Antibacterial Activity of Oxolinic Acid on *Pseudomonas glumae*

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Abstract

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo[1,3]dioxolo[4,5-g] quinoline-7-carboxylic acid, Starnar®) is a synthetic bactericide, developed from quinoline derivatives. Oxolinic acid completely inhibited the growth of gram negative bacteria including *Pseudomonas glumae*, *P. plantarii*, *P. avenae*, *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* at the concentration of 0.4 µg/ml or below *in vitro*. Oxolinic acid was effective against various isolates of *P. glumae* from rice grains collected from various districts of Japan. Antibacterial activities of oxolinic acid on *P. glumae* varied depending on the growth phase of the bacterium. Activity of oxolinic acid was bactericidal for the bacterium at the lag phase, but was bacteristatic for the bacterium at the early logarithmic phase and decreased after the middle logarithmic phase. Oxolinic acid sometimes induced abnormal elongation of *P. glumae* cells at the early logarithmic phase.

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Key words: oxolinic acid, *Pseudomonas glumae*, growth phase, bactericidal activity.

INTRODUCTION

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo[1,3]dioxolo[4,5-g] quinoline-7-carboxylic acid, Starnar®, Fig. 1) is a synthetic bactericide developed for the control of bacterial disease on plants. Oxolinic acid is one of quinoline derivatives which have a high antibacterial activity against gram negative bacteria^{5,11}. I have shown that oxolinic acid is effective for the control of bacterial grain rot of rice (*Pseudomonas glumae*), black leg of potato (*Erwinia carotovora* subsp. *atroseptica*), fire blight (*E. amylovora*) and soft rot (*E. carotovora* subsp. *carotovora*)⁴. Oxolinic acid has been used in Japan for the control of bacterial seedling rot of rice (*P. glumae*) in the form of seed treatment, and for the control of bacterial grain rot of rice (*P. glumae*) and soft rot of vegetables (*E. carotovora* subsp. *carotovora*) in the form of spray treatment.

Antibacterial activities of quinoline derivatives such as ciprofloxacin, nalidixic acid, pipedimic acid and miloxacin have been reported to be bactericidal^{1,2,6,8,12}. On the other hand, Zeiler¹³ has reported that ciprofloxacin shows different antibacterial activities on *Escherichia coli* KL16 at the logarithmic phase and the stationary phase of the growth. I report here that antibacterial activity of oxolinic acid is either bactericidal or bacteristatic depending on the growth phase of *P. glumae*.

MATERIALS AND METHODS

Chemical. Oxolinic acid (technical compound, 99%) was used throughout all experiments. Oxolinic acid was dissolved in NaOH solution whose concentration was three-tenth times that of oxolinic acid used in tests. There was no influence of NaOH at any concentration used in tests on the growth of *P.*

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Fig. 1. Structure of oxolinic acid (5-ethyl-5,8-dihydro-8-[1,3]oxolo[4,5-g]quinoline-7-carboxylic acid, Starner®).

glumae.

Bacteria. *Pseudomonas glumae* Pg-4 was obtained from Ehime Prefectural Agricultural Experiment Station. Other isolates of *P. glumae* were isolated from rice grains collected from various districts of Japan, and were obtained from National Kyushu Agricultural Experiment Station. For the isolation of bacterium each grain was ground in a mortar and a pestle with 10 ml of sterile water at room temperature. Aliquots (0.1 ml) of the original solution and its 10-fold dilution were dispensed onto S-PG selective medium¹⁰. Bacterial colonies were isolated after incubation for 2 days at 40°C. Other bacteria used were obtained from Agricultural Science Research Laboratory, Takarazuka Research Center, Sumitomo Chemical Co., Ltd.

Minimum inhibitory concentration (MIC). Bacteria incubated overnight in peptone-yeast liquid medium (peptone: 5 g, yeast: 2 g, deionized water: 1 liter, PY medium) were suspended in PY medium to give the density ranging from 1×10^2 to 5×10^2 colony forming unit (cfu)/ml. Bacterial suspensions (0.1 ml) were spread onto agar plates (Nutrient agar "Nissui" Nissui Seiyaku Co., Ltd) containing oxolinic acid at various concentration. MICs were determined based on colony emergence after incubation of 48 hr at 30°C.

Antibacterial Activity of oxolinic acid on growth of *P. glumae*. *P. glumae* Pg-4 was incubated for 24 hr in PY medium and washed three times with PY medium by centrifugation at 5,000 rpm for 5 min. Pellet was suspended in PY medium and the bacterial density was adjusted. Then oxolinic acid was added to the suspension to give the final concentration of 0.1 to 2.5 µg/ml. The bacterial suspensions were incubated at 32°C for 24 hr. Serial dilutions of the bacterial suspension at various times after the onset of incubation were plated on Nutrient Agar plates and the bacterial density was determined by counting colonies after 48 hr of incubation at 40°C.

Antibacterial Activity of oxolinic acid on each phase of *P. glumae* growth. *P. glumae* Pg-4 at stationary phase was suspended in PY medium to give the bacterial density of 10^6 cfu/ml. The bacterial suspension was incubated at 32°C for 24 hr, and oxolinic acid was added at 0, 6, 9 and 12 hr after the onset of incubation to give the final concentration of 5 µg/ml. The bacterial density at 24 hr after the onset of incubation was determined by plating serial dilutions of the bacterial suspension on Nutrient Agar plates after 48 hr of incubation at 40°C.

RESULTS

Minimum inhibitory concentration

Oxolinic acid completely inhibited the growth of gram negative bacteria such as *Erwinia* spp. and some *Pseudomonas* spp. including *P. glumae*, *P. avenae*, *P. plantarii* and *P. solanacearum* at the concentration of 0.4 µg/ml while higher concentration of oxolinic acid was required to inhibit the growth of *Xanthomonas* spp. (6.3 µg/ml) and *P. syringae* (25.0 µg/ml) (Table 1). Sensitivity of bacteria to oxolinic acid was different among *Pseudomonas* spp.

Oxolinic acid also had strong antibacterial activity against 23 isolates of *P. glumae* and MIC was below 0.4 µg/ml (Table 2).

Antibacterial Activity on growth of *P. glumae*

When initial density of the bacterium was 2×10^8 cfu/ml, the bacterial density decreased to 10^3 to 10^4 cfu/ml during 3, 6 and 9 hr after incubation in the culture with 0.1, 0.5 and 2.5 µg/ml oxolinic acid (Fig. 2). Thereafter the bacterial densities in the cultures with 0.5 and 2.5 µg/ml oxolinic acid remained constant,

Table 1. Antibacterial activity of oxolinic acid

Bacteria	MIC ($\mu\text{g/ml}$) ^{a)}
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	25.0
<i>Curtobacterium flaccumfaciens</i> pv. <i>oortii</i>	25.0
<i>Agrobacterium tumefaciens</i>	6.3
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	0.4
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	0.4
<i>Erwinia ananas</i>	0.4
<i>Pseudomonas avenae</i>	0.4
<i>Pseudomonas glumae</i>	0.4
<i>Pseudomonas plantarii</i>	0.4
<i>Pseudomonas solanacearum</i>	0.4
<i>Pseudomonas syringae</i> pv. <i>lachrymans</i>	25.0
<i>Pseudomonas syringae</i> pv. <i>maculicola</i>	25.0
<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	25.0
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	1.6
<i>Xanthomonas campestris</i> pv. <i>citri</i>	6.3
<i>Xanthomonas campestris</i> pv. <i>oryzae</i>	6.3
<i>Xanthomonas campestris</i> pv. <i>pruni</i>	6.3
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	6.3

a) Minimum inhibitory concentration.

Table 2. Antibacterial activity of oxolinic acid against isolates of *P. glumae* from rice grains collected from various districts of Japan

Districts	MIC ($\mu\text{g/ml}$) ^{a)}
Yamagata Pref. Yahata-machi	0.2
Niigata Pref. —	0.4
Shizuoka Pref. Iwata-shi	0.4
Shizuoka Pref. Haibara-shi	0.4
Aichi Pref. Inazawa-shi	0.4
Aichi Pref. Nissin-cho	0.4
Gifu Pref. Gifu-shi	0.4
Gifu Pref. Ohno-cho	0.4
Shiga Pref. Kohra-cho	0.4
Shiga Pref. Kohoku-cho	0.4
Kyoto Pref. Fukuchiyama-shi	0.4
Wakayama Pref. Kanaya-cho	0.4
Tottori Pref. Aotani-cho	0.4
Tottori Pref. Nishihaku-cho	0.4
Shimane Pref. Tsuwano-cho	0.4
Kagawa Pref. Zentsuji-shi	0.4
Ehime Pref. Hohjoh-shi	0.4
Ehime Pref. Uwa-cho	0.4
Kohchi Pref. Ohtsuki-cho	0.4
Kohchi Pref. Nakamura-shi	0.4
Kohchi Pref. Tano-cho	0.2
Saga Pref. Karatsu-shi	0.2
Miyazaki Pref. Kitakata-cho	0.1

a) Minimum inhibitory concentration.

but the bacterial density in the culture with 0.1 $\mu\text{g/ml}$ oxolinic acid started to increase after 12 hr of incubation.

When initial density of the bacterium was 10^8 cfu/ml, the bacterial density decreased to 10^4 and 10^6 during 6 and 9 hr after incubation in the culture with 0.1 and 0.5 or 2.5 $\mu\text{g/ml}$ oxolinic acid (Fig. 2). And

Fig. 2. Antibacterial activity of oxolinic acid on *P. glumae* Pg-4 in liquid culture. A: Initial density of the bacterium was 2×10^5 cfu/ml. B: Initial density of the bacterium was 1×10^5 cfu/ml. Concentration of oxolinic acid: ○, untreated control; ●, 0.1 µg/ml; ■, 0.5 µg/ml; ▲, 2.5 µg/ml.

Fig. 3. Antibacterial activity of oxolinic acid on *P. glumae* Pg-4 at different growth phases. Oxolinic acid was added at 0, 6, 9 or 12 hr after the onset of incubation to the final concentration of 5 µg/ml. Arrows indicate application time with oxolinic acid.

the bacterial densities increased after 12 hr of incubation in the culture with 0.1 and 0.5 µg/ml oxolinic acid, but not in the culture with 2.5 µg/ml oxolinic acid.

P. glumae cells sampled at each time point after treatment with oxolinic acid were tested for their sensitivity to oxolinic acid. All bacterial cells did not form colony on agar plates containing 0.4 µg/ml oxolinic acid.

Antibacterial activity on different growth phases of *P. glumae*

The bacterial density (10^6 cfu/ml) at the onset of incubation decreased to 7×10^4 cfu/ml after 24 hr of incubation in the culture with 5 µg/ml oxolinic acid. When oxolinic acid was applied at the early logarithmic phase (6 hr after incubation), the growth of bacterium was inhibited and the bacterial density remained 2×10^6 cfu/ml after 24 hr of incubation (Fig. 3). On the other hand, oxolinic acid had little antibacterial activity when it was applied at 9 and 12 hr after incubation (after the middle logarithmic phase) when the bacterial densities were 3×10^9 and 5×10^{10} , respectively.

DISCUSSION

Generally, quinoline derivatives are reported to have bactericidal activity^{1,2,6,8,12)}. On the other hand, Zeiler¹³⁾ has reported that ciprofloxacin, one of the quinoline derivatives, shows different antibacterial

Fig. 4. Abnormal elongation of *P. glumae* Pg-4 cells treated with oxolinic acid at the concentration of 5 μ g/ml. The bacterium at the initial density of 10^8 cfu/ml was incubated at 32°C in PY medium containing 5 μ g/ml of oxolinic acid for 24 hr.

activities on *E. coli* KL16 depending on its growth phases. Bactericidal activity of ciprofloxacin on *E. coli* KL16 at the logarithmic phase is strong and rapid, while that at the stationary phase is weak and slow. But activity of ciprofloxacin on *E. coli* Neumann at any growth phase is bactericidal. And the pattern seen with *E. coli* KL16 seems to be rare in the observation of clinical isolates of *E. coli*. The present results demonstrated that the antibacterial activity of oxolinic acid was also different depending on the growth phase of *P. glumae*. But in contrast with activity of ciprofloxacin on *E. coli* KL16, activity of oxolinic acid on *P. glumae* at the lag phase was bactericidal and that at the early logarithmic phase was bacteristatic. And oxolinic acid showed little antibacterial activity on *P. glumae* after the middle logarithmic phase.

Smith^{2,6)} has reported on lower bactericidal activity of nalidixic acid on *E. coli* KL16 and one curious feature of this activity, whose *E. coli* KL16 treated with nalidixic acid commenced to elongate abnormally. In this test, I observed similar abnormal elongation of *P. glumae* cells. When initial density of the bacterium was 10^8 cfu/ml, the bacterial cells elongated abnormally with 0.5 μ g/ml oxolinic acid (Fig. 4). I could not observe a septum in the elongating cells. After the elongating cells were incubated in PY medium without oxolinic acid for 24 hr, no elongation cells were observed. The emergence of elongating cells of *P. glumae* at the early logarithmic phase may result from inhibition of cell division by oxolinic acid. Activity of oxolinic acid was bacteristatic for the bacterium at the early logarithmic phase, and the cell division of the treated bacterium was inhibited and the cells were still alive. So the bacterial cells may elongate abnormally.

Quinoline derivatives inhibit DNA gyrase activity which introduces negative supercoils into bacterial DNA^{3,7,9)}. Inhibition of DNA gyrase activity leads to inhibition of DNA reproduction and cell division in the bacterium, leading to death of the bacterium. Therefore antibacterial activity of quinoline derivatives is thought to be bactericidal. However, in this test, bactericidal activity of oxolinic acid on *P. glumae* depended on the growth phase of the bacterium. Results presented here suggested that antibacterial activity of oxolinic acid depended on not only inhibition on DNA gyrase activity but also many factors in sensitivity of the bacterium to the chemical, that is, growth phase of the bacterium, population of the bacterium and permeability of the chemical in the bacterial cell membrane. Further studies are necessary to clarify the relationship of antibacterial activity of oxolinic acid with inhibition on DNA gyrase activity and these factors.

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和 文 摘 要

曳地康史： *Pseudomonas glumae* に対するオキシロニック酸の抗菌特性

キノリン系合成化合物であるオキシロニック酸 (5-ethyl-5,8-dihydro-8-oxo[1,3]dioxolo[4,5-g]quinoline-7-carboxylic acid, スターナ®) は、植物病原細菌のうちグラム陰性細菌、とくに *Pseudomonas glumae*, *P. plantarii*, *P. avenae*, *P. solanacearum* などの *Pseudomonas* 属菌や *Erwinia* 属菌に高い抗菌活性を示した。*P. glumae* 各分離株に対するオキシロニック酸の MIC は 0.1-0.4 µg/ml であった。その作用性は、誘導期に対しては殺菌作用が強く、対数増殖期初期に対しては増殖抑制効果が強いが、対数増殖中期以降に対する効力は低いことが明らかとなった。また、オキシロニック酸処理によって、*P. glumae* の細胞形が異常に伸長する現象が認められた。