Microbial Degradation of Chlorhexidine by Clinical Isolates

MASARU UYEDA, TAEKO SHIOZAKI, TAKAKO ENOKIDA, KEITA WATANABE,
KAORI KOMIYA, KAZUMI YOKOMIZO and KEITAROU SUZUKI

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi,
Kumamoto 862, Japan

(Received February 7, 1996)

Chlorhexidine degradation by clinical isolates such as *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Alcaligenes faecalis*, *Alcaligenes xylosoxidans*, *Serratia marcescens* and *Pseudomonas* sp. strain No. A-3 (as a control organism) was investigated after enrichment with chlorhexidine.

By this enrichment, *P. aeruginosa* (Nos. 1-3), *A. faecalis*, *A. xylosoxidans* (No. 2) and *S. marcescens* IFO 3046 smoothly acquired resistance to a high concentration of chlorhexidine.

Among them, only *S. marcescens* IFO 3046 showed the same degradation pattern as the control organism, which degraded chlorhexidine *via* a modified degradation pathway. The other strains were thought to have a different resistance system from that of *Pseudomonas* sp. strain No. A-3 and *S. marcescens* IFO 3046.

Key words — chlorhexidine ; microbial degradation ; clinical isolates

Introduction

In our laboratories, some bacteria which are able to degrade chlorhexidine (CH) or are resistant to it were isolated from activated sludge about ten years ago. *Pseudomonas* sp. strain No. A-3 (*Ps.* A-3) among these bacteria was able to degrade and utilize CH as a sole nitrogen source for its growth. Purification of CH degradatives has revealed that CH was degraded *via* two pathways (one is a modified degradation pathway and another is a direct degradation pathway) by this strain (Fig. 1).¹⁾

Recently, it has been reported that many microorganisms are resistant to antibiotics²⁾ or disinfectants.³⁾ Therefore, we investigated whether or not clinical isolates could degrade CH. At first, all clinical isolates and *S. marcescens* IFO 3046 were enriched with CH to acquire CH resistance. The CH resistant strains thus obtained were examined regarding CH degradation. The degradation pathway of these clinical isolates was compared to that of *Ps.* A-3. CHDI-B (see Fig. 1) is an

indicator compound in the modified degradation pathway by *Ps.* A-3. In this study, we examined exclusively the formation of CHDI-B by these clinical isolates.

Materials and Methods

Disinfectant — Chlorhexidine gluconate (20% solution) was purchased from Sumitomo Chemical Industries, Ltd. and was used without further purification.

Bacterial Strains — *Ps.* A-3, *Pseudomonas aeruginosa* (3 strains), *Pseudomonas cepacia* (2 strains), *Alcaligenes faecalis*, *Alcaligenes xylosoxidans* (2 strains) and *Serratia marcescens* (2 strains: one is obtained from IFO) were used in this study. All strains except *Ps.* A-3 and *S. marcescens* IFO 3046 were supplied by Kumamoto University Hospital.

Determination of MIC and CH Enrichment

— Minimum inhibitory concentrations (MIC) of CH against each strain were determined as follows: One milliliter volumes of a two-fold strength of brain heart infusion bouillon (BHIB, Nissui Co., Ltd.) and two-fold serial dilutions of

Fig. 1. Degradation Pathway of Chlorhexidine by *Pseudomonas* Sp. Strain No. A-3

CH concentrations were inoculated with 5 μ l of a cell suspension (equivalent to 3 loopfuls/2 ml) of the strains, and the MIC was determined after incubation for 24 h at 37°C. The CH solution of more than 125 μ g/ml made a precipitate with BHIB, so bacterial growth could not be recognized clearly. In this case, 2 loopfuls of each turbid culture were transferred to CH-free BHIB and incubated for 24 h at 37°C.

A CH enrichment culture of each organism was carried out to acquire CH resistance. Two loopfuls of the culture containing the highest concentration of CH in which cellular growth was observed were transferred into a medium containing higher concentrations of CH,⁴⁾ and the culture was incubated for 1 or 2 d at 37°C. This cultivation was repeatedly carried out to generate CH resistance.

Culture Conditions and Preparation of Intact Cells—For preparation of the intact cells of *Ps. A-3* for the microbial degradation of CH, the strain was cultured in a nitrogen-limited growth medium (30 ml) containing K-phosphate buffer (6 mM, pH 7.5), glucose (1 mg/ml), a modified Pridham and Gottlieb trace salt solution ($\text{FeSO}_4 \cdot \text{H}_2\text{O}$ is 0.005 mg/ml and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ and CaCl_2 are 0.0005 mg/ml, respectively), and chlorhexidine (0.1 mg/ml) as a nitrogen source at 28°C on a rotary shaker (180 rpm). After cultivation for 3 d, the cells were harvested by centrifugation (12000 rpm, 10 min) at 5°C and washed with sterilized water. The cells thus obtained were employed as intact cells of *Ps. A-3* in the incubation mixture for CHDI-B formation. The number of intact cells in the cell suspension was determined by colony counting on a bouillon agar plate. The intact cells of each clinical isolate were prepared by almost the same method as *Ps. A-3*.

Incubation Mixture for CHDI-B and CHDI-BR Formation—The intact cells were resuspended with distilled water to give an optical density of 0.2 at 660 nm. The incubation mixture (30 ml) containing the intact cells, K-phosphate buffer (10 mM, pH 7.5), CuSO_4 (0.001 mM), NAD^+ (0.2 mM), pyruvate (0.5 mg/ml), glucose (10 mg/

ml), BHIB powder (1 mg/ml) and CH (100 μ g/ml) was incubated at 37°C in a 100 ml-Erlenmeyer flask for a desired length of time. After incubation, the reaction mixture was adjusted to pH 3 with HCl. Five milliliters of this acidic solution was passed through a Celite column (bed volume: 0.75 ml) and concentrations of CH, CHDI-B and CHDI-BR in the eluate were measured by HPLC. The incubation mixture was determined by the remarkable stimulation of CHDI-B and CHDI-BR formation when pyruvate, NAD^+ and Cu^{2+} ion were added to the incubation mixture.

Measurement of CH, CHDI-B and CHDI-BR—HPLC was done using a jacketed stainless steel analytical column (250 $\times$ 4 mm i.d.) packed with LiChroCARTLi-Chrosorb RP-select B (7 μ m). The mobile phase was $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ (7:3, v/v, pH 3.7) containing 5 mM 1-heptanesulfonic acid. The concentrations of CH, CHDI-B and CHDI-BR were measured by HPLC on an apparatus equipped with a UV detector set at 254 nm.⁵⁾ The injection volume for this study was 5 μ l of sample solution. The quantification of each compound was achieved by the use of a calibration curve for each.

Results and Discussion

Acquisition of CH Resistance

Table I shows the process of acquisition of CH resistance by *P. aeruginosa* No. 3. The initial MIC of CH against *P. aeruginosa* No. 3 was determined to be 7.8 μ g/ml. The bacteria which were cultured in 3.9 μ g/ml of CH were transferred into media containing the higher concentrations of CH. The strain became resistant to CH, which acquired resistance to 1000 μ g/ml as MIC after 5 d. To attain CH resistant strains, the same method was used for the other strains.

Table II indicates the acquisition degree of CH resistance by various strains. The acquisition degree of CH resistance was calculated by dividing the MIC against a resistant strain by that against prototroph. For example, the CH resistant *S. marcescens* IFO 3046 in Table II had acquired 1282-fold higher

TABLE I. Process of Acquisition of CH Resistance by *P. aeruginosa* No.3

MIC ^{a)} ($\mu\text{g/ml}$)	Incubation time (d) ^{b)}				
	0	1	2	3	5
10000	—	—	—	—	—
5000	—	—	—	—	—
2500	—	—	—	—	—
1250	—	—	—	—	—
1000	—	—	—	—	—
500	—	—	—	—	+
250	—	—	—	+	
125	—	—	+		
62.5	—	+			
31.3	—	+			
15.6	—	+			
7.8	—	+			
3.9	+				
2.0	+				
0	+				

a) Higher CH concn. above 125 $\mu\text{g/ml}$ made precipitate with BHIB, so each culture was transferred to CH-free BHIB and MIC was determined after incubation for 24h (see text). b) Time to acquire CH resistance. +, growth observed; —, growth not observed.

Fig. 2. Diagram to Show Acquisition of CH Resistance

—□—, *P. aeruginosa* No. 1; —◇—, *P. aeruginosa* No. 2; —○—, *P. aeruginosa* No. 3; —△—, *P. cepacia* No. 1; —▢—, *P. cepacia* No. 2; —◊—, *A. xylosoxidans* No. 1; —⊕—, *A. xylosoxidans* No. 2; —▽—, *S. marcescens* No. 1; —■—, *S. marcescens* IFO 3046; —◆—, *A. faecalis*; —*—, *Ps. A-3*.

TABLE II. Acquisition Degree of CH Resistance by Clinical Isolates

Strain	<i>P. aeruginosa</i>			<i>P. cepacia</i>		<i>A. faecalis</i>	<i>A. xylosoxidans</i>		<i>S. marcescens</i>		<i>Ps. A-3</i>
	No.1	No.2	No.3	No.1	No.2		No.1	No.2	No.1	IFO 3046	
MIC against prototroph ($\mu\text{g/ml}$)	15.6	15.6	7.8	2.0	31.3	62.5	7.8	31.3	1250	3.9	2.0
MIC against resistant strain ($\mu\text{g/ml}$)	500	5000	1000	31.3	250	5000	125	5000	5000	5000	10000
Acquisition degree of CH resistance ^{a)}	32	320	128	16	8	80	16	160	4	1282	5000

a) The value was calculated by dividing the MIC against the resistant strain by that against the prototroph.

resistance than the prototroph strain. The acquisition degree of CH resistance of *A. xylosoxidans* and *S. marcescens* were particularly different from each other. The acquisition degree of CH resistance of *A. xylosoxidans* No. 2 (value 160) was higher than that of the strain No. 1 (value 16). The difference was caused by MIC values against the resistant strains. On the other hand, the acquisition degree of *S. marcescens* No. 1 was very low because the prototroph itself already showed very high MIC (the clinical isolate had already become resistant to CH). These

results were in good accordance with the report that the susceptibility of Gram-negative bacteria to commonly used disinfectants varied strain to strain as indicated by Gotou *et al.*⁶⁾ The time courses of various isolates in acquiring CH resistance are shown in Fig. 2. Some isolates such as *A. faecalis*, *P. aeruginosa* Nos. 2 and 3 and *S. marcescens* (also *Ps. A-3*) acquired resistance to about 1000 $\mu\text{g/ml}$ as MIC in only a few d.

Comparison of Various Strains on CH Degradability

The strains that acquired higher CH

Fig. 3. Time Course of CH Degradation by Various Clinical Isolates

0.1% BHIB was supplemented to each incubation mixture except *Ps. A-3*. —●—, CH; —△—, CHDI-B; —○—, CHDI-B+CHDI-BR; —■—, cells/ml.

resistance, *P. aeruginosa* No. 2, *P. cepacia* No. 2, *A. faecalis*, *A. xylosoxidans* No. 2 and *S. marcescens* IFO 3046 were examined for CH degradability in the next step. *Ps.* A-3 was used as a positive control (Fig. 3).

Ps. A-3 was observed to form CH degradative products with a decrease in CH. Among these strains, *S. marcescens* IFO 3046 appeared to form CH modified degradative products such as CHDI-B and CHDI-BR. As shown in Fig. 4, the HPLC chart of CH degradatives by *S. marcescens* showed almost the same pattern as that by *Ps.* A-3 under the same condition. Therefore, we inferred that the strain had the same CH modified degradative pathway as *Ps.* A-3. On the other hand, the other strains formed a trace amount or no CH modified degradative products, although BHIB or peptone was supplemented for the incubation mixture to improve their growth (Fig. 3). These strains were thought to have a different resistance system than that of *Ps.* A-3 as a CH-degradation pathway. *S. marcescens* No. 1 had already become resistant to CH in the hospital, therefore, the clinical isolate might be alive under an envi-

ronment where the higher concentration of CH exists. *A. faecalis*, *P. aeruginosa* No. 2, *A. xylosoxidans*, *S. marcescens* IFO 3046 and *Ps.* A-3 acquired CH-resistance quickly and the degree of resistance of these isolates was very high (MIC above 5000 $\mu\text{g/ml}$). If they settled in the hospital, they would become CH-resistant strains and may cause an infection in the hospital.

The resistance mechanism to CH by gram-negative bacteria has been investigated by many researchers. That mechanism was not yet fully understood. However, as one of the mechanisms, we reported previously that the lipid content in the cells of CH-resistant *S. marcescens* were higher than those of the prototroph cells; therefore, the difference in the lipid content was thought to be significant in relation to structural differences of the cell envelope of CH resistant strains.⁴⁾ In this paper, we reported that *S. marcescens* also has a CH modified degradative pathway similar to that of *Ps.* A-3. *S. marcescens* has two mechanisms for CH-resistance, therefore, the strain may acquire resistance quickly, highly and stably.

Fig. 4. Comparison of HPLC Chromatograms

Column, LiChrosorb RP-select B (7 μm , 250 $\times$ 4 mm i.d.); flow rate, 0.8 ml/min; detection, OD 254 nm; mobile phase, $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ (7 : 3, v/v, pH 3.7) containing 5 mM 1-heptanesulfonic acid.

Acknowledgments We thank Prof. Nobuhisa Yamane of the Medical School of Kumamoto

University for the supply of clinical isolates.

References

- 1) Y. Kido, H. Kodama, F. Uraki, M. Uyeda, M. Tsuruoka and M. Shibata, *Eiseikagaku*, **34**, 10 (1988) ; *idem*, *ibid.*, **34**, 319 (1988) ; H. Kodama, T. Hashimoto, M. Tsuruoka, Y. Kido, M. Uyeda and M. Shibata, *ibid.*, **34**, 408 (1988) ; Y. Kido, H. Kodama, F. Uraki, M. Uyeda, M. Tsuruoka and M. Shibata, *ibid.*, **34**, 97 (1988) ; Y. Kido, H. Kodama, Y. Isohama, M. Uyeda and M. Shibata, *ibid.*, **36**, 139 (1990) ; M. Uyeda, T. Shiosaki, K. Yokomizo, K. Suzuki, F. Uraki, T. Tanaka and Y. Kido, *Jpn. J. Toxicol. Environ. Health*, **42**, 121 (1996).
- 2) B.R. Lyon and R. Skurray, *Genetic Basis Microbiol. Rev.*, **51**, 88 (1987) ; H. Yasuda, Y. Ajiki, T. Koga, H. Kawada and T. Yokota, *Antimicrob. Agents Chemother.*, **37**, 1749 (1993) ; K. Hiramatsu, *Bioscience and Industry*, **52**, 11 (1994).
- 3) H.N. Prince, W.S. Nonemaker, R.C. Norgard and D.L. Prince, *J. Pharmaceut. Sci.*, **67**, 1629 (1978) ; G. Nicoletti, V. Boghossian, F. Gurevitch, R. Borland and P. Morgenroth, *J. Hosp. Infect.*, **23**, 87 (1993) ; T. Shiraishi and Y. Nakagawa, *Postgrad. Med. J.*, **69**, (Suppl. 3), 70 (1993) ; P.A. Gandhi, A.D. Sawant, L.A. Wilson and D.G. Ahearn, *Appl. Environ. Microbiol.*, **59**, 183 (1993).
- 4) M. Uyeda, M. Shibata, T. Hitoshi and A. Miura, *Chemotherapy*, **29**, 733 (1981) ; M. Uyeda, M. Sakata, M. Shibata and T. Hitoshi, *ibid.*, **30**, 286 (1982).
- 5) C.E. Huston, P. Wainwright and M. Cooke, *J. Chromatogr.*, **237**, 457 (1982).
- 6) S. Gotou, Y. Kaneko and K. Kawasaki, *Rinsho Byori*, **25**, 684 (1977).