

# Isolation of novel probiotic Clostridiales from the chicken GI tract that have anti-pathogen activity

B.B. Oakley<sup>1\*</sup>, R. Chalghoumi<sup>2</sup>, J.K. Garrish<sup>3</sup>, J.E. Line<sup>3</sup> & B.S. Seal<sup>4\*</sup>

<sup>1</sup>College of Veterinary Medicine, Western University of Health Sciences, Pomona, CA 91766 USA; \*boakley@westernu.edu

<sup>2</sup>University of Carthage, College of Agriculture - Mateur, Department of Animal Science, Tabarka Road, 7030 Mateur, Bizerte, Tunisia

<sup>3</sup>USDA Agricultural Research Service, Richard B. Russell Agricultural Research Center, 950 College Station Road, Athens, GA, 30605, USA

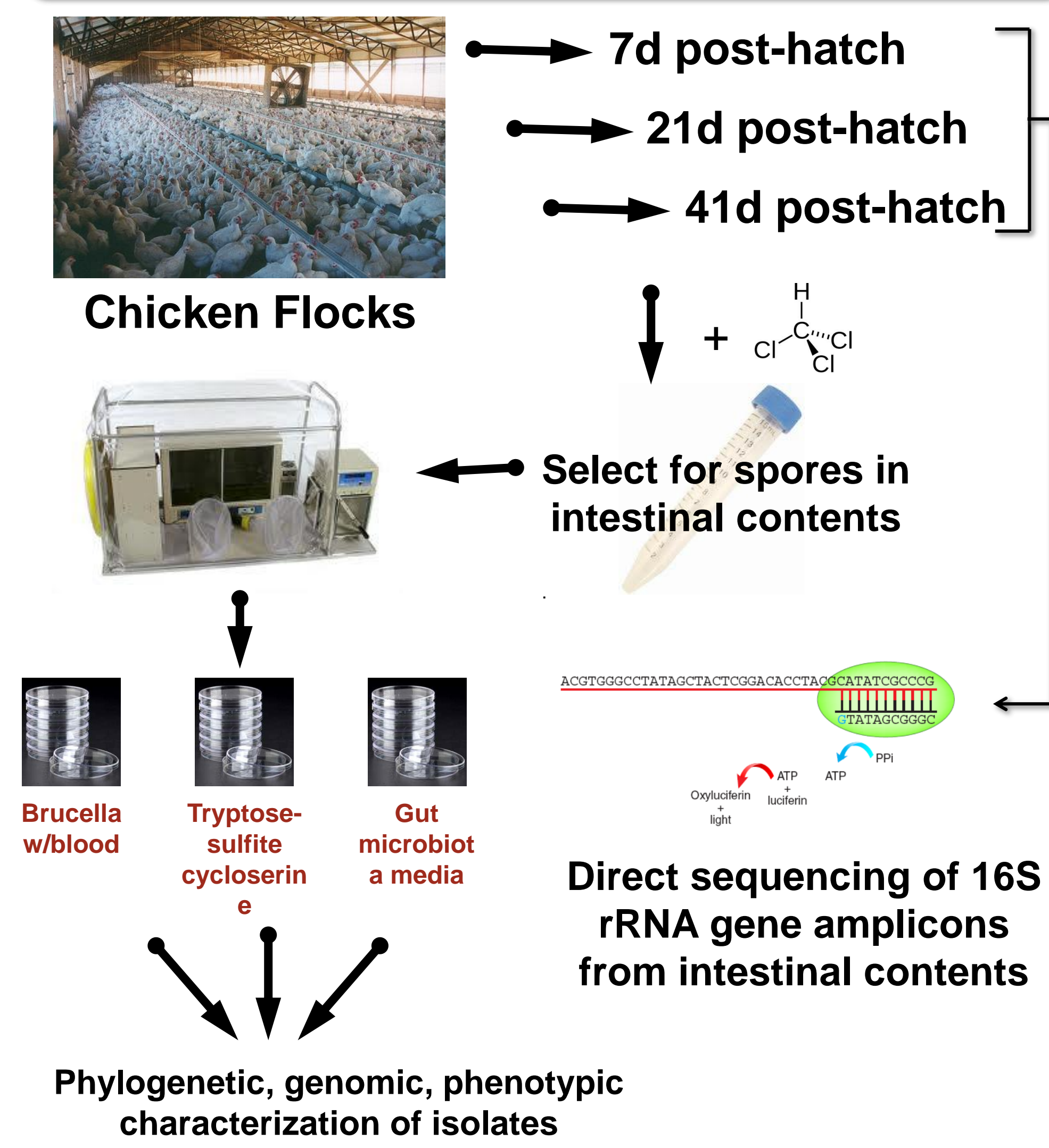
<sup>4</sup>Biology Program, Oregon State University Cascades Campus, Bend, OR 97702 USA; \*sealb@oregonstte.edu

## ABSTRACT

The removal of antibiotics during food-animal production will require development of new strategies to maintain animal health, optimize nutrition and control pathogens. Utilizing naturally occurring bacteria as probiotics has several advantages including viability, ability to compete against ecologically similar taxa and a natural rationale for regulatory approval. Indigenous species of non-toxin producing, anaerobic bacteria of the Clostridiales promote anti-inflammatory immune responses in the mammalian gut by activating T-regulatory cells and these bacteria make up a large proportion of the monogastric animal intestinal microflora. Our hypothesis is that selecting spore-forming, non-toxin producing bacterial taxa closely related to known pathogens offers potential for competitive exclusion of pathogenic bacteria. Consequently, three percent chloroform treatment of chicken gastrointestinal contents was completed for one hour to remove vegetative bacterial cells followed by anaerobic culture of surviving spores. Approximately 40 axenic isolates were obtained that were distinct from one another morphologically and phenotypically based on Gram staining and sulfide production. Moreover, by genomic analyses some of the strains represent novel species based on the standard of less than 97 percent similarity comparing the full-length 16S rRNA gene sequences to their known closest cultured representative species, primarily Clostridia. Growth reduction utilizing lawns of *Clostridium perfringens*, *C. septicum* and *C. difficile* was demonstrated by zones of inhibition produced by eight cecal and five mid-gut newly identified isolates when placed on the respective pathogen lawns. At this time the antimicrobial mechanism is unknown, although several interesting genes such as potential prophage holins and endolysins were identified by sequencing genomes of the newly obtained isolates. Results from these investigations further demonstrate that newly identified, potential probiotic bacterial cultures can be isolated and identified for future use to improve animal and human health.

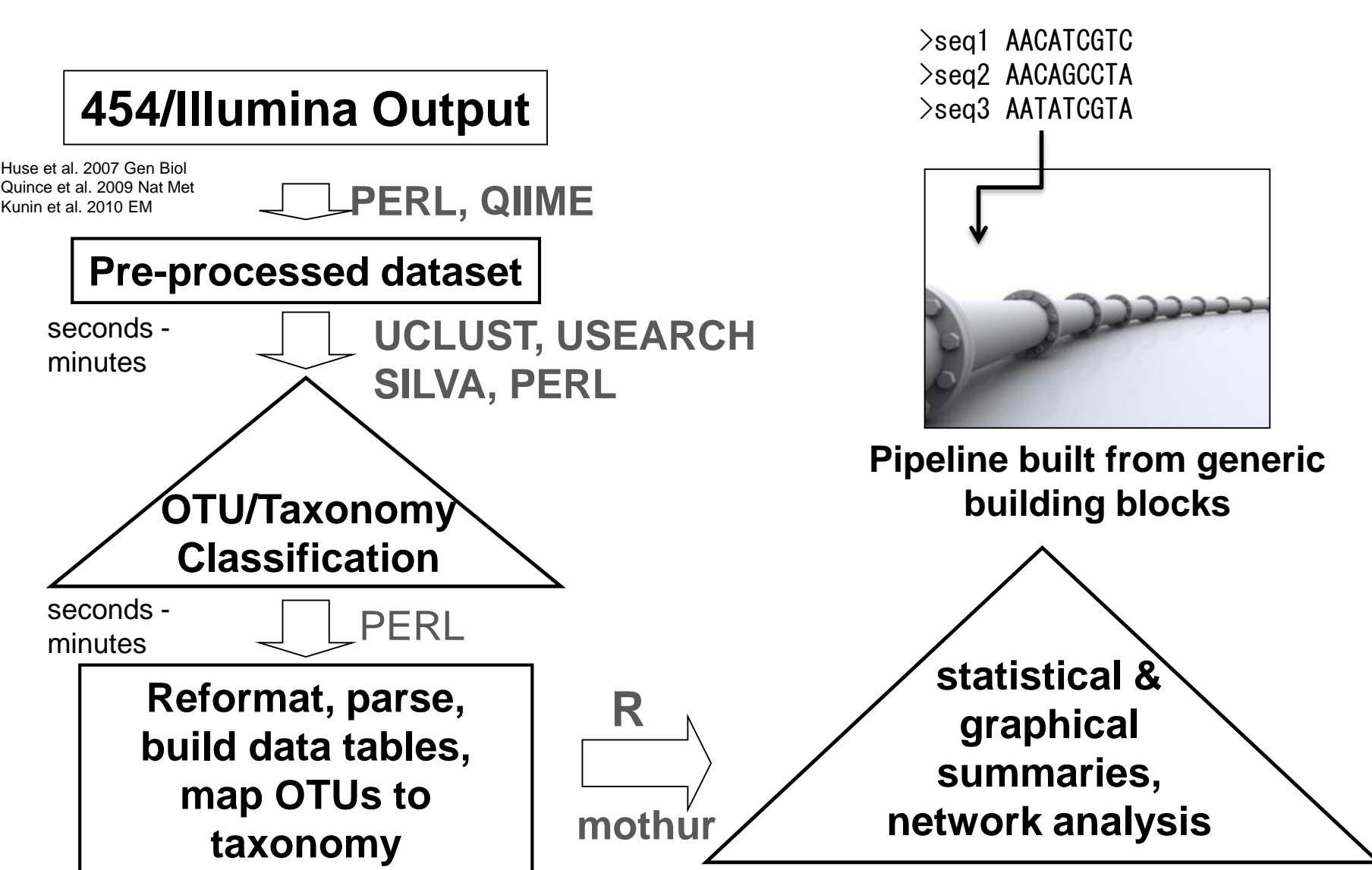
Furthermore, two axenic cultures selected for further characterization belonged to the genus *Clostridium* and represent potentially novel species within Cluster 1 based on full-length 16S rRNA gene sequences. Phylogenetic reconstruction of cpn60 (pfam00118) showed c2-6 in a clade with *C. saccharoperbutylacetonicum* and *C. beijerinckii*, and c1-8 most similar to the chitinolytic *C. paraputrificum*, *C. celatum*, and *C. butyricum*. Genome sequencing of these two strains (N50 values of 129255 kb and 470109 kb respectively) has revealed several interesting characteristics including several bacteriophage holin and endolysin genes. Strain c1-8 contains an annotated bacteriophage endolysin with significant similarity to chitinases, endopeptidase, and R-type bacteriocins or "diffocins" that may be alternate bactericidal agents. Phenotypic characterizations of isolates revealed anti-microbial activity against *C. perfringens*, *C. septicum*, and *C. difficile*.

## EXPERIMENTAL OUTLINE



Genomic DNA was sequenced on an Illumina MiSeq instrument with PE250 reads and data processed using standard genomics tools including trimmomatic, the fastx toolkit, flash, assembly with Velvet and Spades, and annotation with RAST.

## ANALYTICAL PIPELINE FOR MICROBIOME DATA



Oakley et al. 2012 ISMEJ  
Schloss et al. 2009 AEM

## RESULTS

### Microbiome composition

Surveys of poultry GI microbiome show communities dominated by Clostridiales

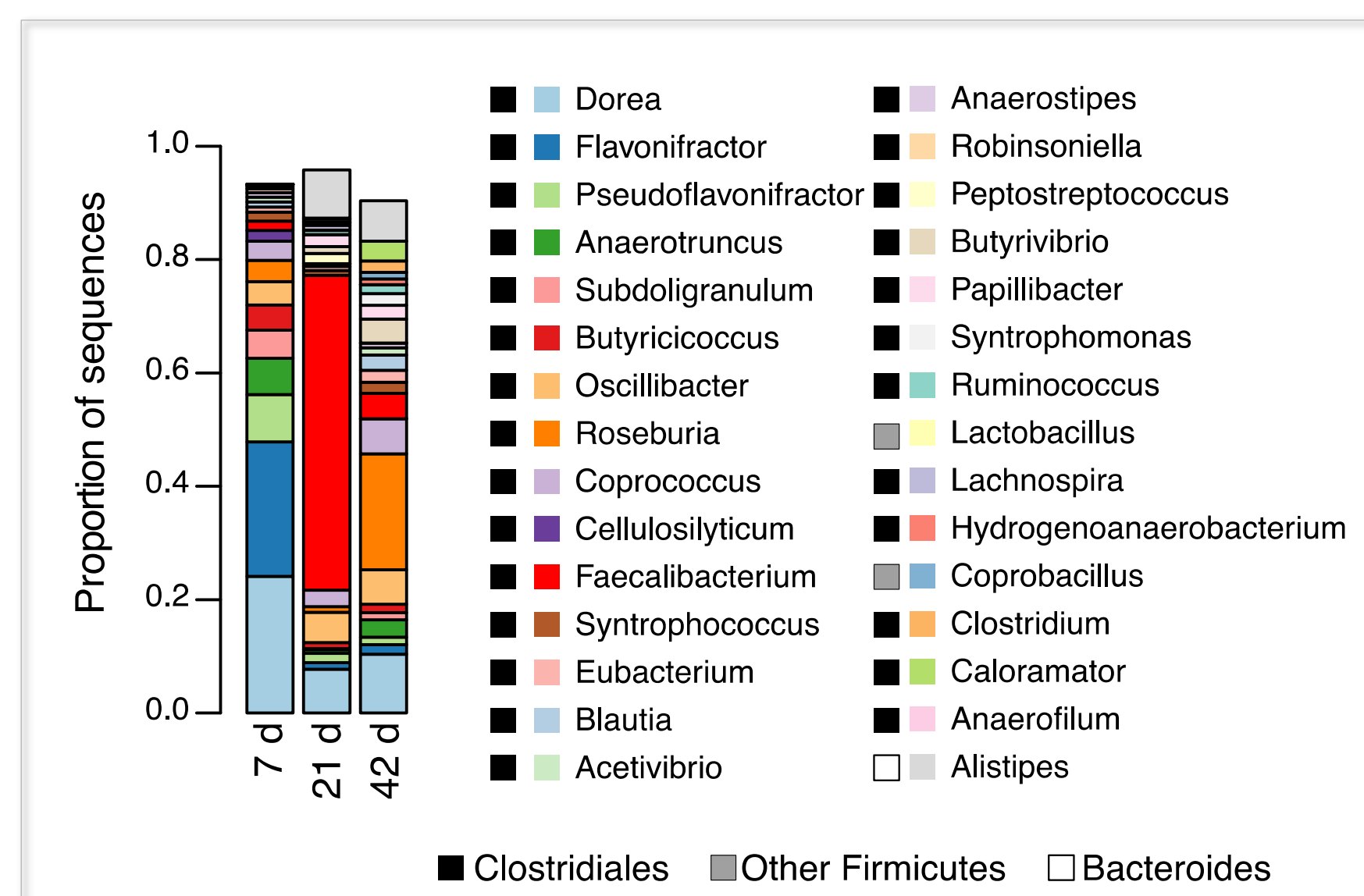


Figure 1. Typical taxonomic composition of chicken cecal microbiome at 1 wk, 3 wk, and 6 wk post-hatch. Sequences were classified against the v115 of the Silva database using usearch; use of the RDP classifier produced essentially similar results with minor differences in nomenclature. Only sequences with a total relative abundance greater than 5% are shown.

## RESULTS

### Isolation of spore-forming bacteria

Approximately 40 axenic isolates obtained.

Several strains may represent novel species based on low (<97%) similarity of 16S rRNA sequences to closest cultured representative.

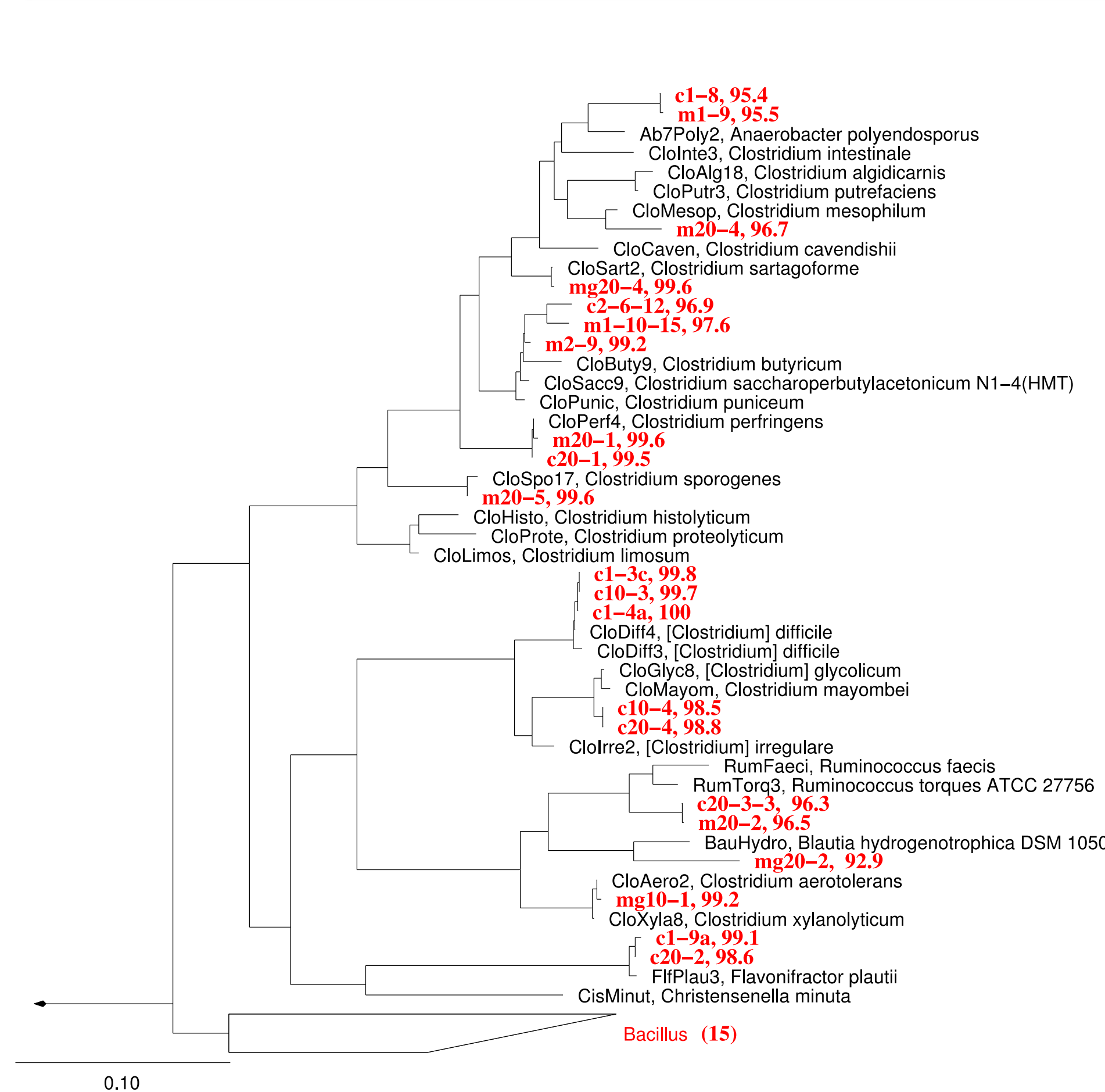


Figure 2. 16S rRNA-based phylogeny of newly-isolated Clostridia. Tree represents maximum-likelihood phylogenetic reconstruction based on full-length (>1400 bp) 16S rRNA gene sequences. Taxa shown in red represent axenic cultures obtained as part of this study with representative nearest cultured isolates from v115 of the Silva database shown in black. Numbers after taxa represent % identity to closest cultured representative as determined by global usearch against a comprehensive reference database. Approximately 15 strains most closely related to *Bacillus* were also isolated as indicated by the collapsed clade at the bottom of the figure.

Growth inhibition of at least one of *C. perfringens*, *C. septicum*, or a *C. difficile* NAP1 strain was observed for eight strains from ceca and five from mid-gut samples.

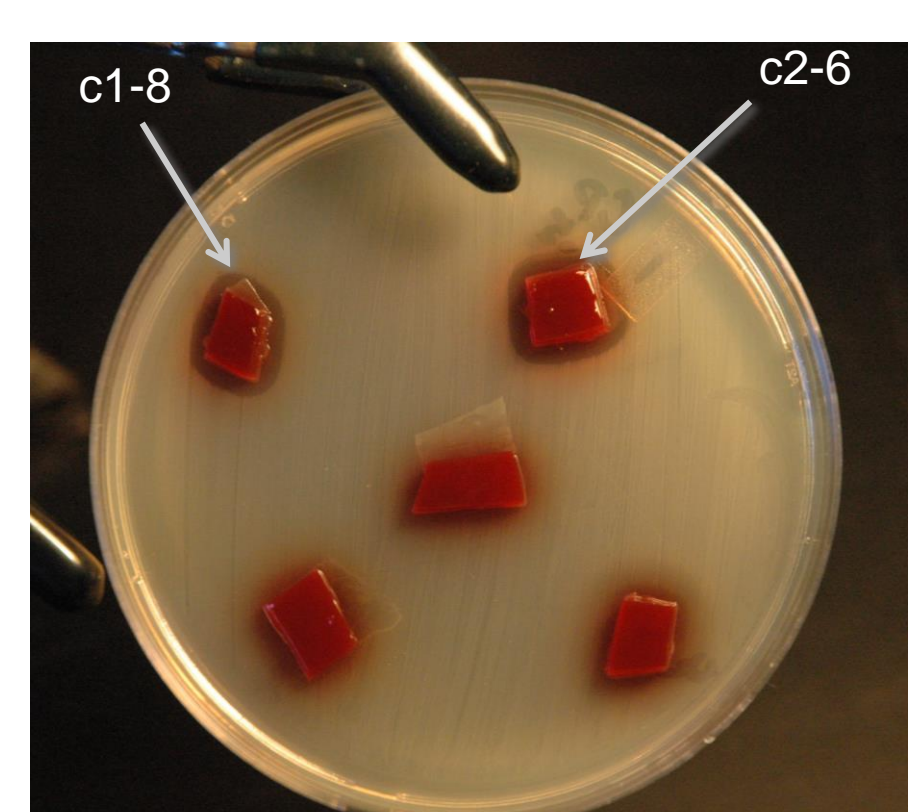


Figure 3. Growth inhibition of *C. perfringens*. Strains c2-6 and c1-8 (shown with arrows) demonstrated anti-microbial properties when placed on a lawn of *C. perfringens*. The causal mechanisms underlying this phenomenon are still unknown.

## RESULTS

### Genomic and phenotypic characterizations of novel isolates

Novel taxa are distinct from one another morphologically, phenotypically and genomically

Figure 3. Morphological and phenotypic comparisons of strains c1-8 and c2-6. A) Gram stain of c1-8. B) Gram stain of c2-6. Not variable morphology and sporulation forms. Lead acetate assay show C) positive sulphide production for c1-8 with positive control, and D) no sulphide production for c2-6 with negative control.

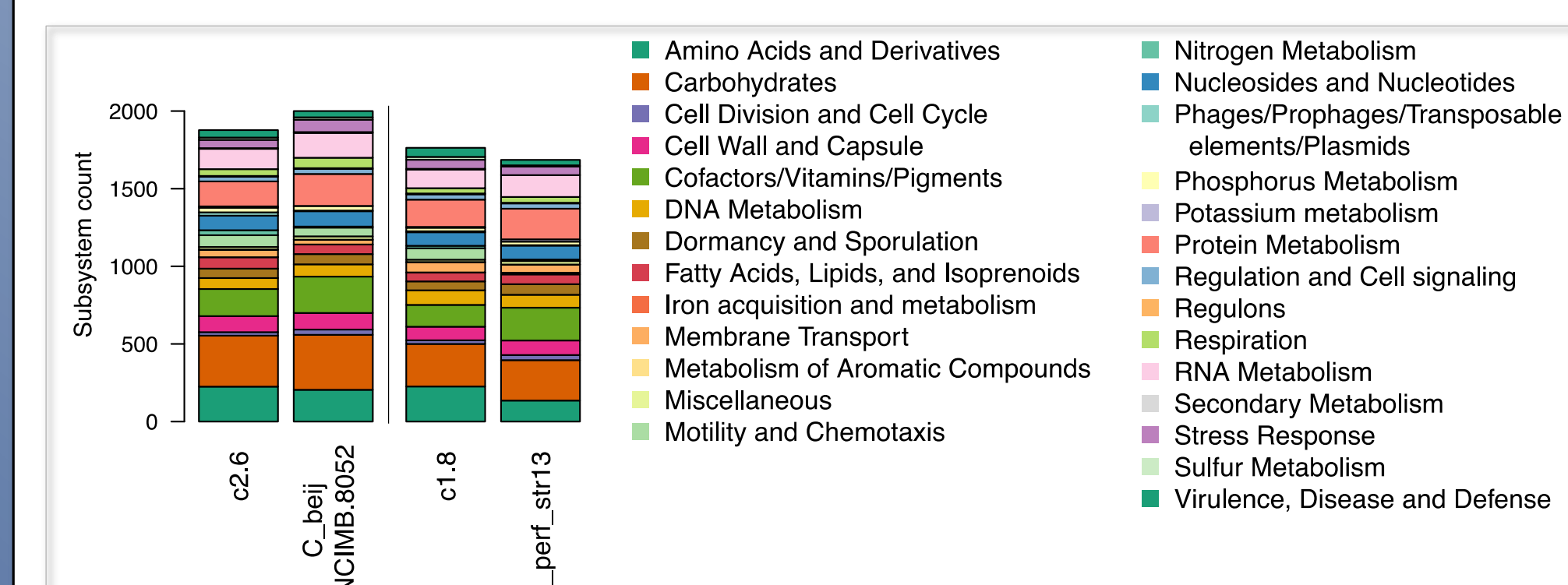
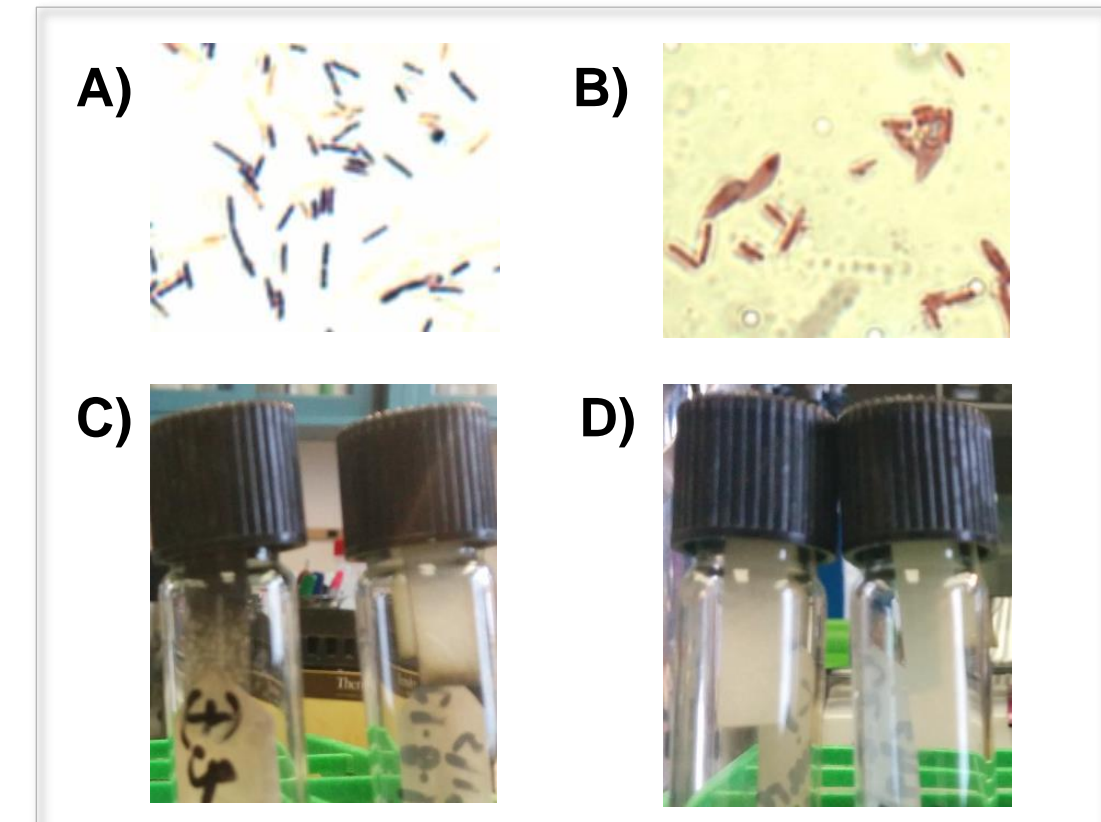


Figure 4. Genomic overview of subsystem classifications for strains c2-6 and c1-8 compared to their nearest genomic neighbors, *Clostridium beijerinckii* NCIMB 8052, and *C. perfringens* str. 13, respectively. Subsystems were classified by RAST with genomic assemblies as performed above.

Several interesting genes of bacteriophage origin including holins and endolysins

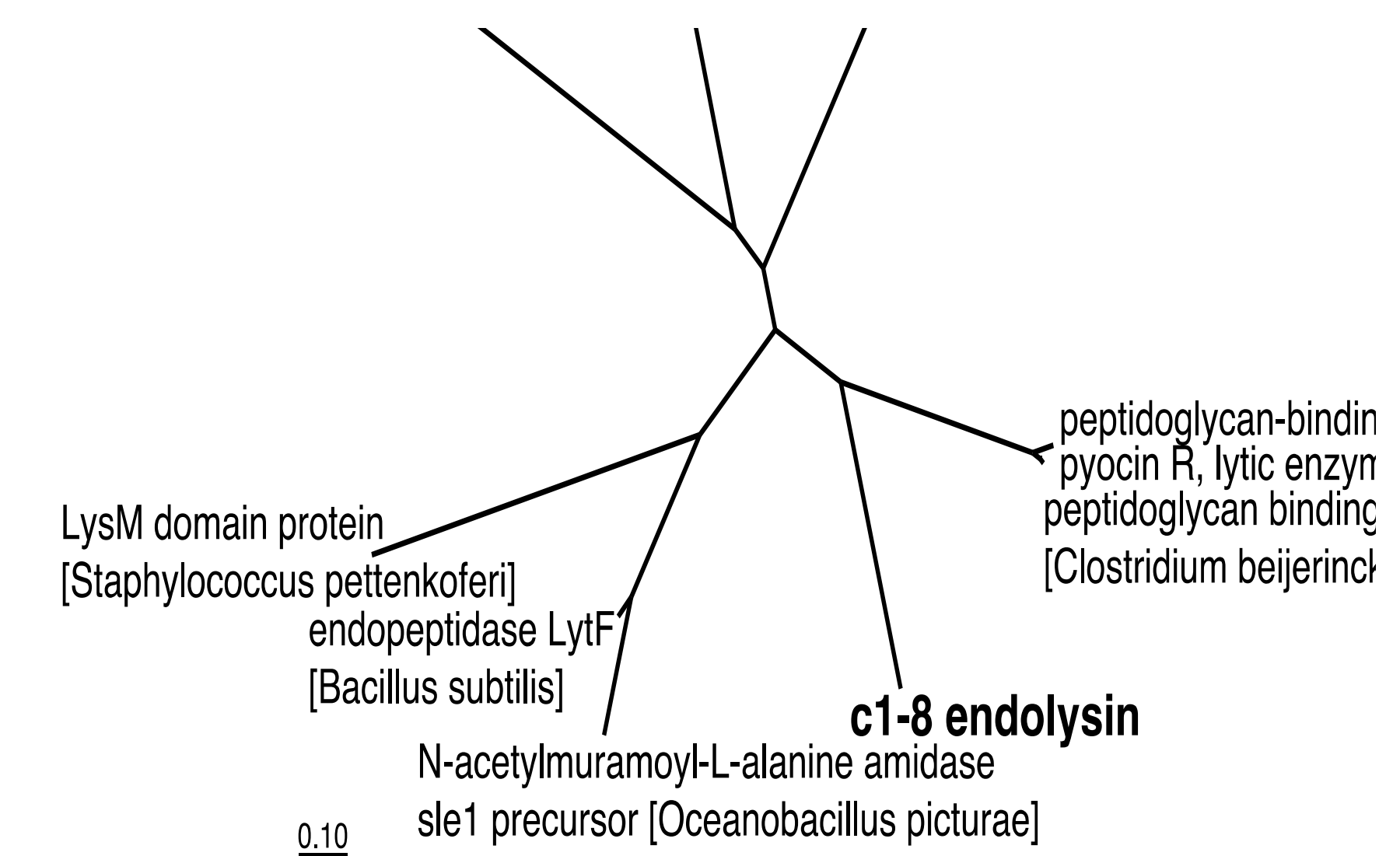


Figure 5. Phylogeny of nearest-neighbors to putative phage endolysin annotated in genome of strain c1-8. Tree represents a maximum-likelihood phylogenetic reconstruction from aligned protein sequences of annotated genes selected as best matches from blastp and psi-blast search of the nr database, RAST, and IMG.

## CONCLUSIONS

- High-throughput sequencing of 16S rRNA gene amplicons demonstrated that the poultry GI microbiome is dominated by members of the Clostridiales, many of which have important roles in bird health and disease.
- Clostridiales and other spore-formers such as *Bacillus* can be brought into cultivation by killing vegetative cells and selecting for spore-forming bacteria.
- Several isolates most closely related to *Clostridia* likely represent novel species not cultured previously.
- Characterizations of isolates revealed anti-microbial activity against *C. perfringens*, *C. septicum*, and *C. difficile*.
- Genome sequencing of several strains indicated a potential for sulphide production which was confirmed phenotypically for at least one axenic isolate.
- Strain c1-8 contains an annotated bacteriophage endolysin with amino acid sequence similarity to chitinases, endopeptidases, and R-type bacteriocins or "diffocins" that could potentially be an alternative antimicrobial agent.

## ACKNOWLEDGEMENTS

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