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**Note**

# The Properties of Bacterial Adaptation to Mono- and Bis-Quaternary Ammonium Compounds

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**The adaptation of bacteria to mono- and bis-type quaternary ammonium compounds (QACs) was carried out and studied. The minimum inhibitory concentration (MIC) of bis-QACs against the bacteria in contact with bis-QACs was nearly the same as that against the control. However, the MIC of mono-QAC against the strains adapted to mono-QAC was higher than that against the control. Moreover, bis-QACs were effective against even the strains adapted to mono-QAC. As the cell surface of the strains adapted to mono-QAC changed to hydrophilic, the difference in the bacterial adaptation was thought to arise from a potent property of bis-QACs whose antimicrobial characteristics are independent of the change in the cell surface hydrophobicity.**

*Key words* : Quaternary ammonium compound/Bis-QAC/Bacterial adaptation/Resistance to disinfectant/Cell surface hydrophobicity.

Quaternary ammonium compounds (QACs), which have the ammonium or pyridinium cation moiety in their molecule, have been used widely as general disinfectants. Many studies on the synthesis and antimicrobial characteristics of various kinds of QACs have been reported up to this time (Baley et al., 1997; Devinsky et al., 1991 and 1996; Kourai et al., 1994; Maeda et al., 1998, 1999a and 1999b; Okazaki et al., 1996 and 1997; Yoshida et al., 2000 and 2001). Since QACs have a comparatively lower toxicity and an excellent antimicrobial effect against various kinds of bacteria, they have been used broadly in the food- and textile-industrial, and medical fields. They have also been used in domestic settings. For example, cethylpyridinium chloride (CPC) is used as a disinfectant in personal hygiene products and has been added to oral products (Holbeche and Reade, 1978; Meier et al., 1996).

In recent years, the appearance of bacteria resistant to QACs has been reported. QAC-resistant bacteria are reported for benzalkonium chloride abbreviated as BAC (Langsurd and Sundheim, 1997; Nakashima et al., 1987; Sakagami et al., 1989), CPC (Irizarry et al., 1996; Tattawasart et al., 1999), didecyldimethylammonium bromide (Méchin et al., 1999), benzyldimethyltetradecylammonium chloride (Guérin-Méchin et al., 1999) and dimethyldodecylammonium chloride (Jones et al., 1989). Most of these QAC resistant bacteria are Gram-negative bacteria. Therefore, to elucidate the resistance mechanism against QACs, much attention has been paid to the difference between the cell surface structure of Gram-negative bacteria and that of Gram-positive bacteria. The cell surface structures of Gram-negative bacteria are more complicated than those of Gram-positive bacteria. Furthermore, fatty acids and lipopolysaccharides in the outer membrane interrupt the contact of various materials to the bacterial cell surfaces (Russell and Gould, 1988). As a matter of fact, some QAC-resistant strains have changed the fatty acid composition of the membrane (Guérin-Méchin et al., 1999 and 2000; Jones et al., 1989; Méchin et al., 1999; Sakagami et al., 1989). These

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reports indicated that Gram-negative bacteria easily adapt to QACs by changing the cell surface structures. Thus, it is necessary to develop new disinfectants with potent antimicrobial activity that can avoid such bacterial adaptation.

It was previously reported that new type QACs, bis-QACs, have a wide range of antimicrobial activity (Maeda et al., 1999a and 1999b; Okazaki et al., 1997; Yoshida et al., 2000 and 2001). Moreover, as the bacteriostatic activity of these bis-QACs is little affected by the hydrophobicity of the bacterial cell (Okazaki et al., 1997), it is suggested that this characteristic is one of the important factors to prevent the appearance of QAC-resistant bacteria.

The purpose of this study is to investigate the difference between the properties of bacterial adaptation to mono-QAC and to bis-QACs. This study focused on the change of the bacterial cell surface hydrophobicity and the interaction between QACs and the bacterial cell surface.

Mono-QAC, *N*-dodecylpyridinium iodide abbreviated as P-12 (Kourai et al., 1980), and bis-QACs, 4, 4'-( α , ω -polymethylenedithio)bis(1-alkylpyridinium iodide)s (Okazaki et al., 1997), 4,4'-(1,6-hexamethylenedioxydicarbonyl)bis(1-dodecylpyridinium iodide) (Maeda et al., 1999b), *N,N'*-hexamethylenebis(1-dodecyl-4-carbamoylpyridinium iodide) (Yoshida et al., 2000) and 4,4'-(tetramethylenedicarbonyldia-

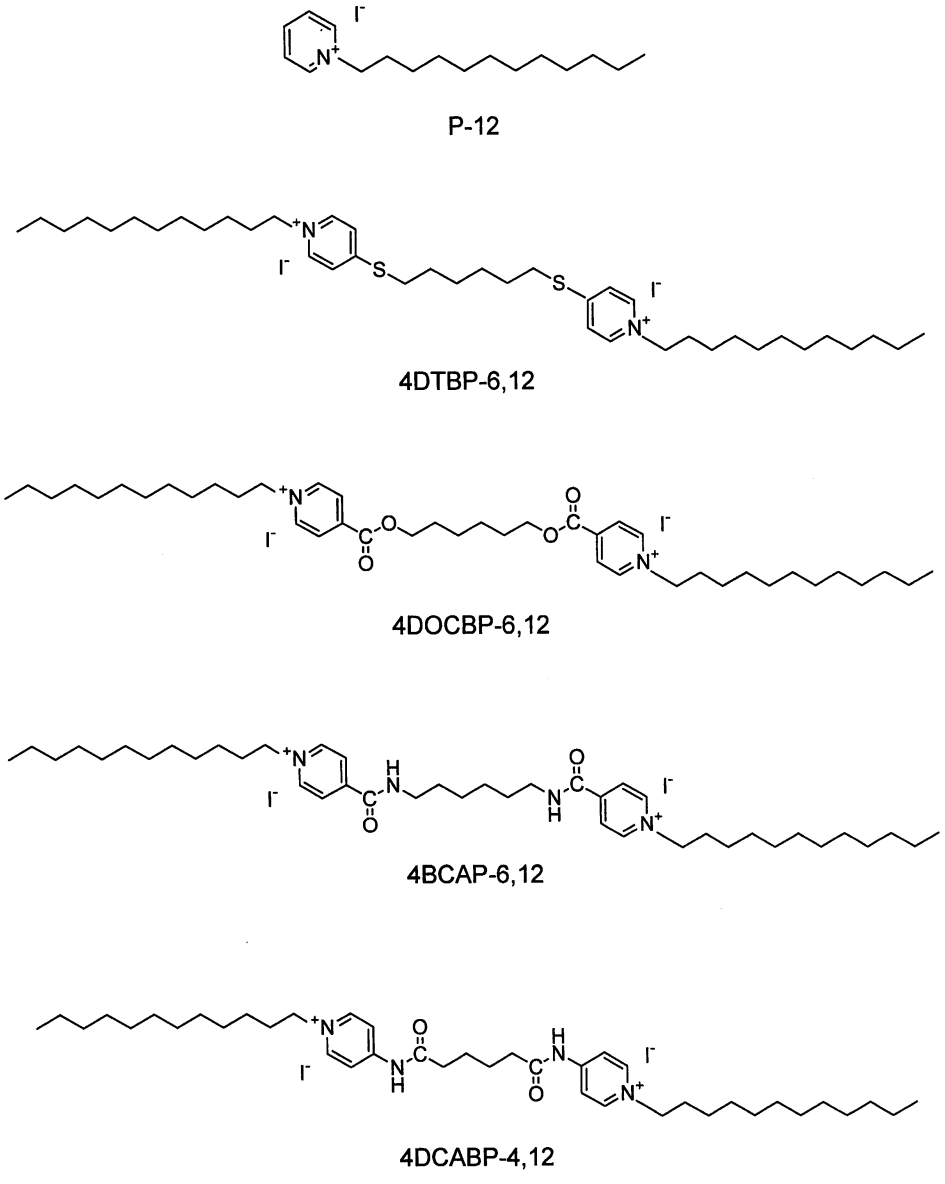


**FIG. 1**. Chemical structures of QACs.

mino) bis (1-dodecylpyridinium iodide) (Yoshida et al., 2001), abbreviated as 4DTBP-6,12, 4DOCBP-6,12, 4BCAP-6,12 and 4DCABP-4,12, respectively, used in this study were synthesized in our laboratory (Fig. 1). Other chemicals for this study were of commercially available reagent grade and used without further purification. Two Gram-negative bacteria strains, *Pseudomonas aeruginosa* ATCC 10145 and *Escherichia coli* IFO 12713, and two Gram-positive bacteria strains, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* IFO 12732 were employed for the experiment. They were cultured according to a previous report (Maeda et al., 1999a). The minimum inhibitory concentration (MIC) of various QACs was measured basically according to the standard broth dilution method (Maeda et al., 1996). The tested QACs were prepared as an aqueous solution (for mono-QAC) or 80% (v/v) ethyl alcohol solution (for bis-QACs), then diluted in steps with nutrient broth (NB, Becton Dickinson, Sparks, MD, USA) to the prescribed concentrations. Bacteria with adapted resistance to QACs were developed by using the standard broth dilution method according to the previous report (Tabata et al., 2002). QAC dilution series were made by NB to the prescribed concentration. The preincubated culture of test bacteria at 37℃ was diluted to a concentration of $1.0\times10^6$cells/ml with NB. A 0.5ml portion of the cell suspension was added to an equal volume of each of the QAC dilution series. After incubation for 24h at 37℃, the bacteria which grew in the highest concentration from the dilution series were used in the following, and the process was repeated for 7 cycles. The measurement of cell surface hydrophobicity and calculation of a hydrophobicity index (HI) were performed according to the previous report (Kourai et al., 1989). Cells in the stationary-phase were used in this measurement. The oil-phase used in this measurement was *n*-hexyl alcohol for Gram-negative bacteria or *n*-hexadecane for Gram-positive bacteria.

The adaptation of various bacteria (*P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus*) to QACs (P-12, 4DTBP-6,12, 4DOCBP-6,12, 4BCAP-6,12 and 4DCABP-6,12) was investigated. In general, the adaptation of Gram-negative bacteria to QACs was easily achieved owing to the adjustment of cell surface components (Jones et al., 1989; Russell et al., 1986; Szumala et al., 1986). *Staphylococcus* spp., the Gram-positive bacteria, were also able to adapt to QACs due to the acquisition of plasmids containing QAC-resistant determinants (Bjorland et al., 2001; Heir et al., 1995 and 1999). Figure 2 shows the adaptation profiles of *P. aeruginosa* ATCC 10145 to P-12, 4DTBP-6,12, 4DOCBP-6,12, 4BCAP-6,12 and

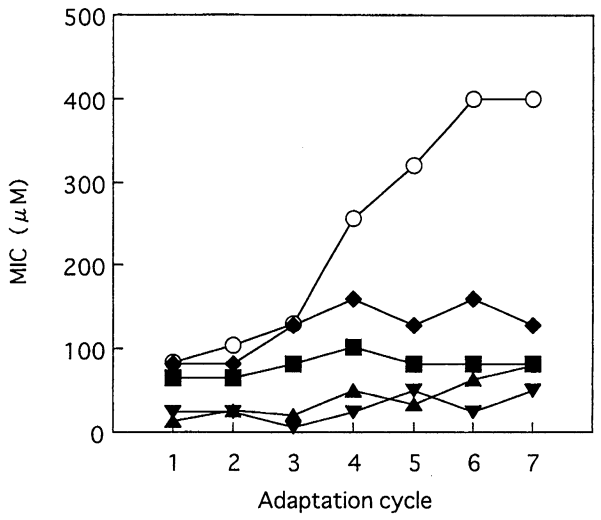


**FIG. 2**. Relation between the adaptation cycle and the MIC of QACs against *Pseudomonas aeruginosa* ATCC 10145. Symbols : ○, P-12; ■, 4DTBP-6,12; ▲, 4DOCBP-6,12; ◆, 4BCAP-6,12; ▼, 4DCABP-4,12.

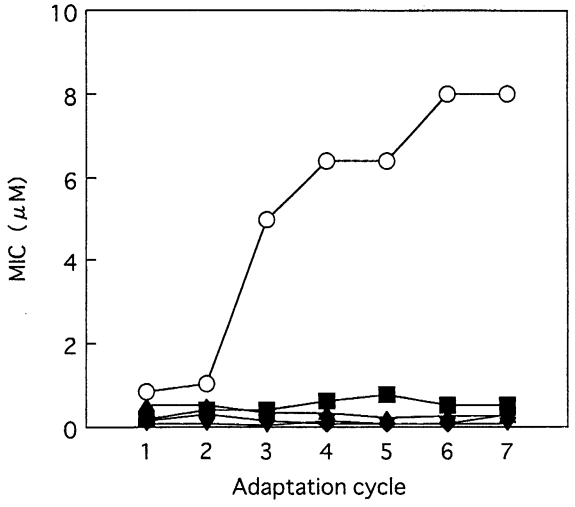


**FIG. 3**. Relation between the adaptation cycle and the MIC of QACs against *Staphylococcus aureus* IFO 12732. Symbols : ○, P-12; ■, 4DTBP-6,12; ▲, 4DOCBP-6,12; ◆, 4BCAP-6,12; ▼, 4DCABP-4,12.

4DCABP-4,12. Since only the MIC of P-12 against test bacteria increased gradually after 7 cycles of contact with various QACs, it was revealed that *P. aeruginosa* easily adapted to P-12. On the other hand, the MIC of bis-QACs against *P. aeruginosa* did not increase. Similarly to *P. aeruginosa*, *E. coli* had adapted to P-12, but not to bis-QACs (data not shown). Giving the same results as Gram-negative bacteria, *S. aureus* adapted to P-12 only (Fig. 3). The same tendency in the adaptation to QACs was also seen in the result of *B. subtilis* (data not shown). These results

**TABLE 1**. MIC of P-12 against stationary-phase bacteria in contact with various bis-QACs.

| Strain | MIC of P-12 (μM)[a] | | | | |
|---|---|---|---|---|---|
| | Control | 4DTBP-6,12 | 4DOCBP-6,12 | 4BCAP-6,12 | 4DCABP-4,12 |
| *P. aeruginosa* ATCC 10145 | 125-250 | 250 | 250 | 250 | 250 |
| *E. coli* IFO 12713 | 62.5 | 62.5-125 | 62.5-125 | 62.5-125 | 62.5 |
| *B. subtilis* ATCC 6633 | 7.8 | 7.8 | 7.8 | 7.8-15.6 | 7.8 |
| *S. aureus* IFO 12732 | 1.0-2.0 | 3.9-7.8 | 0.5-1.0 | 1.0-2.0 | 0.5-2.0 |

[a] MIC was determined by a broth dilution method after incubation for 24h at 37℃. The measurement was repeated three times and MIC was shown as a range of results.

**TABLE 2**. MIC of various bis-QACs against stationary-phase bacteria.

| Strain | | MIC (μM)[a] | | | | |
|---|---|---|---|---|---|---|
| | | P-12 | 4DTBP-6,12 | 4DOCBP-6,12 | 4BCAP-6,12 | 4DCABP-4,12 |
| *P. aeruginosa* ATCC 10145 | Control | 125 | 12.5-50.0 | 250-100 | 25.0-50.0 | 25.0-100 |
| | adapted to P-12 | 500 | 25.0-50.0 | 50.0-100 | 25.0-50.0 | 100 |
| *E. coli* IFO 12713 | Control | 62.5 | 3.1-6.3 | 6.3-12.5 | 6.3-12.5 | 6.3-12.5 |
| | adapted to P-12 | 250 | 3.1 | 6.3 | 3.1 | 6.3 |
| *B. subtilis* ATCC 6633 | Control | 7.8 | 0.8-1.6 | 1.6-3.1 | 0.8-1.6 | 1.6 |
| | adapted to P-12 | 31.3 | 0.8 | 1.6-3.1 | 0.8-1.6 | 0.8-1.6 |
| *S. aureus* IFO 12732 | Control | 1.0-2.0 | 0.4-0.8 | 0.8-1.6 | 0.4-0.8 | 0.4-0.8 |
| | adapted to P-12 | 15.6 | 0.8 | 1.6-3.1 | 1.6 | 1.6 |

[a] MIC was determined by a broth dilution method after incubation for 24h at 37℃. The measurement was repeated three times and MIC was shown as a range of results.

**TABLE 3**. Cell surface hydrophobicity of stationary-phase bacteria in contact with P-12.

| Strain | Hydrophobicity (log HI)[a] | | |
|---|---|---|---|
| | Control | 4 cycles | 7 cycles |
| *P. aeruginosa* ATCC 10145 | −0.76 | −0.87 | −0.86 |
| *E. coli* IFO 12713 | −0.81 | −0.87 | −1.05 |
| *B. subtilis* ATCC 6633 | −1.39 | −1.50 | −2.09 |
| *S. aureus* IFO 12732 | −0.92 | −1.66 | −1.87 |

[a] Partition coefficients of bacterial cells between oil-phase and physiological saline.

suggest that both Gram-negative and Gram-positive bacteria easily adapt to mono-QAC but find it more difficult to adapt to bis-QACs. In other words, the contact with bis-QACs does not cause the bacterial adaptation to them. From these results, it is strongly implied that the contact mechanisms of bis-QACs were quite different from those of mono-QAC.

Table 1 shows the MIC of P-12 against the bacteria which were in contact with various bis-QACs in 7 cycles. Compared with the control, the MIC against Gram-negative bacteria, *P. aeruginosa* or *E. coli*, did not vary significantly. Gram-positive bacteria, *B. subtilis* or *S. aureus*, also had nearly the same value as that of control. These results reveal that the contact with various bis-QACs do not affect the adaptive resistance of bacteria unlike the contact with P-12.

Table 2 indicates the MIC of various bis-QACs against the bacteria which were in contact with P-12 for 7 cycles. The MIC of P-12 against the strains of *P. aeruginosa, E. coli, B. subtilis* and *S. aureus* adapted to P-12 were about 4.0, 4.0, 4.0 and 12 times higher than that against each control, respectively. However, the MIC of bis-QACs against each strain adapted to P-12 tended to be nearly the same as the control MIC. It is conceivable that bis-QACs are effective against the strains adapted to P-12. These results are considered to arise from the difference between the properties of antimicrobial action of bis-QACs and those of mono-QAC. It was reported that bis-QACs have potent antimicrobial activity against not only Gram-positive bacteria but also Gram-negative bacteria (Mąeda et al., 1999b; Okazaki et al., 1997). It is suggested that these desirable characteristics of bis-QACs greatly depend on the unique bis-type structure. The details of antimicrobial mechanism of bis-QACs are currently under study, and one of the potent antimicrobial actions of bis-QACs is proved to be due to the quickness of their antimicrobial action

(unpublished data).

Further investigation focusing on the change of the cell surface characteristics was carried out. This was based on the idea that the antimicrobial action of QAC mainly damages the bacterial cell surface structures. Table 3 shows the cell surface hydrophobicity of the strains of *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus* as controls, or in contact with P-12 in 4 or 7 cycles. The cell surface of Gram-negative bacteria (*P. aeruginosa* and *E. coli*) and Gram-positive bacteria (*B. subtilis* and *S. aureus*) gradually changed to hydrophilic with the adaptation to P-12. These results suggest that the cell surface components such as membrane lipids and/or lipopolysaccharide (Gram-negative bacteria) or lipoteichoic acid (Gram-positive bacteria) changed with the adaptation to P-12. In fact, the change of fatty acid composition of membrane was reported for the other QAC resistant *P. aeruginosa* (Guérin-Méchin et al., 1999; Jones et al., 1989; Méchin et al., 1999; Sakagami et al., 1989). From the results of this study, it is suggested that the bacteria was able to adapt to P-12 owing to the hydrophilic change of the cell surface. In spite of these hydrophilic changes of bacterial cell surface with P-12 adaptation, bis-QACs were still effective against such adapted bacteria (Table 2). It is suggested that this excellent characteristic of bis-QACs contributes to the fact that their antimicrobial activity is higher than that of mono-QAC regardless of the kind of bacteria that is involved with various levels of cell surface hydrophobicity (Maeda et al., 1998).

Since the antimicrobial action of bis-QACs is different from that of mono-QACs and the antimicrobial activity of bis-QACs is superior to other domestic disinfectants such as BAC or CPC (data not shown), it is conceivable that bis-QACs are useful for the control of pathogenic bacteria even if the bacteria had adapted to QAC (in other words, QAC-resistant bacteria). Moreover, even repeated use of bis-QACs did not cause bacteria to adapt to them. These characteristics of bis-QACs are advantageous when disinfection must be regularly carried out in various fields and the appearance of the pathogenic and/or resistant bacteria often is a concern.

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