitable for *S. pneumoniae*. *C. accolens* produces a lipase, likely extracellular, that releases FFAs from host TAGs. Some of these FFAs have antibacterial activity and might impede the colonization and proliferation of *S. pneumoniae* and possibly other FFA-susceptible colonizers.

(Fig. 1) (18). The recent identification of an OTU associated with 6-week-old breast-fed infants that resembles *C. accolens* raises the possibility that *C. accolens* might be present in many young infants (41). Collectively, *Corynebacterium* spp. are widely distributed across various skin sites, as well as on mucosal surfaces of the upper respiratory tract (18, 21–31). In addition, other pathobionts are also susceptible to FFAs, e.g., *S. aureus* (45, 59, 76) and *S. pyogenes* (60). Thus, our current model in which *Corynebacterium* spp. contribute to modifying the local habitat via release of antibacterial FFAs might be generalizable to other sites where *Corynebacterium* spp. are in higher relative abundance, such that the local concentration of FFAs reaches a level that can shape bacterial community structure.

By demonstrating that *C. accolens* liberates FFAs from human skin surface TAGs and identifying LipS1 as a primary *C. accolens* TAG lipase, this work expands the repertoire of bacterial contributors to FFAs on human skin. Historically, FFA release from skin surface TAGs is attributed to both human-produced and bacteria-produced lipases (77). In support of a role for host lipases, a recent study revealed that *in vitro* human sebocytes produce FFAs in the absence of bacteria (78). The role of *P. acnes*-produced lipases was initially based on a 1971 paper in which Marples and colleagues showed that a 99% reduction in the *P. acnes* population on adult forehead skin corresponded to a 50% decrease in FFA content, thus establishing that *P. acnes* (formerly known as *Corynebacterium acnes*) plays a significant role in regulating FFA content on human skin (79). Follow-up studies have identified the *P. acnes* TAG lipases (68, 80, 81). However, the findings of our study and others are consistent with lipases from *C. accolens* and other skin microbes also playing an important role in liberating FFAs from SSLs, e.g., *Staphylococcus epidermidis* and *S. aureus* both produce TAG lipases (45, 66, 67). Interestingly, some strains of *P. acnes* (82) and *S. aureus* (45) are also susceptible to the toxic effects of some FFAs released from human sebum and skin surface TAGs, which might limit the role of these bacteria in FFA release.

We speculate that the ability of *Corynebacterium* spp. to release FFAs from host surface TAGs is especially important in populations and at sites where *Propionibacterium* spp. are less abundant than *Corynebacterium* spp., such as skin surfaces of prepubertal children (18, 21). For example, the genus *Corynebacterium* is the most abundant member of the *Actinobacteria* in the microbiota of the pediatric nostril (18, 21) and of the pediatric nasopharynx, a mucosal surface (20, 41); although communities at both sites are

dominated by the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. In these settings, *Corynebacterium* spp. might play an important role, along with host lipases, in regulating epithelial surface FFA content.

In contrast to prepubertal children, the nostril microbiota of adults under 65 years of age does not commonly contain pneumococcus and is often dominated by the phyla *Actinobacteria*, in particular *Corynebacterium* and *Propionibacterium* spp., and *Firmicutes*, in particular *Staphylococcus* spp. (21–31). This composition appears to carry over into the mucosal surfaces of the nasal cavity and nasopharynx (29, 83, 84), although the mucosal surface of the nasal cavity seems to host a greater diversity of bacterial genera than the nostrils (29). These observations raise the possibility that increased sebum production beginning with puberty (85) gives an advantage to microbes in the nostrils, and on other oily or moist skin sites, that both secrete TAG lipases and are relatively resistant to FFAs, which would fit with bacterial community composition at these sites in adults (21). It also raises the possibility that topical application of TAGs in circumstances where TAG production is naturally lower might sculpt a microbial community dominated by *Corynebacterium* and/or *Propionibacterium* spp.

In addition to impacting *S. pneumoniae* directly, the *C. accolens* release of FFAs might impact the host immune system, leading to indirect effects on the composition of the bacterial microbiota. For example, using a human sebocyte cell culture line, Gallo and colleagues show that lauric, palmitic, and oleic acids increased expression of the human beta-defensin-2 gene (hBD-2). The hBD-2 antimicrobial peptide (AMP) is found on skin (86) and is antibacterial toward pneumococcus (87), suggesting an additional indirect mechanism by which release of FFAs might contribute to protection against *S. pneumoniae* colonization.

Treating the surfaces of human body sites as individual ecosystems implies the existence of a complex network of both microbe-microbe and microbe-host interactions (42). Based on our findings, we predict that on human surfaces *C. accolens* functions as a mutualist, and not merely as a commensal, by altering the local host surface environment in a manner that can partially shape the composition of nasal and skin microbiota and by helping to protect against colonization by pathobionts, such as *S. pneumoniae*. This interaction is part of a growing list of examples of skin/nasal commensals impacting pathobiont growth/behavior (73, 88–90), and the outcome of this interaction could have important implications for the human host. We predict that a greater understanding of the molecular mechanisms underlying commensal-pathobiont interactions, in particular those that occur between *Corynebacterium* spp. and pathobionts belonging to the phylum *Firmicutes*, will facilitate the development of alternative approaches to interfere with pathobiont colonization and/or infection.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids are listed in Table S2 in the supplemental material, and additional growth information is in Text S1 (a further description of our study's materials and methods) in the supplemental material. As the base medium for all coculture assays, 10-ml aliquots of BHI agar containing 200 U/ml of bovine liver catalase (BHIC; EMD Millipore, Billerica, MA) was added to 100-mm by 15-mm petri dishes. Calculation for the addition of catalase was based on the following spec sheet information: activity of ≥5,000 U per mg dry weight. On the day of use, one of the following was spread onto the agar medium: 50 μl of 100-mg/ml glyceryl trioleate (triolein; catalog number T7140;

Sigma-Aldrich) solubilized in chloroform; 50 μl of 100-mg/ml glyceryl trilinoleate (trilinolein; catalog number T9517; Sigma-Aldrich) emulsified in ethanol; 50 μl of chloroform or 50 μl of ethanol. (Sapienic acid is abundant on human skin [61], but we were unable to locate a commercially available TAG containing sapienic acid.) For consistent results, we observed that it is best to use agar medium that has been prepared within 1 to 2 days of use.

Ethics statement. Under a protocol amendment approved by the Harvard Pilgrim Health Care Human Studies Committee, and with approval from the Forsyth Institute Institutional Review Board, nasopharyngeal swabs were analyzed from children >6 months and <7 years of age. These samples were collected as part of an ongoing study of *S. pneumoniae* colonization, as described in references 16, 17, 38, and 39.

Identification of pediatric nasopharyngeal bacterial microbiota. We received a randomly selected subset of ~200 of the ~1,000 nasopharyngeal swabs (calcium alginate) collected from October 2008 to April 2009 as part of a study of pneumococcal colonization in Massachusetts (16, 17, 38). For each swab, we received information on the age group (<2 years or 2 to ≤7 years) of the participant and whether the swab was positive for *S. pneumoniae* by cultivation. Swabs had been originally shipped overnight and inoculated onto sterile agar medium and then stored in STGG (skim-milk, tryptone, glucose, and glycerol) with 10% glycerol at −70°C. In 2010, to harvest DNA, swabs were thawed, removed from the storage medium, and aseptically cut from the remaining wire applicator into a PowerSoil tube. To maximize recovery of bacterial cells, the storage medium was then centrifuged at 17,000 × g for 10 to 20 min, after which most supernatant was discarded, leaving behind 50 μl. The remaining 50 μl was vortexed to resuspend any bacterial cells before adding 45 μl to the PowerSoil tube containing the swab. DNA was harvested with the MoBio PowerSoil DNA isolation kit per the Human Microbiome Project protocol (section 7.8.2) (28) with minor modifications, including bead beating in a FastPrep24 for 30 s at 5 m/s for more rapid cell lysis.

From the ~200 DNA samples, we selected a representative subset of 45 that had been amplified by PCR with ARISA primers 1406F-FAM and 125R (91). We submitted these, along with the negative control of a processed sterile swab, for 16S rRNA gene V1-V3 titanium pyrosequencing at the Broad Institute (Cambridge, MA). Sequencing and processing from raw sequences to an OTU table was done as described in reference 92. Twenty-seven samples, of which nine were culture-positive for pneumococcus, had >1,000 high-quality sequences each (minimal number of sequence/sample, 1,503). After filtering in QIIME (93) (using the MacQIIME 1.4 package), first to remove samples with <1,000 sequences and second, to remove OTU sequences that were not present more than once in one sample, sequences were subsampled to the minimum of 1,503 reads/sample. Data from these 27 samples were analyzed using linear discriminant analysis effect size (LEfSe) (40) with pneumococcal colonization by cultivation as class and age group (<2 years versus 2 to <7 years) as the subclass. Sequences (pre-chimera check) are accessible in the short read archive (SRA) under biosample accessions SAMN04323356 through SAMN04323382.

Chemical structures. Chemical structures were produced using ChemDraw Professional version 15 (PerkinElmer).

Growth assays. *C. accolens* KPL1818 cells were transferred from BHI Tw80 agar medium into sterile 1× phosphate-buffered saline (PBS) and vortexed for 2 min. Cells were diluted to an optical density at 600 nm (OD$_{600}$) of approximately 0.3 to 0.4, 25 μl of which was added to 25 ml BHI broth and vortexed. Aliquots of 5 ml were then dispensed into 15-ml Falcon tubes (Corning Life Sciences) to ensure an equal inoculum for each biological replicate. We then added 5 μl of one of the following to each tube: 100 mg/ml of triolein in chloroform, 100 mg/ml of trilinolein in ethanol, chloroform, or ethanol, resulting in a final concentrations of 112.9395 μM and 113.7165 μM for triolein and trilinolein, respectively. The tubes were incubated aerobically at 37°C with shaking at 200 rpm. Sterile medium controls for all four types of final media were included. Following 48 h of incubation, the OD$_{600}$ was recorded using sterile BHI

without additives as a reference medium. After confirming that the sterile medium controls were not turbid, measurements of BHI containing TAG were used to correct the OD_{600} values for the corresponding growth tube, since the TAGs have slight absorbtion at OD_{600}. Data represent nine biological replicates carried out on four separate days. Analysis of variance (ANOVA) was performed, and *P* values were adjusted for multiple testing by using Tukey's test.

Side-by-side cocultivation assay. *C. accolens* cells were resuspended in 1× PBS to an OD_{600} of 0.3 to 0.4, after which a 5-μl aliquot was inoculated onto the appropriate solid medium (see above). *C. accolens* was then grown for ~42 h at 37°C, after which a 5-μl suspension of *S. pneumoniae* in 1× PBS (OD_{600} of 0.3 to 0.4) was inoculated adjacent to *C. accolens*. We also developed the alternative membrane-based assay for *C. accolens* excretion of antipneumococcal compounds (described in the materials and methods of Text S1 in the supplemental material). For both assays, to show that in the absence of *C. accolens* BHI supplemented with triolein, trilinolein, chloroform, or ethanol supported pneumococcal growth, *S. pneumoniae* was inoculated onto each medium alone that had been prepped in exactly the same manner. Plates were held at room temperature for ~4 to 6 h after pneumococcal inoculation and then incubated overnight at 37°C with 5% CO_2; this minimized pneumococcal autolysis prior to observation and photographic documentation of results.

Methanol extracts of *C. accolens* CFCM and sterile medium controls. We used LB SeaKem LE agarose (Lonza) spread with triolein for methanol extraction experiments. Triolein is not soluble in aqueous solution, so 50 μl of triolein (100 mg/ml in chloroform) was added to each plate and spread with a sterile spreader to generate as homogenous a layer as possible. Plates were dried for ≥30 min before use to ensure evaporation of residual solvent. After drying, a sterile, 0.2 μm, 47-mm-diameter polycarbonate membrane (EMD Millipore) was placed atop each agarose plate using sterile tweezers. Using a sterile swab, freshly grown *C. accolens* KPL1818 cells were transferred from BHI Tw80 agar to the membrane and a lawn was spread. The membrane permits passage between cells and medium of nutrients, waste products, and secreted compounds/enzymes. Agarose plates were incubated for 72 h at 37°C, after which the membranes (and cells) were removed and discarded. After incubation with or without cells, agarose medium was moved to a sterile glass bottle to which 75 ml of high-performance liquid chromatography/Optima-grade methanol (Fisher Scientific) was added. The bottles were gently agitated and placed at 4°C overnight (~17 h). Following extraction at 4°C, the methanol was decanted into 50-ml Falcon tubes and spun at 5,000 × *g* for 10 min to pellet any residual agarose or debris. A single, 1-ml aliquot of each clarified methanol extract was reserved for LC/MS analysis. The remainder of each methanol extract was concentrated using a Büchi Rotavapor R-215 to a volume of ≤0.5 ml, adjusting the vacuum pressure as needed to maintain a relatively constant evaporation rate. Sterile medium controls were incubated alongside the experimental plates and processed the same to demonstrate that FFAs were not abundant in unconditioned medium. The approximate "fold concentrate" of each extract was calculated by dividing the starting volume of agar (25 ml) by the remaining volume of methanol extract (e.g., 25 ml agarose/0.5 ml methanol extract = 50× concentrated).

Disk-diffusion assays with methanol extracts. Aliquots of 20 μl of an ~50×-concentrated extract were assayed for antipneumococcal activity. Methanol was added to bring the final volume of each aliquot to 20 μl. Each extract was then placed on a 6-mm sterile filter disk, dried for ≥20 min, and then placed on a freshly plated lawn of *S. pneumoniae* 603 on BHIC agar medium. Lawns were prepared by transferring pneumococcal cells with a sterile swab from CNA agar onto BHIC agar. For consistency, the BHIC medium was prepared 2 days prior to use by dispensing 10-ml aliquots of medium into 100-mm by 15-mm petri dishes. Plates were incubated overnight at 37°C in a 5% CO_2 incubator overnight. The ZOI was measured as the smallest diameter of the area of no growth. If a ZOI was not uniform, the shortest diameter of no growth was reported.

LC/MS analysis of methanol extracts. Methanolic extracts were initially analyzed using a ThermoFinnigan Quantum electrospray ionization triple-quadrupole mass spectrometer operating in negative-ion mode. Data were analyzed by using Xcaliber 2.0.7 software (Thermo Scientific). Extracts from unconditioned medium, and cell-free conditioned media (CFCM) were introduced by direct line infusion for several minutes for each and the limited mass spectrum from *m/z* 50 to 500 recorded continuously. The spectrum of unconditioned medium was subtracted from the spectrum for CFCM using the Xcaliber software to identify peaks in the spectrum unique to CFCM. CFCM and unconditioned medium from four different preparations were analyzed by this method, and the resulting subtracted spectra were highly similar. High-resolution mass analysis of CFCM was performed on a ThermoScientific Orbitrap XL mass spectrometer operating in negative-ion mode and equipped with an IonMax source housing, a standard electrospray ionization probe, and a 50-μm-inner diameter high-flow metal needle kit. The Orbitrap was calibrated in negative mode over a mass range of *m/z* 265 to 1,780 by using a mixture of SDS, taurocholic acid, and Ultramark-1621 (Thermo-Pierce). Resolution at *m/z* 500 was 30,000 and the maximum injection time was 100 ms. For determination of the concentration of oleic acid in methanolic extracts, 3.7 nmol of heptadecanoic acid (HdA; a $C_{17:0}$ fatty acid) was added to 60 μl of extracts or authentic synthetic oleic acid and then analyzed via LC/MS. Ultraperformance liquid chromatography was performed with an Acquity UPLC apparatus (Waters) coupled to a Quantum mass spectrometer operating in multiple reaction mode monitoring transition of *m/z* 281 to *m/z* 281 at 10 eV for HdA and *m/z* 269 to *m/z* 269 at 10 eV for oleic acid, respectively. The volume of injection was 10 μl. Mobile phase A was 1 mM triethylammonium acetate in water, and mobile phase B was 0.1% acetic acid in methanol. Extracts were chromatographed on a Kinetex C_8 column (50 by 2.1 mm; 2.6 μm; Phenomenex) with a constant flow of 250 μl/min. After a 0.5-min hold at 1% mobile phase B, the solvent gradient was ramped to 99% B over 2 min, then held at 99% B for another 2.5 min, and returned to 1% B over 1 min and held at 1% B for 2 min prior to starting the next injection. The concentration of oleic acid was calculated from the ratio of the peak area for the *m/z* 281 peak at 3.58 min to the peak area for the *m/z* 269 peak at 3.60 min, multiplied by 3.7 nmol (amount of internal standard added), and divided by 60 μl (volume of extract used). In comparing the concentrations of oleic acid detected in CFCM versus sterile medium controls, we considered the control and the experimental sample as paired samples, to account for any possible batch effects of media that were prepared on separate days. We analyzed these data with a two-tailed, paired *t* test (at a *P* level of <0.05).

Fatty acid susceptibility. We purchased FFAs from Sigma-Aldrich, except for sapienic acid, which was purchased from Matreya (Pleasant Gap, PA). For each FFA, 10 μl of a stock solution of 10 mg/ml in 200-proof ethanol (100 μg total) was pipetted onto a sterile, 6-mm filter disk (Whatman). The control in each experiment was a disk spotted with 10 μl of 200-proof ethanol. Disks were dried for ≥40 min prior to placement on a freshly plated lawn of *S. pneumoniae* prepared and incubated as described above. The ZOI was measured and documented as described above. For consistency, 25-ml aliquots of the BHIC medium were dispensed into 100-mm by 15-mm petri dishes.

Construction of an in-frame deletion of *lipS1*. Regions extending approximately 1 kb adjacent to the 5′ and 3′ ends of the putative TAG lipase gene, from nucleotides 281230 to 282573, encoded in the KPL1818 genome (nucleotide GenBank accession AXMA01000001.1), were PCR amplified from *C. accolens* KPL1818 genomic DNA with primers designed such that the 5′ fragment ended with a 21-bp random scar sequence and a GC content of 54% and the 3′ fragment started with the same 21-bp scar sequence, followed by the stop codon (oKL516-519 [see Table S3 in the supplemental material]). After digestion with PstI followed by treatment with calf intestinal phosphatase for 1 h each at 37°C (both NEB), pJSC232 (94), which replicates in *Escherichia coli* and not in *Corynebacterium* spp., was cleaned up by using the QIAquick PCR purification kit. The two PCR fragments were purified, assembled with PstI-opened pJSC232 by Gibson

assembly (95), and then transformed into NEB 5-alpha competent *E. coli* cells according to the manufacturer's instructions. Clones on LB-kanamycin plates were screened using primers flanking the insertion site (oKL520-521 and also oKL516-519 [Table S3]). The resulting pJSC232-derived deletion plasmid (pSB121) was purified and verified by PCR and restriction enzyme digestion. Competent cells were prepared, and pSB121 was introduced into *C. accolens* KPL1818 by electroporation as previously described (96), except that the growth temperature was 37°C and all media contained 1% Tw80 (see Text S1 in the supplemental material for more details regarding our materials and methods). Transformants that had integrated the nonreplicating plasmid in the genome by a single homologous recombination event were selected using the kanamycin resistance marker on the vector backbone. Kan-resistant transformants were then grown nonselectively in BHI-sorbitol and subsequently plated on BHI agar–1% Tw80 with 5% sucrose, which should permit growth of only clones that have lost the *sacB*-containing vector backbone (i.e., a negative selection marker for sucrose sensitivity) by a second homologous recombination event. Successful deletions were identified by PCR with primers oKL522 and oKL523 (see Table S3) that anneal outside the regions of homology used in the deletion construct, and correct deletion was verified by sequencing (Macrogen USA).

Construction of the *lipS1* complementation vector pLB502. The complementation vector pLB502 was constructed using the Gibson assembly method and the *Corynebacterium* spp.-*E. coli* shuttle vector pCGL0243 (97) as detailed in Text S1 in the supplemental material. To ensure that the promoter region was included, we cloned 949 bp upstream of the predicted start codon listed with NCBI, along with the predicted ORF for *lipS1*. In addition to the ORF, a His_6 tag fused to the C terminus was introduced in-frame immediately downstream of the ORF, followed by the stop codon (see Fig. S5 in the supplemental material). Gibson reactions were transformed into *E. coli* NEB 5-alpha cells, transformants were selected on LB agar containing 40 μg/ml of kanamycin, and Kan-resistant transformants were screened by PCR with primers oKL381 and oKL382 (see Table S3 in the supplemental material), which amplify the multiple-cloning site in pCGL0243, to detect candidates harboring pLB502. A single PCR-positive clone was inoculated into LB broth with kanamycin and grown at 37°C overnight with shaking at 200 rpm. Plasmid (pLB502) purified from this culture (or cultures from the frozen stocks) was sequenced to verify the correct insert (see Table S3 for primers).

Complementation of the *lipS1*-deficient mutant strain. Plasmids pCGL0243 and pLB502 were transformed separately into both wild-type *C. accolens* KPL1818 and an isogenic *lipS1*-deficient mutant (KPL2503), generating the following strains: KPL2489, KPL2478, KPL2504, and KPL2505 (see Table S2 in the supplemental material). Electroporations were performed using one of two methods: (i) as described above with the exception that transformants were selected for on BHI Tw80 sorbitol plates with 20 μg/ml kanamycin, or (ii) as described above with the exceptions that heat shock was not performed, the outgrowth medium did not contain sorbitol, and transformants were selected on BHI Tw80 with 20 μg/ml kanamycin. With both electroporation methods, Tw80 was used to support the growth of lipid-requiring *C. accolens*, because FFA can be liberated from Tw80 by an esterase. Successful transformation of *C. accolens* with each plasmid was verified by PCR amplification of the plasmid's multiple-cloning site (oKL562 and oKL563 [see Table S3 in the supplemental material]).

Growth of *C. accolens* KPL1818 mutants with triolein or Tw80 as the sole source of fatty acid. Colonies of each strain were resuspended separately in sterile 1× PBS to an OD_{600} of 0.3 prior to inoculating 5 μl of the suspension into 5 ml of BHI broth supplemented with 5 μl of a 100-mg/ml triolein solution or 1% Tw80; medium for strains with pCGL0243 or pLB502 included 20 μg/ml kanamycin. Cultures were grown at 37°C with shaking (200 rpm), and the OD_{600} was measured after 48 h. Each independent growth experiment ($n = 3$) included a sterile medium control. Data comparing growth of the LipS1$^-$ mutant and the wild-type strain were analyzed using a Welch two-sample *t* test. Data comparing growth of strains carrying pCGL0243 or pLB502 were analyzed via an ANOVA and Tukey's test when applicable.

Statistical analyses. As described above, ANOVA and *t* tests, with corrections for multiple comparisons if applicable (Tukey test), were performed using R version 3.2.0 (RStudio version 0.98.1103) and GraphPad Prism version 6.01 (La Jolla, CA). Figures were plotted in R, and indicators of significance (asterisks and bars) were added using Inkscape version 0.91.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01725-15/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.02 MB.
Figure S2, PDF file, 1.5 MB.
Figure S3, PDF file, 0.03 MB.
Figure S4, PDF file, 0.02 MB.
Figure S5, PDF file, 1.4 MB.
Table S1, PDF file, 0.04 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.05 MB.

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