Biocontrol Science, 2002, Vol. 7, No. 3, 147−153

Original

Characterization of *Pseudomonas aeruginosa* Resistant to a Quaternary Ammonium Compound

ATSUSHI TABATA, TAKUYA MAEDA, HIDEAKI NAGAMUNE, AND HIROKI KOURAI*

Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Minamijosanjima-cho, Tokushima 770-8506, Japan

Received 21 September 2001/Accepted 23 March 2002

We developed the resistance of *Pseudomonas aeruginosa* ATCC 10145 to *N*-dodecylpyridinium iodide (P-12), a quaternary ammonium compound (QAC), by using a standard broth dilution method and compared its characteristics with those of the sensitive strain. The minimum inhibitory concentration (MIC) against the resistant strain was 8 times higher than that against the sensitive strain and the adapted resistance to P-12 was stable. Population analysis showed that there were some variations in the extent of the resistance of *P. aeruginosa* to P-12. The cell surface of the resistant strain was more hydrophilic than that of the sensitive strain. In order to investigate the relationship between the resistance to P-12 and the biofilm formation of *P. aeruginosa*, alginic acid was isolated and its concentration was determined. The results revealed that the alginic acid was produced only in the resistant strain. Moreover, the observation by scanning electron microscope (SEM) of both strains showed that many clusters of bacteria were seen only in the resistant strain. These results suggested that not only the hydrophilic change of cell surface but also the biofilm formation is involved in the resistance of *P. aeruginosa* to P-12.

Key words : Quaternary ammonium compound/*Pseudomonas aeruginosa*/Disinfectant resistance/Cell surface hydrophobicity/Biofilm.

INTRODUCTION

Our awareness of the importance of cleanliness has increased and large amounts of disinfectants are used in various fields nowadays. This tendency is notable in the food and textile industries, and in hospitals requiring a strictly clean environment. Quaternary ammonium compounds (QACs) have been used widely as disinfectants. There have been many reports on the synthesis and antimicrobial characteristics of various kinds of QACs (Baley et al., 1997; Devinsky et al., 1985, 1991, 1996; Kourai et al., 1994b, 1995; Maeda et al., 1996, 1998, 1999a, 1999b; Okazaki et al., 1996, 1997, 1999; Yoshida et al., 2000). The antimicrobial characteristics of *N*-dodecylpyridinium iodide (P-12), a member of the QACs, have also been studied (Kourai et al., 1994a). Cetylpyridinium chloride (CPC), which has a similar structure to P-12, is added to the toothpaste as an antimicrobial agent. Thus, QACs have an important role in our daily life.

In recent years, serious concerns have been raised regarding the appearance of resistant bacteria (Langsrud et al., 1997; Nagai et al., 1996; Sidhu et al., 2001). A typical resistant species is *Pseudomonas aeruginosa*. Specifically, *P. aeruginosa* is able to grow in high concentrations of QACs (Jones et al., 1989). This characteristic makes it difficult to control *P. aeruginosa*. Moreover, *P. aeruginosa* is able to form a biofilm that protects it from attacks by drugs and changes in the environments. Actually, this biofilm formation was reported in the food industry field (Zottola et al., 1994). According to this report, it was suggested that biofilm formation is one of the important factors involved in the resistance of *P. aeruginosa* to

**Corresponding author. Tel* : +81-88-656-7408, Fax: +81-88-656-9148.

disinfectants.

It was reported that the fatty acid composition of QAC resistant *P. aeruginosa* is changed (Dubois-Brissonnet et al., 2001; Guérin-Méchin et al., 1999, 2000; Méchin et al., 1999; Sakagami et al., 1989), but other factors contributing to the resistance of *P. aeruginosa* to QACs have not been reported. If present conditions continue unchanged, it will be extremely difficult to control the QAC resistant *P. aeruginosa*. Because CPC is added to the toothpaste as an antimicrobial agent, the danger of the appearance of QAC resistant *P. aeruginosa* exists close to us. Countermeasures against the QAC resistant *P. aeruginosa* are immediately required. The present study is an attempt to investigate the mechanism of the resistance in *P. aeruginosa* against one of the QACs, P-12.

MATERIALS AND METHODS

Chemicals

N-dodecylpyridinium iodide was synthesized in our laboratory as described previously (Kourai et al., 1980) and is abbreviated as P-12 (FIG. 1). Other

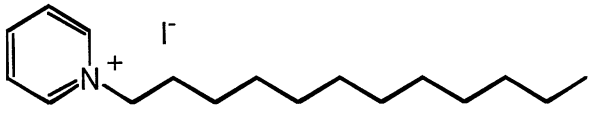

FIG. 1. Chemical structure of *N*-dodecylpyridinium iodide (P-12).

chemicals for this study were of commercially available reagent grade and used without further purification.

Organism and culture condition

P. aeruginosa ATCC 10145 was employed for the experiment. It was cultured basically according to a previous report (Maeda et al., 1999a).

Antimicrobial activity

The minimum bactericidal concentration (MBC) and the minimum inhibitory concentration (MIC) of P-12 were basically measured according to the standard broth dilution method (Maeda et al., 1996). For the measurement of MIC, P-12 solution was diluted with nutrient broth (NB, Difco Laboratories, Detroit, MI, USA) to the prescribed concentrations.

Development of *P. aeruginosa* with adapted resistance to P-12

P. aeruginosa ATCC 10145 with adapted resistance to P-12 was developed by using the standard broth dilution method.

A 1.25-fold P-12 dilution series was made by adding 4ml of the P-12 solution to 1ml of NB, consecutively. The preincubated culture of *P. aeruginosa* at 37 ℃ was diluted to a concentration of 1.0×10^6cells/ml with NB. A 0.5ml portion of the cell suspension was added to an equal volume (0.5ml) of each of the P-12 dilution series. After incubation for 24h at 37℃, the bacteria which grew in the highest concentration of the dilution series were used in the following process.

To investigate the stability of the adapted resistance of *P. aeruginosa* to P-12, each adapted culture was transferred into fresh NB and incubated for 24h at 37℃. Then, the MIC against these cultures was determined as described above.

Analysis of growth properties

The preincubated cell culture in 5ml of L-broth was transferred into a flask containing 100ml of NB, and incubated at 37℃ in a water bath shaker (100 strokes/min). A culture sample was taken at every hour from 1h to 12h. The growth was determined by measuring the optical density at 660nm with a spectrophotometer (UV-160, Shimadzu Co., Kyoto). In the case of the resistant strain, the incubation was done in NB containing 200 μM or 800 μM of P-12.

Population analysis

Five milliliters of preincubated culture were transferred into 100ml of NB containing 200 μM or 800 μM of P-12 or no P-12. After incubation for 18h at 37℃ in a water bath shaker (100 strokes/min), the culture was diluted to make the concentration 1.0×10^6cells/ml with NB, and the aliquots of 0.5ml cell suspensions were mixed with an equal volume (0.5ml) of NB containing 200 μM, 400 μM, 600 μM, 800 μM, 1000 μM or 1200 μM of P-12 or no P-12. Further incubation was carried out for 24h at 37℃, cultures were diluted 10-fold with SCDLP medium (Wako, Osaka) and the aliquots of 0.1ml of suspensions were spread on SCDLP agar plates (Wako, Osaka). Each plate was incubated for 24h at 37℃ and the number of colonies was counted.

Measurement of cell surface hydrophobicity

The measurement of cell surface hydrophobicity and calculation of the hydrophobicity index (log HI) were performed basically according to a previous report (Kourai et al., 1989). Cells in the stationary-phase were used in this measurement.

Determination of alginic acid concentration

Alginic acid was extracted from *P. aeruginosa* strains and their concentrations were measured

according to a previous method (Franklin et al., 1993).

Amplification and DNA sequencing of the *algD* gene

Chromosomal DNA was prepared from the sensitive strain and the P-12 resistant strain on the CsCl gradients (Sambrook et al., 1989). The *algD* gene was amplified using *Ex Taq* DNA polymerase (TaKaRa, Shiga) and amplification primers *algD*-Fw (5'-GCTATGTCGGTGCAGTATGTG-3'), which anneals upstream of *algD* coding region, and *algD*-Bw (5'-TCGAAGATGCGGAACTCGTAG-3'), which anneals downstream of the *algD* gene. The mixture was treated for 5min at 94℃ followed by 35 cycles of 1min at 94℃, 1min at 59℃ and 1min at 72℃, before finishing the reaction with 10min at 72℃. The amplified *algD* fragments were sequenced using an ALF red DNA Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

Ultrasonic treatment

A suspension of the preincubated cells was added to 100ml NB and incubated for 2h at 37℃ in a water bath shaker (100 strokes/min). The cells in the exponential-phase were harvested by centrifugation (6000×g, 5min, 4℃), washed once (6000×g, 5min, 4℃) and suspended in ice-cold sterilized water. The concentration was adjusted to 1.0×10^6cells/ml with ice-cold sterilized water, then sonicated using an ultrasonic homogenizer (UT-51N, SHARP, Osaka).

Scanning electron microscopy

The sensitive strain and the P-12 resistant strain were observed with a scanning electron microscope (SEM) according to a method previously reported (Maeda et al., 1998).

RESULTS AND DISCUSSION

P. aeruginosa with adapted resistance to P-12

The development of the adapted resistance of *P. aeruginosa* ATCC 10145 to P-12 is shown in FIG. 2. The constant increase of MIC was saturated at the ninth adaptive cycle and the maximum MIC was 1024 μM at the eleventh. The value was approximately 8 times higher than that of the sensitive strain. To determine the stability of the adapted resistance, the cells were incubated in fresh NB overnight at 37℃ and its MIC was determined in the same way. The adapted resistance to P-12 was stable even if the resistant strain was incubated in the fresh NB, and a decrease in MIC was not observed (FIG.2).

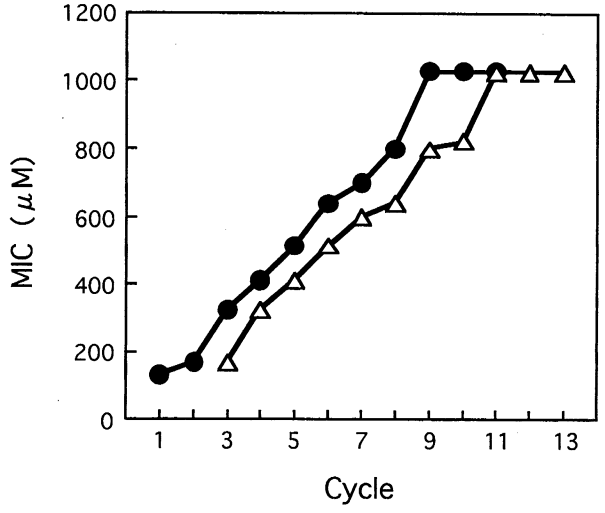

FIG. 2. Relationship between the P-12 treatment cycle and MIC of P-12 against *Pseudomonas aeruginosa* ATCC 10145. Symbols: ●, MIC of P-12 against the sensitive strain; △, MIC of P-12 against the strain incubated in fresh NB.

Growth properties of the resistant strain

The growth properties of the sensitive strain and the resistant strain (11 cycles of adaptation to P-12) were investigated (FIG.3). The growth of the resistant strain was delayed compared with that of the sensitive strain. However, in the stationary-phase, the cell concentration of the resistant strain grown in the NB without P-12 was nearly equal to that of the sensitive strain. One of the reasons why the growth of the resistant strain was decreased in the exponential growth-

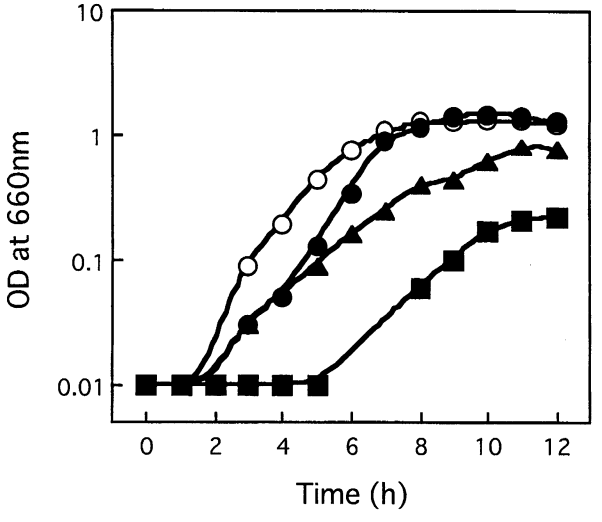

FIG. 3. Growth curve of the sensitive and the resistant strains against P-12. Symbols: ○, Sensitive strain; ●, Resistant strain (grown in NB without P-12); ▲, Resistant strain (grown in NB containing 200 μM of P-12); ■, Resistant strain (grown in NB containing 800 μM of P-12).

phase is thought to be the functional loss of protein biosynthesis systems. The optical density of the resistant strain in the stationary-phase decreased with increase in the concentration of the co-existent P-12. These results suggest that some changes occurred in the growth state of the resistant strain by contact with P-12. These changes would be not only a negative change for the resistant strain (e.g. the functional loss of protein biosynthesis systems or the following decrease of growth), but also positive (e.g. the production of alginic acid). In the NB containing 800 μ M of P-12, the growth was delayed for about 5 hours. It seems that the selection of more resistant cells occurred at this time lag. That is to say, there are differences in the degree of the resistance to P-12. In order to reveal them, a population analysis was carried out (FIG. 4). Though the sensitive strain died out in the NB containing 200 μ M of P-12, the resistant strain did not decrease so much. This result indicates that the degree of resistance to P-12 in the sensitive strain is quite different from that of the resistant strain. The number of the resistant strain decreased with increase of P-12 concentration. However, the resistant strain preincubated in the NB containing 800 μ M of P-12 easily died out. It suggests that the growth state of the cells preincubated in the NB containing 800 μ M of P-12 was unstable. As a whole, it is thought that the highly adapted resistance observed in *P. aeruginosa* was a result of the selection brought about by contact with a high concentration of P-12 in the NB.

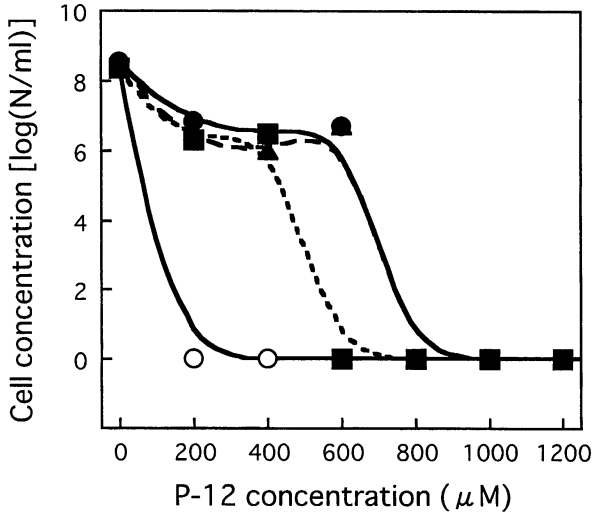

FIG. 4. Population analysis of the sensitive and the resistant strains against P-12. Symbols: ○ (a solid line), Sensitive strain; ● (a solid line), Resistant strain (preincubated in NB without P-12); ▲ (a broken line), Resistant strain (preincubated in NB containing 200 μ M of P-12); ■ (a dotted line), Resistant strain (preincubated in NB containing 800 μ M of P-12).

TABLE 1. MIC and MBC of P-12 against *Pseudomonas aeruginosa* ATCC 10145 (the sensitive strain) and the P-12 resistant strain.

Strain	MIC (μ M)	MBC (μ M)
Sensitive	131	41.9
Resistant	1024	64.8

The MIC and MBC against the sensitive strain and the resistant strain were determined (TABLE 1). Both the MIC and MBC against the resistant strain were higher than those against the sensitive strain and the difference in the MIC between the sensitive strain and the resistant strain was larger than that in the MBC. This is thought to be due to the differences in the measurement system (Maeda et al., 1998). This result suggests that the adaptation of *P. aeruginosa* to P-12 may be influenced by certain materials contained in the NB for the MIC assay.

Hydrophobic interaction and the resistance to P-12

The change in the characteristics of the bacterial cell surface brought about by contact with P-12 was investigated. The result showed that the cell surface of the resistant strain (log HI=-0.279) was more hydrophilic than that of the sensitive strain (log HI =0.198). The factor most likely affecting the cell surface hydrophobicity is thought to be lipopolysaccharide (LPS). LPS consists of hydrophobic lipid and hydrophilic polysaccharides, and is a component of the outer membrane of gram-negative bacteria. The hydrophilic character of the resistant strain may be due to the extension of the polysaccharide chain or an increase of the amount of LPS. In any case, it seems that such a change related to LPS obstructs the contact of P-12 to bacterial surfaces.

Relationship between the biofilm formation and the resistance to P-12

Biofilm is the term for the bacterial crowd that is formed with alginic acid and oligosaccharides. Biofilm has been a serious problem in the industries and hospitals since the bacteria within it exhibit high resistance against various disinfectants. The type of strain of *P. aeruginosa* does not produce alginic acid. However, it was reported that many clinical strains of *P. aeruginosa* change to produce it (Deretic et al., 1986).

It was microscopically shown that only the P-12 resistant strain of *P. aeruginosa* easily formed a bacterial crowd (FIG. 5). This result suggests that some connection exists between the P-12 resistance and the formation of biofilm. To verify this suggestion, the

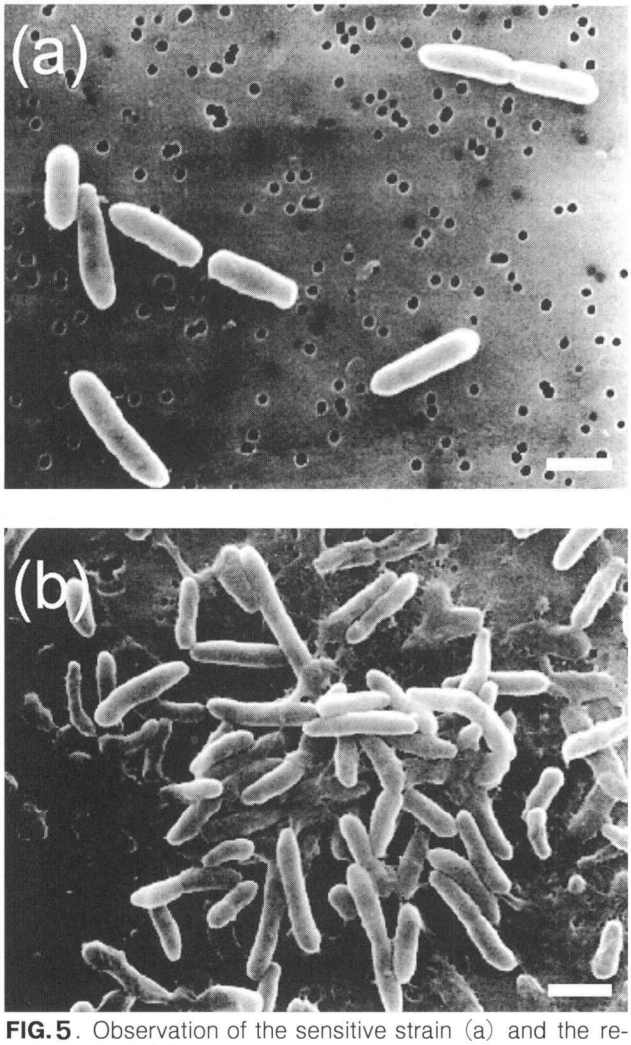

FIG. 5. Observation of the sensitive strain (a) and the resistant strain (b) against P-12 by scanning electron microscope (SEM). Bar markers represent 1 μm.

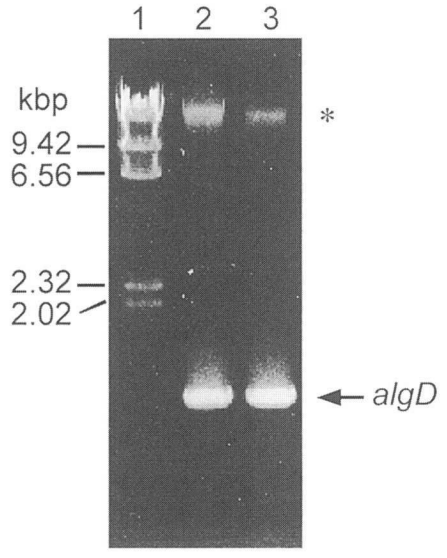

FIG. 6. PCR amplification analysis of *algD*. Lanes: 1, Marker (λ DNA digested with *Hin*dIII); 2, Sensitive strain; 3, Resistant strain. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel, stained with 1mg/l ethidium bromide solution and photographed under UV light. The asterisk indicates the chromosomal DNA as a template of PCR.

amount of alginic acid was measured. The alginic acid was found to be produced only in the resistant strain (2.57×10^{-6} μg/cell). This result suggests that the adaptive resistance of *P. aeruginosa* to P-12 was attained by the change to produce alginic acid. The alginic acid is synthesized by the action of some enzymes encoded on the alginic acid biosynthesis genes. One of the alginic acid biosynthesis genes, *algD* is essential for the alginic acid biosynthesis, and it is transcriptionally regulated (Berry et al., 1989). To confirm the presence of *algD* on the chromosomal DNA of the sensitive strain and the resistant strain, PCR amplification and DNA sequencing for *algD* were done. Because the major band was amplified at the correct size (FIG. 6), *algD* was present on the chromosomal DNA of the both strains. Also, as the amplified partial DNA sequences of *algD* were the same as each other (data not shown), it is obvious that the resistant strain originated in the starting strain of *P. aeruginosa*. These results suggest that the resistant strain is induced to produce alginic acid due to some of the genetic activation of alginic acid biosynthesis gene(s). The measurement of the amount of alginic acid was also done in the resistant strain grown in the NB containing 500 μM of P-12 (half of MIC). The amount of alginic acid extracted from this culture (2.57×10^{-6} μg/cell) was the same as that from the culture grown in the absence of P-12. These results suggest that the production of alginic acid is not inducible but constitutive in the P-12 resistant strain. It is also strongly suggested that biofilm formation is one of the factors in the adaptive resistance of *P. aeruginosa* to P-12.

To further investigate the relationship between the biofilm formation and the P-12 resistance, the culture of the resistant strain was sonicated to destroy the biofilm and then, the MIC was measured. The sonication conditions were decided from a preliminary test, these conditions enabled more than 80% of the sonicated bacteria to grow (data not shown). The MIC (819 μM) and MBC (42.4 μM) against sonicated bacteria decreased in comparison with those of non-sonicated bacteria. These results suggest that the bacteria dispersed from the biofilm were directly attacked by P-12. Therefore, the biofilm formation is regarded as one of the factors in the adaptive resistance of *P. aeruginosa* to P-12. However, since greater differences in MIC between the sensitive

strain and the resistant strain are shown in TABLE 1, it is thought that the other factor(s) may participate in the adaptation. It is thought that the drug resistance of bacteria is related to the enzymatic degradation of antibacterial drugs, the change of outer membrane protein(s) or the change in the permeability of antibiotics (Dever et al., 1991). Therefore, the adaptation of *P. aeruginosa* to P-12 would be related to not only the change of cell surface hydrophobicity and the biofilm formation but also these factors, i.e. the enzymatic degradation and/or the change of outer membrane protein(s) or permeability.

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