SUPPLEMENTAL METHODS

Bacterial cultivation. *C. accolens* strains were revived from glycerol stocks on Brain Heart Infusion agar (BHI, BD Diagnostic Systems, Franklin Lakes, NJ) containing 1% Tween 80® (Tw80; Sigma-Aldrich, St. Louis, MO) and incubated aerobically at 37°C for ~2 days prior to starting each assay. *Streptococcus pneumoniae* strains were revived from glycerol stocks on Columbia CNA agar with 5% Sheep Blood (BD Diagnostic Systems) and incubated aerobically at 37°C with 5% CO_2 overnight prior to starting each assay. *E. coli* strains were grown at 37°C overnight using either lysogeny broth (LB) or LB agar. Kanamycin concentrations were 40 μg/ml or 50 μg/ml for *E. coli* and 15 μg/ml or 20 μg/ml for *C. accolens*. For preparation of BHI broth or BHI agar, the BHI portion (and any additives) was filtered through 0.2 micron filters, prior to use or prior to mixing with autoclaved agar (if solid medium).

Phylogenetic analysis and assignment of *Corynebacterium sp*. KPL 1818 as *C. accolens* KPL1818. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (1). In brief, partial *rpoB* sequences were generated in silico using primers C2700F and C3130R which allow species identification of *Corynebacterium* spp. (2). Primers C2700F and C3130R were aligned on the *C. accolens* CIP 104783 *rpoB* sequence in SnapGene viewer 2.8.2 (GSL Biotech, Chicago, IL). The resulting 446 bp top strand sequence (beginning with forward primer C2700F) was used to identify corresponding *Corynebacterium* spp. sequences in the NCBI nucleotide collection with BLAST (3). Sequences of the following *Corynebacterium* spp. were selected from the first 100 hits*: accolens* (*n*=3), *maginleyi,*

striatum, aurimucosum, minutissimum, singulare, phocae, propinquum, pseudodiphtheriticum, mastitidis, testudinoris, vitaeruminis, spheniscorum, glutamicum, diphtheriae, ulcerans, riegelii, thomssenii and *sundsvallense*. The KPL1818 corresponding sequence was retrieved by using the whole-genome sequence contigs database with limitation by *Corynebacterum* spp. *D. cinnamea* P4 was used as an outgroup. All sequences were imported into MEGA and were aligned by ClustalW using default parameters. Phylogeny reconstruction was done using Maximum likelihood method with 1000 bootstrap replications.

Partial sequencing of *rpoB* was also used to verify the genetic background of all constructed complementation mutants and the wild-type strain KPL1818. PCR and sequencing of the *rpoB* gene was performed using primers C2700F and C3130R (2). The PCR reaction mixture contained 25 µl of 2x GoTaq Green Master Mix (Promega, Madison, WI) and 0.5µM final concentration of each primer (Integrated DNA Tech Inc., Dallas, TX) in a total volume of 50 µl. Bacteria for colony PCR were inoculated directly into the mastermix from an agar plate using a sterile tip. The following cycling conditions were used: primary denaturation and enzyme activation for 2 min at 95°C, followed by 30 cycles of 98°C for 30 s, 56°C for 30 s, 72°C for 2 min and ending with a final extension for 10 min at 72°C. PCR products were cleaned up using QIAquick PCR Purification Kit (Qiagen) after verification of correct band size on a 0.8% agarose gel prior to Sanger-sequencing.

Membrane-based cocultivation assay. We also developed a simpler assay to test for *C. accolens* excretion of antipneumococcal compounds in which the medium is conditioned by growth of *C. accolens*, which is then removed prior to inoculation of *S.*

pneumoniae. Using a sterile swab, *C. accolens* cells were transferred from a BHI Tw80 plate onto a 0.2 micron, 25 mm polycarbonate membrane filter (EMD Millipore) placed atop of the coculture medium. Following ~42 h incubation at 37°C, the cells and filters were removed and a *S. pneumoniae* lawn was plated by transferring cells from a CNA agar plate with 5% sheep blood using a sterile swab. The lawn was incubated as described for the side-by-side assay.

LB agarose preparation. We used LB agarose spread with triolein for methanol extraction experiments because of concern that crude extracts of sterile BHI agar might contain antibacterial activity. We autoclaved 2x (3%) SeaKem® LE agarose (Lonza) then cooled it to 50-60°C. Meanwhile, 2X LB broth (20g/L tryptone, 20 g/L NaCl, 10 g/L yeast extract) was prepared, filter sterilized and warmed to 50-60°C. Sterile 2x agarose and sterile 2x LB broth were mixed and a serological pipette was used to dispense 5 ml aliquots of LB agarose to 60 mm x 15 mm petri dishes. Following at least overnight of drying, triolein was spread on top of the agar.

Conserved Domain Database (CDD) Analysis. The *C. accolens* KPL1818 genome (RefSeq accession NZ_AXMA00000000; in FASTA format for protein) was queried against the CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) using the Batch-CD Search function. We performed two separate 'Live' searches, one of the Pfam database and one of the COG database. The search parameters were as follows: Expect value Threshold was set to 0.01, maximum number of hits was set to 500 and retired sequences were included. This analysis was most recently repeated on August 26, 2015 using a version of the KPL1818 genome downloaded on the same date.

***C. accolens* electroporation.** In brief, 150 µl of competent bacterial cells were thawed on ice and were mixed with 1-1.5 µg of plasmid DNA in a 0.2 cm gap Gene Pulser cuvette (BioRad, Hercules, CA). After electroporation at 25 µF, 200 Ω, and 2.5 kV cells were resuspended in BHI broth with 9.1 g/100ml sorbitol and heat shocked at 46°C for 6 min. After growth for 1 h at 37°C, cells were plated on BHI Tw80 sorbitol plates containing 15 µg/ml kanamycin.

Construction of pLB502. A fragment including 949 bp upstream of the predicted start codon listed at NCBI along with the predicted ORF for *lipS1* was amplified from *C. accolens* KPL1818 genomic DNA using the primers oKL508 and oKL509 and Phusion® Hi-Fidelity DNA polymerase (NEB, Ipswich, MA) following the manufacturer's instructions. The oKL508-509 **(Table S3)** PCR product was purified using the Qiagen PCR Purification Kit and then used in Gibson assembly along with *Sal*I-digested, CIP-treated pCGL0243 (*Corynebacterium spp. – E. coli shuttle vector)*. Following 1 h incubation, 5 µl were transformed into NEB 5-alpha cells following the manufacturer's instructions. Five µl of a Gibson assembly, which included the above components except for the purified *lipS1* PCR product, was also transformed into *E. coli,* as a negative control.

Standard molecular biology. PCR products used for cloning were purified using the QIAquick PCR Purification Kit. Plasmids used for transformation and/or as sequencing templates were purified using using QIAprep Spin Miniprep Kit (Qiagen).

REFERENCES

1. Tamura K, Stecher G, Peterson D, Filipski A, & Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30(12):2725-2729. doi:10.1093/molbev/mst197
2. Khamis A, Raoult D, & La Scola B (2004) *rpoB* gene sequencing for identification of *Corynebacterium* species. *J Clin Microbiol* 42(9):3925-3931. doi:10.1128/JCM.42.9.3925-3931.2004
3. Altschul SF, Gish W, Miller W, Myers EW, & Lipman DJ (1990) Basic Local Alignment Search Tool. *J Mol Biol.* 215(3):403-410.