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Isolation and genome characterization of poly(3-hydroxy-butyrate)-producing *Brevibacillus* sp. from dairy cattle

A.J. Kim[1], M.-G. Kang[1], M.-G. Kim[1], K. Jang[1], and Y. Kim[1*]

[1]*Seoul National University, Seoul, Republic of Korea;* [*]*Corresponding author:* ykeys2584@snu.ac.kr

Introduction

Rumen microbiome contribute to dairy cattle performance traits, including feed efficiency and methane emission (Mousa et al. 2025). Host genetic background shapes the rumen environment through differences in physiology and feeding behavior and these host-dependent conditions impose the selective pressure for microbial growth and metabolism (MahalaKalaand Kumar 2022). Microbial traits that promote persistence under these conditions shift fermentation end-products and hydrogen flux linking microbial function to methane output and feed efficiency. Recent studies revealed that host-dependent rumen conditions associate with variation in rumen microbial community structure and functional potential across Holstein cohorts (Martínez-Álvaro et al. 2022). However. gene-level mechanisms that explain condition-dependent microbial function in the rumen, including regulation across feeding-cycle perturbations, remain unclear.

Poly(3-hydroxybutyrate) (PHB) is an intracellular storage polyester synthesized by many bacteria. PHB accumulation buffers carbon flux and redox imbalance during environmental nutrient transitions and supports redox homeostasis (Choi et al. 2020). The rumen undergoes recurrent shifts in substrate availability and physicochemical constraints across feeding cycles (He et al. 2024). Defining the PHB biosynthetic pathway provides mechanistic basis of bacterial adaptation strategy in the rumen.

This study aimed to isolate a Holstein rumen bacterium showing PHB-associated storage metabolism and define the genetic basis of that trait at the pathway level. A PHB-accumulating strain was isolated from Holstein rumen fluid, and intracellular PHB production was confirmed. Genome sequencing identified the PHB biosynthetic locus and assigned the corresponding PHA synthase class. The study establishes a gene-level framework for future analysis of host-linked rumen microbial function by defining a PHB-associated adaptive trait in a Holstein rumen isolate.

Materials & Methods

Holstein rumen fluid was diluted and plated on nutrient-stress agar. From 26 strains, six colonies were isolated and *Brevibacillus* sp. SLAM10 was selected after Nile-Red staining. Isolates were cultured in nutrient-stress broth and optical density 600nm (OD_{600}) was measured. SLAM10 cells collected at 2448 and 72 h were stained with Nile Red imaged by fluorescence microscopy and quantified by intensity-threshold segmentation of Nile Red-positive regions (spot count per cell spot are a total spot area per cell). Polymer extracted from SLAM10 biomass was analyzed by Fourier Transform Infrared Spectroscopy (FT-IR) and compared with a commercial PHB standard. The SLAM10 genome was sequenced, assembled, and annotated the *phaC* locus was compared with reference Class IV PHA synthase clusters and pairwise amino-acid identities of PhaC and FabG homologs were calculated.

Results

Identification of a PHB-accumulating Holstein rumen isolate. Twenty-six rumen-derived isolates were plated on nutrient-stress agar and six formed visible colonies (Fig. 1A). Among

these, *Brevibacillus* sp. SLAM10 displayed strong intracellular Nile Red-positive signal as discrete red fluorescent inclusions (Fig. 1B). SLAM10 also grew robustly in nutrient-stress media, reaching the stationary phase at 48 h and remaining stable through the subsequent incubation period (Fig. 2A). The intracellular polymer was extracted from SLAM10 biomass (Fig. 1C) and analyzed by FT-IR (Fig. 1D). The extracted material reproduced the characteristic PHB absorption bands observed in a commercial PHB standard. Spectral similarity was confirmed by a Pearson correlation coefficient > 0.99 between the SLAM10 polymer and the PHB standard.

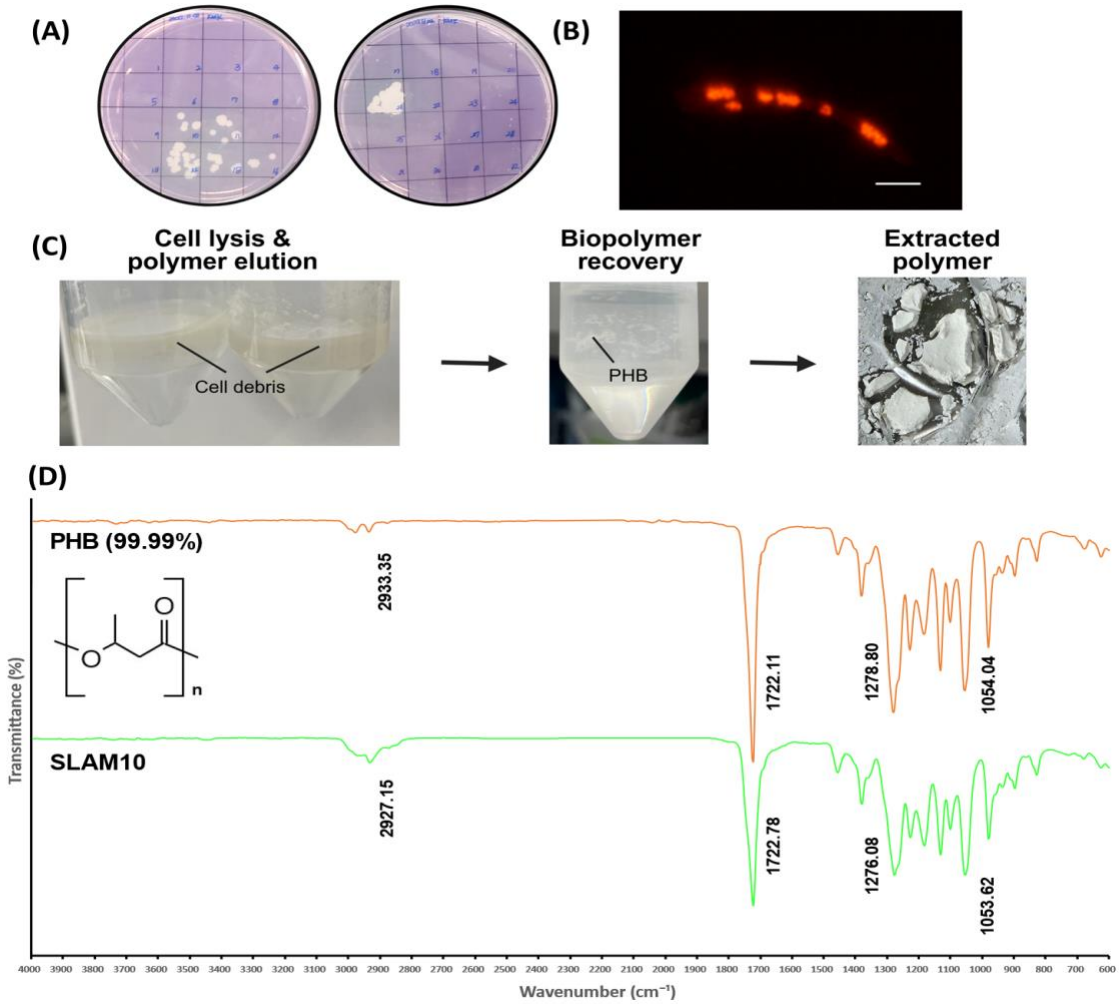

Figure 1. Isolation and PHB confirmation of Brevibacillus sp. SLAM10. (A) Isolation of PHB-producing bacteria. Six strains formed visible colonies on the nutrient-stress agar plate. (B) Red fluorescent-stained granules in SLAM10. Scale bar2µm. (C) Biopolymer extraction from SLAM10. (D) FT-IR spectra of the standard PHB and polymer extracted from SLAM10.

Growth-associated dynamics of a PHB-linked storage phenotype. To map the timing and intracellular pattern of PHB accumulation, SLAM10 cells were sampled along the growth curve and stained with Nile Red (Fig. 2A and B). The images were processed by intensity-based detection of Nile Red-positive regions and the number of red spots per cell and the spot area were quantified at the single-cell level (Fig. 2C). At 24 h (early log phase) red fluorescence was sparse and weak. At 48 h (early stationary phase), the signal was distributed across the cytoplasm with strong variation in spot size and spot number between cells (Fig. 2D). By 72 h (late stationary phase) the signal shifted to a more localized pattern, with fewer larger spots that

were frequently observed near the mid-cell and poles (typically 1-3 spots per cell). From 48 h to 74 hthe spot count per cell decreased (Fig. 2D) but the spot area increased (Fig. 2E), resulting the high total Nile Red-positive area per cell at 72 h (Fig. 2F).

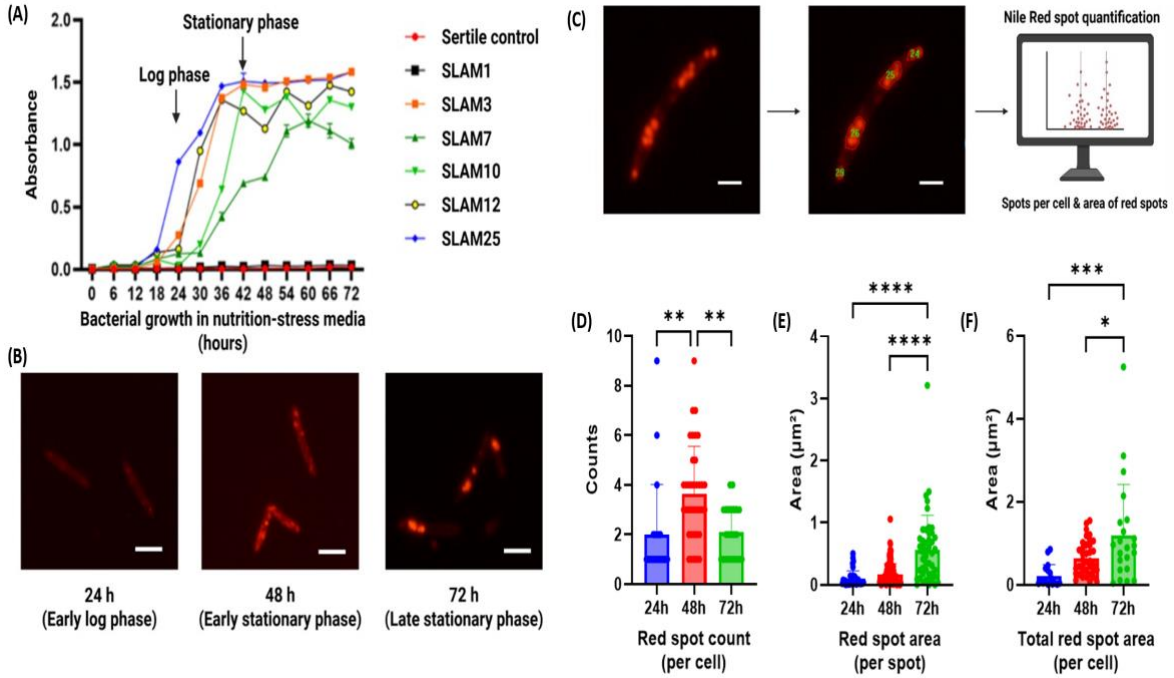

Figure 2. PHB accumulation dynamics in Brevibacillus sp. SLAM10. (A) Growth of six rumen-derived isolates in nutrient-stress broth, measured as OD_{600} over time. SLAM10 entered log phase at 18-24 h and reached stationary phase at 42-48h. (B) Nile Red-stained SLAM10 cells after 24 h48 hand 72 h in nutrient-stress media. Scale bars5 µm. (C) Image-processing for Nile Red spot quantification using intensity-threshold detection of Nile Red-positive regions. Scale bars2 µm. (D-F) Quantification of Nile Red-positive regions: spot count per cell (D), spot area (E), and total spot area per cell (F). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Genome features of the PHB-associated locus. Whole-genome annotation identified multiple genes linked to PHB metabolism across the SLAM10 genome, including precursor supply reductase activity polymerization and transcriptional control (Fig. 3A). To define the PHB synthesis module and its regulatory context, the *phaC* locus was examined and compared with type-strain clusters. SLAM10 showed a Class IV-like gene orientation with *phaR* and *phaQ* in the locus and a *fabG* homolog occupying the canonical *phaB* position (Fig. 3B). The amino acid identities of SLAM10 PhaC to Class IV PHA synthases were 51.9 and 48.4% (Fig. 3C). Among SLAM10 *fabG* paralogs, the *phaB*-position *fabG* copy showed the highest amino acid identities (59.1 and 55.1%) to reference PhaB proteins.

Discussion

SLAM10 was isolated as a PHB-producing rumen bacterium to connect PHB metabolism with adaptation to shifts in nutrient availability and redox balance. SLAM10 grew rapidly and reproducibly under nutrient-stress culture and intracellular PHB granules became prominent in late stationary phase. Late stationary phase is associated with nutrient depletion and oxidative stress. The timing of PHB accumulation, together with a Class IV-like PhaC module, supports a model in which SLAM10 stores surplus carbon as PHB when nitrogen and trace nutrients limit cell division. The condition-dependent appearance of PHB also points to a regulated switch that controls storage initiation. The SLAM10 genome encoded *phaR* and *phaQ*

candidate regulators of *phaC* gene expression in response to environmental signals. Future work should define the signals that modulate *phaR/phaQ* expression and relate that timing to PHB accumulation. This framework supports further study of PHB-associated storage metabolism in rumen microbial adaptation.

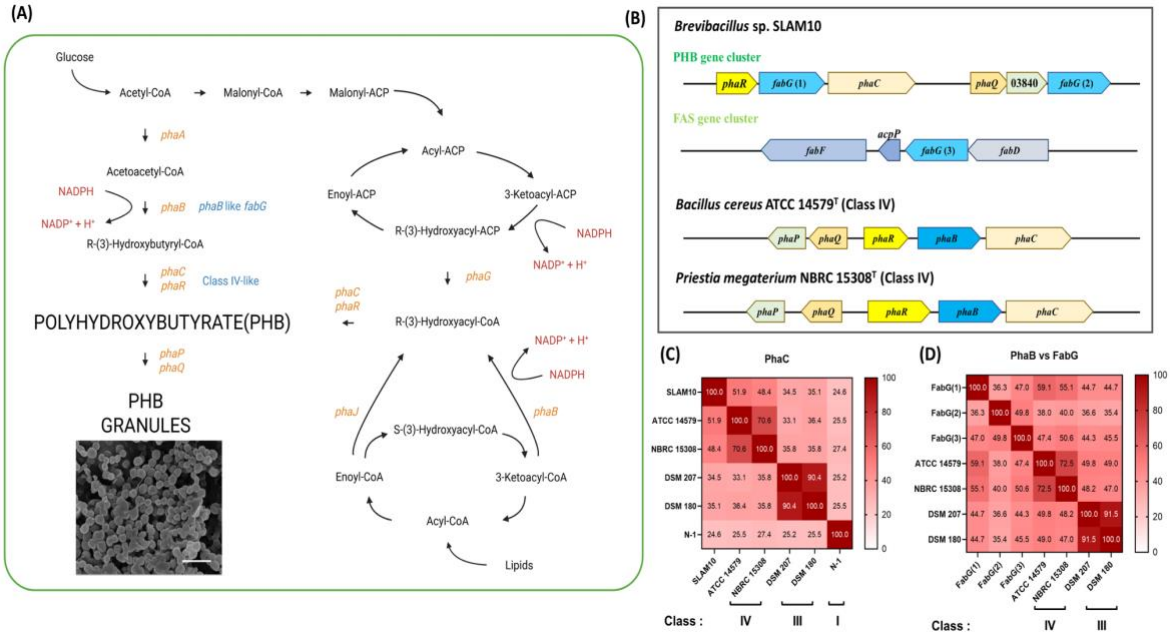

Figure 3. PHA biosynthetic genes in Brevibacillus sp. SLAM10. (A) PHB biosynthetic pathway in SLAM10. (B) Organization of the PHB biosynthetic gene cluster in SLAM10 and comparison with representative Class IV PHA synthase strains (Bacillus cereus ATCC 14579^T and Priestia megaterium NBRC 15308^T). The positions of three fabG copies in SLAM10 are also indicated; two within the PHB locus (fabG1 and fabG2) and one within the fatty acid synthesis locus (fabG3). (C and D) Pairwise amino acid identity matrix comparing SLAM10 PhaC and FabG proteins with reference strains.

Conclusions

A fast-growing PHB-accumulating bacterium was isolated from Holstein rumen fluid. The timing and intracellular pattern of PHB accumulation were defined and the core genes locus organization and biosynthetic pathway linked to PHB production were identified. SLAM10 provides a tractable Holstein rumen isolate for studying PHB-associated storage metabolism and a gene- and pathway-level framework for future functional analysis of rumen microbial adaptation.

Acknowledgements

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