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Isolation and Characterization of Transposon-Insertional Mutants from *Paenibacillus polymyxa* E681 Altering the Biosynthesis of Indole-3-Acetic Acid

Quyet Tien Phi · Sang-Ho Oh · Yu-Mi Park · Seung-Hwan Park · Choong-Min Ryu · Sa-Youl Ghim

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Abstract We screened a mini-Tn*10* insertional mutant library of the spore-forming bacterium *Paenibacillus polymyxa* E681 with variable indole-3-acetic acid (IAA) productivity. Four mutants, of which two showed a decrease in IAA production and the other two showed an increase in IAA production, were finally selected. Further analyses demonstrated different levels of IAA intermediates from culture supernatant of wild-type strain and mutants. In addition, mutants showed different promotions on the early growth of 10-day-old maize in terms of the increase in shoot and root weights. DNA fragments flanking the transposon insertion in four mutants were cloned and sequenced. The target sites of insertion were gene *gpr1*, disrupted at two sites, 49 bp downstream of the *spo0F* gene, and *relA/spoT* homologue, which codes for GPR1/FUN34/YaaH family protein, stage 0 sporulation protein F, and RelA/SpoT domain protein, respectively. This evidence suggests that there may be a number of genes involved in the regulation of IAA biosynthesis of *P. polymyxa*.

Q. T. Phi · S.-H. Oh · Y.-M. Park · S.-H. Park
Department of Microbiology, Kyungpook National University, Daegu 702-701, Korea

S.-H. Park · C.-M. Ryu
Genome Research Center, KRIBB, Daejon 305-600, Korea

S.-Y. Ghim (✉)
Department of Microbiology, College of Natural Sciences, Kyungpook National University, 1370 Sankyug-Dong, Buk-Gu, Daegu, Korea
e-mail: ghimsa@mail.knu.ac.kr

Introduction

Indole-3-acetic acid (IAA), a major plant growth hormone of the auxin class, has been found to be produced by numerous organisms including of plants, bacteria, fungi, and algae. The identification of IAA intermediates demonstrated five different pathways of IAA biosynthesis in bacteria [15]. Many reports have described the factors involved in the level of IAA biosynthesis, which are both genetic and environmental factors [15]. Related to the effects of genetic elements, the location of IAA biosynthetic genes, mode of expression, transcriptional regulator RpoS, and two-component system GacS/GacA have been shown to affect the level of IAA production in some bacteria [8, 16, 19]. By transposon-insertional mutagenesis, several genes and proteins from bacteria have been reported to be relevant to the level of IAA biosynthesis, mainly involved in transcriptional regulators, transport system proteins, outer membrane proteins, and cytochrome *c* biogenesis genes [11, 23].

Paenibacillus polymyxa, a Gram-positive bacterium, has been proved to dependently synthesize IAA from the main precursor tryptophan (Trp) [10]. However, studies on the identification and characterization of the key genes and proteins related to this process in the bacterium have not been conducted. *P. polymyxa* E681 used in this experiment was isolated from the rhizosphere of winter barley grown in Korea [18]. This strain is known to form endospores, suppress plant diseases, produce antimicrobial compounds, secrete diverse degrading enzymes, and produce phytohormones [18]. The bacterium was previously reported to produce IAA dependent on exogenous Trp via only the indole-3-pyruvic acid (IPA) pathway. In addition, the enzyme indole- 3-pyruvate decarboxylase (IPDC), the key enzyme in the IPA pathway, from the bacterium was

functionally characterized [17]. The full genome of the bacterium, about 5 Mb in size, was recently sequenced by the Genome Research Center at Korea Research Institute of Bioscience and Biotechnology (unpublished data) [14]. This represents vast progress in molecular and genetic studies on bacterium-plant interactions and genes involved in IAA biosynthesis as well.

In this work, tranposon-insertional mutants from *P. polymyxa* E681 were screened for decreases and increases in levels of IAA production compared to the wild-type strain. Inserted sites in selected mutants and the effects of mutants on IAA-intermediate production and maize growth promotion were investigated and are discussed.

Materials and Methods

Bacterial strains and growth conditions *P. polymyxa* strain E681 was previously isolated from the rhizosphere of winter barley cultivated in Korea [18]. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani (LB). For IAA production, *P. polymyxa* strains were grown at 30°C with shaking at 180 rpm in DM medium consisting of (g/L) glucose (5.0), $(NH_4)_2SO_4$ (1.0), NaCl (2.0), $MgSO_4.7H_2O$ (0.2), $MnSO_4.4H_20$ (0.007), $CaCl_2.2H_20$ (0.008), L-arginine (0.7), L-glutamine (0.1), histidine (0.3), biotin (0.001), thiamine (0.001), and 50 mM potassium phosphate buffer, pH 7.2, supplemented with 0.01% Trp. Antibiotics were added at the following final concentrations: spectinomycin (Spec), 800 μg/ml for *P. polymyxa* mutants and 100 μg/ml for preserving Spec-resistant plasmids in *E. coli*.

Transposon mutagenesis and screening of various IAA-producing mutants A random insertion library was constructed by introducing the mini-Tn*10* delivery vector pIC333 into *P. polymyxa* strain E681 cells as described previously [20]. Strain E681 carrying the pIC333 plasmid was cultured at 25°C in tryptic soy broth (TSB; Difco, Detroit, MI, USA) medium containing Spec until the begining of the exponential growth phase, the temperature was elevated to 37°C to eliminate the plasmid, and the cultures were incubated for an additional 4 h. Diluted cultures were spread on TSB agar plates containing Spec and grown colonies were then screened for variations in IAA production.

Colorimetric assay for indolic compound production Bacteria were cultivated for 20 h in DM medium without Trp, and then a 20-μl aliquot was transferred into 5 ml of fresh DM medium supplemented with 0.01% Trp, obtaining a final cell density of $\log_5$ colony-forming units (CFU)/ml. After 28 h of cultivation, the density of cultures was measured spectrophotometrically at 600 nm and bacterial cells were removed from the culture medium by centrifugation (5000 *g*, 10 min). Eighty-microliter aliquots of the supernatant were slightly mixed with 160 μl of Salkowski's reagent (0.5 M $FeCl_3$:35% $HClO_4$, 2:100) in a 96-well plate. After 25 min of incubation at room temperature, the mixture was measured for absorbance at 530 nm in a Sensident Scan (Labsystems, Helsinki, Finland). The concentration of IAA in the culture was determined by comparison with a curve of standard IAA purchased from Sigma (St. Louis, MO, USA). IAA levels produced by bacteria were calculated for each $\log_{10}$ CFU.

Quantification of indolic compounds by HPLC Twenty-five milliliters of the bacterial supernatants was acidified to pH 2.5 with concentrated H_3PO_4 and extracted two times with equal volumes of ethyl acetate. The organic solvent phase was evaporated at 37°C and the dried compound was dissolved in 1.0 ml of 100% methanol. A 25-μl aliquot of the filtered extract was injected into an Atlantis C18 reverse column (5 μm, 250 × 4 mm; Atlantis Water, Milford, MA, USA) equipped with a differential UV detector absorbing at 280 nm. The mobile-phase solvent used for reverse-phase chromatography was 72% solvent A (1% acetic acid) and 28% solvent B (100% methanol). The flow rate was set at 0.6 ml min^{-1} and quantitative estimates of IAA and other indolic compounds were based on the HPLC analysis. Authentic IAA intermediates purchased from Sigma-Aldrich (St. Louis, MO, USA) were used as the standards.

Inoculant preparation and bacterization of maize seeds The plant used in the experiment was maize (*Zea mays* L.), provided by Gyeongsangbuk-Do Agricultural Research and Extension Services, Korea. Maize seeds were surface-sterilized with 1.2% NaClO for 40 min, rinsed three times with sterile distilled water, and air-dried on a clean bench. Mutants and wild-type strain E681 were grown in 200 ml of TSB broth medium for about 24 h at 28°C with shaking at 150 rpm, centrifuged, washed in sterile saline solution, measured at optical density 600 nm, and resuspended in 50 ml of prepared 1.0% methyl cellulose (MC) to obtain a final concentration of $\log_{10}$ CFU/ml. Fifty *Zea mays* L. seeds were submerged in 50 ml of bacterial inoculum in 200-ml Erlenmeyer flasks. Control seeds were submerged in 50 ml of 1.0% MC. Flasks were incubated at 25°C on a rotary shaker at 70 rpm for 2 h to allow bacterial cells to adhere to seeds. After incubation, excess inoculum was removed and seeds were immediately planted in plastic pots filled with sterile commerce-artificial soil and distilled water. Seedlings were grown in a growth chamber under 14 h of light at 25°C and 10 h of darkness at 20°C at 65% humidity. Bacteria were applied to the seed at approximately $\log_8$ CFU/seed, as determined by plate counts on TSA medium.

PCR amplification and sequencing of mini-Tn10 inserted genes Chromosomal DNA from various IAA-producing mutants was digested with *Hin*dIII and the DNA fragments were then ligated with T4 DNA ligase. The ligation mixture was transformed into *E. coli* XL1-Blue strain and transformants were screened for resistance to Spec. DNA plasmids were then extracted from *E. coli* transformants. DNA fragments flanking the mini-Tn*10* insertion locus from isolated plasmids were sequenced using the pair of primers Tn*10*-left, 5′-CGATATTCACGGTTTACCCAC-3′, and Tn*10*-right, 5′-CGTTGGCCGATTCATTAATGC-3′ [5]. To verify the presence of the mini-Tn*10* insertion, the plasmids were treated with *Bam*HI to determine a 2.2-kb *Bam*HI-treated fragment, the characteristic of the mini-Tn*10* insertion.

Statistical analysis Experiments on plant growth were performed on the basis of a completely randomized design. All experiments were repeated three times with three replicates and each replicate contained 15 plantlets. Data were statistically analyzed by analysis of variance (ANOVA) and means were compared using Duncan's multiple-range test ($p \leq 0.05$) using the software package SPSS v.14.1 for Windows (SPSS Inc., Chicago, IL, USA). Standard deviation (SD) was recorded.

Results

Screening of mutants varying in IAA production About 3,000 mutants obtained from independently made transposon libraries were initially screened for variation in IAA production by colorimetric assays. Eleven mutants showing an increase or decrease in IAA production were selected and then stored at −70°C until use. IAA levels produced by the 11 selected mutants were regularly analyzed by HPLC, and 4 mutants of which 2 stably showed a decrease in IAA production and the other 2, interestingly, showed an increase in IAA production—were finally obtained (Table 1). The other seven mutants did not show stable IAA biosynthesis and are not mentioned in the next experiments. The names of mutants that showed a decrease and an increase in IAA production were designated to start with *auxd* and *auxi*, respectively. In the initial screening by colorimetric assays, mutants absolutely deficient in IAA biosynthesis were not found. Inverse PCR amplifying the DNA regions flanking mini-Tn*10* using primers Tn*10*-left and Tn*10*-right showed only a single band for each selected mutant. This suggests that each of the four mutants had only a single transposon inserted (data not shown).

Time course of IAA-intermediate accumulation in mutant cultures IAA and intermediates accumulated in culture supernatants of the wild-type strain and mutants were measured by HPLC at specific time intervals and the growth of bacteria was monitored by measuring optical density at 600 nm (Fig. 1). *P. polymyxa* E681 was known to produce IAA via the IPA biosynthetic pathway dependent on the primary precursor Trp [17]. To characterize the effects of mini-Tn*10* transposon insertions on IAA-intermediate biosynthesis by mutants, the production of IPA and tryptophol (TOL), a specific by-product of the IPA pathway, was also assayed in the culture filtrates at certain intervals (Fig. 1).

Table 1 IAA biosynthesis by *P. polymyxa* mutants in DM medium

Mutant[a]	IAA (mg)[b]	Percentage (%)
auxd40	1.18	35.6
auxd404	1.21	36.5
auxi203	5.17	156.2
auxi444	5.38	162.5
Wild type	3.31	100.0

[a] Mutants were tested for IAA production by colorimetric assays once a month in stock cultures stored at −70°C

[b] Data are the mean of three separate experiments by colorimetric assays (calculated for $\log_{10}$ CFU)

Effect of the wild-type strain and mutants on growth of maize Inoculation of maize seedlings with *P. polymyxa* E681 and mutants showed effects on plant growth regarding increases in the shoot and root weights at 10 days after sowing (Table 2). The shoot and root length of bacterially treated plants were not significantly different from those of control plants. However, the bacterization of maize seeds with the wild-type strain and mutants resulted in significant increases in fresh root and shoot weights compared with the untreated control ($p < 0.05$), leading to increases in total fresh weight ranging from 17.4% to 30.3%.

Amplification and sequencing of the genes involved in IAA biosynthesis The DNA fragments flanking the mini-Tn*10* transposon insertions in four mutants were recovered by transformation into *E. coli* and the DNA-inserted sites were sequenced. BLAST analysis of the DNA sequences flanking the transposon insertion sites revealed that mini-Tn*10* was inserted into two coding regions with homology to known and hypothetical genes and one intergenic region (Table 3). Since the full genome of *P. polymyxa* E681 has recently been sequenced by the Genome Research Center, Korea Research Institute of Bioscience and Biotechnology (unpublished data) [14], the DNA sequences disrupted by mini-Tn*10* from mutants were further compared with the complete genome sequence of *P. polymyxa* E681, available at http://www.gem.re.kr (Table 3).

Analysis of transposon-inserted genes In mutants *auxd40* and *auxd404*, the mini-Tn*10* transposon was

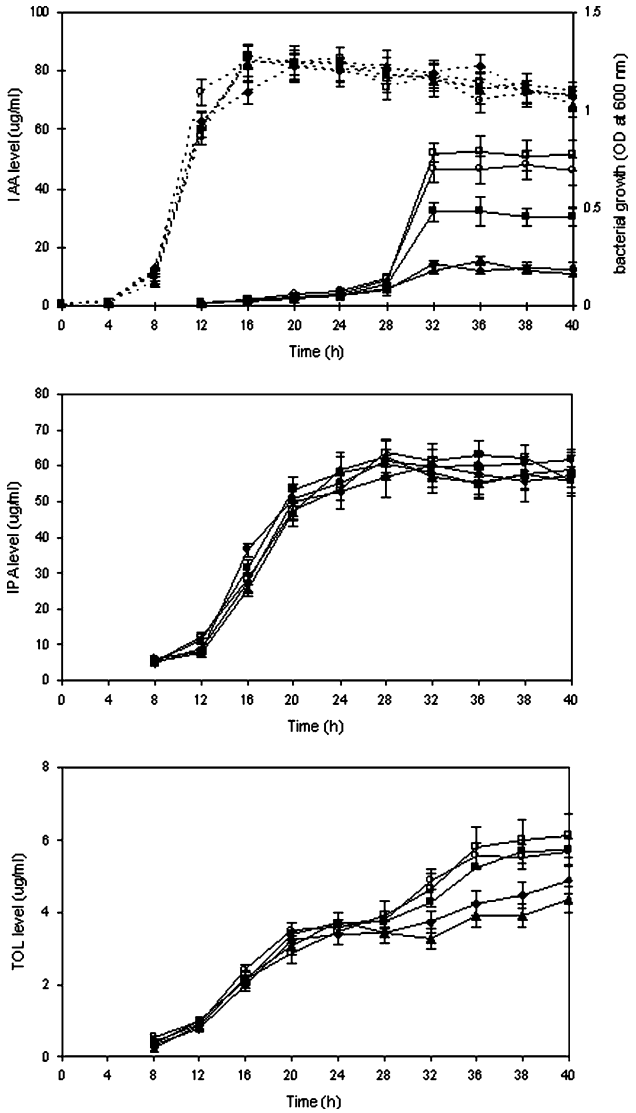

Fig. 1 Growth (- - -) and IAA-intermediate production (—) of the wild-type strain and mutants of *P. polymyxa* E681 in DM medium supplemented with Trp. Wild-type strain E681 (■), *auxd40* (◆), *auxd404* (▲), *auxi203* (○), and *auxi444* (□). (**A**) Bacterial growth and IAA production; (**B**) IPA production; (**C**) TOL production

inserted at two different positions of a putative 621-bp ORF encoding a protein belonging to the poorly characterized GPR1/FUN34/YaaH family protein. The predicted amino acid sequence of the *gpr1* gene product showed high homology with the GPR1/FUN34/YaaH family protein of *Bacillus thuringiensis* str. Al Hakam, *B. cereus* E33L, and *B. cereus* ATCC 10987, with identities of 69%. This protein family, commonly found in prokaryotes and lower eukaryotes, has not been characterized in bacteria yet. These proteins are predicted to be localized in the bacterial membrane involving transport and their function remains to be elucidated [4], whereas in yeasts and fungi this novel protein family has been demonstrated to be involved in ammonia secretion, acetate uptake, and the adaptation process to acetic acid [1, 4, 13].

In mutant *auxi203*, the transposon was inserted in a site located 49 bp downstream of the TAG stop condon of the ORF encoding a protein of 122 amino acid residues. This deduced amino acid sequence showed a high homology to stage 0 sporulation protein F, a phosphotransferase involved in the signaling pathway of controlling sporulation, of *Geobacillus thermodenitrificans* NG80-2, *Geobacillus kaustophilus* HTA426, and *Bacillus weihenstephanensis* KBAB4, with identities of 74%, 73%, and 73%, respectively. Analyses of this ORF sequence showed that the TGA stop codon of this ORF was followed by a possible ρ-independent transcription terminator. The stem-loop sequence corresponding to the putative *spo0F* terminator was AGAACTC TGTTAACACAACAGGGTTTT, with an energy of the potential hairpin of −8.9 kcal. The transposon-inserted site in mutant *auxi203* was consequently three nucleotides following the possible stem-loop structure on mRNA. The ORF adjacent to *spo0F* is transcribed in the opposite direction, encoding a protein of 113 amino acid residues. The predicted amino acid of this protein showed homology to the BEACH domain-containing protein and hypothetical protein Rpic12DDRAFT_3701 of ameba *Dictyostelium discoideum*

Table 2 Effects on plant growth of inoculation of maize seedlings with *P. polymyxa* E681 and mutants

Parameter	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Total fresh weight (g)
Untreated	28.41 ± 2.17^{a}	25.19 ± 3.22^{ab}	0.80 ± 0.081^{a}	0.29 ± 0.05^{a}	1.09^{a}
auxd40	29.35 ± 1.74^{a}	24.16 ± 2.14^{ab}	0.91 ± 0.078^{abc}	0.37 ± 0.038^{ab}	1.28^{ab}
auxd404	27.23 ± 1.67^{a}	24.47 ± 2.97^{ab}	0.90 ± 0.089^{abc}	0.38 ± 0.041^{ab}	1.26^{ab}
auxi203	28.58 ± 2.13^{a}	23.49 ± 2.09^{ab}	0.91 ± 0.071^{abc}	0.37 ± 0.032^{ab}	1.26^{ab}
auxi444	30.58 ± 2.43^{a}	26.53 ± 2.73^{ab}	0.98 ± 0.101^{ac}	0.45 ± 0.067^{abc}	1.42^{abc}
Wild type	28.52 ± 1.9^{a}	25.41 ± 2.95^{ab}	0.91 ± 0.084^{abc}	0.38 ± 0.045^{ab}	1.27^{ab}

Note. Data are mean ± standard deviation. Mean values within a column followed by the same superscript letter(s) are not significantly different according to Duncan's multiple-range test ($p < 0.05$). Data were measured for 10-day-old maize

Table 3 Homology analysis of mini-Tn*10* insertion sites in IAA mutants

Mutant	Disrupted gene[a]	Insertion site[b]	Putative ORF function	Highest indentity (%)[c]	Accession no.
auxd40	*gpr1*: GPR1/FUN34/YaaH family protein (207)	24	Contains six transmembrane regions involved in transport	*Bacillus cereus* E33L (69%)	EU283811
auxd404	*gpr1*: GPR1/FUN34/YaaH family protein (207)	106	Contains six transmembrane regions involved in transport	*Bacillus cereus* E33L (69%)	EU283811
auxi203	*spo0F:* Stage 0 sporulation protein F (122)	49 bp downstream from stop codon TGA of *spo0F* gene	Responsible for activation of sporulation genes	*Bacillus cereus* subsp. cytotoxis NVH 391-98 (73%)	EU283812
auxi444	*relA*: RelA/SpoT domain protein (246)	95	(p)ppGpp synthetase & hydrolase	*Alkaliphilus oremlandii* OhILAs (60%)	EU283810

[a] The number in parentheses indicates the length (amino acids) of the corresponding protein

[b] Deduced amino acid position of the mini-Tn*10* interruption in the *P. polymyxa* E681 deduced protein

[c] Organism and protein designation for the amino acid sequence showing the highest homology score using BLAST against proteins in public databases available at the NCBI

AX4 and *Ralstonia pickettii* 12D, with identities of 30% and 28%, respectively.

In the mutant *auxi444*, the deduced amino acid sequence of the disrupted gene product, a protein consisting of 246 amino acids, showed a high homology to the RelA/SpoT domain protein of *Alkaliphilus oremlandii* OhILAs, *Alkaliphilus metalliredigens* QYMF, and *Bacillus halodurans* C-125, with identities of 60%, 58%, and 58%, respectively. The RelA/SpoT protein is responsible for the synthesis and hydrolysis of guanosine 3′-diphosphate 5′-triphosphate (pppGpp) during the stringent response and is conserved in Gram-positive and Gram-negative bacteria. In *E. coli*, two proteins RelA and SpoT are involved in stress-induced (p)ppGpp accumulation, in which the former is a ribosome-associated (p)ppGpp synthase responding mainly to uncharged tRNAs that accumulate as a result of amino acid limitation and the latter is a bifunctional (p)ppGpp synthase and hydrolase, probably regulating (p)ppGpp levels in response to most conditions other than amino acid limitation [3, 22].

Discussion

The results of this study indicate that the mini-Tn*10* insertions occurred within the *P. polymyxa* DNA regions that probably up- and down-regulate the expression of the IAA biosynthetic gene(s) or IAA secretion (Table 1). All four mutants and the wild-type strain exhibited almost-identical growth curves, suggesting that the divergence in IAA production and transposon insertions might not influence the growth rate of bacteria. A mutant completely deficient in IAA biosyntheis was not found. This is consistent with previous reports demonstrating no mutants showing complete abolishment of IAA biosynthesis by insertions of transposons into the *A. brasilense* choromosome [6]. Although the accumulation of IAA in the cultures of mutants and wild-type was different at late stationary phase, the levels of IPA remained almost constant, suggesting that the diverse deprivation of IPA which was decarboxylated to indole-3-aldehyde (IAAld) was sufficiently converted from Trp. In addition, the TOL levels produced by all bacterial strains were different in the late stationary phase (Fig. 1). These results indicate that the insertions of transposons affected only the production of IAA and TOL, and not the production of IPA.

The influences of seed inoculation with *P. polymyxa* E681 and mutants on growth of maize were investigated under growth chamber conditions (Table 2). Seedlings treated with mutants and wild-type strain showed significant increases in plant root and shoot weights but did not exhibit significant increases in root and shoot lengths in comparison with untreated plants. Since the different IAA levels were produced by mutants in vitro, the bacterial strains might influence root hormone levels by producing IAA and/or other hormones in the rhizosphere, which are then absorbed by the root. Consequently, the root and shoot weights were divergently enhanced by IAA, producing various mutants. The results also show that the varying increases in root and shoot weights were not correlated with IAA levels produced by corresponding mutants in vitro. These might be due to other unknown effects of the transposon-inserted genes on bacterial physiology beyond the alteration in IAA production.

The same gene coding for a GPR1/FUN34/YaaH family protein was disrupted at different positions in mutants *auxd40* and *auxd404* (Table 3). Among members of the GPR1/FUN34/YaaH family, the functions of only two proteins, Gpr1p from *Y. lipolytica* and Ady2p from *S. cerevisiae*, were identified [1, 4, 13]. The physiological role

of both genes is related to the intracellular presence of acetate, in which Gpr1p is required for adaptation of *Y. lipolytica* cells to acetic acid, and might involve acetate export, preventing intracellular accumulation of the acid [1, 13]. Ady2p was demonstrated to be a key determinant of the kinetics of acetate transport in *S. cerevisiae*. This study showed that bacterial indole-3-acetic acid, which has a residue acidic structure somewhat similar to the structure of acetic acid, might be intracellularly accumulated in *P. polymyxa* cells. The bacterial protein Grp1 was, consequently, able to assist to transport IAA to extracellular environment. Hence, the inactivation of protein Gpr1 by transposon-insertion in mutants *auxd40* and *auxd404* probably inhibited the secretion of IAA, resulting in lower IAA levels in culture supernatants than that produced by the wild-type strain.

In mutant *auxi203*, a transposon was inserted into an intergenic regionat a position located three nucleotides following the stem-loop sequence, a putative ρ-independent transcription terminator of the *spo0F* gene (Table 3). This, therefore, might affect the transcription of the *spo0F* gene. In the *spo0F* mutant from *B. subtilis*, Spo0F, a phosphotransferase involving the signaling pathway controlling sporulation, was poorly phosphorylated by KinA and KinB, and sporulation was greatly reduced [7]. IAA production and expression of the key gene *ipdC* in *A. brasilense* have been demonstrated to be increased under carbon limitation, a reduced growth rate, and an acidic pH [12, 21]. In this study, *P. polymyxa* E681 progressively produced IAA in the late stationary phase, when the carbon source was nearly exhausted and the cell density started to decrease. Therefore, the mutation in *spo0F* from mutant *auxi203* might elongate the stationary phase, resulting in increased expression of the *ipdC* gene and, consequently, increased IAA production. Since studies on bacterial IAA biosynthesis by Gram-positive bacteria at the genetic level have been limited, this experiment might provide a preliminary result and the linkage between the sporulation process along with corresponding genes and IAA production from these bacteria requires further elucidation.

A transposon was inserted into an ORF coding for the RelA/SpoT domain protein in mutant *auxi404*, leading to an increase in IAA production (Table 3). To date, there has been no report indicating direct links between RelA/SpoT protein and IAA biosynthesis in bacteria. In *E. coli*, accumulation of ppGpp during glucose limitation is still found in a *relA*-null mutant, but cells with double-null mutations of *relA* and *spoT* genes have completely lost ppGpp-synthetic activity [2]. It has been shown that ppGpp is implicated in the control of *rpoS* expression as a positively acting signal molecule. In double-null *relA* and *spoT* mutants the expression of RpoS was reduced 20 to 50 times compared with that in the wild-type strain [9]. Glick et al. proved that stationary-phase σ factor RpoS could positively regulate trancription of the *ipdC* gene in *Pseudomonas putida* GR12-2, leading to higher IAA biosynthesis [16]. Similar results of upregulated biosynthesis of IAA also were observed when introducing RpoS into *E. cloacae* CAL2 [19]. Hence, the mutation in the *relA*/*spoT* homologue from mutant *auxi444* probably helped to enhance the expression of RpoS, consequently leading to an increase in IAA production.

Collectively, these data suggest that transposon-inserted mutants from *P. polymyxa* E681 alter IAA-intermediate production as well as maize growth promotion. To understand the complete effects of these mutations on IAA production, bacterial physiology, and plant growth, gene complement experiments, biochemical assays, and trial tests on plant growth promotion deserve further studies. This is one of the first reports presenting the effects of several gene disruptions in Gram-positive bacteria on the biosynthesis of IAA.

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