原著論文

Reduction of benzalkonium chloride-resistant mutants of *Pseudomonas aeruginosa* PAO1 in the absence of benzalkonium chloride

Yoichi Yamada[1) *], Daisuke Ueda[2)], Toshiyuki Kudo[1)],
Tomofusa Tsuchiya[2)] and Sumiko Shiota[1)]
[1)] *Department of Molecular Biology, School of Pharmacy, Shujitsu University,*
[2)] *Department of Infection Control Laboratory, College of Pharmaceutical Sciences, Ritsumeikan University*
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Abstract

Pseudomonas aeruginosa is one of the major causes of nosocomial infection. Treatment of diseases caused by *P. aeruginosa* by antibiotics is difficult due to its antibiotic resistance. Thus prevention of *P. aeruginosa* infection by using disinfectants is desired. For efficient use of disinfectants, it is important to elucidate characteristics of disinfectant-resistant mutants.

In this study, we isolated benzalkonium chloride-resistant mutants from *P. aeruginosa* PAO1 and characterized them. Generation time of the mutants and that of the parent were the same or almost the same. No apparent change in the colony forming unit (CFU) ratio between the mutant and the parent was observed under co-cultivation conditions in the absence of benzalkonium chloride during the exponential phase of growth. However, when cell growth entered the stationary phase, the CFU ratio between the resistant mutant and the parent drastically decreased. We tested the effect of nutrient on the decrease of the CFU ratio of the mutant, and found that the mutant decreased under poor nutrient conditions.

Keywords: *Pseudomonas aeruginosa*, benzalkonium chloride, resistance, reduction

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium and exists in the environments such as soil, water and animal skin[1)]. *P. aeruginosa* is famous for causing nosocomial infections to compromised hosts at clinical sites[1)]. It shows both intrinsic resistance and acquired resistance against many antibiotics and disinfectants[2-5)]. It is difficult to treat *P. aeruginosa* infections by antibiotics due to their drug resistance[5)].

To prevent infections caused by *P. aeruginosa*, appropriate usage of disinfectants is important. Appropriate usage means to disinfect the following medical care providers, medical devices and environments. Furthermore, appropriate usage also prevents the appearance of disinfectant and antibiotic resistant bacteria[3), 6), 7)]. According to the package insert, bacteria should not cause troubles. Some outbreaks were attributed to improper dilution or storage conditions with cotton or gauze[3), 4), 8)].

Benzalkonium chloride, one of the most frequently used disinfectants in the world, is a quaternary ammonium compound. Benzalkonium is a cationic disinfectant which is used to on skin and medical equipment. Insertion of benzalkonium to a bacterial cell membrane leads to the disruption of the membrane and denaturation of its proteins[9)]. Benzalkonium chloride takes effect on Gram-positive bacteria and Gram-negative bacteria, but has no effect on tubercle bacillus, viruses and spores[9)].

Resistance mechanisms for benzalkonium chloride are known in *P. aeruginosa*. It is a substrate of PmpM, multidrug efflux pump, belonging to the MATE family[10)]. PmpM also confers resistance to many cationic compounds, ethidium bromide, acriflavin and so on. Disinfectants containing benzalkonium chloride attack cellular membranes and also cause the induction of the multidrug efflux pump, MexCD-OprJ[11)]. Decreased permeability of benzalkonium chloride depending on the changing of membrane properties is also reported[12-14)]. Biofilm formation affects the resistance of not only disinfectants, but also many antibiotics[15)].

In this study, we isolated benzalkonium chloride resistant mutants of *P. aeruginosa* PAO1 with two methods. These mutants were characterized and their growth rate was calculated. To examine the survivability of the resistant mutant, the mutant and *P. aeruginosa* PAO1 were co-cultivated and the ratio of the mutant was calculated. This mutant showed less survivability in a low nutrient medium.

Experimental procedures

Materials. Benzalkonium chloride and carbenicillin were purchased from Nacalai tesque Inc. Acriflavine, alkyl -diamino - ethylglycine hydrochloride, Chlorhexidine gluconate and norfloxacin were purchased from FUJIFILM Wako Pure Chemical Corp., Ltd. Imipenem was purchased from LKT Laboratories Inc.

Bacterial strain and Growth. *P. aeruginosa* PAO1 and its mutants were grown in L broth (1.0 % tryptone, 0.5 % yeast extract, and 0.5 % NaCl) (Nacalai tesque Inc., Japan) or Mueller-Hinton broth (Difco, Sparks, U. S. A) supplemented with 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+} (CAMHB: Cation-adjusted Mueller Hinton broth) or minimum salt Tanaka broth (34 mM NaH_2PO_4, 64 mM Na_2HPO_4, 20 mM $(NH_4)_2SO_4$, 0.3 mM $MgSO_4$, 1 μM $FeSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$ (pH 7.0)) supplemented with 0.2% of glucose, fructose, or glycerol, and incubated at 37 ^oC[16)].

Drug susceptibility test. The minimum inhibitory concentration of liquid two-fold dilution method (MIC_{liq}) was determined in CAMHB according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI)[17)]. Cells in the medium (5 x 10^5 cells/mL) were incubated at 37 ^oC for 20 hr, and the growth was judged. The minimum inhibitory concentration of agar plate dilution method (MIC_{plate}) was determined for benzalkonium chloride according to the recommendations of

the CLSI[17]. Cells (1.0 x 10^7 cells/mL) were inoculated on CAMHB agar supplemented with benzalkonium chloride and incubated at 37 ºC for 20 hr and the growth was judged.

Isolation of benzalkonium chloride-resistant mutants. Culture of *P. aeruginosa* PAO1 were plated onto L-agar plate containing benzalkonium chloride and incubated at 37 ºC for 48 hr. In the same time, serial dilutions of cell suspension were plated on L-agar plates to calculate the total cell numbers and isolation frequency. To confirm the acquisition of benzalkonium chloride resistance, mutants were streaked on L-agar plates containing the appropriate concentration of benzalkonium chloride. Isolation of benzalkonium chloride - resistant mutants with L broth was performed basically according to Loughlin and Tabata mothods[6),13)]. A 1.25-fold benzalkonium chloride dilution series from 1/4 x MIC_{liq} (16 μg/mL) was prepared with L broth. Ten to the seventh cells of *P. aeruginosa* PAO1 were added to 4 mL of dilution series in glass tubes and incubated for 24 hr at 37 ºC with shaking. The bacteria which grew in the highest concentration were used for passage culture. After several passages, bacterial cultures were streaked on the L-agar plates and a few mutants were isolated.

Growth measurement. 10^4 cells were added to 5 mL of L broth and cultured in L-type tube TN-5L (ADVANTEC, Japan) at 37 ºC for 48 hr with shaking at 20 rpm. Turbidity of $O.D._{600}$ was measured.

Colony forming unit (CFU) count of *P. aeruginosa* PAO1 and mutants in co-cultivation. *P. aeruginosa* PAO1 (10^4 cells) and mutant (10^4 cells) were added to 5 mL of L broth and cultured in L-type tube TN-5L (ADVANTEC, Japan) at 37 ºC for 48 hr with shaking at 20 rpm. Turbidity of $O.D._{600}$ was measured. CFU was counted using L-agar plates or L-agar plates containing 512 μg/mL of benzalkonium chloride.

Table 1. MIC of benzalkonium chloride resistant mutants.

P. aeruginosa	MIC_{plate}	MIC_{liq}						
strains	BC	BC	CHX	ADECG	CBPC	IPM	NFLX	ACF
PAO1	1024	64	8	64	32	1	1	128
BC12	2048	128	16	32	64	2	1	128
BC13	4096	128	8	256	32	0.5	1	128
BC21	2048	128	64	64	32	1	2	256
BC6961	n.t.	256	8	256	32	2	0.5	256
BC6963	n.t.	256	32	256	32	2	0.5	256
BC7251	n.t.	256	32	256	128	4	4	256
BC7252	n.t.	256	32	256	32	4	4	256

MIC; minimum inhibitory concentration (μg/mL), n.t.; not tested, BC; benzalkonium chloride, CHX; chlorhexidine, ADECG; alkyldiaminoethylglycine hydrochloride, CBPC; calbenicillin, IPM; imipenem, NFLX; norfloxacin, ACF; acriflavine

Results and Discussion

Isolation of resistant mutants of benzalkonium chloride. MIC_{plate} of benzalkonium chloride in *P. aeruginosa* PAO1 was determined to be 1024 μg/mL. Plates containing 1024, 2048 and 4096 μg/mL of benzalkonium chloride were used for selection of resistant mutants. Three mutants, BC12, BC13 and BC21, were selected from the plate containing 1024 μg/mL, 1 x MIC_{plate}, of benzalkonium chloride. The isolation frequency was calculated to be 2.26×10^{-9}. Judging from this frequency, these mutants might have one mutation in their chromosome[18]. Because no mutants were selected from plates containing higher concentrations than 1024 μg/mL of benzalkonium chloride, minimum bactericidal concentration of benzalkonium chloride should be 2048 μg/mL.

MIC_{liq} of benzalkonium chloride of *P. aeruginosa* PAO1 was determined to be 64 μg/mL (Table 1). *P. aeruginosa* PAO1 was inoculated in L broth containing 16 μg/mL, 1/4 x MIC_{liq} of benzalkonium chloride and serial dilution media were used for passage culture. Finally bacterial cultures of benzalkonium chloride-resistant mutants were obtained from L broth containing 696 μg/mL and 725 μg/mL of benzalkonium chloride. Each of 2 mutants, BC6961 and BC6963, BC7251 and BC7252, were isolated individually.

Drug susceptibility of benzalkonium chloride-resistance mutants. MIC_{liq} of several antimicrobial agents were determined (Table 1). All mutants showed elevated MIC_{liq} of benzalkonium chloride. Four mutants, BC6961, BC6963, BC7251 and BC7252, isolated from broth showed higher resistance than those isolated from plates, BC12, BC13 and BC21. Because passage culture was used for isolation of mutants from broth, they might have several mutations.

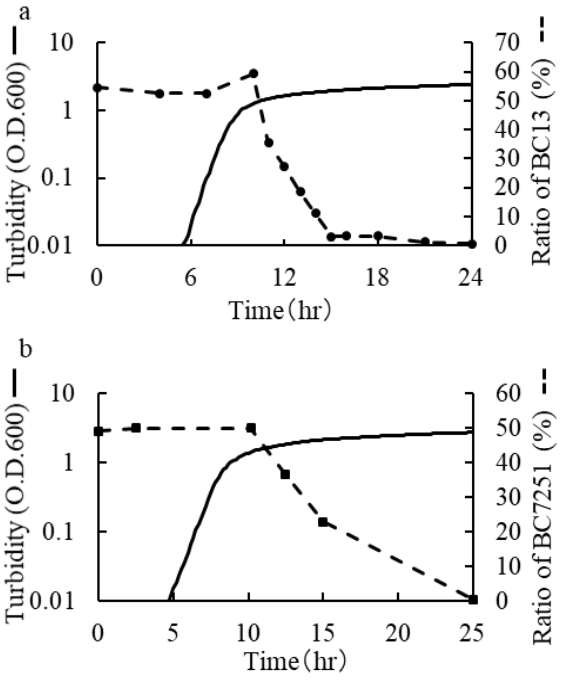

Fig. 1. Ratio of mutants in co-culture with *P. aeruginosa* PAO1. 10^4 cells of each strain were inoculated in L broth and $O.D._{600}$ was measured. CFUs were calculated at each time point. a, *P. aeruginosa* PAO1 and BC13. b, *P. aeruginosa* PAO1 and BC7251.

BC21 and mutants obtained from liquid culture showed resistance to acriflavine, a dye, which is known to be a substrate of multi-drug efflux pumps[10,11,19-21]. Thus, some multi-drug efflux pumps may be upregulated in these mutants. BC7251 and BC7252 showed resistance to imipenem [22]. Imipenem is known to be a substrate of multi-drug efflux pump, MexAB-oprM[23]. MexAB-OprM might be up-regulated in these mutants. The contents like phospholipids (PL) and fatty and neutral lipids (FNL) in the cell wall were increased and they prevented permeation of benzalkonium chloride in resistant *P. aeruginosa*[24]. The amount of these content

might be increased.

Comparison of generation time of mutants and parental strain PAO1. It seems likely that resistant mutant may disappear in the absence of the antimicrobial agent because generation time of the mutants is often longer than that of the wild type, due to the cost for working the resistance mechanism. BC13, the highest resistance mutant of MIC_{plate} of benzalkonium chloride from the plate method, and BC7251, a mutant isolated from the liquid method were selected for further analysis. Multi-drug efflux pumps and alternation of membrane component are known mechanisms for benzalkonium chloride resistance in bacteria[10), 11)].

The growth of BC13 and BC7251 in L broth were measured and generation time was calculated (Table 2). The generation time of *P. aeruginosa* PAO1, BC13 and BC7251 were calculated to be 26.6 min, 26.6 min and 27.0 min, respectively. Unexpectedly, generation times of both mutants were almost the same as that of parental *P. aeruginosa* PAO1. Thus, mutations in BC13 and BC7251 may not affect cell growth under our experimental conditions. Cellular shapes of PAO1, BC13 and BC7251 were checked by microscopically with Gram-staining, and differences were not observed in either of them (data not shown).

Table 2. Generation time of *P. aeruginosa* PAO1, BC13 and BC7251

P. aeruginosa	Generation time (min)	
	Average	S. D.
PAO1	26.6	2.3
BC13	26.6	1.0
BC7251	27.0	1.1

Ratio of viable cells between mutants and *P. aeruginosa* PAO1 under co-cultivation. Since the presence of drugs leads to the selection of drug-resistant mutants, we thought that the absence of drugs also led to the selection of the drug sensitive strain. Because drug-resistant mutants use their energy for maintaining their drug-resistant mechanism, that 'waste of energy' might affect the activity of them in the absence of drugs. But nobody tried to confirm the selection of the drug sensitive strain before.

Cell viability between resistance mutants and *P. aeruginosa* PAO1 under co-cultivation in the absence of benzalkonium chloride was measured. Thinking from generation times of BC13 and BC7251, mutants are thought to survive in co-culture with *P. aeruginosa* PAO1. Equal amounts, 10^4 cells, of BC13 and PAO1 were co-cultivated in the absence of benzalkonium chloride, and the ratios of viable cell numbers of the mutants to that of the parental were calculated (Fig. 1(a)). Since the cell density for counting colonies was much lower than that for measuring MIC_{plate}, 512 μg/mL of benzalkonium chloride was enough to prevent the growth of *P. aeruginosa* PAO1 on L-agar plate. As expected from the generation times, the ratio between BC13 and PAO1 was not changed in the logarithmic phase. Surprisingly, the ratio decreased when the bacterial growth reached to the stationary phase. CFU of BC13 and PAO1 were about 4.3×10^7 and 6.1×10^9 at 24 hr. BC7251 showed similar results with BC13 (Fig. 1(b)).

Ratio of the mutant in passage co-culture with *P. aeruginosa* PAO1. Ratio of BC13 drastically decreased in the stationary phase. It is suggested that the resistant cell BC13 was not suitable for growth under low-nutrient conditions. Therefore,

Table 3. Constant rate of death of *P. aeruginosa* PAO1 and BC13

CFU measuring plate	Independent culture		Co-culture
	PAO1	BC13	PAO1 + BC13
L plate	0.062 hr^{-1}	0.067 hr^{-1}	0.063 hr^{-1}
L pate containing BC (512 μg/mL)	-	0.36 hr^{-1}	0.35 hr^{-1}

BC; benzalkonium chloride

the co-culture method with passage was applied for calculating the ratio of BC13 to PAO1 (Fig. 2). The co-cultivated culture of BC13 and PAO1 was calculating the ratio of BC13 to PAO1 (Fig. 2). The co-cultivated culture of BC13 and PAO1 was diluted with the fresh medium when it reached to the stationary phase to supply nutrients, and the cultivation continued. Again, the culture was diluted with the fresh medium upon reaching to the stationary phase. The CFU ratio of BC13 to PAO1 was about 50 % in the logarithmic phase and in the early stationary phase regardless of the passage co-culture. The ratio decreased after cells entered the stationary phase similar to the result shown in Fig. 1(a) (Fig. 2). This means that BC13 can grow as fast as the parental strain with enough nutrients, but the mutant dies much faster than the parent under nutrient-poor conditions. We tested this possibility as shown in the later section.

Comparison of constant rate of death. The constant rates of death in the stationary phase for *P. aeruginosa* PAO1 and BC13 were calculated (Table 3). The rate for PAO1 in the independent culture and that for PAO1 and BC13 in the co-culture were almost the same, 0.062 hr^{-1} and 0.063 hr^{-1}. Also, the rate for BC13 in the independent culture and that for PAO1 and BC13 in the co-culture were almost the same, 0.36 hr^{-1} and 0.35 hr^{-1}, measuring CFU when using L plate containing 512 μg/mL of benzalkonium chloride. Surprisingly, this rate for BC13 in the independent culture was 0.067 hr^{-1}, almost the same as the rate for PAO1, in the independent culture measuring CFU with L plate. Because both constant rates of death were the same, *P. aeruginosa* PAO1 and BC13 were not affected by each other. Some metabolites in BC13 cells might accumulate in the stationary phase and that might cause dropping in the CFU ratio of BC13[25]. The constant rates of death of BC13 calculated when using L plate containing benzalkonium chloride was faster than that calculated when using L plate. There is a possibility that the cause of decrease in

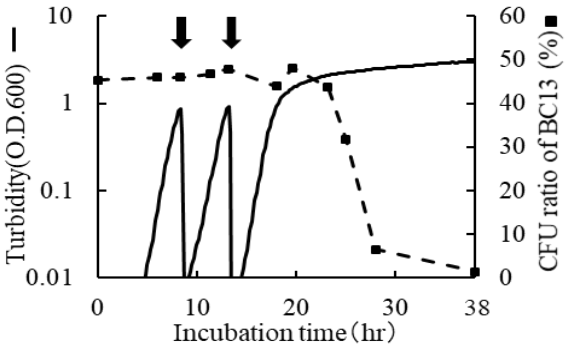

Fig. 2. Ratio of mutant in passage co-culture with *P. aeruginosa* PAO1. 10^4 cells of *P. aeruginosa* PAO1 and BC13 were inoculated in L broth and $O.D._{600}$ was measured. When $O.D._{600}$ reached to 1.0, about 10^4 cells were inoculated in fresh L broth (arrows). CFUs were calculated at each time point.

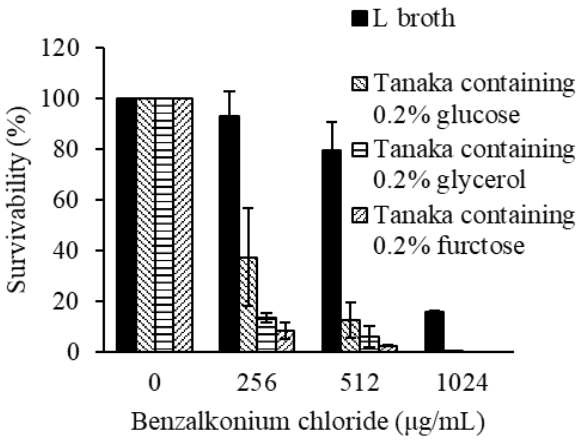

Fig. 3. Survivability of BC13 on the plate. BC13 cells were cultured in L broth and appropriate amounts of cells were plated to agar plates containing benzalkonium chloride and each carbon source. The cell survivability was calculated. Error bars represent standard deviation of three independent experiments.

of BC13 CFU ratio in co-cultivation is not an accumulation of metabolites in BC13 and suggests that poor nutrition prevent showing benzalkonium resistance phenotype.

Effect of nutrient conditions. We hypothesized that the lower amount of nutrition in the stationary phase might affect the survivability of BC13. Glucose, fructose, or glycerol, were chosen as a carbon source for measuring the growth rate. The generation time of BC13 grown in minimum salt Tanaka medium supplemented with 0.2% of glucose, fructose, or glycerol were calculated to be 45.8 min, 71.5 min and 69.9 min, respectively. The generation times grown in these media are much longer than that in L broth. These results mean that glucose is the best carbon source for BC13 among these sugars, and L broth is a better medium than minimum salt Tanaka medium supplemented with any of the three kinds of sugars. Similar generation times were observed with wild type PAO1 (data not shown). BC13 cells were grown and CFU was counted on L-agar plate containing each concentration of benzalkonium chloride (Fig. 3). Viable cell ratio of BC13 in L-broth containing less than or equal to 512 μg/mL of benzalkonium chloride was almost the same. But those in Tanaka medium containing even 256 μg/mL of benzalkonium chloride showed lower ratio compared to that in Tanaka medium without benzalkonium chloride. Decreasing viable cell ratio depends on the concentration of benzalkonium chloride and the kind of carbon sources. The survivability in Tanaka medium containing glucose, the most useful carbon source, was the highest. These results suggest that benzalkonium chloride resistance of BC13 becomes lower when the amount of nutrient is decreased in the medium.

Mechanisms of benzalkonium chloride resistance are not clear in these mutants yet. It seems that nutrition is a key for showing resistance to benzalkonium chloride for these mutants. The further study is needed to clear the correlation of drug resistant mechanisms and nutrition. Poor nutrient condition might make the benzalkonium chloride resistant *P. aeruginosa* difficult to produce enough amounts of multidrug efflux pumps, PL, FNL for showing resistant phenotype[10),11),19-24)]. The selection of the drug-sensitive strain might occur in a usual environment. This is the first report that the susceptible bacteria (parental strains) are selected when resistant bacteria and susceptible bacteria (parental strains) coexist in the absence of drugs. Taken together, these kinds of resistant mutants will disappear by keeping the environment under very low nutritional conditions or, if possible, free from nutrition.

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