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Bacillus coagulans

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DEFINITION

The *Bacillus coagulans* species is a lactic-acid producing, Gram-positive, spore-forming, rod-shaped bacterium that is aerobic to microaerophilic. *Bacillus coagulans* is manufactured as a spore preparation of the specified strains of *Bacillus coagulans*. 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 coagulans*.

- **Strain GBI-30, 6086:***Bacillus coagulans* GBI-30, 6086 is a pure, specific strain of *Bacillus coagulans*.
- **Strain Unique IS-2:***Bacillus coagulans* Unique IS2, is a pure, specific strain of *Bacillus coagulans*.

[NOTE—The nucleic acid-based identification method detailed in the monograph only applies to strains GBI-30, 6086 and Unique IS-2.]

IDENTIFICATION

• **A. MICROSCOPIC IDENTIFICATION**

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

Acceptance criteria: The spores are seen as small, oval-shaped, retractile, and terminal bodies at the end of each rod-shaped vegetative cell. The presence and number of vegetative cells with spores depends on the growth conditions.

Change to read:

• **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 sample preparation reagent.²

Sample: The preparation of the *Sample* requires isolation of colonies grown on plates in the test for *Enumeration*. Surface colonies grown in the *Enumeration* test should be carefully removed as follows from incubated *Glucose yeast extract agar medium* plates following counting of the colonies as described in the Assay. Dispense 100 µL of *Buffer* into a 2.0-mL microcentrifuge tube with a screw cap closure. [NOTE—Dispense the *Buffer* according to the manufacturer’s instructions.] Using a sterile loop, carefully remove the cells from a single surface colony and suspend the cells in the microcentrifuge tube. Cap the tube, and vortex for 10–30 s or until the cells are homogeneously suspended in the *Buffer*. Heat for 10 min in a water bath held at 100°, and then centrifuge for 3 min at 16,000 × g. Carefully transfer the supernatant to a new microcentrifuge tube. This solution contains the genomic DNA. Before analysis, prepare the *Sample* by diluting an aliquot of this solution with sterile water.

Primer sets: See [Table 1](#).

Table 1. Primer Sets and Acceptance Criteria

Strain	Primer Set ^a	Primer Sequence	Acceptance Criteria ^b
GBI-30, 6086	1	Forward (5'–3') AAGAAGTGGATGTGGGCCGTT C and Reverse (5'–3') CTTTTGCCATGCCAGCATCC. The annealing temperature for primer set is 65.0°.	Amplification product of 376–399 base pairs.
	2	Forward (5'–3') GCCTGATGCAGGGCTTTTCCT and Reverse (5'–3') CCGTCCGCTTCCGTTAAGCCG. The annealing temperature for primer set is 70.0°.	▲Does not produce an▲ (RB 1-Jul-2023) amplification product of 1255–1331 base pairs.

Strain	Primer Set ^a	Primer Sequence	Acceptance Criteria ^b
	3	Forward (5'–3') TTATAGGCGGTAGCAGGGATC and Reverse (5'–3') CGATTGTTTTCCGAAGCA. The annealing temperature for primer set is 61°.	Amplification product of 329–339 base pairs.
Unique IS-2	1	Primer set IS-3: Forward (5'–3') GTCAAGGTCATCAGCTATCTGC and Reverse (5'–3') TCAGTGGTCACGAACATCTCA. The annealing temperature for primer set is 64.0°.	Amplification product of 480–504 base pairs.
	2	Forward (5'–3') GTCTGCGAAAGTTGAGTTATAG TTTAAAGAATG and Reverse (5'–3') ATGATGTGCCAGTTTGTGATG ATATTAACTG. The annealing temperature for primer set is 62.0°.	Amplification product of 280–297 base pairs.
	3	Forward (5'–3') GATGAAAGACATTTGGGGTCG GAGATG and Reverse (5'–3') TCAGTGGTCCCGAGATGTTCTA AAAGAC. The annealing temperature for primer set is 65.0°.	Amplification product of 440–452 base pairs.

^a DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

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

Primer dilution

Strain GBI-30, 6086: Dilute to 2 µg/mL in sterile water and store between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v).

Strain Unique IS-2: Dilute to 0.008 µg/ml in sterile water and store between –20° and –80°.

Polymerase chain reaction (PCR) sample preparation and amplification: See [Table 2](#). For each primer set, perform PCR on each PCR sample preparation using a thermal cycler.³

Table 2. PCR Sample Preparation and Amplification

Strain	Primer Sets	PCR Sample Preparation ^a	PCR Amplification Cycle
GBI-30, 6086	1, 2, and 3	For each primer set, prepare a PCR sample preparation that contains 7.5 µL of PCR master mix, 1 µL of diluted primer left (approximately 400 ng), 1 µL of diluted primer right (approximately 400 ng), 1 µL of diluted <i>Sample</i> containing template DNA (approximately 10–100 ng), and 4.5 µL of sterile water.	• Step 1: Incubate at 98° for 30 s • Step 2: Incubate at 98° for 10 s • Step 3: Incubate at specific primer set annealing temperature for 10 s • Step 4: Incubate at 72° for 45 s Repeat steps 2–4 for 30 cycles, followed by a final extension.



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Strain	Primer Sets	PCR Sample Preparation ^a	PCR Amplification Cycle
			at 72° for 5 min with a hold at 4°.
Unique IS-2	1, 2, and 3	For each primer set, prepare a PCR sample preparation that contains 10.0 µL of PCR master mix, 1 µL of diluted primer left (approximately 10 µM), 1 µL of diluted primer right (approximately 10 µM), 1 µL of diluted Sample containing template DNA (approximately 50–90 ng), and 7.0 µL of sterile water.	• Step 1: Incubate at 98° for 30 s • Step 2: Incubate at 98° for 30 s • Step 3: Incubate at specific primer set annealing temperature for 30 s • Step 4: Incubate at 72° for 30 s Repeat steps 2–4 for 30 cycles, followed by a final incubation at 72° for 5 min with a hold at 4°.

^a For master mix, use New England Biolabs Phusion High-Fidelity PCR Master Mix with GC Buffer (<http://www.neb.com>), DreamTaq Green PCR Master Mix (2x) (<http://www.thermofisher.com>), or other commercial sources.

PCR negative control: Prepare each strain as directed for the PCR sample preparations in [Table 2](#), replacing the 1 µL of diluted *Sample* with 1 µL of nuclease-free water.

PCR positive control: Prepare each strain as directed for the PCR sample preparations in [Table 2](#), either replacing the *Sample* with 1 µL of genomic DNA of the reference strain or replacing the strain-specific primers with species-specific universal primers of *Bacillus coagulans*.

Analysis: Analyze the products of the PCR amplification for each *PCR sample preparation* (see [Table 2](#)) and for the *PCR positive control* and *PCR negative control* by using an automated on-chip electrophoresis system with a DNA kit.⁴ Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 2% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain 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 suitable for determining the size of linear double-stranded DNA fragments amplified with each *Primer set*. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons. If using the 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. Analysis of the *PCR negative control* must result in the absence of any amplification products. 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

Peptone diluent: Prepare a solution of 0.1% peptone⁵ in water (w/v) and adjust with lactic acid solution 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 into sterile containers as needed for preparing samples.

Trace mineral solution: Prepare a solution containing the mineral concentrations shown in [Table 3](#) in deionized water.

Table 3. Preparation of Trace Mineral Solution

Reagent	Quantity (mg/mL)
Sodium chloride	10
Iron (II) sulfate, heptahydrate	18
Manganese (II) sulfate, monohydrate	16
Zinc sulfate, heptahydrate	1.6