

APPENDIX 5- GENETIC STABILITY STUDY

Genetic Stability Study on *Bacillus subtilis* R0179

Executive Summary

Genetic stability testing of bacterial strains is often required for regulatory documentation. The best method to evaluate genetic stability in bacteria is using Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR). We used this technique to determine the genetic stability of *Bacillus subtilis* R0179 over 20 transfers in the laboratory. We did not see any genetic drift with any of the 5 primers indicating that this strain is stable during repeated cultivation.

Objective

To evaluate the genetic stability of bacterial culture *B. subtilis* R0179 over 20 transfers by RAPD PCR.

Materials and Methodology

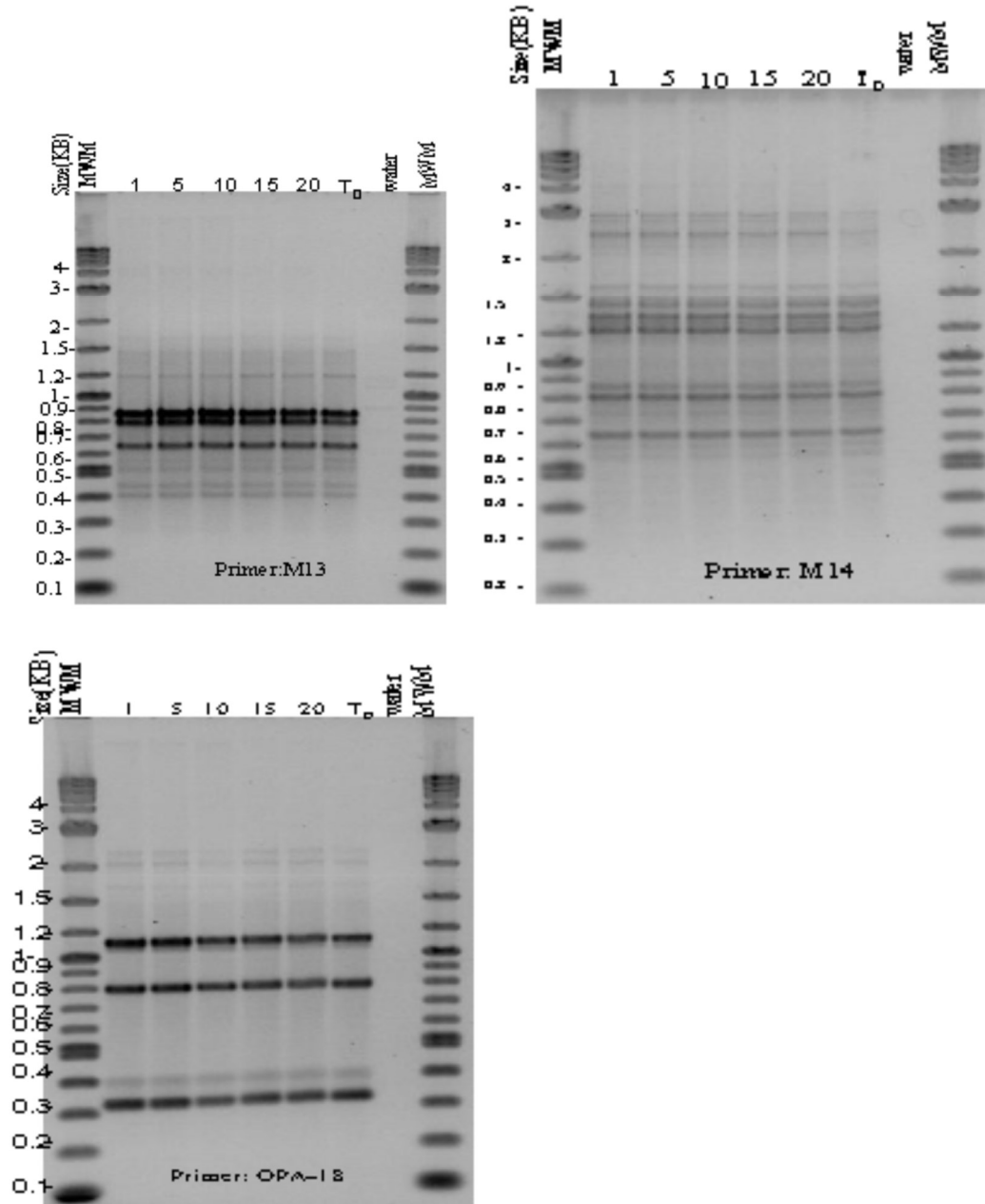
Frozen stock culture of *B. subtilis* R0179 was streaked on Tryptic-Soy agar (Oxoid CM0131) to ensure the selection of pure colonies. The agar plate was incubated under aerobic condition at 37°C for 24 hours. This is the 1st transfer. A single colony isolate was selected from this plate and streaked on a new agar plate; this process was repeated until 20 transfers were completed. Glycerol stock was prepared (6017M) for culture collection and DNA extraction (RM-31) was performed for each transfer 1, 5, 10, 15, and 20, respectively.

The DNA extraction was performed on 1 mL of overnight culture with the DNeasy DNA extraction kit (Qiagen, 69504) following protocol RM-31 which includes several modifications for the lysis of Gram positive bacteria.

Strain Identification by RAPD PCR

The identification was performed by RAPD PCR using the OPA-2, OPA-18, M13, M14 and OPL-16 primers (protocol RM-20). Briefly, each 20 L PCR contained: 1X Polymerase buffer, 0.2 mM dNTP mix, 1pmol/L of primer, 1L of 20ng DNA, and 0.05U/L Recombinant Taq DNA polymerase (M0267L, NEB). The PCR conditions were as follows: 94°C for 5 minutes followed by 35 cycles of: heating at 94°C for 30 seconds, annealing at 42°C for 1minute, amplifying at 72°C for 1 minute, followed by 7 minutes at 72°C and then the samples were held at 4°C. Water was used as a negative control. Ten L of each PCR product was run on a 15 cm 1.5% Agarose gel at 90V for 3 h.

Results



Photos of RAPD-PCR gels using primers: M1, M14 and OPA-18.

Conclusion

The RAPD-PCR analysis on DNA extracted from *B. subtilis* R0179 transfer 1, 5, 10, 15, and 20 and compared with the DNA from the R0179 mother culture (T0) shows that there is no changes in the

DNA profiles based obtained using 5 different primers over 20 transfers. R0179 genetic integrity has not been affected by storage or repeated cultivation. The RAPD-PCR profile assessment of *B. subtilis* R0179 indicates that it is genetically stable with repeated cultivation.

APPENDIX 6 - ENUMERATION OF *BACILLUS SUBTILIS* R0179 IN FOOD APPLICATIONS

Objective:

Enumerate viable *Bacillus subtilis* R0179 in food products (solid or liquid form).

Responsibilities:

- It is the responsibility of the person(s) doing the test to apply the proper procedures to prepare the required materials and equipment used in this test.
- It is the responsibility of the person(s) doing the test to work under strict aseptic conditions.

Material:

- Culture media: TSA- Merck 1.05458.5007
- Phosphate Buffer Solution (PBS: 0.1% Soya Peptone, 0.121% k_2HPO_4 , 0.034% KH_2PO_4 and bring volume to one (1) litre using dH_2O). Ensure that the PBS is at room temperature at time of use.

Sample preparation:

- Prepare a representative sample of the food product and dilute it in dilution 1:10 of PBS (example 30g in 270ml). Blend it for 1 minute and agitate at 100 rpm for 6 minutes. This step can be skipped if it is a liquid product and start directly with the dilutions using a representative sample of 25ml in 225 ml of PBS. This dilution is 10^{-1} . Agitate the liquid solution vigorously for 30 seconds.
- Transfer 1.0 ml of the 10^{-1} dilution in a test tube containing 9.0 ml of peptone water. Vortex to homogenize. This dilution is 10^{-2} .
- Repeat the last step until the required dilution is obtained (usually 10^{-5}).

Procedure:

- Transfer 0,1ml aliquots of required dilutions were spread on already fixed Triptycal Soy Agar (TSA) plates. All dilutions are plated in triplicate.
- The aliquots were spread on the agar by shaking with cross movement with small sterile latex beads. Invert dishes when the liquid as been absorb by the culture media.
- Incubate aerobically at $37^{\circ}C \pm 2^{\circ}C$ for 48 hours and count the colonies. Only dishes containing between 30 and 300 colonies are valid.
- Viable cell count (C.F.U.) per g =
$$\frac{\text{number of colonies in the 3 dishes} \times \text{dilution factor}}{3}$$

APPENDIX 7 - EVALUATION OF *BACILLUS SUBTILIS* R0179 FOR BILE SALT HYDROLASE ACTIVITY

Objective

To investigate bile salt hydrolase (BSH) activity in *Bacillus subtilis* R0179.

Background

Various bacteria can hydrolyze bile salts. It has been targeted as an important criterion for selection. An agar plate assay has been used to detect bile salt hydrolase activity in various lactic acid bacteria by supplementing the media with taurodeoxycholic, taurocholic, or taurochenodeoxycholic acids. Bile salt hydrolysis manifested at two intensities, either: (i) the formation of precipitate halos around colonies or (ii) the formation of opaque granular white colonies.

Materials and Methodology

Preparation of Test Plates

Prepare TSA agar (Oxoid, CM0131) Sterilize at 121°C for 15 minutes then pour 20mL of reconstituted TSA molten agar cooled to 45°C into petri dishes. Allow at least 10 minutes to solidify the agar. This is the control plates. Prepare TSA agar (Oxoid, CM0131) supplemented with 0.5% (wt/vol) of Sodium taurodeoxycholate hydrate (TCA, Sigma). Sterilize at 121°C for 15 minutes then pour 20 mL of reconstituted TSA molten agar + 0.5% TCA cooled to 45°C into petri dishes. Allow at least 10 minutes to solidify the agar.

Bile salt hydrolase assay

Frozen stock culture of *B. subtilis* R0179 were streaked on Tryptic Soy agar (TSA) to ensure the selection of pure colonies. The plate was incubated in aerobic condition at 37°C for 24 hours. Using a sterile loop streaked the bacterial culture sample across on each test plates in quadrant and incubated under the same condition.

Results

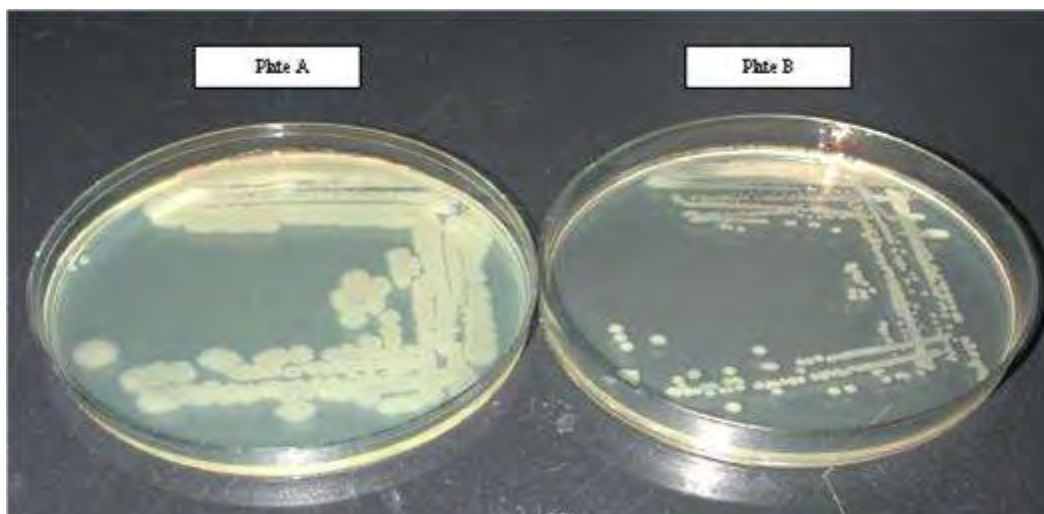


Figure 1: (Plate A) *B. subtilis* R0179 streak on TSA as control plate. (Plate B) *B. subtilis* R0179 streak on TSA +0.5% TCA

Conclusion

Colonies of *B. subtilis* R0179 are generally large, spreading and irregularly shaped due to the exopolysaccharides they form, as observed in plate A. However, in the presence of sodium taurodeoxycholate hydrate (TCA), the strain forms small colonies with no precipitate halos. Therefore, *Bacillus subtilis* R0179 does not produce bile salt hydrolase. The bile salt inhibited the growth of the strain and prevented the production of exopolysaccharides.

APPENDIX 8 - LETTER FROM THE FOOD DIRECTORATE OF HEALTH CANADA REGARDING THE “NON-NOVELTY” STATUS OF *B. SUBTILIS* R0179

Health
CanadaSanté
CanadaHealth Products
and Food BranchDirection générale des produits
de santé et des aliments

Novel Food Section
Bureau of Microbial Hazards
251 Sir Frederick Banting Driveway
PL# 2204E
Tunney's Pasture
Ottawa, Ontario
K1A 0L2

August 30, 2011

Solange Henoud
Regulatory Affairs Manager
Institut Rosell – Lallemand
8480 St. Laurent Boulevard
Montreal, Quebec
H2P 2M6

Dear Ms. Henoud,

This will refer to your request concerning the use of the *Bacillus subtilis* strain R0179 as a food ingredient. Officers of the Food Directorate, Health Products and Food Branch, have reviewed the information provided to determine the potential novelty of this product as defined in Division 28 of the *Food and Drug Regulations*.

The information provided included a description of the specific *B. subtilis* strain, its origin, history of use in food, expected consumption, data on general safety, and a description of the manufacturing process.

Based on the information provided regarding the history of food use for *B. subtilis* as a species, we have concluded that this product would not be considered a novel food and therefore is not subject to pre-market notification under B.28.002 of the *Food and Drug Regulations*. It should be noted that this opinion is only in regard to the novelty of the *B. subtilis* R0179 strain. It is the continuing responsibility of a manufacturer or importer to ensure that its products are in compliance with all applicable statutory and regulatory requirements. The sale of a food or food ingredient that poses a hazard to the health of consumers would contravene the provisions of the *Food and Drugs Act*.

In the event that you or your clients want to market this ingredient as a probiotic, please note that the use of the term “probiotic/probiotics” in food labelling and advertising should be accompanied by a validated statement about a specific health effect or benefit of the probiotic strain. This is explained in the *Guidance Document – The Use of Probiotic Microorganisms in Food* (http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/probiotics_guidance-orientation_probiotiques-eng.php).

For guidance on what information is required in documenting the health effect or benefit of a food or food ingredient, please see our *Guidance Document for Preparing a Submission for Food*



Food Health Claims at:

http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/health-claims_guidance-orientation_allegations-sante-eng.php#a1-8. Inquiries may be made to the Nutrition Labelling and Claims Section of the Food Directorate by email: healthclaims-allegations-sante@hc-sc.gc.ca.

Sincerely,



Kirsten Mattison
Acting Chief, Evaluation Division
Bureau of Microbial Hazards
Food Directorate

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration
**GENERALLY RECOGNIZED AS SAFE
(GRAS) NOTICE** (Subpart E of Part 170)

Transmit completed form and attachments electronically via the Electronic Submission Gateway (*see Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (*HFS-200*), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission (*Check one*)
 New Amendment to GRN No. _____ Supplement to GRN No. _____

2. All electronic files included in this submission have been checked and found to be virus free. (*Check box to verify*)

3. Most recent presubmission meeting (*if any*) with FDA on the subject substance (*yyyy/mm/dd*): _____

4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (*Check one*)
 Yes If yes, enter the date of communication (*yyyy/mm/dd*): _____
 No

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Solange Henoud		Position or Title Regulatory Affairs Director	
	Organization (<i>if applicable</i>) Lallemand Health Solutions			
	Mailing Address (<i>number and street</i>) 17975 rue des Gouverneurs 923 Water Street #66			
City Mirabel		State or Province Quebec (QC)	Zip Code/Postal Code J7J 2K7	Country Canada
Telephone Number 1-514-573-7067		Fax Number	E-Mail Address shenoud@lallemand.com	
1b. Agent or Attorney (if applicable)	Name of Contact Person James T. Heimbach		Position or Title President	
	Organization (<i>if applicable</i>) JHeimbach LLC			
	Mailing Address (<i>number and street</i>) 923 Water Street #66			
City Port Royal		State or Province Virginia	Zip Code/Postal Code 22535	Country United States of America
Telephone Number 1-202-320-3063		Fax Number	E-Mail Address jh@jheimbach.com	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Bacillus subtilis strain R0179

2. Submission Format: *(Check appropriate box(es))*

- Electronic Submission Gateway Electronic files on physical media
 Paper
If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

- Yes *(Proceed to Item 5)* No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

- a) GRAS Notice No. GRN _____
 b) GRAS Affirmation Petition No. GRP _____
 c) Food Additive Petition No. FAP _____
 d) Food Master File No. FMF _____
 e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

- Scientific procedures *(21 CFR 170.30(a) and (b))* Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? *(see 21 CFR 170.225(c)(8))*

- Yes *(Proceed to Item 8)*
 No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

- Yes, information is designated at the place where it occurs in the submission
 No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

- Yes, a redacted copy of the complete submission
 Yes, a redacted copy of part(s) of the submission
 No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Addition as an ingredient in a specified list of conventional food products at up to 10E9 cfu/serving.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

- Yes No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

- Yes No, you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes No

Did you include this other information in the list of attachments?

Yes No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Lallemand Health Solutions
(name of notifier)
has concluded that the intended use(s) of Bacillus subtilis strain R0179
(name of notified substance)
described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Lallemand Health Solutions
(name of notifier) agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Office of JHeimbach LLC at 923 Water Street #66, Port Royal VA 22535, USA
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official,
Agent, or Attorney

Printed Name and Title

James T. Heimbach, President, JHeimbach LLC

Date (mm/dd/yyyy)

04/29/2021

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667.pdf	Administrative
	GRASforBacillussubtilisstrainR0179.pdf	Administrative
	SignatureNicolosi.pdf	Administrative
	SignaturePariza.pdf	Administrative
	SignatureThomas.pdf	Administrative
	LallemandBsubtilisCoverLetter20210429.pdf	Administrative

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, PRASStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.