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Add the following:

^Bacillus clausii

DEFINITION

Bacillus clausii is a Gram-positive, rod-shaped, spore-forming, motile, facultative aerobic alkaliphilic bacterium. *Bacillus clausii* is manufactured as a spore preparation consisting solely of a specified strain of *Bacillus clausii* that has been spray dried. The formulated ingredient may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of the specified strain of *Bacillus clausii*.

- **Strain UBBC07:** *Bacillus clausii* UBBC07 is a pure, specific strain of *Bacillus clausii*.

[NOTE—The nucleic acid-based method detailed in *Identification B* applies only to *Bacillus clausii* UBBC07.]

IDENTIFICATION

• A. MICROSCOPIC IDENTIFICATION

Analysis: Proceed as directed in the Assay and perform microscopic examination.

Acceptance criteria: Rod-shaped vegetative cell. Ellipsoidal spores may be present at the end of each vegetative cell depending on the growth conditions.

• B. NUCLEIC ACID-BASED IDENTIFICATION

[NOTE—In all cases for *Identification B*, “sterile water” refers to sterile, nuclease-free water acceptable for use in molecular biology.¹]

Buffer: Use a molecular biology-grade 10 mM Tris pH 8.0, 1 mM EDTA buffer.²

Sample: The preparation of the *Sample* requires isolation of colonies grown on plates in the test for *Enumeration*. Colonies grown in the *Enumeration* test should be carefully removed as follows from incubated *BHI agar medium* plates following counting of the colonies as described in the Assay. Dispense 2 mL of *Buffer* into a microcentrifuge tube. Using a sterile loop, carefully remove the cells from the agar (select 1 large colony if appearing on the surface, or 2–3 colonies if small- or medium-sized colonies appear, or extend the incubation for a few more hours to produce surface colonies), and suspend the cells in the microcentrifuge tube containing *Buffer*. Perform DNA extraction with a suitable extraction kit.³ Before analysis, prepare the *Sample* by diluting an aliquot of this solution with sterile water to obtain 50–90 ng of DNA/μL.

Primer sets:⁴ See [Table 1](#).

Table 1. Primer Sets and Acceptance Criteria

Primer Set	Primer Sequence	Acceptance Criteria ^a
UBBC02	Forward (5'-3') TGTATCTAATCCAATTGTCGGTTCGTCCAT and Reverse (5'-3') AGATGTGTATGAATAGTGTATGGAGAGTG AGA. The annealing temperature for primer set is 64.0°.	Amplification product of 516 base pairs
UBBC03	Forward (5'-3') ATTTGATGACGACGATTATTATGCACCACT CTA and Reverse (5'-3') AGACTTAGCTAGGGCTGGAACCAATGCTT GGTA. The annealing temperature for primer set is 68.7°.	Amplification product of 447 base pairs

^a Acceptance criteria for each primer set must be met to prove strain identity.

Primer dilution: Primers should be diluted to 10 μM in sterile water and stored between –20° and –80°.

Polymerase chain reaction (PCR) sample preparations: For each primer set, prepare a *PCR sample preparation* that contains 10 μL of PCR master mix,⁵ 1 μL of diluted forward primer (10 μM), 1 μL of diluted reverse primer (10 μM), 1 μL of diluted sample containing template

DNA (approximately 50–90 ng), and 7 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of diluted sample with 1 µL of nuclease-free water.

PCR positive control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of diluted sample with 1 µL of genomic DNA of the reference strain or replacing the strain specific primers with species specific universal primers of *Bacillus clausii*.

PCR amplification: Perform PCR on the *PCR sample preparation*, *PCR positive control*, and *PCR negative control* using an appropriate thermal cycler.⁶ Incubate at 98° for an initial 30 s, at 98° for another 30 s, at the specific primer set annealing temperature for 30 s, and at 72° for 30 s. Repeat steps 2–4 for 30 cycles, followed by a final elongation at 72° for 5 min with a hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR positive control* and *PCR negative control* using gel electrophoresis. Prepare a 1.5% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris hydrochloride, 1% glacial acetic acid, and 1 mM EDTA) or 0.5X tris-borate–EDTA buffer, or use a commercially available one with the same specifications. Stain the gel with 0.5 mg/mL of ethidium bromide in water and destain with deionized water. [CAUTION—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)⁷ suitable for determining the size of linear double-stranded DNA fragments amplified by the different primer sets described in [Table 1](#). The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons. Alternatively, analysis and visualization may be accomplished using an automated on-chip electrophoresis system with a DNA kit.⁸ Follow the manufacturer's instruction for analysis. Analysis of the *PCR negative control* must result in the absence of any amplification products. If using genomic DNA of the strains as positive control, analysis of *PCR positive control* must result in the amplification of specific base pairs as indicated in the *Acceptance criteria* (see [Table 1](#)). If using species specific universal primers as positive control, analysis of *PCR positive control* must result in the amplification of specific base pairs as found in published literature. If the results are not as expected, preparation of the *PCR sample preparations*, the *PCR positive control*, and the *PCR negative control* must be repeated, followed by PCR amplification and analysis.

Acceptance criteria: See [Table 1](#).

ASSAY

• ENUMERATION

Saline solution: 0.9% sodium chloride in water (w/v). Adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, and then allow to cool in the unopened autoclave. Dispense in sterile containers as needed for preparing samples.

Brain heart infusion (BHI) agar medium: Prepare as shown in [Table 2](#) or use a suitable commercially available agar (see [Table 2](#)).⁹

Table 2. Preparation of BHI Agar Medium

Reagent	Quantity (g)
HM infusion powder ^a	12.5
BHI powder ^b	5.0
Protease peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Agar	15.0
pH	7.4 ± 0.2

^a HM infusion powder is commercially available from HiMedia Laboratories (www.himedialabs.com). Meat infusion powders from other commercial sources may be equivalent.

^b BHI powder is available from www.himedialabs.com or other chemical or microbiological suppliers.

Suspend *BHI agar medium* in 1 L of purified water in an approximately sized conical flask or beaker (sufficiently large enough to prevent boiling over). Cover the flask or beaker with aluminum foil and heat with stirring to boil on a hot plate. Allow to boil for 1 min to completely dissolve the medium, and then autoclave the medium at 121° for 15 min. Cool to 45° and use immediately. Boiled *BHI agar medium* also may be aseptically transferred to individual media bottles in 100- or 200-mL aliquots before sterilization and use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the *BHI agar medium* before use).]