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Plant Terpenes Enhance Survivability of Polychlorinated Biphenyl (PCB) Degrading *Pseudomonas pseudoalcaligenes* KF707 Labeled with *gfp* in Microcosms Contaminated with PCB

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Abstract Polychlorinated biphenyls are toxic pollutants and their degradation is quite slow in the environment. Recently, interest in bioremediation using PCB-degrading bacteria has increased. In a previous report, plant terpenes (*p*-cymene, (S)-(-)-limonene, α-pynene, and α-terpinene) have been found to be utilized by a PCB degrader and to induce the biphenyl dioxygenase gene in pure culture. In this study, *Pseudomonas pseudoalcaligenes* KF707, a PCB-degrading Gram-negative soil bacterium, was used to determine whether the terpene stimulation of PCB degrader occurred in the natural environment. First, *P. pseudoalcaligenes* KF707 was genetically tagged using a transposon with *gfp* (green fluorescent protein) as a reporter gene. The population dynamics of *P. pseudoalcaligenes* KF707 harboring *gfp* gene in a PCB-contaminated environment was examined with or without terpenoids added to the microcosm. About 10–100-fold increase was found in the population of PCB degraders when terpene was added, compared with control (non-terpenes samples and biphenyl added samples). It was proposed that the *gfp*-monitoring system is very useful and terpenes enhance the survivability of PCB degraders in PCB-contaminated environments.

Key words: *Pseudomonas pseudoalcaligenes* KF707, terpene, green fluorescent protein (GFP), polychlorinated biphenyls (PCBs), bioremediation

Polychlorinated biphenyls (PCBs) have been used commercially since 1930 as dielectric and heat exchange fluids as well as for a variety of other applications. Although the production and widespread usage of PCBs have been banned in the industrialized world for almost twenty years, contamination of soil, toxicity, bioconcentration, and persistence are serious problems [3]. Recently, interest in bioremediation using PCB-degrading bacteria has increased [6, 16, 18], including *P. pseudoalcaligenes* strain KF707 [4, 6]. Biphenyl (Bp)-utilizing bacteria are widely distributed in the environment, and several biphenyl-utilizing bacteria are able to co-metabolize various PCB components [9]. Recent studies revealed that dioxygenases of *P. pseudoalcaligenes* KF707 involved in the initial oxygenation of aromatic hydrocarbons are multi-component enzymes [5, 14].

Microorganisms to perform beneficial tasks such as bioremediation of toxic compounds and biological control are increasingly released. Development of recombinant DNA techniques has demonstrated the potential for genetic engineering of various microorganisms which are to be released into natural environments. However, information on survival and recombinant gene expression of released genetically engineered microorganisms (GEMs) in the environment is limited. Therefore, it would be useful to develop a simple and reliable reporter gene system in which survival and expression can be monitored [15]. The development of sensitive methods for monitoring bacteria in laboratory model systems and natural environments using reporter genes such as *gfp* [13] and *gus* [21] is of great importance. The GFP of the jellyfish *Aequorea victoria* was proven to be a valuable tool for studying a variety of biological problems. GFP is useful for examining biological phenomena, because cells can be studied nondestructively and without the addition of exogenous substrates. Additionally, GFP-marked cells can be visualized by using standard microscopes, equipped with commonly available fluorescent filter sets [13].

Plant terpenes [*p*-cymene, (S)-(-)-limonene, α-pynene, and α-terpinene] are nontoxic natural substances and their structures are similar to PCB congeners. In addition, plant terpenes are known to stimulate PCB degradation. In previous

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reports, plant terpenes were shown to increase PCB degradation in pure culture conditions [7, 12] and soil microcosms [11]. In this study, another PCB degrading Gram-negative bacterium, *P. pseudoalcaligenes* KF707, was marked with *gfp* as a reporter gene. Then, *gfp*-harboring cells were released into PCB-contaminated soil microcosms to study their survivability in the presence of added plant terpenes, compared with controls (absence of plant terpenes), to examine their stimulating effect on the survivability of the PCB degrader.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Escherichia coli strain JB143, containing pJBA36 with mini-Tn5 transposon and *gfp,* was kindly provided by Jens Bo Andersen [1] and maintained on a modified Luria Bertani (LB) [19] agar plate with 50 μg ml^{-1} kanamycin. *P. pseudoalcaligenes* KF707 was provided by Dr. Furukawa (Kyushu University, Fukuoka, Japan). The PCB degrader was maintained on tryptic soy agar (TSA) (Difco, MD, U.S.A.) supplemented with kanamycin at 200 μg ml^{-1} for the transformants. Precultures of *P. pseudoalcaligenes* KF707 were grown in tryptic soy broth (TSB) (Difco, MD, U.S.A.) at 150 rpm and 30°C. The PCB degrader was routinely maintained on mineral salts (MS) agar using inverted plates with biphenyl crystals placed on the lid. MS composition was described by Furukawa and Miyazaki [5]. Then, the liquid cultures were grown in shaking flasks containing liquid medium (100–200 ppm biphenyl dissolved in hexane) on a rotary shaker at 150 rpm and 30°C.

Growth experimentation was performed using *P. pseudoalcaligenes* KF707 *gfp* harboring strains, and was compared to their wild-type strain. In the growth rate experiment, PCB degraders were cultured in 100 ppm BP containing MS liquid medium. To determine biodegradation of the PCB congener, 4,4'-dichlorobiphenyl (4,4'-DCBp) was used as a substrate [4]. Resting cell assay was used to determine degradation of PCB congeners, as described by Gilbert and Crowley [7]. Ring fission products for each substrate were monitored using the supernatants at 434 nm (4,4'-DCBp) spectrophotometrically [7, 12].

Utilization Test of Terpenes

PCB-degrading bacteria use plant terpenes as sole carbon sources [7]. Plant terpenes [*p*-cymene, (S)-(-)-limonene, α-pynene, and α-terpinene] 200 ppm were added to MS broth. Then, the PCB degraders were cultured and their growth was detected at $O.D._{600}$ by a spectrophotometer.

Electrotransformation and Selection of Useful *gfp* Mutants

P. pseudoalcaligenes KF707 electrocompetent cells were prepared and transformed as described previously with a minor modification [2]. Approximately 0.5 μg of plasmid DNA was mixed with 60 μl of electrocompetent cells, added to a 2 mm gap electroporation cuvette, and electroporated at 1.8 kV, 25 mA, and 4.5 ms with the Gene Pulser II apparatus (Invitrogen, CA, U.S.A.). The electroporated cells were diluted into 500 μl of TSB medium and grown for 2 h before being plated on TSA supplemented with kanamycin (final concentration was 200 μg ml^{-1}). The plasmid pJBA36 was a suicide plasmid harboring the mini-Tn5 transposon (Fig. 1). It is maintained only in strains that have the λ pir. The transposase located outside of the mini-Tn5 operation makes the transposon move. Once it moves, it remains stable in the recipient cell's replicon [20]. An epifluorescent microscope (Leica, Wetzlar, Germany), equipped with digital camera (Nikon, NY, U.S.A.), was used to visualize fluorescent cells on slide and bacterial colonies on TSA plates.

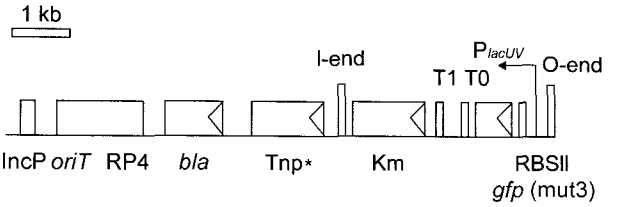

Fig. 1. Structure of pJBA36.
bla, a gene encoding β-lactamase (ampicillin resistance gene); Km, kanamycin resistance; Tnp*, transposase; *gfp* (mut3), mutated *gfp*; RBSII, ribosome binding site of phage T5; T0, terminator from phage lambda; T1, terminator from the *rrnB* operon of *E. coli*; P_{lacUV5}, inducible (IPTG) promoter, composed of the phage T7-promoter combined with two *lac* operators [1].

Southern Blot Analysis

Southern blot analysis was used to determine the insertion of *gfp* into *P. pseudoalcaligenes* KF707 genomic DNA. Isolation methods for genomic DNA and Southern blot procedure are described by Sambrook and Russell [19]. For Southern blot analysis, undigested and restriction enzyme-digested genomic DNA from *P. pseudoalcaligenes* KF707 parent strain, *gfp*-harboring strains, and pJBA36 plasmid DNA were used. A Digoxigenin-labeled probe was made by incorporating DIG-11-dUPT (Boehringer, Mannheim, Germany) by random priming method using plasmid DNA pJBA36 as a template. The hybridized probes were immunodetected with anti-digoxigenin-AP and were visualized with the colorimetric substrates NBT/X-phosphate, as in the DIG DNA-labeling and detection Kit (Boehringer, Mannheim, Germany) [20].

Microcosm Study

Soil samples were taken from In-Kyoung Lake, Inha University, Incheon, Korea and homogenized by a 425-μm pore size sieve. Ten grams of the soil were placed in 50-ml vials, and the soil was contaminated with 4,4'-DCBp to

a concentration of 200 ppm. Moisture content of the microcosm was 20%. *P. pseudoalcaligenes* KF707 *gfp*-harboring strains (10^9 cells per one vial) were inoculated in the microcosm and incubated at 4°C (cold condition when the cells were released in cold environment) and 30°C (optimum culture condition) for 2 months. Samples were divided into four parts; 50 ppm of Bp and 200 ppm of 4,4'-dichlorobiphenyl (DCBp), 200 ppm of 4,4'-DCBp and 50 ppm of *p*-cymene, 200 ppm of 4,4'-DCBp and 50 ppm of α-terpinene, and non-carbon source part such as control. Culturable counts were determined as cfu (colony forming unit) g^{-1} soil, as described by Unge *et al.* [22]. Each sample was diluted by sterile water and spread on TSA. *gfp*-harboring strains were counted under epifluorescent microscope.

RESULTS AND DISCUSSION

In this study, the PCB degrader *P. pseudoalcaligenes* KF707 was successfully tagged with the *gfp* gene by electrotransformation. *gfp*-Harboring strain colonies were identified by an epifluorescent microscope under UV light. As shown in Fig. 2, *P. pseudoalcaligenes* KF707 *gfp*-harboring strain was easily detected on TSA plates based on their green fluorescence.

In order to determine the effect of *gfp* expression on cell metabolism, the wild-type KF707 cells and the *gfp*-harboring strain were compared in terms of growth rate and PCB degradation. As shown in Fig. 3, it was found

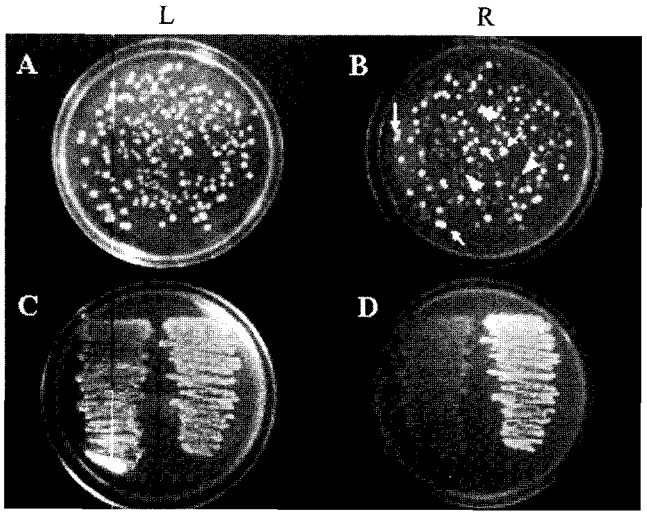

Fig. 2. Selection of *P. pseudoalcaligenes* KF707 *gfp*-harboring strain.
(**A**, **B**) Wild-type strains and the *gfp*-harboring strains were spread on TSA plate after mixing (the arrows, wild-type strain colonies; the arrowheads, *gfp*-harboring strain colonies). (**C**, **D**) Wild-type strains (left) and the *gfp*-harboring strains (right) were streaked on TSA plates. (L: under visible light; R: under UV light.)

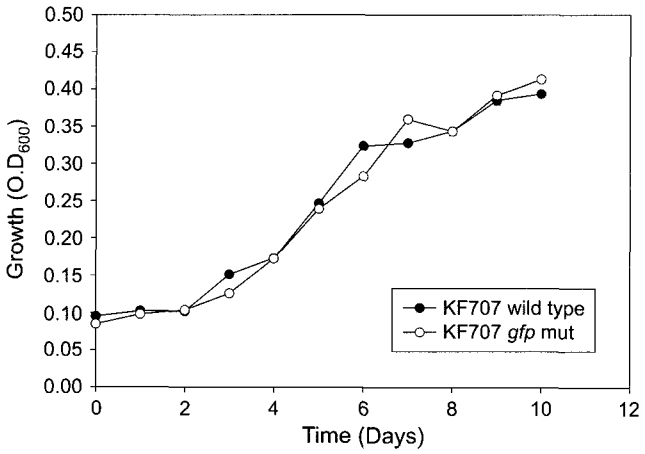

Fig. 3. Growth curves of *P. pseudoalcaligenes* KF707 (filled circles) and the *gfp*-harboring strains (open circles) in MS broth containing biphenyl at 30°C.

that the *gfp*-harboring strain had an almost identical growth curve compared with the wild-type, suggesting that *gfp* labeling appeared not to have effected on any of the microorganism's metabolic functions. Resting cell assay was performed to determine and compare PCB degradation between the wild-type KF707 cells and the *gfp*-harboring strain [7]. Since *P. pseudoalcaligenes* KF707 was shown to use 7 PCB congeners, including 4,4'-DCBp, as a sole carbon source [4], 4,4'-DCBp was used to perform resting cell assay. Ring fission products as the substrate were monitored by analyzing the culture supernatants spectrophotometrically at 434 nm (4,4'-DCBp) [7]. As shown in Fig. 4, degradation kinetics of the two strains were identical, indicating that GFP expression did not affect the PCB degradation.

To determine *gfp* insertion in genomic DNA of *P. pseudoalcaligenes* KF707, Southern blot analysis was

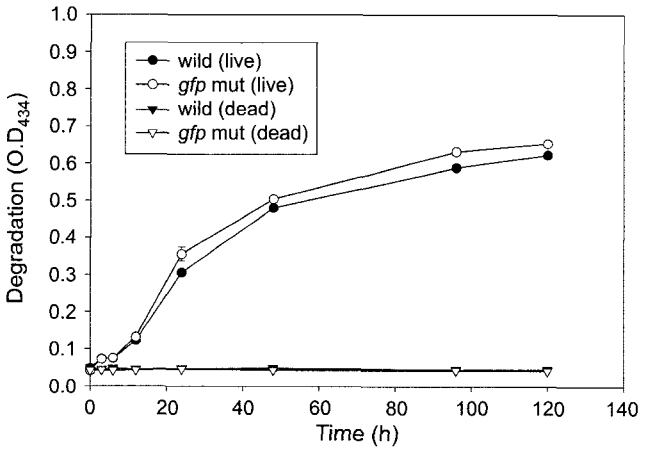

Fig. 4. Degradation of 4,4'-DCBp by *P. pseudoalcaligenes* KF707 (●) and the *gfp*-harboring strain (○) in biphenyl-containing MS broth at 30°C.

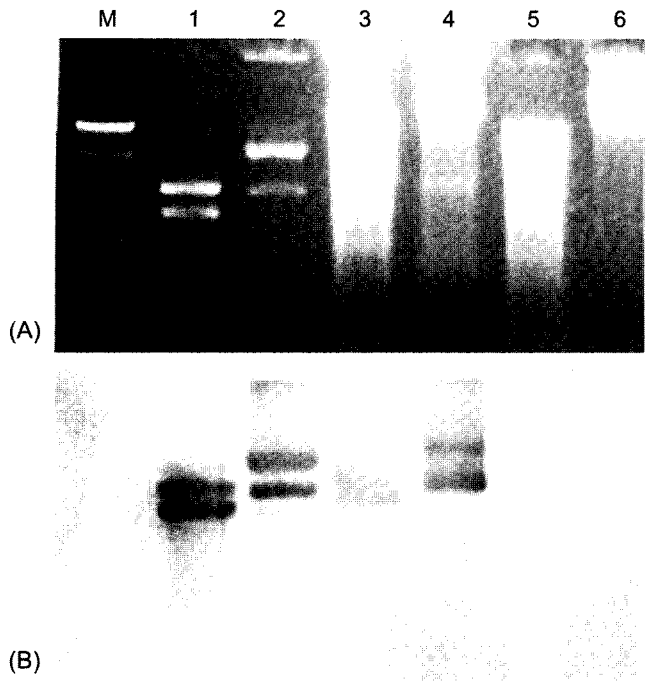

Fig. 5. Southern analysis of total genomic DNA of *P. pseudoalcaligenes* KF707 and the *gfp*-harboring strain cells along with pJBA36 as a control.
(**A**) Agarose gel picture of DNA samples. (**B**) Colorimetric image of Southern blot of the above gel probed with pJBA36. Lanes 1, 3, and 5, digested DNA samples; Lanes 2, 4, and 6, undigested DNA samples with *Kpn*I; Lane M, λ/*Hin*dIII size markers. Lanes 1 and 2 were pJBA36, Lanes 3 and 4 were the *gfp*-harboring strain, and Lanes 5 and 6 were the wild-type.

used. As shown in Fig. 5, undigested high molecular size genomic DNA as well as *Kpn*I-digested DNA hybridized with the DIG-labeled *gfp* probe, whereas DNA from the parent strain (undigested and cleaved with *Kpn*I) did not show any hybridization signal. However, the hybridization patterns of the *gfp*-harboring strain were similar to those of the plasmid (lanes 1 and 2). It appeared that the *gfp* was not inserted into the genomic DNA of *P. pseudoalcaligenes* KF707; instead, the introduced pJBA36 was maintained in KF707 as a plasmid. The reason why the transposition did not occur was not clear. The stability of plasmid pJBA36 in *P. pseudoalcaligenes* KF707 was tested under selective and nonselective conditions. Plasmid survival was assessed during 200 generations by plating diluted aliquots on TSB medium with or without kanamycin and comparing duplicate colony counts on selective or nonselective TSA plates [8]. pJBA36 was found to be stable in *P. pseudoalcaligenes* KF707 over 200-generation times (data not shown).

When the microbes are to be released into the environment for bioremediation, they must be able to compete successfully with the indigenous bacteria for the available nutrient and must be able to survive in the presence of toxic chemicals and predators such as bacteriophage and protozoa [6]. Using the *gfp*-harboring strain, we attempted to determine whether plant terpenes could enhance the survivability of PCB-degrading bacteria in microcosms. Terpenes are natural substances which have structural similarities to those of aromatic hydrocarbons. It has been known that plant terpenes stimulate *bph* gene and enhance the growth of PCB-degrading Gram-positive bacteria such as *Arthrobacter* sp. B1B [7] and *Rhodococcus* sp. T104 [12]. Natural terpenes have characteristics such as nontoxicity to humans and wide distribution in the environment [12]. In a previous report, it was suggested that Gram-negative bacteria *Ralstonia eutropha* H850 was not able to grow on monoterpenes (e.g. *p*-cymene, α-pinene, α-terpinene, and abietic acid) as sole carbon sources, because of their toxicity [11]. However, monoterpenes are effective inducers for the PCB degradation pathway of Gram-negative bacteria [17]. Gram-positive bacteria have tolerance to high concentration of terpenes and use them as sole carbon sources or inducers for a certain metabolic activity [10, 23].

In this study, it was found that KF707 was able to grow in the presence of plant terpenes as sole carbon sources, which is in contrast to the previous report on *R. eutropha* H850. As shown in Fig. 6, the growth of wild-type KF707 and the *gfp*-harboring strain were enhanced in MS medium supplemented with *p*-cymene and α-terpinene during 6-day incubation. Since KF707 was able to grow in the presence of plant terpenes, especially *p*-cymene and α-terpinene, these compounds were used in the microcosm study to monitor their stimulatory effects on the survivability of PCB degrader. Microcosms contaminated with PCB in the presence or absence of terpenes were inoculated with the *gfp*-harboring strain (10^9 cells per microcosm). Microcosms were placed in incubators (30°C, optimized condition for PCB degrader) and cold chamber (4°C, the condition for PCB degrader released to cold environment). In the samples supplemented with plant terpenes, the survivability of PCB degrader was considerably enhanced. As shown in

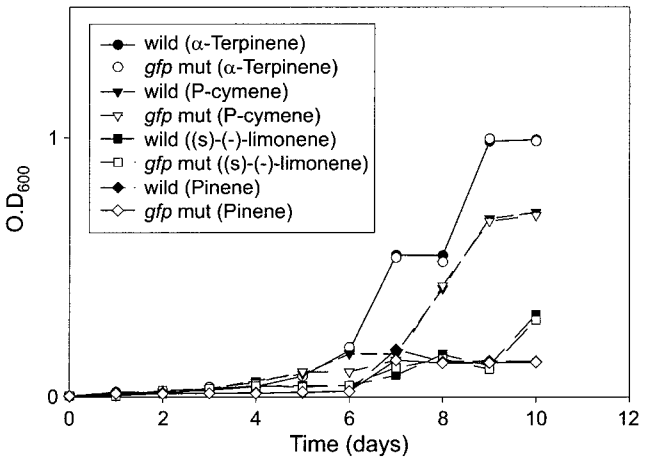

Fig. 6. Utilization of various plant terpenes as a sole carbon by *P. pseudoalcaligenes* KF707 and the *gfp*-harboring strain.

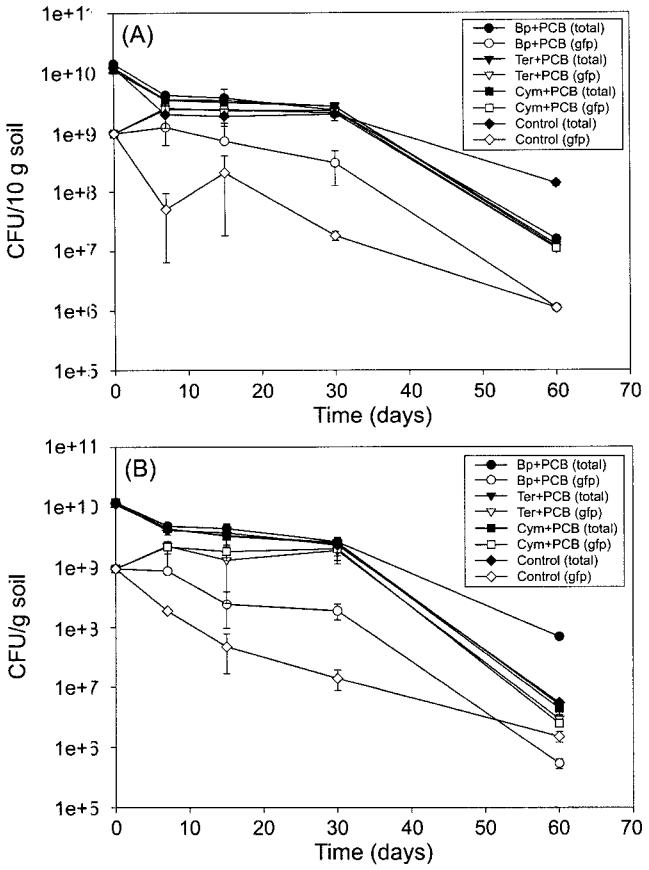

Fig. 7. Population dynamics of KF707 *gfp*-harboring strains at 30°C (A) and 4°C (B).
Filled symbols are total cell count while open symbols are *gfp*-harboring strain of KF707. (○, ●), Bp+PCB; (▽, ▼), PCB+terpinene (Ter); (□, ■), PCB+cymene (Cym); (◇, ◆), controls with no addition. PCB was 4,4'-DCBp.

Fig. 7A, during the 2-month period, the number of the PCB degraders maintained about 10-100-fold higher in the presence of terpenes. In Fig. 7B, the population of PCB degraders placed in the cold chamber had a similar tendency with the samples placed at 30°C. Therefore, it is suggested that plant terpenes would enhance survivability of PCB degraders in environments contaminated with PCB. As shown in Figs. 7A and 7B, PCB degrader was dominant in microcosms contaminated with PCB, probably because PCB was toxic to indigenous soil bacteria. Taken together, natural substrates such as plant terpenes can be used for the purpose of PCB decontamination. It was also demonstrated that *gfp*-labeling was effective in *in situ* detection and enumeration of PCB degrader in nonsterile soil microcosm.

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