



Occurrence of phenotypic variation in *Paenibacillus polymyxa* E681 associated with sporulation and carbohydrate metabolism

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ABSTRACT

We report the phenotypic variation in *Paenibacillus polymyxa* E681 (E681), a plant growth-promoting rhizobacterium (PGPR) isolated from a winter barley root in Korea. Phenotypic variation (F-type) occurred when E681 (B-type) was grown in the media, and F-type was generated from B-type. B- and F-types were characterized by their morphological, Biolog, and GC-MIDI analyses. F-type cells altered the original biological capacity of B-type cells on endospore and flagella formation, changes in pH in culture, and carbon utilization. In growth curve analysis, B-type variants recovered bacterial growth as the variation occurred after the decline phase, but F-type variants did not. To determine this cause, we conducted comparative proteome analysis between B- and F-types using two-dimensional gel electrophoresis (2-DE). Of the identified proteins, 47% were involved in glycolysis and other metabolic pathways associated with carbohydrate metabolism. Therefore, our findings provide new knowledge on the mechanism of phenotypic variation and insights into agricultural biotechnology.

1. Introduction

Phenotypic variation enables population diversity in several bacterial species, thereby increasing bacterial fitness under certain environmental conditions [1]. Phenotypic variation can be defined as variable expression patterns exhibited by bacterial cells within an isogenic population [2]. It is a natural adaptive process employed by various bacterial species to increase population diversity and fitness during environmental changes that can lead to the alteration of the expression of several proteins, thereby resulting in phenotypic variation [1]. Phenotypic variation has been described for many bacteria, displaying different ecological behaviors, and has been documented in several *Bacillus* species, including closely related *B. subtilis* strains [3,4]. These phenotypic variations often include seemingly unrelated properties, such as motility, aggregation, pigmentation, and metabolic properties in their vicinity [5,6]. A more extreme survival strategy is the formation of endospores, such as those produced by the gram-positive bacterium, *Paenibacillus polymyxa*, in response to nutrient exhaustion. *P. polymyxa* is a rhizosphere-competent bacterium that promotes plant growth; controls plant disease, and induces stress resistance [7]. Additionally, the regulation of biological traits by phenotypic variation has been reported in some strains of *Bacillus* spp., isolated from various rhizospheres [8,9].

Soil microorganisms compete with each other for nutrients and their ecological niche on roots, as they depend on organic substances in the soil [10]. Among root-associated microorganisms, plant growth-promoting rhizobacteria (PGPR) have been extensively studied. PGPR exert beneficial effects on plants by suppressing disease development and improving plant productivity [11,12]. For applications of PGPR, it is first necessary to understand the mechanisms of disease suppression and growth promotion. Many studies have reported the production of antibiotic compounds by PGPR involved in microbial growth inhibition [13]; in contrast, Volfson et al [14]. observed no effect on plant-growth promotion due to phenotypic variation in bacteria.

Paenibacillus polymyxa E681, isolated from the roots of winter barley, is a PGPR strain that can colonize the rhizosphere of cucumber, pepper, sesame and *Arabidopsis thaliana*, conferring beneficial effects to these plants both *in vitro* and field conditions [13]. The strain E681 produces antimicrobial compounds that protect plants against various phytopathogens. In addition, protects plant tissue from biotic and other environmental stresses [15]. *Streptomyces* spp. and *Pseudomonas* spp. can suppress plant pathogens, but lacking the long-term storage [16]; therefore *Bacillus* spp. and *Paenibacillus* spp. are being considered as strong candidates to produce antimicrobial peptides that suppress the growth of the plant pathogens [17]. Some *P. polymyxa* strains are

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important for plant growth as they facilitate the production of plant growth regulators, such as cytokinins and auxins [18], by colonizing the roots [19,20].

Furthermore, gram-positive bacteria, such as *Bacillus subtilis* and *P. polymyxa* are capable of utilizing carbohydrates as a primary source of carbon and energy under *in vitro* conditions [21]. The bacterial utilization of carbohydrates depends heavily on their chemical composition [22]. Since bacteria can regulate carbohydrate metabolism under various conditions, such as soil, water, vegetable matter, and rhizosphere environments, they have been used to evaluate the efficiency and liability of organic carbon [23]. Furthermore, certain lactic acid bacteria have been found to metabolize various carbohydrates competitively to colonize numerous ecosystems through various metabolic pathways [24]. They are naturally well established group of microorganisms widely used in food industry and of well-documented impact on human health [25]. This has provided a deeper understanding of bacterial carbohydrate metabolism and other phenotypic traits, including arabinose and melibiose utilization genes in *Lactococcus lactis* [26] and glyoxylate cycle and D-galacturonate degradation in *Paenibacillus* strains [27]. Previously, Zheng et al [28]. showed that genes lost in heterofermentative species are mostly associated with carbohydrate metabolism, which justifies genotype-phenotype association studies at the orthogroup level. To date, among many reports on PGPR-elicited growth promotion, reports on the effects of bacterial phenotypic variation in *Paenibacillus* species have been limited. Therefore, the objective of this study was to characterize the phenotypic variation in *P. polymyxa* by analyzing changes in the protein expression pattern and variations expressed as phenotypes.

2. Materials and methods

2.1. Microorganisms and culture conditions

The bacterium *P. polymyxa* E681 (E681) used in this study was originally isolated from winter barley roots in Korea [29]. To analyze the phenotypic variation of the colonies from the wild-type strain E681, a single colony was inoculated into tryptic soy broth (TSB; Difco Laboratories, Detroit, USA) and incubated at 28 °C for 4 days under shaking conditions at 180 rpm. From this culture, a 100 µL aliquot was diluted appropriately, spread on freshly prepared tryptic soy agar (TSA) plates, and incubated for 48 h. These plates produced two types of bacterial colonies, named B-type and F-type, for the wild-type and variant forms, respectively.

2.2. Comparison of B- and F-type of E681 using BIOLOG and GC-MIDI

B- and F-type E681 colonies were tested for the utilization of 95 carbon sources using the BIOLOG program [30]. Briefly, bacterial cells cultured on bacterial universal growth (BUG) agar supplemented with 0.25% maltose and 0.9% thioglycolate (BUG+M + T) at 28 °C for 48 h were suspended in an inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, and 0.01% gellan gum), inoculated onto microplates (Biolog GP MicroPlate™), and incubated at 28 °C. After 24 and 48 h of incubation, the absorbances of the plates were read with a MicroLog 3-Automated Microstation system (Biology Inc., Hayward, CA, USA). The bacterium was identified using the MicroLog Gram-positive database (version 4.0; Biology Inc.). Gas chromatography of fatty acid methyl esters (GC-FAME) was conducted to confirm bacterial identification. The bacteria were cultured on TSA plates at 28 °C for 48 h. The colonies were harvested and placed in screwcap culture tubes, and 1 mL of saponification reagent (45 g NaOH, 150 mL methanol, and 150 mL distilled water) was added. A methylation reagent (325 mL 6.0 N hydrochloric acid and 275 mL methanol) was added after heat treatment, and fatty acids were extracted with extraction solvent hexane/methyl tertiary butyl ether (MTBE), mild base (10.8 g of NaOH in 900 mL distilled water), and a saturated NaOH solution. The fatty acid composition was

analyzed using the Sherlock system, followed by the generation of a similarity index for the isolates that corresponded to a microorganism in the database (MIDI Library version, TSBA 4.0, Library Generation system software version 4.0; MIDI Inc., Newark, USA). Additionally, the VITEK2 automated system (bioMerieux, Marcy l'Etoile, France) was used for biochemical identification of both B- and F-type cells. Bacterial cells were grown for 48 h at 28 °C in TSA and emulsified in 0.45% sterile NaCl solution according to the manufacturer's instructions. The prepared cell suspensions (150 µL) were loaded onto VITEK2 gram positive (GP) cards, and the data were analyzed using the AES parameter version of VITEK2 (03.01).

2.3. Assessment of growth conditions for phenotypic variation of E681

To examine bacterial growth and the phenotypic variation of E681, B-type cells were cultured in 100 mL of fresh TSB in a 250 mL Erlenmeyer flask and incubated at 30 °C under shaking conditions at 180 rpm for 7 days. Bacterial population growth and colony phenotypes were recorded by plating 10-fold serial dilutions of the cells on TSA. The total colony forming units (CFU)/mL and phenotypic variants were detected every 24 h during the growth period. Both types of E681 were incubated in TSB at 28 °C for 3 days and the pH of the medium was monitored daily.

2.4. Assay of sporulation efficiency

To assess endospore formation in E681, B- and F-type cells were cultured on TSA plates and incubated for 5 days at 28 °C. The cells were harvested from a solid medium using phosphate-buffered saline (PBS) and then centrifuged at 13,000 × g for 10 min. Two distinct layers (white and brown) appeared in the B-type precipitate, whereas only a single brown layer appeared in the F-type precipitate. The condensed endospores were only detected in the white layer using a phase-contrast microscope (400 ×) (LABOPHOT-2; Nikon, Japan).

2.5. Total RNA extraction, cDNA synthesis, and gene expression through real-time reverse transcription PCR

The total RNA from B- and F-type cells was isolated from the cultures grown in TSB for 3 or 6 days using the RNeasy Mini kit (Qiagen Inc., Hilden, Germany) with on-column DNase I treatment according to the manufacturer's instructions. qRT-PCR was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). For real-time reverse transcription PCR (qRT-PCR), the reaction mixture contained 10 ng of cDNA, SsoAdvanced SYBR Green Supermix (Bio-Rad), and 5 pmol of each forward and reverse primer: hag-F (5'-TCT TCG GGT CTG CGT ATT AA-3') and hag-R (5'-TTG AAC GCC CAG CTC TTT-3') for the flagellin gene (NCBI gene ID: 9,777,150 [31]), sigD-F (5'-GCC GCT CAT TTA AAC CAT TC-3') and sigD-R (5'-GAC GGC CTG ACA CAT ATT C) for the sigma factor D gene (NCBI gene ID: 9,774,665; [31]), and pai1-F (5'-TGA GTC ACT CGA AAT TGA GAG GAT TTA T-3') and pai1-R (5'-CCA TAC GCC GAG CCA GAT TT-3') for the *pai1* gene (NCBI gene ID: 9,775,134). Gene transcription analysis was conducted by qRT-PCR using a CFX Connect Real-Time PCR Detection System (Bio-Rad), with 16S rRNA as a reference gene [32]. Thermal cycling conditions were as follows: denaturation at 95 °C for 3 min for polymerase activation, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. After the final reaction cycle, melting curves were obtained via a temperature ramp from 65 to 95 °C, with 0.5 °C/s increments, to exclude nonspecific products. Each PCR run included a "no template" sample, and all tests were performed in triplicate. The cycle threshold (Ct) value relative to the control sample was considered for the calculation of $\Delta\Delta Ct$ (difference between ΔCt values calculated from the difference between the Ct of the target and the reference gene) for the samples. The PCR efficiency was determined using a standard curve generated by serial dilution of the cDNA (~0.2–20 ng).

2.6. Protein sample preparation

Both B- and F-type E681 cells were incubated for 2 days and two different types of colonies were recovered with ice cold PBS (pH 7.2). The cell suspensions were centrifuged at $5000 \times g$ for 15 min. The bacterial cell pellets were washed twice with ice-cold PBS and sonicated for 10 s using Sonoplus (Bandelin Electronic, Germany). The cell pellets were homogenized directly using a motor-driven homogenizer (Power-Gen125, Fisher Scientific) in a sample lysis solution composed of 7 M urea, 2 M thiourea containing 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), and 2% (v/v) pharmalyte, and 1 mM benzamidine. Proteins were extracted for 1 h at room temperature by vortexing. After centrifugation at $15,000 \times g$ for 1 h at 15 °C, insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis (2-DE). The protein concentration of each sample was assayed by the Bradford method [33] using the Bio-Rad DC protein assay kit, according to the manufacturer's instructions.

2.7. Two dimensional protein gel electrophoresis

Two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were conducted by Genomine Inc. (Pohang, South Korea). IPG dry strips (4–10 NL IPG, 24 cm, Genomine, Korea) were equilibrated for 12–16 h with 7 M urea, 2 M thiourea containing 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte and loaded with 200 µg of the sample. Isoelectric focusing (IEF) was performed at 20 °C using a Multiphore II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences) following the manufacturer's instructions. For IEF, the voltage was linearly increased from 150 to 3500 V during 3 h for sample entry, followed by a constant 3500 V, with focusing complete after 96 kVh. Before the second dimension, the strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% (w/v) DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 × 24 cm, 10–16%). SDS-PAGE was performed using the Hoefer DALT 2D system (Amersham Biosciences) following the manufacturer's instructions. The 2D gels were run at 20 °C for 1700 Vh. Then, the 2-DE gels were stained with coomassie G250 as described by Anderson et al [34]. The digitized images were quantified using PDQuest software (version 7.0, BioRad) according to the manufacturer's protocols. The quantity of each spot was normalized to the total valid spot intensity. Protein spots were selected for the significant abundance variation that deviated over 2 fold in its accumulation levels compared with B- or F-type samples.

2.8. Protein sequencing

Spots, which differed significantly in volume between B- and F-type variants, were excised from the gel and digested with trypsin (Promega, Madison, WI), mixed with α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, and subjected to MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics). Spectra were collected from 300 shots per spectrum over an *m/z* range of 700–4000 and calibrated by two-point internal calibration using trypsin auto-digestion peaks (*m/z* 842.5094, 2211.1040). The peak list was generated using FlexAnalysis 3.0 (Bruker Daltonics). The search program MASCOT, developed by the Matrix science (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), was used for protein identification by peptide mass fingerprinting (PMF).

2.9. Statistical analysis

Data were subjected to analysis of variance using JMP software (SAS Institute Inc., Cary, NC, USA). The significance of the B- and F-treated

growth parameters was determined by the magnitude of the F value at $P < 0.05$. When a significant F value was obtained for treatments, separation of the means was accomplished using Fisher's protected least significant difference (LSD) at $P < 0.05$.

3. Results

3.1. Occurrence of phenotypic variation in E681

Previous studies have shown that phenotypic variation is regularly observed when cultured the wild-type E681 cell suspensions on a solid medium, and two types of colonies (B- and F-type) have been reported [35]. Phenotypic variation occurred in B-type led to the generation of F-type. F-type showed no variation to other types and did not revert to B-type under our experimental conditions (Fig. 1). A typical E681 colony has an opaque, milky-white, with a round, bald and shiny surface (Fig. 1A). This phenotypic variation was observed by spreading four-day-old culture suspensions on TSA plates. Typical colonies of the E681 strain, hereafter referred to as B-type, were bald, shiny, convex, and round in shape (enlarged image shown by dotted arrows); however, a few colonies showed phenotype switching into flat morphologies, known as phenotype variants, hereafter referred to as F-type (Fig. 1B; enlarged image shown by dotted arrows). The colony shapes associated with F-type were translucent and round, with a scalloped edge and flat with a dull surface (Fig. 1B). This phenotypic variation was irreversible (B-type → F-type; Fig. 1C), while no further variation of F-type was observed from the phenotypic variant after a third subculturing of phase II (F-type → F-type; Fig. 1D) from the initial culture. At this stage, no detectable reversion from the F- to the B-type was observed when the cells were examined under different culture conditions, including different media (Table S1).

3.2. Morphological characteristics, BIOLOG and GC-MIDI analysis of B- and F- types in E681

The colony morphology and physiological and biochemical characteristics of both B and F-types are summarized in Table 1. The biochemical characteristics of both B- and F-types were assessed using BIOLOG and VITEK 2 system, which suggested that both isolates belonged to *Paenibacillus polymyxa* E680. According to the results of BIOLOG, F-type could utilize six types of carbon sources (glycerol, 2-deoxy adenosine, adenosine, thymidine, monomethyl succinate, and uridine), while B-type did not. We also found differences in the biochemical activities of F-type, including L-aspartic acid arylamidase activity, alanine arylamidase activity, and O/129 resistance (comp. vibrio.) in (VITEK 2). Additionally, GC-FAME analysis identified B- and F-types as *P. polymyxa* consisting of 16 fatty acids with minor differences in fatty acid profiles (Table 1).

3.3. Effect of bacterial growth conditions on phenotypic variations

Regarding bacterial growth, the B-type showed a pattern in which the number of populations recovered after the decline phase, but F-type showed no recovery during the decline phase. In detail, 24 h after entering the stationary phase, the growth of B-type declined after day 3 and again started to increase after day 4 (Fig. 2A). In contrast, 24 h after entered the stationary phase, the growth of F-type started to decline on day 5, and the population did not recover until day 7 (Fig. 2B). When B-type cells were cultured in a liquid medium for 3 days, followed by spreading on a solid medium, no phenotypic variation occurred. Later, phenotypic variation (F-type) began to appear from the day 4 of culture and reached a maximum level on day 7 (Fig. 2C). When the phenotypic variation was very active on day 4, the bacterial population declined dramatically. On day 3, the population was 9.4×10^6 CFU/mL, while on day 4; it was 1.3×10^5 CFU/mL. In fact, 98.6% of the cells observed on day 3 died on day 4, and majority of the remaining viable cells were

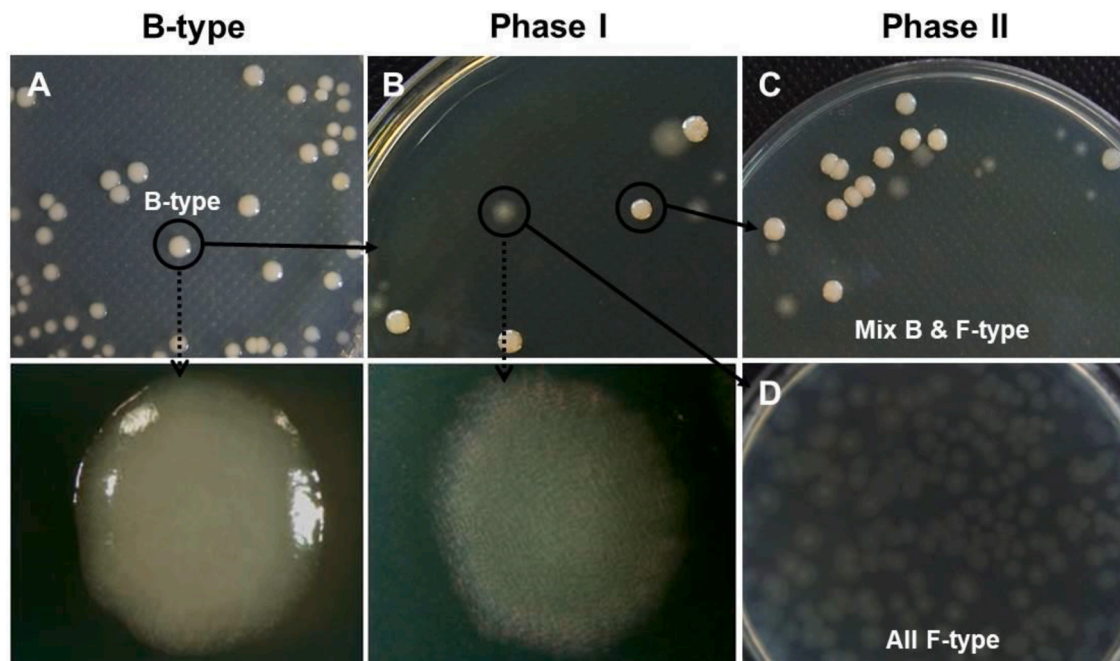


Fig. 1. Phenotypic variation of *Paenibacillus polymyxa* E681 on sub-culturing at different generations. (A) The B-type colonies of E681 grown on TSA plate switched to phenotypic variant (F-type) on successive subculturing in the 2nd generation (Phase I). (B) When cultured onto the fresh TSA plates, the colony from the 1st generation formed two types of colonies (B- and F-type). The enlarged images of B- and F-type are shown by dotted arrows. (C) In the 2nd generation (Phase I), B-type showed two types of colonies (B- and F-type). The F-type did not revert to any other phenotype. Each experiment contains three replicates, and the experiment was performed at least two times.

transformed into variation, suggesting that phenotypic variation occurred during this phase. However, F-type showed no variation until day 7 (Fig. 2D) and did not revert to B-type or any other type. Interestingly, B-type showed a pattern of population recovery after a short decline phase, while the F-type cells died in the decline phase. The population of B-type did not fall below 1.3×10^5 CFU/mL and recovered to 4.3×10^6 on day 7 (the last day of measurement). F-type showed slightly faster growth than B-type at three temperatures (Fig. 2E). At 20 °C, B- and F-types exhibited a doubling time of 48.0 ± 0.1 and 41.4 ± 3.0 min, respectively.

3.4. Effect of phenotypic variation on sporulation

A noticeable difference between the B- and F-types was the ability to form endospores (Fig. 3A). After the end of the exponential growth phase, prespore formation and lysis of the spores were initiated in the B-type colonies on TSA. When the harvested bacterial cells were centrifuged in Eppendorf tubes, the cells separated into two layers (white and brown). Phase-contrast microscopic observations revealed that endospores were present only in the white layer (lower part), and a few endospores were present in the brown layer (upper layer), mainly containing cell debris. In the case of F-type cells, only vegetative cells were present in the brown layer, but no endospores were observed, except for cell debris. Upon increasing the incubation time, the number of mature spores drastically increased in the B-type colonies, whereas the F-type colonies did not form endospores and were self-destructed by autolysis.

3.5. Expression of genes involved in sporulation and flagellum synthesis

Sporulation deficiency in F-type was supported by the analysis of expression levels of a gene homologous to *pai1*, which is involved in the downregulation of sporulation and degradative-enzyme production. The deduced amino acid sequence of the *pai1* homolog (PPE_02381/PPE_RS11580) in E681 encodes the protease synthase and sporulation-

negative protein PAI 1. During 6 days of bacterial culture, the over-expression of *pai1* in B-type was detected only on day 3 during the late stationary phase (Fig. 3B). However, the induced expression of *pai1* gene started to decrease drastically from day 4 onwards. In contrast, in F-type, no significant difference was observed in the expression levels of *pai1* throughout the 6-day bacterial culture. From the growth curve (Fig. 2C); *pai1* gene expression was upregulated just before switching of B-type to F-type. These results suggest that the *pai1* gene plays a crucial role in converting B-type into F-type at a particular stage, preventing endospore formation.

The increased number of flagella in F-type was confirmed by analyzing the expression levels of the genes linked to bacterial motility; *hag*, which encodes the flagellin structural protein, and *sigD*, which encodes a sigma factor to control flagella synthesis. The expression of both *hag* and *sigD* genes in F-type was upregulated compared to that in B-type, except on day 3 (Fig. 3C). The expression of *hag* and *sigD* genes was upregulated in B-type on day 3, whereas the *hag* gene was highly expressed on day 1 of incubation in F-type. In B-type, the *hag* gene was upregulated on day 3 when phenotypic variation occurred. This suggests that B-type becomes hyper-flagellated after a particular period of incubation, a characteristic feature of phenotypic variation (F-type), which can be used for bacterial swarming to migrate across solid surfaces. The pH of the culture medium of B- and F-types was monitored for 3 days (Fig. 3D). The pH of the medium changed from neutral to acidic conditions, leading to a greater decrease in the pH of the culture medium of F-type than that of B-type. The pH of the B-type and F-type culture media was 6.4, and 5.7, respectively, at the 3-day culture in TSB.

3.6. Proteomics analysis of B- and F-types

Protein spots on 2-DE were visualized within the molecular weight (MW) range of 10–200 kDa at isoelectric points (pI) of 4–10 (Fig. 4A and 4B). Proteomic analysis was performed in duplicate using different biological replicates. In the first experiment, 215 and 325 spots were counted on the maps of B- and F-types, respectively, using the PDQuest

Table 1
Phenotypic characteristics of B- and F-types in *P. polymyxa* E681.

Test	B-type	F-type
Colony morphology	Convex	Flat
Cell shape [#]	Rod	Rod
Swarming ability [#]	-	+
Endospore formation	+	-
Biofilm formation [#]	+	-
Number of Flagella [#]	A few	plenty
Lipase activity (Fig. S2)	+	-
Antifungal capacity [#]		
<i>Rhizoctonia solani</i>	++	+
<i>Cylindrocarpum destructans</i>	++	+
Antibacterial activity [#]		
<i>E. coli</i> (DH5 α)	+	-
Indole test [#]	++	+
Plant growth promotion	++	+
Metabolic activity through BIOLOG GP microplate assay		
Substrate	B-type	F-type
Glycerol	-	+
2-deoxy adenosine	-	+
Adenosine	-	+
Thymidine	-	+
Monomethyl succinate	-	+
Uridine	-	+
Biochemical characteristics using Vitek 2		
Biochemical test (substrate)	B-type	F-type
L-ASPARTIC ACID Arylamidase	+	-
Alanine Arylamidase	+	-
O/129 Resistance (comp.vibrio.)	-	+
Cellular fatty acid profiles based on GC-FAME		
GC-FAME Short hand name of fatty-acid	Percent fatty acid (%)^a	
	B-type	F-type
11:0 2OH*	0.13 - 0.19	0 - 0.11
13:0 ISO	0 - 0.12	0.1 - 0.22
13:0 ANTEISO	0 - 0.15	0.09 - 0.22
14:0 ISO	1.28 - 1.47	1.23 - 1.73
14:00*	1.05 - 1.27	1.32 - 1.44
15:0 ISO*	6.44 - 6.45	6.9 - 7.74
15:0 ANTEISO*	51.85 - 52.94	57.62 - 63.96
16:1 w7c alcohol	1.1 - 1.18	1.06 - 1.18
16:0 ISO*	6.31 - 7.37	4.98 - 5.54
16:1 w11c	2.43 - 2.35	2.63 - 2.78
16:00*	3.45 - 4.46	2.23 - 3.17
ISO 17:1 w10c	0.85 - 0.95	0.78 - 0.99
17:0 ISO*	3.56 - 4.38	2.21 - 3.28
17:0 ANTEISO	13.31 - 18.16	9.28 - 13.63
17:00*	1.02 - 1.03	0.48 - 0.66
18:00*	0.41 - 1.44	0 - 0.39

Note: -, negative reaction; +, positive reaction.

[#] Results were described by Lee et al. (2020).

software, of which 167 spots were significantly different between the two types. In the second experiment, 398 and 458 protein spots were counted on the maps of B- and F-types, respectively, of which 218 spots were significantly different between the two types. Among these, 53 proteins (49 upregulated and 4 downregulated) were selected for identification by MALDI-TOF analysis. This selection was based on the abundance levels (fold change >1 or <1 in common in replicates) and the position of the proteins relative to the spots. Only spots that could be excised without the risk of cross-mixing were selected for identification. The identities of the selected proteins obtained by MALDI-TOF analysis are listed in Table 2. The results showed that in some cases, two or more spots differing in their pI or MW were corresponded to the same protein. For example, enolase (2-phosphoglycerate dehydratase) was detected in four spots (1402, 1403, 1605, and 1625). This suggests that different isoforms of the same protein exist or the protein undergoes a possible post-translational modification, such as phosphorylation or glycosylation. A total of 53 protein spots were identified as 18 specific proteins (Table 2). By applying this protein list to the string map, it was confirmed that 8 proteins were linked to each other and that two types of glycerol dehydrogenase (GDH) were linked to each other (Fig. 4C).

To systematically study the phenotypic variation in E681, we

combined the list of differentially expressed proteins from 2-DE and the products of differentially expressed genes (DEGs) from previously conducted RNA-sequencing [35]. Both methods revealed that 7 out of 18 proteins were upregulated (Table 2). These upregulated proteins were 6, 7-dimethyl-8-ribityllumazine synthase (ribE), which is involved in riboflavin biosynthesis; butanediol dehydrogenase (BDH), which is involved in butanoate metabolism, and other gene products (succinate dehydrogenase flavoprotein subunit, ATP synthase subunit alpha, and hexulose-6-phosphate synthase), which are involved in carbohydrate metabolism, such as glycolysis, the TCA cycle, the pentose phosphate pathway, and ATP production. Additionally, 2-DE analysis showed that flagellin was overexpressed, which is consistent with *hag* gene over-expression in the RNA-seq results.

3.7. Differential carbohydrate metabolism in B- and F-type variants

A closer look at the differentially expressed proteins in both types indicated that a major fraction of the upregulated proteins is involved in carbon metabolism through glycolysis, TCA cycle, oxidative phosphorylation, and the pentose phosphate pathway (Fig. 4D). These proteins were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase (ENO), GDH, pyruvate dehydrogenase E1 component subunit beta (PDH β), and succinate dehydrogenase flavoprotein subunit (SDH), and upregulated in F-type. Transaldolase 2 (TAL) and hexulose-6-phosphate synthase (HPS) are involved in the pentose phosphate pathway. ATP synthase subunits alpha and beta (ATP α and ATP β) involved in energy generation were upregulated in F-type. The stress protein, superoxide dismutase (SOD), was upregulated in F-type. SOD is an enzyme that converts superoxide radicals to oxygen and hydrogen peroxide and has an adaptive function in ensuring the viability of bacteria under harsh environmental conditions [36,37]. To scavenge reactive by-products of oxygen, which occur in the energy production process, antioxidant superoxide dismutase, an antioxidant protein, was highly expressed in F-type. Additionally, butanediol dehydrogenase (BDH) and inosine-uridine, preferring nucleoside hydrolase (IUNH), were highly expressed in F-type. Energy production by carbohydrate metabolism is of great significance for bacterial growth and antioxidant potential [38]. Over 47% of the identified proteins in 2-DE were involved in glycolysis and other metabolic pathways associated with carbohydrate metabolism and all the proteins were upregulated in F-type.

4. Discussion

Generally, microorganisms can enhance their survival under harsh environmental conditions by increasing their diversity at the population level. According to the definition, phenotypic variation occurs at a frequency rate >10⁻⁵ switches/cell/generation, where the phase variation has been displayed for many bacterial ecological behaviors [39,40]. This phase variation is necessary to generate intra-population diversity to increase bacterial fitness during niche adaptation. In E681, the bald and convex-shaped colony (B-type) switched to the flat-shaped colony, a phenotypic variant (F-type) on TSA after multiple subcultures. During phenotypic variation, the variants are generally unstable and capable of reverting to the original phenotype [39]; however, in some cases, the switch is irreversible [41,40]. Consistent with the results of our study, the reversion of F-type to B-type was rarely observed, even after multiple subcultures. In the growth curve of B-type, the population growth was reduced at the declined phase recovered from day 4 when the phenotypic variation occurred. F-type did not change to either B-type or any other type, suggesting that the phenotypic variation may help sustain the population of E681.

The most prominent feature of this phenotypic variation is a deficiency in the endospore-producing ability. Bacterial endospores can resist a variety of harsh environmental conditions [42]. *P. polymyxa* can form endospores, which may be potential biocontrol agent. E681 suppressed fungal pathogens both *in vitro* and *in vivo* and increased plant

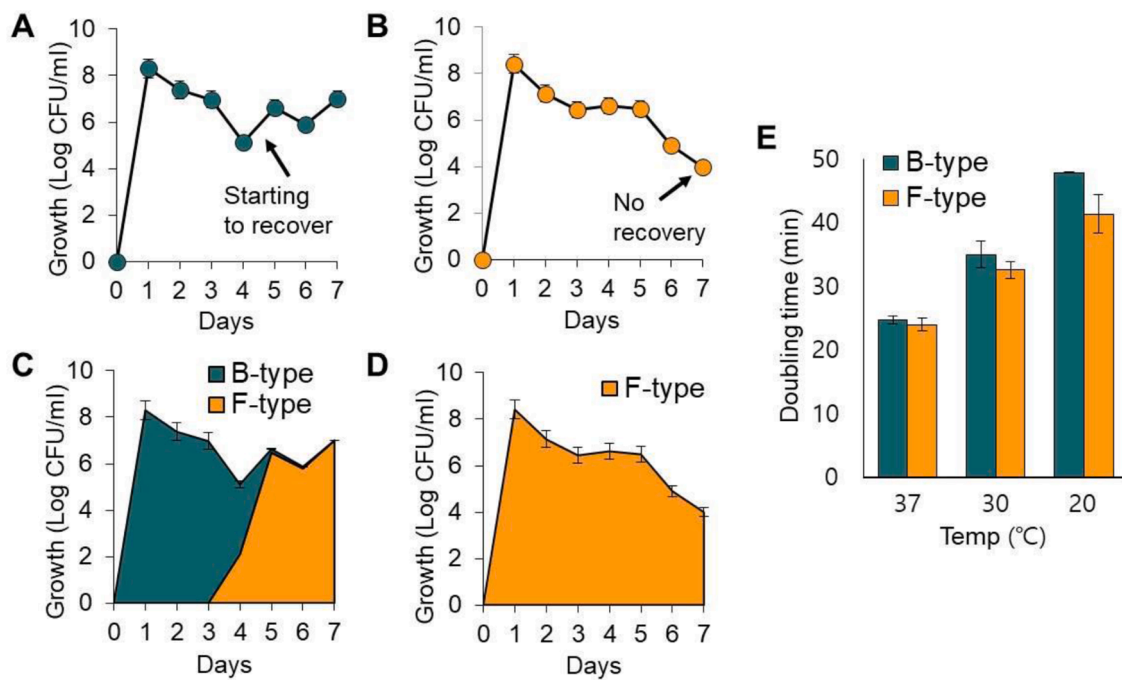


Fig. 2. Bacterial cell growth at different duration of time. The growth curves of two types of E681, wild type (B-type; A) and phenotypic variant (F-type; B) were tested for growth population. The conversion of B-type to F-type starts on the 3rd day after incubation at 30 °C (C), and when F-type cultured onto TSB, did not show any variation until seven days (D). All colonies were of only F-type. (E) The cell doubling time of B- and F-types at different temperatures. Results represent means ± SD from duplicated independent experiments. Each experiment contains three replicates and the experiment was repeated at least once with similar results.

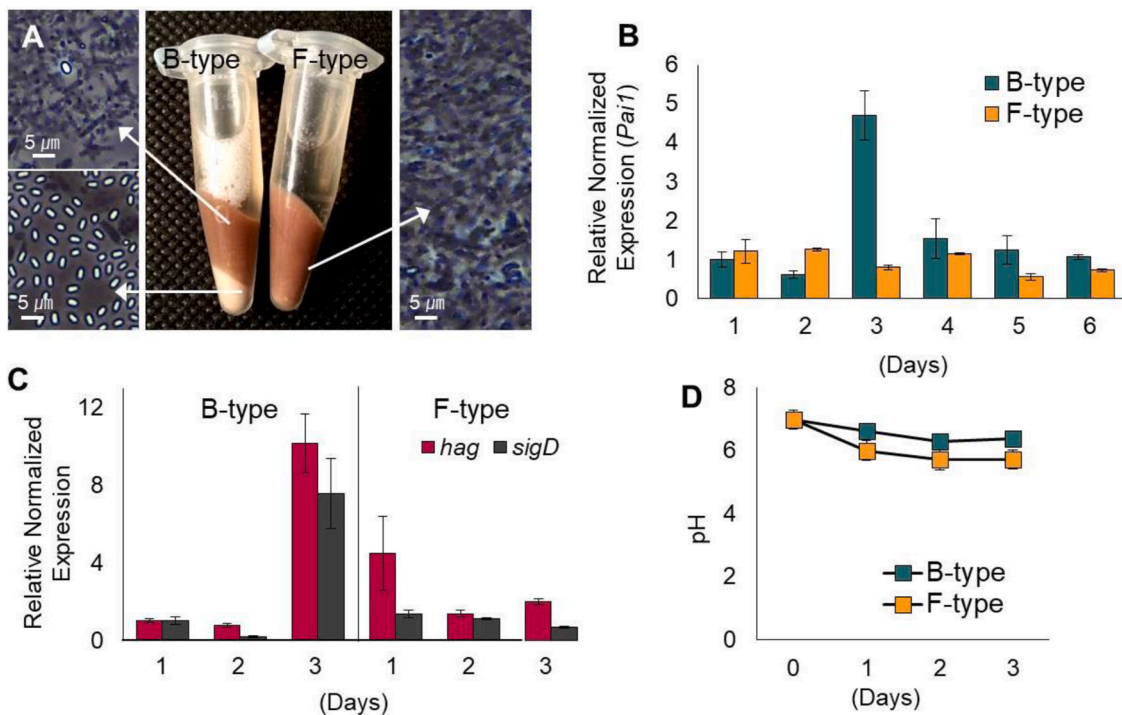


Fig. 3. Phenotypic variation on sporulation, relative expression of endospore formation and flagella related genes. Collection of B-type and F-type cells from the solid medium, tryptic soy agar (TSA) plates by centrifugation after 6 days of incubation. (A) When B-type cells were precipitated, two distinct layers (white and brown) were appeared, while there was only brown layer in the F-type precipitate. Endospore formation, and a large proportion of B-type cells had endospores, but no endospores were detected in F-type after 9 days of culturing on TSA at 30 °C. The phase contrast microscopic observations showed that endospore colonies were found greater number in B-type than in F-type, while the F-type colonies were destructed by autolysis and did not form endospores. (B) Relative expression of *Pai1* gene for endospore formation during phenotypic variation was found greater in B-type than F-type on the 3rd day, and later started reducing gradually. (C) Relative expression of flagella related *hag* and *sigD* genes of B-type and F-types detected by qRT-PCR analysis. (D) pH variation was observed both in B- and F-types in TSB for 3-days of incubations.

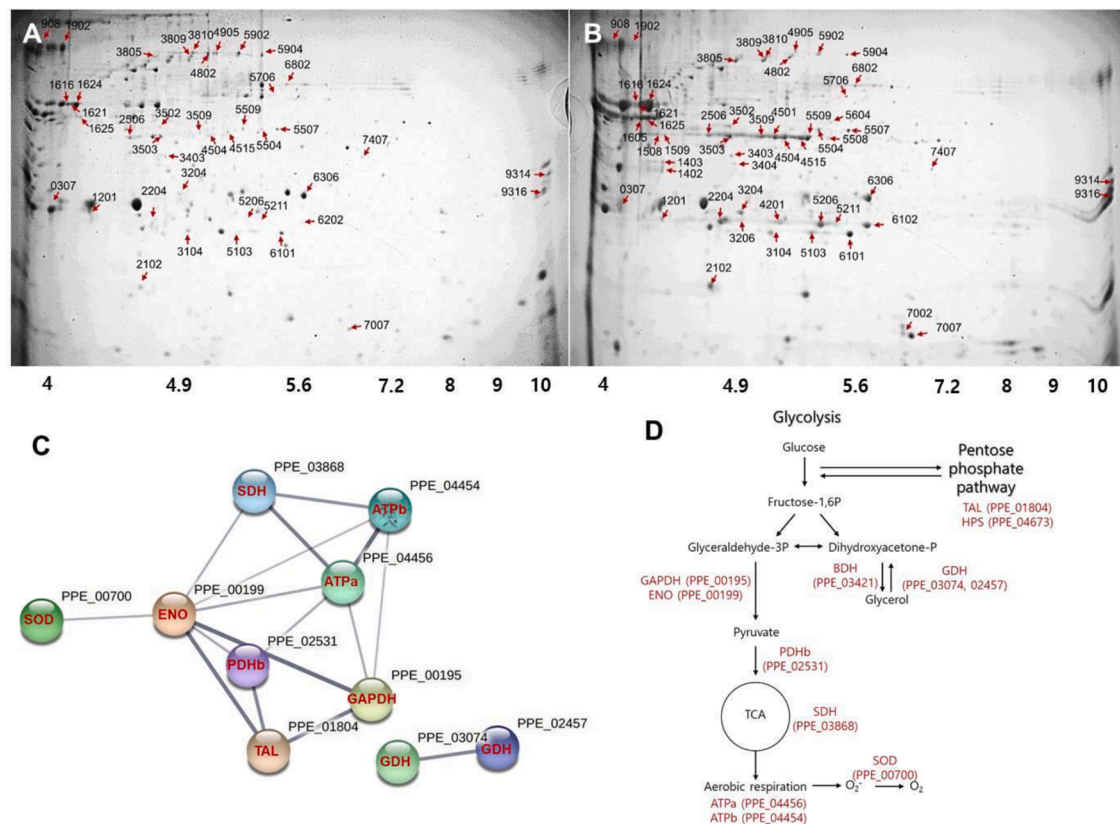


Fig. 4. (A, B) Comparative 2-DE gels showing the proteome of E681. B-type (left) and F-type (right). Proteins occurred on a pH 4–7 linear IPG strip, separated by 2-DE and stained with Coomassie Blue. Arrows represent differentially expressed proteins spots. (C) The interaction network among the upregulated proteins in F-type using STRING map of E681 proteins. Disconnected proteins were not shown in the network. Line thickness indicates the strength of data support. (D) The central carbohydrate metabolic network of E681. Upregulated enzymes identified using 2-DE were depicted in red. BDH: (S,S)-butanedioyl dehydrogenase, GDH: glycerol dehydrogenase; TAL-transaldolase; HPS: hexulose-6-phosphate synthase; GAPDH-glyceraldehyde 3-phosphate dehydrogenase; ENO-enolase; PDH: pyruvate dehydrogenase; SDH: succinate dehydrogenase; ATPa-ATP synthase alpha subunit; ATPb-ATP synthase beta subunit; SOD-superoxide dismutase.

growth in some crops, including *Arabidopsis thaliana* [19,43]. The strain E681 produces polymyxin and fusaricidin, which have excellent antimicrobial activities against gram-negative bacteria and plant pathogenic fungi, respectively [44]. However, as per the results of our study, an attention is required when developing a biocontrol agent using E681, because of the conversion of B-type (wild type) to F-type (phenotypic variation) after 4 days of incubation, the biological activities such as antibacterial activity are reduced along with lack of formation of endospores. According to our previous study, where *P. polymyxa* exhibited phenotypic variation and the incidence was different for each isolate [35]. Phenotypic variation for sporulation deficiency was supported by analysis of the expression levels of a gene that was annotated to *pai1*, which is known to be involved in the downregulation of sporulation and production of degradative enzymes [45]. Our results suggest that B-type changes to F-type in the medium at a nutrient-depletion stage under environmentally stressed conditions and the variants lose their endospore-forming ability. Huo et al [46]. reported that the sporulation of *P. polymyxa* SQR-21 occurred due to different environmental factors. B-type variant, an endospore former is found to be an ideal type to protect the crop plants from the pathogen stress, and increases the growth promotion; while F-type variant is not involved to protect the crop plants from the pathogen stress due to the lack endospore forming ability. Our results are supported by a previous report [15], which has stated that endospore forming bacteria possesses long-term storage for their activity. The strain SQR-21 was also used as a biocontrol agent against major plant fungal pathogens. Phenotypic variation occurred on days 3 and 4 correlated with very high expression of the *pai1* gene. The *pai1* gene expression was induced at a greater level just before

phenotypic variation. These results suggest that the *pai1* gene plays a crucial role in converting B-type into F-type at a particular stage, preventing endospore formation.

In contrast, bacterial swarming describes the migration of cells across solid surfaces, powered by flagella. The swarming pattern has been studied in several other *Paenibacillus* spp., such as *P. dendritiformis*, *P. aeruginosa*, and *B. subtilis*, where the event was regulated by multiple environmental factors [47]; including the production of surfactants on a solid surface [48]. This is consistent with our previous study, where B-type cells exhibited no swarming motility behavior, whereas F-type cells showed robust swarming ability [35]. The flagella on the surface of bacteria are controlled by the expression of the *hag* and *sigD* genes. The flagellum density was observed by transmission electron microscopy (TEM), and we found that F-type possessed more flagella than B-type. The number of flagella on the F-type cells was greater than that on the B-type cells. Transcriptome and proteome analyses revealed that the flagellin gene (*hag*) was upregulated in F-type. This result suggests that under harsh conditions, B-type converts F-type with numerous flagella to search for nutrients for survival. Furthermore, the culture medium of F-type was found to be more acidic than that of B-type, suggesting that the most upregulated proteins of F-type were located within a pH range between 4.5 and 6.0 in 2-DE. Among these proteins, some were related to the flagella apparatus such as flagellin and hook proteins, which were reported as moderately acidic proteins (pI < 5) [49].

To better understand the physiological changes between B- and F-types, we investigated the profile of differentially expressed proteins related to phenotypic variation by performing a comparative proteomic analysis in B- and F-types. The data from the proteomic analysis

Table 2
MALDI-TOF analysis for the identification of proteins.

	Spots no.	Protein name	2DE Protein matching	Sequence coverage (%)	Mascot score	mass	pI	F/B (FC)	Gene ID	RNA-Seq COG class	Log ₂ FC	p-value	
1	5504/ 5507	glyceraldehyde-3-phosphate dehydrogenase	GAPDH	gi 308,067,011	48/36	166/ 58.5/ 210 58.3	5.4/ 5.6	2.2/2.0	PPE_00195	G	—	—	
2	1402/ 1403 /1605/ 1625	Enolase (2-phosphoglycerate dehydratase)	ENO	gi 308,067,015	45/43 /58/73	273/ 118 /190/ 275	46.6/ 49.0 62.7	4.4/ 4.4 /4.3/ /	551.4/ 745.0 9662.4/ 33.2	PPE_00199	G	—	—
3	6101	superoxide dismutase	SOD	gi 308,067,487	70	182	32.9	5.6	2.9	PPE_00700	P	—	—
4	3206/ 5206 /5211/ 6202	Transaldolase (fructose-6-phosphate aldolase)	TAL	gi 308,068,573	66/68 /50/69	165/ 180 /289/ 176	35.6/ 34.6 34.4	4.9/ 5.4 /5.5/ 5.8	693.6/ 8.8 /4.2/7.3	PPE_01804	G	—	—
5	3403	pyruvate dehydrogenase E1 component subunit beta	PDHb	gi 308,069,294	77	366	50.8	4.9	3.6	PPE_02531	C	1.46	0.000
6	7002/ 7007	6,7-dimethyl-8-ribityllumazine synthase	ribE	gi 308,069,553	73/88	97/135	21.2/ 21.0	6.2/ 6.4	665.0/ 3.21	PPE_02793	H	2.71	0.000
7	3502	leucyl aminopeptidase	AP	gi 308,069,703	61	110	59.8	4.8	4.3	PPE_02945	E	—	—
8	1508/ 1509 /2204/ 4201	glycerol dehydrogenase	GDH	gi 308,069,829	42/41 /27/42	213/ 180 /267/ 176	57.0/ 56.8 35.1	4.4/ 4.5 5.2	607.8/ 1015.5 1247.8	PPE_03074	C	—	—
9	2506/ 3503	glycerol dehydrogenase	GDH	gi 308,069,220	56/51	220/ 103	56.9/ 56.4	4.7/ 4.9	5.3/2.6	PPE_02457	C	—	—
10	5508/ 5604	oligopeptide-binding protein oppA	OPPA	gi 308,069,896	34/36	268/ 144	56.3/ 61.0	5.5/ 5.5	809.4/ 1104.3	PPE_03143	E	—	—
11	7407	glutamyl-aminopeptidase	AP	gi 308,070,002	27	357	47.1	6.6	17.6	PPE_03251	G	—	—
12	4515/ 3204 /3509/ 4501 /4504/ 5103 /5509	Butanediol dehydrogenase	BDH	gi 308,070,172	64/53 /70/56 /68/31 /58	212/ 284 /277/ 180	56.0/ 37.0 56.3/ 56.2	5.3/ 4.9 /5.0/ /5.2/ 5.3 /5.3	19.6/2.9 /90.6/ 3205.5 2.5 /5.8	PPE_03421	ER	1.13	0.000
13	6802	succinate dehydrogenase flavoprotein subunit	SDH	gi 308,070,598	40	212	83.8	5.6	2.8	PPE_03868	C	1.14	0.000
14	307/ 6306	Flagellin	HAG	gi 308,071,102	39/41	132/ 236	37.8/ 39.8	4.2/ 5.8	0.2/1.4	PPE_04409	N	2.21	0.000
15	0908/ 1616 /1624/ 1902 /2102/ 3805 /3809/ 3810 /4802/ 4905 /5902/ 5904 /9314/ 9316	copper amine oxidase	AOC	gi 308,071,125	24/25 /18/19 /22/32 /46/41 /44/37 /46/44 /25/19	171/ 207 /169/ 192 /231/ 233 /303/ 326 /309/ 173 /215/ 185 /218/ 160	125.9/ 69.6 /69.1/ 124.3 /26.3/ 106.7 /106.6/ 109.9 110.4/ 116.6 /113.4/ 113.1 /43.9/ 39.9	4.1/ 4.3 /4.4/ 4.3 /4.7/ 4.9 /5.1/ 5.2 /5.2/ 5.2 /5.4/ 5.6 /9.7/ 9.7	0.0/0.4 /20.7/ 0.0 /1.7/1.2 /4.3/ 12.4 /3.0/1.2 /50.5/ 11.0 /1.6/1.9	PPE_04432	Q	—	—
16	1621	ATP synthase subunit beta	ATPb	gi 308,071,147	62	218	69.7	4.3	1.6	PPE_04454	C	—	—
17	5706	ATP synthase subunit alpha	ATPa	gi 308,071,149	45	143	75.6	5.6	4.5	PPE_04456	C	1.15	0.000
18	3404	inosine-uridine nucleoside N-ribohydrolase	IUNH	gi 308,071,302	38	208	48.6	4.9	492.0	PPE_04610	F	—	—
19	1201	Hexulose-6-phosphate synthase	HPS	gi 308,071,365	75	160	35.7	4.4	3.3	PPE_04673	G	1.54	0.000

FC: fold change; '—': not DEGs.

indicated that the increased expression of specific proteins could play potential roles in the phenotypic variation of E681 based on their biological functions according to gene ontology (GO) and KEGG database and suggested that high levels of energy metabolism, stress resistance, and transportation were involved during bacterial adaptation under unfavorable environmental conditions. Many proteins involved in carbohydrate metabolism, such as glycolysis, the citrate cycle, the pentose phosphate pathway, and butanoate metabolism, were highly expressed in F-type. Energy production by carbohydrate metabolism is of great significance for bacterial growth and antioxidant potential [38]. SOD is an enzyme that converts superoxide radicals to oxygen and hydrogen peroxide and has an adaptive function in ensuring the viability of bacteria under harsh environmental conditions [36,37]. Over 47% of the identified proteins in 2-DE were involved in glycolysis and other metabolic pathways associated with carbohydrate metabolism and all the proteins were upregulated in F-type.

Inosine-uridine preferring nucleoside hydrolase (EC:3.2.2.1) (IUNH) (PPE_04610) was highly expressed in F-type. IUNH is a base-specific inosine-uridine preferring nucleoside hydrolase that cleaves the N-glycosidic bond of ribonucleosides in the purine salvage pathway [50]. According to KEGG analysis, IUNH functions in purine metabolism in E681, converting guanosine to guanine, xanthosine to xanthine, inosine to hypoxanthine, or adenosine to adenine (Fig. S1). The upregulation of IUNH in the 2-DE analysis is consistent with the BIOLOG results in the Table 1. Because of IUNH induction, the nucleosides undergo rapid catabolism and serve as excellent carbon sources for E681. RNA-seq results from a previous study showed that genes included in the inosine monophosphate biosynthesis (M00048) module in purine metabolism were overexpressed in F-type. Oligopeptide-binding protein oppA was also upregulated in F-type. The Opp system is considered essential for nutrition [51]. Among transport systems, the Opp system belongs to the family of ATP-binding cassette (ABC) transporters, which hydrolyze ATP to drive transport [52]. Similar results were observed in the RNA-seq analysis using E681. ‘Transport’ was the most abundant subcategory identified as differentially enriched among GO biological process term, and the majority of genes were ABC transporter permeases.

P. polymyxa has been demonstrated to ferment butanediol on a commercial scale [53]. Although butanediol dehydrogenase (BDH) was upregulated at the transcriptomic and proteomic levels, the function of BDH in E681 remains unclear. According to Zhang et al [54], BDH is involved in butanediol fermentation by *Paenibacillus subtilis* SC2, whereas *Corynebacterium glutamicum* has been reported to consume glucose to excrete glycerol under oxygen deprivation by BDH [55,56]. Two types of GDH were overexpressed in F-type, which were found to interact with each other in the STRING map. This supports our Biolog data (Table 1) that, unlike B-type, F-type could use glycerol as a carbon source. The ability of bacteria to efficiently exploit various nutrient sources is crucial for their survival and growth [57].

Riboflavin (vitamin B₂), an essential cofactor in all organisms, plays an important role as the precursor of flavin mononucleotide (riboflavin 5'-monophosphate, FMN) and flavin adenine dinucleotide (FAD), which function as coenzymes for a wide variety of enzymes involved in intermediate metabolism [58]. The direct biosynthetic precursor of riboflavin, 6,7-dimethyl-8-ribityllumazine, is synthesized by the enzyme 6,7-dimethyl-8-ribityllumazine synthase [59]. Proteome and transcriptome analyses showed that this enzyme was upregulated in F-type. The proteins related to energy production, purine salvage pathway and riboflavin metabolism were overexpressed in F-type (Fig. S2). This type of metabolic modification could support rapid cell proliferation.

5. Conclusion

Cumulatively, our results suggest that E681 not only employs dormancy mechanisms via the formation of endospores for survival in unfavorable environments but also initiates processes that allow for

migration via increased motility in F-type by utilizing various carbon sources more than B-type, resulting in the activation of carbohydrate metabolism. This motility is mediated via the downregulation of sporulation, thereby allowing for increased production of flagella and other metabolic pathways. The novel findings of this study contribute to the unraveling of overall mechanism of phenotypic variation in E681 and the reliability of the use of this microorganism for potential industrial applications.

Author contributions

Y.M.L. and S.Y.K. designed the experimental setup and performed experiments. K.B.R. wrote the manuscript with input from all authors. Y. H.J. supervised the project.

Conflict of Interest

The authors have no financial conflict of interest to declare

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00719.

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