Safety characterization of ART24, a novel live biotherapeutic product in development for the prevention of CDI, by *in silico* and *in vitro* testing

Michelle O' Donnell,[1] Calum J. Walsh,[2] Rodrigo E. Mendes,[3] Colin Hill,[4] R. Paul Ross,[4] Mary C. Rea,[2] Ronald Farquhar,[5] and Laurent Chesnel[5]

[1]University College Cork, Ireland; [2]Teagasc Food Research Centre, Moorepark, Fermoy, Ireland; [3]JMI Laboratories; [4]APC Microbiome Ireland, University College Cork, Ireland; [5]Artugen Therapeutics USA, Inc.

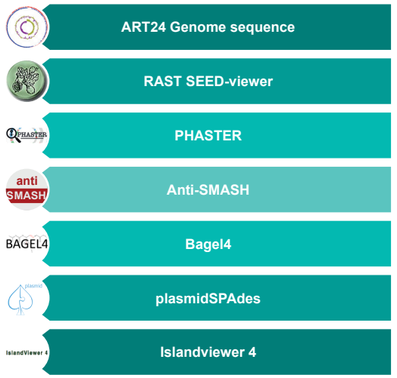
Background

- ART24 is being investigated as a live biotherapeutic product (LBP) treatment to prevent recurrence of *Clostridioides difficile* infection (CDI) following successful antibacterial therapy. ART24 was isolated from a human fecal sample, identified, and purified. ART24 is a member of the *Bacillus amyloliquefaciens/B. velezensis* group. Further, ART24 is considered a member of the 'operational group *B. amyloliquefaciens*', which consists of the soil-borne *B. amyloliquefaciens*, and plant associated *B. siamensis* and *B. velezensis*, which is synonymous with *B. amyloliquefaciens* subsp. plantarum. The human carriage of *B. amyloliquefaciens* as well as the genome of ART24 itself were investigated as a means to identify potential safety and toxicity concerns prior to animal and human testing

Materials/methods

- The whole closed, assembled, and annotated ART24 genome was investigated for functional operons that contain known antimicrobial gene content, plasmid, phage content, and virulence factors using antiSMASH, BAGEL4, plasmidSPAdes, PHASTER, IslandViewer4, and RAST SEED viewer (**Figure 1**)

Figure 1. Bioinformatics workflow



- In addition, standard susceptibility testing of ART24 against 16 antibiotics was determined using Clinical and Laboratory Standards Institute (CLSI)-recommended broth microdilution method and MIC results interpreted using current interpretative breakpoints
  - Broth microdilution. Isolates were tested for antimicrobial susceptibility using the reference broth microdilution method per CLSI M07 (2018) guidelines. JMI Laboratories produced frozen-form 96-well panels containing cation-adjusted Mueller-Hinton broth per CLSI M45 (2015) for all antimicrobials. In addition, metronidazole was tested under anaerobic conditions in supplemented brucella broth + 5% laked horse blood per CLSI M11 (2018) guidance
  - MIC values obtained against ART24 were validated by concurrently testing CLSI-recommended (M100, 2019) ATCC quality control (QC) reference strains. These strains included: *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC 29213, and *Bacteroides fragilis* ATCC 25285. The inoculum density during susceptibility testing was monitored by bacterial colony counts
  - Categorical interpretations for MIC results obtained against ART24 used CLSI M45 (2015) breakpoint criteria or European Food Safety Authority (EFSA; 2012) microbiological cut-off values, where published. Acceptable QC results were those from CLSI (M100, 2019)
- Finally, available metagenomic datasets from human fecal samples were mined for the presence of *B. amyloliquefaciens* and *B. velezensis* using Kraken2, Bayesian Re-estimation of Abundance with KrakEN (Bracken), and curatedMetagenomicData to better understand the presence of the 'operational group *B. amyloliquefaciens*' in the general population. *B. amyloliquefaciens* and *B. velezensis* are both considered a surrogate for presence of ART24 as part of the same operational group in these analyses of metagenomic datasets from fecal samples
  - Kraken2 is a k-mer-based classifier that works by aligning paired end FastQ metagenomic reads against a database of NCBI RefSeq genomes
  - Bracken was used to adjust the Kraken2 output used for the human fecal microbial communities from inner Mongolia and China datasets
  - The R package curated MetagenomicData containing data from a number of metagenomic datasets were chosen where the MetaPhlAn classification table was available

Results

- *In silico* analysis indicates that the ART24 genome displays the expected genetic properties related to *Bacillus* subspecies, and that off-target activities of virulence and antibiotic resistance mechanisms are unlikely
  - A total of 5 prophage regions were identified by PHASTER analysis of the ART24 genome, which were classified as two intact regions and three incomplete regions
  - There were no plasmids present in the genome of ART24
  - RAST/SEED and IslandViewer4: no virulence factors or homologs were identified – while there may be some virulence or antimicrobial resistance genes, there is no complete operon
- Up to 10% of the genome is dedicated to antimicrobial production
  - ART24 encodes the genetic information allowing for the synthesis of up to 12 known antimicrobials
  - The antimicrobials identified using antiSMASH are known lipopeptides (surfactin and fengycin) and the three known polyketides (PKS) produced by *B. subtilis* group bacteria: bacillaene, difficidin, and macrolactin. Other known or associated *B. subtilis* group antimicrobials were also identified using this analysis. These include the siderophore bacillibactin; and the non-thiotemplate non-ribosomal peptide bacilysin (**Table 1**)

Table 1. Antimicrobials identified using antiSMASH

Region	Type	Most similar known cluster		Similarity	MIBiG BGC-ID
Region 1	lanthipeptide	Locillomycin	nrps-t1pks	35%	BGC0001005
Region 2	NRPS	Surfactin	NRPS	86%	BGC0000433
Region 3	PKS-like	Butirosin	saccharide	7%	BGC0000693
Region 4	terpene				
Region 5	transAT-PKS	Macrolactin	other	100%	BGC0000181
Region 6	transAT-PKS,NRPS	Bacillaene	other	100%	BGC0001089
Region 7	NRPS,transAT-PKS,betalactone	Fengycin	other-nrps	100%	BGC0001095
Region 8	terpene				
Region 9	T3PKS				
Region 10	transAT-PKS	Difficidin	other	100%	BGC0000176
Region 11	NRPS, bacteriocin	Bacillibactin	NRPS	100%	BGC0000309
Region 12	other	Bacilysin	other	100%	BGC0001184

MIBiG, minimum information about a biosynthetic gene cluster; NRPS, non-ribosomal peptide synthase; PKS, polyketide synthase; transAT-PKS, transacyltransferase polyketide synthase; T3PKS, type 3 polyketide synthase

  - The BAGEL4 analysis showed four areas of interest with a match to known ribosomally synthesized and post-translationally modified peptides (RiPPs) or bacteriocin-encoding genes. The areas of interest of the ART24 genome that match known sequences by individual area of interest (AOI) are shown in **Table 2**

Table 2. Results of BAGEL4 analysis

AOI	Class
6666666282745.0.AOI_01	266.1;amylocycicin
6666666282745.0.AOI_02	132.2;LCI
6666666282745.0.AOI_03	11.3;colicin
6666666282745.0.AOI_04	Lanthipeptide_class_IV

AOI, area of interest

- ART24 displays low MICs to all antibiotics tested, which resulted in susceptible categories where interpretative criteria are available (**Table 3**)
- *In silico* analysis to identify *B. amyloliquefaciens/B. velezensis* gene content in metagenomic datasets showed that the operational group is present in human fecal samples analyzed at an overall low percentage relative abundance, variable between geographic regions, and was not linked with any disease state in the datasets tested (**Table 4**)

Table 3. Antimicrobial antibiotic susceptibility tests

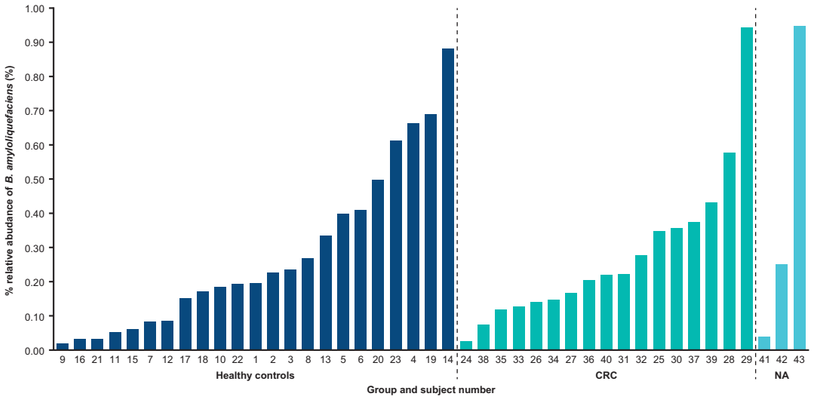
Antimicrobial	ART24	
	MIC (mg/L)	Susceptibility
Ampicillin	≤0.03	S
Chloramphenicol	2	S
Ciprofloxacin	≤0.03	S
Clindamycin	0.25	S
Erythromycin	0.12	S
Fidaxomicin	4	-
Gentamicin	≤0.06	S
Kanamycin	≤0.5	S
Linezolid	0.5	-
Metronidazole (anerobic)	**64**	**-**
Neomycin	≤0.12	-
Penicillin	≤0.03	S
Quinupristin-dalfopristin	2	-
Rifampicin	0.25 (S)	S
Streptomycin	1	S
Tetracycline	2	S
Trimethoprim	≤0.25	S
Vancomycin	0.5	S

Categorical interpretations using CLSI M45 (2015) breakpoint criteria, as available. MIC interpretations for kanamycin and streptomycin used European Food Safety Authority cut-off values (2012)
-, no interpretative criteria available

Table 4. *In silico* mining for *B. amyloliquefaciens* in metagenomic datasets

Gold study ID/ accession number	Study name	Description	# subjects positive for *B. amyloliquefaciens* (%)	# subjects positive for *B. velezensis* (%)
https://www.ebi.ac.uk/ena/data/view/PRJEB12449	Colorectal Cancer and the Human Gut Microbiome: Reproducibility with Whole-Genome Shotgun Sequencing	Colorectal Cancer and the Human Gut Microbiome: Reproducibility with Whole-Genome Shotgun Sequencing	43/110 (39%)	Not reported in dataset
https://www.ebi.ac.uk/ena/data/view/PRJEB6337	Alterations of the human gut microbiome in liver cirrhosis	Alterations of the human gut microbiome in liver cirrhosis	4/237 (1.69%)	Not reported in dataset
https://www.ebi.ac.uk/ena/data/view/PRJEB6070	Potential of fecal microbiota for early-stage detection of colorectal cancer	Potential of fecal microbiota for early-stage detection of colorectal cancer	10/119 (8.4%)	Not reported in dataset
Gs0133435 PRJNA328899	Human fecal microbial communities from inner Mongolia and China	Unique Features of Ethnic Mongolian Gut Microbiome revealed by metagenomic analysis	103/110 (93.6%)	76/86 (88.4%)
Gs0133434 PRJNA422434	Human fecal microbial communities from Peking University Shenzhen Hospital, China	Human fecal microbial communities from Peking University Shenzhen Hospital, China to study type 2 diabetes	1/365 (0.27%)	Not reported in dataset
Gs0133133 PRJEB12124	Human fecal microbial communities from Shanghai, China	Human fecal microbial communities from Shanghai, China to understand if intestinal flora could be related to differential responds to antidiabetic therapies	126/188 (67%)	126/188 (67%)

Figure 2. Relative abundance in percent from patients included in study ID PRJEB12449 (Colorectal Cancer and the Human Gut Microbiome: Reproducibility with Whole-Genome Shotgun Sequencing displaying the percentage relative abundance of *B. amyloliquefaciens*)



- Using the R package curatedMetagenomicData, 39% of individuals were identified from the cohort of patients in study PRJEB12449 (**Table 4**) as having *B. amyloliquefaciens* present in their gut microbiota. The percentage relative abundance of the species ranged from 0.02–0.95% and was distributed across the groups in the study (**Figure 2**)

Conclusions

The series of *in silico* and *in vitro* testing analyses performed on ART24 did not reveal any obvious safety concerns. ART24 is a promising LBP clinical candidate in Phase 1 clinical development for prevention of recurrent CDI.

Acknowledgments

Layout assistance was provided by McCann Health Medical Communications studio team. This assistance was funded by Artugen Therapeutics, Inc., Concord, MA, USA.

Disclosures

Michelle O' Donnell has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.
Calum J. Walsh has no disclosures to make in relation to this work.
Rodrigo E. Mendes has no disclosures to make in relation to this work.
Colin Hill has received grants and personal fees from Artugen Therapeutics Ltd.; has received grants from Janssen Pharmaceuticals, Kerry Foods, and ADARE Pharmaceuticals outside of this work; and additionally has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.
R. Paul Ross is a paid consultant of Artugen Therapeutics Ltd.; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.
Mary C. Rea is a paid consultant of Artugen Therapeutics Ltd.; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.
Ronald Farquhar is a paid consultant of Artugen Therapeutics Ltd., in which he also holds stock; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.
Laurent Chesnel is an employee of Artugen Therapeutics Ltd., in which he also holds stock; and additionally has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

Laurent Chesnel, PhD, Vice President Research and Development, P: +1- 978 202 4337, E: lchesnel@artugentherapeutics.com