Proceedings of The National Conference
On Undergraduate Research (NCUR) 2017
University of Memphis, TN
Memphis Tennessee
April 6-8, 2017

Isolation of potential novel endospore-containing bacteria from Canada goose feces

Haley R. Keillor and Molly K. Svendsen
Biology Program
Oregon State University Cascades
Tykeson Hall 331, 1500 SW Chandler Avenue
Bend, OR 97702 USA

Faculty Advisors: Dr. Patrick N. Ball and Dr. Bruce S. Seal

Abstract

Non-toxin producing anaerobic bacteria have been found to promote anti-inflammatory immune responses in the monogastric animal gut. Therefore, the isolation of chloroform resistant bacterial strains from Canada goose (*Branta canadensis*) feces could potentially select for spore-forming bacteria with probiotic properties for avian species and isolation of newly identified bacterial species from Canada goose microflora. Three percent chloroform treatment of Canada goose feces was completed for one hour to select for chloroform resistant bacterial cells. Surviving spores were cultured in aerobic and anaerobic conditions to differentiate strains by metabolic strategy. Gram staining revealed twenty-six morphologically distinct endospore containing isolates. Twelve anaerobic Gram positive (three) and negative (nine) isolates were obtained by culturing on Brucella blood with vitamin K and hemin or reinforced clostridial hiveg hydrolysate with L-cysteine, Na acetate and starch agars. Fourteen aerobic Gram positive (four) and negative (ten) isolates were cultured using the two-aforementioned media and nine aerobes could subsequently be propagated on lysogeny broth (LB). Results from these investigations further demonstrate that newly identified, potential probiotic bacterial cultures can be isolated from free-ranging species and identified. Current efforts are focusing on 16S ribosomal RNA sequencing, to determine the identification of our isolates.

Keywords: antimicrobial; axenic bacterial culture; microbial ecology; free-ranging avian

1. Introduction

Molecular techniques have been increasingly utilized to examine a variety of microbiomes because it is understood that, for example most of the gut bacteria such as strict anaerobes, have been unable to be obtained as axenic cultures. Consequently, culture-independent, high-throughput nucleic acid sequencing methods have been utilized to characterize gastrointestinal microbiomes. Recently it was reported that difficult to culture bacteria, especially spore-formers can be isolated in culture.[1] Moreover, indigenous species of non-toxin producing anaerobic bacteria (Gram-positive, spore-forming *Clostridium* spp.) 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.[2] Birds, both domestic and free-ranging, represent monogastric animals wherein the gastrointestinal (GI) tracts harbor a diverse community of microbes that could be a source of potentially useful bacteria that include agriculturally important birds, such as chickens, as well as birds of evolutionary or conservation interest.[3]

Selection of an anaerobic, spore-forming mixture of bacteria utilizing chloroform extraction of mouse feces to remove vegetative cells was utilized to develop a treatment of 17 spore-forming strains of bacteria that were orally administered to mice that attenuated colitis and allergic diarrhea.[4,5] Also, administration of *Clostridia* spp., but not *Bacteroidales*, can protect neonatal mice from bacterial pathogen infection and reduces intestinal pathology upon bacterial pathogen challenge.[6] As stated many free-ranging as well as domestic bird species harbor diverse

communities of microorganisms in their GI tract that play crucial roles in providing the host with nutrition and protection from pathogens similar to other animals such as the mouse.[3] Consequently, our hypothesis was that potential probiotic bacterial cultures can be developed from geese gastrointestinal material based on chloroform extraction of vegetative bacterial cells in geese gastrointestinal or feces samples to select for spore-forming bacteria.

2. Methods and Materials

2.1 Collection Of Biological Samples And Selection Conditions For Bacterial Spore-Forming Bacteria

Fresh feces from geese were aseptically collected among known residential Canada goose (*Branta canadensis*) populations in Bend, OR USA utilizing sterile plastic bags and fecal material was stored in an ultra-cold freezer (minus 80C) until processed for selection of bacterial spores. Goose fecal material was thawed and suspended in phosphate-buffered saline (PBS)[7] using organic solvent-resistant, polypropylene 15ml or 50ml conical centrifuge tubes followed by vortex mixing for five minutes. Subsequently, low-speed centrifugation was conducted at 1,000Xg for five minutes to eliminate solids. Following centrifugation, chloroform was added to a concentration of approximately 3 percent; e.g., 0.3ml per 10ml of fecal suspension or 1.5ml chloroform per 50ml fecal suspension and placed on a laboratory shaker for 30 minutes to eliminate vegetative bacterial cells and select for bacterial spores.[2,8]

2.2 Bacterial Culture And Polymerase Chain Reaction For 16S Ribosomal RNA Products

Aliquots of spore suspensions (150µl) were pipetted on bacterial plates for culture of potential spore-forming bacteria. Specifically, bacterial plates were cultured under anaerobic conditions with sachets (Oxoid, AnaeroGenTM) for four days or aerobically for two days utilizing brucella agar with blood and vitamin K-hemin (BBHK) or using reinforced clostridial agar with L-cysteine, Na acetate, starch without polymyxin B.[9] Gram stains were completed utilizing basic bacteriological procedures[10] and certain Gram-negative bacteria were also cultured on lysogeny broth (LB).[9] DNA extraction of bacterial colonies was accomplished using the QuickExtract™ Bacterial DNA Extraction Kit or the mBio UltraClean® Microbial DNA Isolation Kit. Subsequently genomic DNA was used for the 1X SapphireAmp FAST PCR master mix (Clontech/Takara, Mountain View, CA 94043) for PCR using a high-processivity polymerase to amplify universal broad range 16S targets using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').[11] Thermal cycling parameters were 94˚C for 1 minute, followed by 32 cycles of denaturation at 98˚C for 5 s, 5 s of annealing at 58˚C, and 10 s extension at 72˚C, with a final extension step for 10 min at 72˚C. Subsequently, PCR products were electrophoresed in a 0.8 per cent agarose gel.[7]

3. Results and Discussion

Bacterial culture of potential spore-forming bacteria from goose feces was completed under aerobic and anaerobic conditions to differentiate strains by metabolic strategy. The resultant selection procedures utilizing chloroform treatment and plating resulted in twenty-six morphologically distinct endospore-containing bacterial isolates based on Gram stain. Representative cultures are depicted in Figure 1.

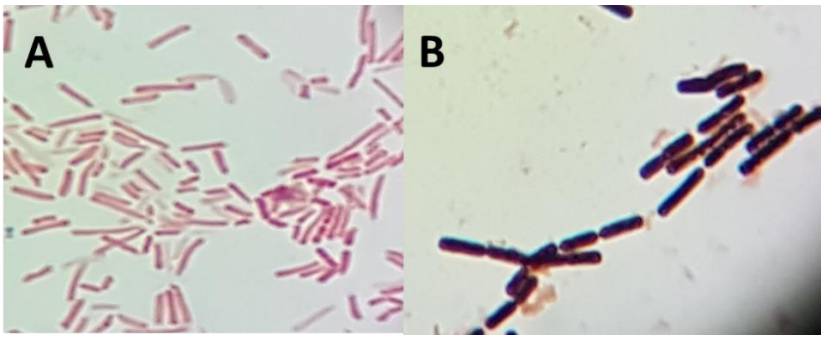

Figure 1. Representative bacterial cultures following chloroform treatment of Canada goose feces and subsequent culture under aerobic and anaerobic conditions. A. Gram Negative Bacteria. B. Gram Positive Bacteria.

Twelve anaerobic Gram positive (three) and negative (nine) isolates were obtained by culturing on Brucella blood with vitamin K and hemin or reinforced clostridial hiveg hydrolysate with L-cysteine, Na acetate and starch agars (Table 1). Fourteen aerobic Gram positive (four) and negative (10) isolates were cultured using the two-aforementioned media and nine aerobes could subsequently be propagated on lysogeny broth (LB) as reported in Table 2. The anaerobic bacteria caused lysis of red-blood cells, while none of the anaerobes could be propagated on LB media. Nine out of the fourteen aerobic bacteria could subsequently be propagated on LB media plates.

Table 1 Anaerobic Bacterial Isolates OSU Cascades Biology

No.	Gram Stain	BBHK[1]	Clostridial[2]	LB[3]	Morphology[4]
01	Positive	Yes	No	No	H β
02	Positive	Yes	Low	No	H β
03	Positive	Yes	Low	No	H β
04	Negative	Yes	No	No	H α
05	Negative	Yes	Yes	No	H α
06	Negative	Yes	Yes	No	H α
07	Negative	Yes	Yes	No	H α
08	Pos/Neg	Yes	No	No	H α
09	Negative	Yes	Yes	No	H α
10	Negative	Yes	Yes	No	H α
11*	Negative	Yes	No	No	H β
12*	Negative	Yes	No	No	H β

[1]BBHK – Brucella blood with Vitamin K and Hemin, Yes or No indicates bacterial growth on specific media type
[2]Clostridial – Reinforced clostridial agar hiveg hydrolysate with L-cysteine, Na acetate, starch
[3]LB – Standard 'Lysogeny Broth' (LB) commonly utilized in the lab for propagating bacteria
[4]Morphology as observed hemolysis type (H) alpha (α) or beta (β)
*Passage resulted in very little growth; most recent passaged plates back in the incubator and new passage on to BBHK from original plates, incubated for 72 hours

Table 2 Aerobic Bacterial Isolates OSU Cascades Biology

No.	Gram Stain	TSA/BBHK[1]	Clostridial[2]	LB[3]	Morphology[4]
01	Positive	Yes	Yes	Yes	
02	Negative	Yes	Yes	Yes	
04	Negative	Yes	Yes; slow	No	
06	Positive	Yes	Yes, slow	No	
07	Negative	Yes	Yes	Yes	
08	Negative	Yes	Yes	Yes	Chromogenic
09	Negative	Yes	Yes	Yes	
10*	Negative	Yes	Yes	Yes	
11*	Negative	Yes	No	No	Motile
12a	Positive	Yes	No	Yes	Motile
12b	Positive	Yes	No	Yes	
13	Negative	Yes	Yes	No	
14	Negative	Yes	Yes	Yes	
15	Negative	Yes	Yes	No	

[1]BBHK – Brucella blood with Vitamin K and Hemin; TSA - tryptone soy agar
[2]Clostridial – Reinforced clostridial agar hiveg hydrolysate with L-cysteine, Na acetate, starch
[3]LB – Standard 'Lysogeny Broth' (LB) commonly utilized in the lab for propagating bacteria
[4]Morphology as exhibiting motility or chromogenic; no hemolytic Gram neagtive isolates were observed
*Passage resulted in very little growth; most recent passaged plates back in the incubator and new passage on to BBHK from original plates, incubated for 72 hours

Following axenic culture of both anaerobic and aerobic Gram-negative and Gram-positive bacteria, genomic DNA was isolated from selected cultures. Subsequently, PCR was completed to obtain 16S rRNA products and representative gel electrophoresis is presented in Figure 2. The resultant PCR products were of the appropriate size, approximately 1.5 kb, for the universal primers used to complete amplification of the 16S rRNA gene.[11] Subsequent nucleotide sequencing will allow for identification of isolated bacteria that have not been previously cultured that will be submitted for whole-genome sequencing and characterization by phenotype.[12,13]

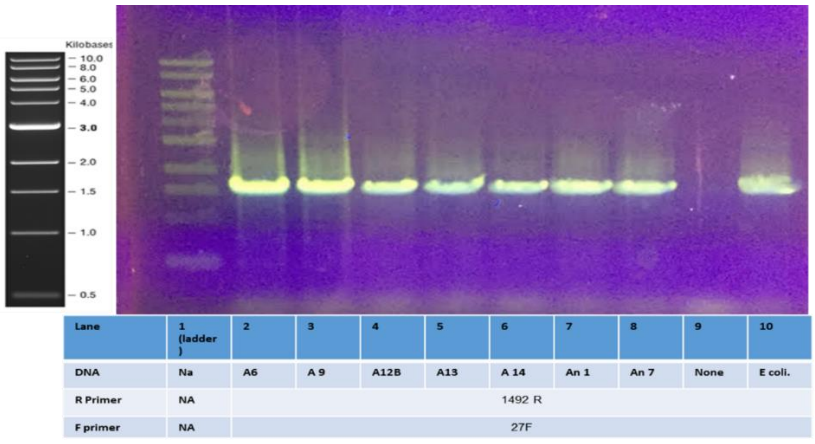

Figure 2. Agarose gel electrophoresis of representative amplification products following polymerase chain reaction of bacterial isolate genomic DNA templates using universal oligonucleotide primers for the 16S ribosomal RNA gene. The isolate designations and oligonucleotide primers are specified in the figure.

Herein several spore-forming, Gram negative bacteria are reported as isolated from goose feces. These bacteria are potentially members of the Firmicutes, a diverse group of microorganisms, that represent a major component of the intestinal microflora.[14,15,16] Although culture of Gram-negative spores is rare, the class *Negativicutes* is currently

divided into one order and two families based 16S rRNA gene sequence phylogenies and can additionally be distinguished based on core protein sequences.[17] These are potentially very interesting bacteria because they are distantly related to *Clostridia*, and are less closely related to other more widely recognized Gram-negative species.[14] The anaerobic, Gram positive spore-forming bacteria are possibly members of the class *Clostridium*[15] and are probably represented among our isolates as well.

4. Conclusions

Although bacteria have been identified in geese[18], there are a variety of spore-forming, Gram-negative and Gram-positive bacteria that remain to be further characterized in culture.[15,16,17] Many of these are now known to improve gut health[2,4,6] and improve feed conversion among domestic animals.[19] This led us to 'bio-prospect' for potential new probiotic, spore-forming bacteria from a free-ranging goose species. The next step is to further identify these isolates phenotypically and by 16S rRNA gene sequencing, then assay for inhibitory properties to known pathogenic microbes.

5. Acknowledgments

Funding was provided to PNB as a start-up grant from the OSU Faculty Innovation Committee and by the Gaskins Fund. Travel funds were provided by the OSU-Cascades Student Fee Committee under the Professional Development funds. Appreciation is extended to other science and bioscience faulty at OSU Cascades for continued sharing of laboratory facilities to conduct undergraduate research. Contact information: pat.ball@osucascades.edu.

6. References

1. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, Goulding D, Lawley TD. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*. 533 (2016):543-546.

2. Atarashi K, Tanoue T, Shima T,et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science.* 331 (2011):337-341.

3. Waite DW, Taylor MW. Exploring the avian gut microbiota: current trends and future directions. *Front. Microbiol.* 6 (2015):673.

4. Atarashi K, Tanoue T, Oshima K, et al. T-reg induction by a rationally selected mixture of *Clostridia* strains from the human microbiota. *Nature.* 500 (2013):232-236.

5. Narushima S, Sugiura Y, Oshima K, Atarashi K, Hattori M, Suematsu M, Honda K. Characterization of the 17 strains of regulatory T cell-inducing human-derived *Clostridia*. *Gut Microbes*. 5 (2014):333-339.

6. Kim YG, Sakamoto K, Seo SU, Pickard JM, Gillilland MG 3rd, Pudlo NA, Hoostal M, Li X, Wang TD, Feehley T, Stefka AT, Schmidt TM, Martens EC, Fukuda S, Inohara N, Nagler CR, Núñez G. Neonatal acquisition of *Clostridia* species protects against colonization by bacterial pathogens. *Science.* 356 (2017):315-319.

7. Wise MG, Siragusa GR. Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *J. Appl. Microbiol*. 102 (2007):1138-1149.

8. Itoh, K. & Mitsuoka. T. Production of gnotobiotic mice with normal physiological functions. I. Selection of useful bacteria from feces of conventional mice. *Zeitschrift für Versuchstierkunde*. 22 (1980):173-178.

9. Chapin K and Murray P. In Murray, Baron, Pfaller, Tenover and Yolken (ed.). Manual of clinical microbiology, 7th ed. (Washington, DC: American Society for Microbiology, 1999).

10. Vandepitte J, Engbaek, K, Rohner, P, Piot P, Heuck CC. Basic laboratory procedures in clinical bacteriology. 2nd ed. (Geneva: World Health Organization, 2003).

11. Lane, DJ. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt E, Goodfellow M, eds. (New York: John Wiley and Sons, 1991).

12. Taboada EN, Graham MR, Carriço JA, Van Domselaar G. Food Safety in the Age of Next Generation Sequencing, Bioinformatics, and Open Data Access. *Front. Microbiol*. 8 (2017):909.

13. Bochner BR, Gadzinski P, Panomitros E. Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res*. 11 (2011):1246–1255.

14. Vesth T, Ozen A, Andersen SC, Kaas RS, Lukjancenko O, Bohlin J, Nookaew I, Wassenaar TM, Ussery DW. *Veillonella*, *Firmicutes*: Microbes disguised as Gram negatives. *Stand. Genomic Sci*. 9 (2013):431-448.
15. Galperin MY. Genome Diversity of Spore-Forming Firmicutes. *Microbiol. Spectr*. 1 (2013):2.
16. Yutin N, Galperin MY. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. *Environ. Microbiol*. 15 (2013):2631-2641.
17. Campbell C, Adeolu M, Gupta RS. Genome-based taxonomic framework for the class *Negativicutes*: division of the class *Negativicutes* into the orders *Selenomonadales* emend., *Acidaminococcales* ord. nov. and *Veillonellales* ord. nov. *Int. J. Syst. Evol. Microbiol*. 65 (2015):3203-3215.
18. Lu J, Santo Domingo JW, Hill S, Edge TA. Microbial diversity and host-specific sequences of Canada goose feces. *Appl Environ Microbiol*. 75 (2009): 5919-5926.
19. Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: Challenges presented for the identification of performance enhancing probiotic bacteria. *Front. Microbiol*. 7 (2016):187.