The effects of Cl\(^{-}\) on the antibiotics productivity of *Pseudomonas* sp. YGJ3 were investigated. YGJ3 produced pyoluteorin and 2,4-diacetylphloroglucinol, depending on the concentration of Cl\(^{-}\) in the culture medium. Cl\(^{-}\) stimulated the biosynthesis of pyoluteorin, thereby repressing 2,4-diacetylphloroglucinol biosynthesis. The cell-free extract from the bacteria grown without Cl\(^{-}\) showed high activity of monoacetylphloroglucinol acetyltransferase, an essential enzyme in 2,4-diacetylphloroglucinol biosynthesis.

**Key words:** chloride ion; pseudomonad; pyoluteorin; diacetylphloroglucinol; acetyltransferase

Fluorescent pseudomonads produce a variety of secondary metabolites, some of which are the useful antibiotics. Chlorinated pyrrole pyoluteorin (PLT) and polyketide 2,4-diacetylphloroglucinol (DAPG) are particularly important due to their potent anti-fungal effects. Pseudomonads producing these antibiotics serve as biocontrol agents against plant pathogens in the rhizosphere. DAPG appears to be more effective than PLT in its broader specificity against various pathogenic microorganisms. To improve the productivity of these antibiotics, nutritional, environmental, and physiological factors have been investigated. PLT and DAPG appear to be biosynthesized independently of each other under the direction of the *plt* and the *phl* gene cluster respectively. A well-conserved operon, *phiACBD*, encodes DAPG synthase, which is assumed to be a multifunctional enzyme, like fatty acid synthase. All the protein products, *PhiACBD*, are required for the synthesis of DAPG via monoacetylphloroglucinol (MAPG), although the proteins have not yet been fully characterized. MAPG acetyltransferase (ATase), catalyzing the conversion of MAPG to DAPG, is an essential component of DAPG synthase. In this study, we examined the effects of halide ions on the biosynthesis of PLT and DAPG in strain YGJ3, a new isolate of *Pseudomonas* sp., with focus on Cl\(^{-}\), an essential ion to PLT.

Ethanol was used as the sole carbon and energy source in bacterial growth, since it is metabolized via acetyl-CoA, which is required for synthesis of both PLT and DAPG. Strain YGJ3 was cultivated at 30°C in a liquid medium (pH 7.0) designated X-medium (X = Cl\(^{-}\), Br\(^{-}\), or I\(^{-}\), which contained 12.5 mm Na\(_2\)HPO\(_4\), 12.5 mm KH\(_2\)PO\(_4\), 1.65 mm MgSO\(_4\), 41.5 mm MnSO\(_4\), 36.0 mm FeSO\(_4\), 200 mm Ca(OH)\(_2\), 0.400 mm CuSO\(_4\), 0.347 mm ZnSO\(_4\), 0.356 mm CoSO\(_4\), 40.0 mm NH\(_4\)Cl, and 170 mm ethanol in distilled water. Ethanol was added just before inoculation. NH\(_4\)F was not examined because of its high toxicity to bacterial growth. When halogen-free medium (N-medium) was prepared, the NH\(_4\)F concentration was adjusted to 40.0 mm with (NH\(_4\))\(_2\)SO\(_4\). Agar plates were prepared by adding 1.5% agar in liquid medium.

Strain YGJ3 was isolated from soil in Gifu, Japan. It was selected for its ability to grow in Cl-medium and to release materials having a \(\lambda_{max}\) at 310 nm, which is characteristic of PLT. YGJ3 was aerobic, fluorescent, Gram-negative, oxidase positive, motile, and rod-shaped. The genomic DNA was extracted and the 16S rRNA gene was amplified by polymerase chain reaction with 27f and 1525r primers, as described by Shinoda et al. The nucleotide sequence, of 1,456 bp, (DDBJ/EMBL/GenBank Nucleotide Sequence Databases accession no. AB517957) showed the highest homology (99.93%) to that of *P. fluorescens* Pf-5. Hence, YGJ3 was identified to be a pseudomonad, and was designated *Pseudomonas* sp. YGJ3. YGJ3 was stored at −80°C in 25% glycerol. At need, stock culture was streaked once on an agar plate of Cl-medium to revive the bacteria, and then the colonies formed were streaked on an agar plate of the same medium as that for liquid culture to remove Cl\(^{-}\).

MAPG was obtained from Tokyo Chemical Industry (Tokyo). PLT and DAPG were isolated from the culture broth. For isolation of PLT, YGJ3 was grown aerobically in 10 l-liter Erlemeyer’s flasks, each containing 100 ml of Cl-medium at 30 °C for 1 d. After the bacterial cells were removed at 4 °C by centrifugation, the supernatant was extracted with CHCl\(_3\). The extract was evaporated at 30 °C. The residue was dissolved in acetone and applied to TLC with silica gel 60 F\(_{254}\) (Merck, Darmstadt, Germany) with CHCl\(_3\)-acetone-MeOH-water (30:1, v/v). The major spot (R\(_f\) = 0.26), visualized by UV, was extracted with acetone (yield, about 14 mg). FT-NMR, EI-MS, FT-IR, and UV spectra of the isolated substance, measured with a JEOL ECA-500 or ECX-400P, a JEOL JMS-700, a Perkin-Elmer System 2000, and a Shimadzu UV-300 respectively, were in accord with those for PLT (data not shown). For isolation of DAPG, YGJ3 was grown in N-medium at 30 °C for 1 d.

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**Note**

The Chloride Ion Is an Environmental Factor Affecting the Biosynthesis of Pyoluteorin and 2,4-Diacetylphloroglucinol in *Pseudomonas* sp. YGJ3

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After removal of the bacterial cells, the supernatant was treated as above. The major spot (Rf = 0.51) in silica gel TLC was extracted with acetone (yield, about 20 mg from 1 liter of culture), and analyzed for identification, as above. The results were consistent with the data reported for DAPG (data not shown). 3, 13 PLT and DAPG were estimated by measuring A310 in CH$_2$OH ($\varepsilon$ = 13 mm$^{-1}$ cm$^{-1}$) and A270 in CH$_3$OH ($\varepsilon$ = 43.3 mm$^{-1}$ cm$^{-1}$) respectively.

HPLC was performed at 45°C on a Shim-pack C$_{18}$ reversed-phase column (0.6 x 15 cm) (Shimadzu, Kyoto, Japan) with a Shimadzu LC-9A liquid chromatograph equipped with a SPD-6AV UV-Vis detector, a CTO-2A column oven, and a C-R6A chromatopac. The flow rate was 0.7 ml/min, and A310 was monitored. For qualitative determination of PLT in the selection of the bacterial strain, elution was done with 80% CH$_3$OH. For quantification of PLT, DAPG, and MAPG, 50% CH$_2$OH-H$_2$PO$_4$ (pH 3.0) was used. Usually, the samples (culture broth or enzymatic reaction mixture) were extracted with CHCl$_3$. The extract was evaporated at 30°C. The residue was dissolved in 200 µl of the elution solution, and 10 µl was analyzed by HPLC. Under these conditions, MAPG, PLT, and DAPG were eluted at about 7.3, 15, and 47 min respectively.

MAPG ATase activity in the cell-free extract was measured as follows: After 24-h-culture at 30°C in N-medium supplemented with halide or PLT, bacterial cells were collected by centrifugation, washed once with 50 mM potassium phosphate, pH 7.0, and suspended in the same buffer. The cells were disrupted at 4°C with a Sonifier (Branson, Danbury, CT), and cell-free extract was prepared by centrifugation at 4°C. The reaction mixture (0.5 ml) contained 50 µl of 13 mM MAPG (dimethylformamide solution) and the cell-free extract (about 8.0 mg, protein) in 50 mM potassium phosphate, pH 7.0. After incubation at 30°C for 1 h, the mixture was extracted 3 times with CHCl$_3$ to measure the formation of DAPG by HPLC. Protein was determined by DC Protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

The effects of halide ions on the productivity of the antibiotics of strain YGJ3 were examined. During growth in Cl-medium, YGJ3 produced PLT in the logarithmic phase (Fig. 1A). YGJ3 did not produce any PLT in the N-medium, but yielded DAPG in the logarithmic phase (Fig. 1B). At the maximal level, about 48 µg (13 µg/ml) of PLT and about 51 µg (11 µg/ml) of DAPG were produced. In the stationary phase, both PLT and DAPG decreased gradually due to degradation to water-soluble compounds. In Br-medium, DAPG (about 43 µg) was produced, while bromine analogs of PLT were not detectable. In I-medium, bacterial growth was considerably inhibited (the doubling time was about 10 h as compared with 6–7 h in Cl- and in N-medium), and no DAPG was produced. MAPG, a precursor of DAPG, was not found in any case.

To confirm the effects of Cl$^-$ on the biosynthesis of DAPG, YGJ3 grown in N-medium was inoculated in Cl-medium in which the concentration of Cl$^-$ varied (Fig. 2A). After 24-h-culture, 0.2 mM Cl$^-$ stimulated the production of PLT and strongly inhibited DAPG production. No accumulation of MAPG was found. Unlike Cl$^-$, Br$^-$ (1–40 mM) was almost ineffective on bacterial growth and DAPG production (data not shown). DAPG production fell by about 60% due to 5 mM I$^-$, and was completely inhibited by 40 mM I$^-$, possibly due to poor bacterial growth. Like Cl$^-$, PLT strongly inhibited DAPG production (Fig. 2B).

To confirm that Cl$^-$ and PLT caused the decrease in DAPG synthase activity, MAPG ATase activity in the cell-free extract was measured. The extract from the N-medium-grown bacteria showed significant activity of MAPG ATase (Table 1). Heat treatment (100°C, 2 min) of the extract completely abolished the activity. Column chromatography of the extract on Superdex 200 (GE Healthcare, Buckinghamshire, UK) showed that the molecular mass of the active fractions was about 300 kDa (data not shown). The activity required no external addition of an acetyl donor such as acetyl-CoA. Possibly, the acetyl group is provided by the enzyme itself. 10 The properties appear to be consistent with those expected of DAPG synthase. MAPG ATase activity was also high in Br- or I (2 mM)-medium-grown bacteria, while it was markedly low in the bacteria grown in Cl-, I (40 mM)-, and in N-medium supple-
Effects of Chloride Ion on Antibiotic Biosynthesis

YGJ3 grown on an agar plate of N-medium was inoculated into 5 ml of N-medium containing various concentrations of (A) Cl\(^-\) or (B) PLT. In B, an ethanol solution of PLT was added to the N-medium in place of ethanol. After 24 h-culture at 30°C, A\(_{600}\) was measured. After centrifugation to remove the bacterial cells, 1 ml of the supernatant was extracted with CHCl\(_3\) to determine PLT (▲) and DAPG (●) by HPLC. Means ± S.D. from three independent experiments are shown.

Effects of Cl\(^-\) and PLT on the Production of DAPG

YMJ3 grown on an agar plate of N-medium was inoculated into 5 ml of N-medium containing various concentrations of (A) Cl\(^-\) or (B) PLT. In B, an ethanol solution of PLT was added to the N-medium in place of ethanol. After 24 h-culture at 30°C, A\(_{600}\) was measured. After centrifugation to remove the bacterial cells, 1 ml of the supernatant was extracted with CHCl\(_3\) to determine PLT (▲) and DAPG (●) by HPLC. Means ± S.D. from three independent experiments are shown.

Table 1. MAPG ATase Activity in Cell-Free Extracts from Bacteria Grown in N-Medium Supplemented with Halide Ions or PLT

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Conc. (mm)</th>
<th>DAPG formed(^*) (nmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>93.5 ± 2.8</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>2</td>
<td>3.8 ± 1.7</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>40</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>2</td>
<td>80.0 ± 1.2</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>40</td>
<td>63.5 ± 1.4</td>
</tr>
<tr>
<td>I(^-)</td>
<td>2</td>
<td>66.0 ± 1.1</td>
</tr>
<tr>
<td>I(^-)</td>
<td>40</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>PLT</td>
<td>0.05</td>
<td>4.3 ± 0.1</td>
</tr>
</tbody>
</table>

\(^*\)Means ± deviations from two independent experiments are shown.

References