Original Article

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Compounds in a particular production lot of tryptic soy broth inhibit *Staphylococcus aureus* cell growth

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Summary ***Staphylococcus aureus* Newman strain and several methicillin-resistant *S. aureus* (MRSA) clinical isolates were grown on agar plates prepared with conventional lots of tryptic soy broth (TSB). Cell growth of these strains was inhibited on agar plates containing TSB of a particular product lot (lot A), whereas the cell growth of *S. aureus* RN4220 strain and several other MRSA clinical isolates was not inhibited. The cell growth of a strain of *S. epidermidis* was also inhibited on agar plates containing TSB of lot A, whereas the cell growth of *Bacillus subtilis*, *Lactococcus lactis*, *Klebsiella pneumonia*, *Salmonella enterica*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Escherichia coli* was not inhibited. Although cell growth of the Newman strain was inhibited on agar plates containing TSB of lot A that was autoclaved in stainless steel or glass containers, cell growth inhibition was not observed when the medium was autoclaved in polypropylene containers. Compounds that inhibited the cell growth of the Newman strain were extracted from a polypropylene tube that was preincubated with liquid medium prepared from TSB of lot A. These findings suggest that polypropylene-binding compounds in TSB of lot A inhibited the cell growth of *S. aureus* Newman strain, some MRSA clinical isolates, and *S. epidermidis*.**

Keywords: MRSA, polypropylene-binding compounds, growth inhibition

1. Introduction

Staphylococcus aureus infects immunocompromised patients and causes opportunistic diseases such as sepsis and meningitis (*1*). At present, half of the *Staphylococcus aureus* strains isolated from clinics in Japan are resistant to methicillin, a beta-lactam antibiotic. Methicillin-resistant *S. aureus* (MRSA) clinical isolates are resistant to various other antibiotics as well, making it difficult to adequately treat patients infected with MRSA (*2*). To overcome MRSA infection, a comprehensive understanding of the mechanisms of drug resistance and pathogenicity based on genetic and biochemical analyses is necessary. Consistent MRSA culture techniques are essential for these analyses.

Tryptic soy broth (TSB) is routinely used for culturing *S. aureus* clinical isolates. We observed that the Newman strain, a standard strain of *S. aureus*, did not form colonies on agar plates prepared with TSB of a particular product lot (lot A), whereas the RN4220 strain, another standard strain of *S. aureus*, did form colonies on the agar plates prepared with TSB of lot A. Here we describe that polypropylene-binding compounds in TSB of lot A inhibited the cell growth of certain *S. aureus* strains.

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2. Materials and Methods

2.1. *Reagents and containers*

Tryptic soy broth (TSB) was purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Product lots of TSB (lot 1291840 [lot A] and lot 1137750 [lot B]) were used. Agar was purchased from Nacalai Tesque (Kyoto, Japan). Polypropylene tubes (50-mL conical tubes) were purchased from Corning Inc. (Corning, NY, USA). Glass conical flasks, glass test tubes, and glass bottles were purchased from Iwaki (Osaka, Japan). A stainless steel kettle (3 L) was purchased from Bestco (Osaka, Japan).

2.2. *Preparation of media*

TSB medium were prepared according to the manufacturer's protocol. Specifically, 30 g TSB powder was dissolved in 1 L water. For preparation of the agar medium, 15 g agar was added to the TSB medium, and the mixture was autoclaved at 121°C for 15 min in the stainless steel kettle, a conical flask, or a polypropylene tube. After autoclaving, the agar medium was poured into 9-cm diameter plastic plates (AS ONE Corporation, Osaka, Japan), cooled, and solidified. In the experiment in which pieces of polypropylene were added to the TSB medium, polypropylene tubes were cut with scissors into 1.5-cm square pieces, and 0.26 g of the pieces were added per 1 mL TSB.

2.3. *Bacteria and growth conditions*

The bacterial strains used in this study are shown in Table 1. In the case of liquid culture, overnight cultures were diluted to 1:100 in TSB liquid medium. The cultures were incubated at 37°C and the turbidities at 600 nm were measured using a spectrophotometer (Shimadzu Corporation). In the case of bacterial culture on agar plates, overnight cultures were appropriately diluted with 0.9% NaCl, then 100 µL of the dilution was spread on a TSB agar plate or streaked on a TSB agar plate using a loop, and the plates were incubated at 37°C.

2.4. *Preparation of acetone extracts of polypropylene-binding compounds in TSB*

Liquid medium prepared with TSB of lot A was autoclaved in a glass bottle, transferred to a polypropylene tube, and incubated for 16 h at room temperature. The liquid was removed by decantation from the tube. Acetone (10 mL) was added and the mixture vortexed. Five milliliters of the acetone extract was transferred to a glass test tube and the acetone was removed by evaporation at 60°C. Liquid media prepared with TSB of another lot (2.5 mL) was added to the dried test tube and used for the experiment.

Table 1. Bacterial strains used in this study

Species	Strains	References
Staphylococcus aureus	Newman	(*6*)
	RN4220	(*7*)
	MRSA3	(*8,9*)
	MRSA4	(*8,9*)
	MRSA5	(*9*)
	MRSA6	(*8,9*)
	MRSA8	(*8,9*)
	MRSA9	(*8,9*)
	MRSA11	(*8,9*)
	MRSA12	(*8,9*)
Staphylococcus epidermidis	ATCC12228	(*10,11*)
Bacillus subtilis	168	(*12*)
Lactococcus lactis	MG1363	(*13*)
	11/19-B1	This study
Enterococcus mundtii	EM1s	This study
Klebsiella pneumonia	ATCC10031	(*14*)
Salmonella typhimurium	ATCC14028s	(*15*)
Serratia marcescens	2170	(*16*)
Pseudomonas aeruginosa	PAO1	(*17*)
Escherichia coli	JM109	(*18*)
	O-157 Sakai	(*19,20*)

3. Results

3.1. *Cell growth inhibition of S. aureus Newman strain on agar plates prepared with TSB of a particular lot*

TSB medium is frequently used to culture *S. aureus*. We observed that *S. aureus* Newman strain did not grow on medium prepared with TSB of a particular lot (lot A). Although Newman strain on agar plates prepared with TSB of another lot did form colonies, it did not form colonies on agar plates prepared with TSB of lot A (Figure 1A). The RN4220 (RN) strain formed colonies on agar plates containing TSB of both lots (Figure 1A). Thus, the cell growth of *S. aureus* RN4220 strain was not inhibited by TSB of lot A.

3.2. *Cell growth inhibition of MRSA strains on agar plates containing TSB of lot A*

We then examined whether MRSA strains grew on agar media prepared with TSB of lot A. All seven strains examined grew on agar plates prepared with TSB of lot B. The cell growth of five strains (MRSA4, MRSA5, MRSA8, MRSA11, and MRSA12) on agar plates prepared with TSB of lot A was markedly inhibited compared to that on plates with TSB of lot B (Figure 1B). Two MRSA strains (MRSA5 and MRSA11), as well as the Newman strain, did not grow at all on agar plates prepared with TSB of lot A. Therefore, cell growth of the majority of MRSA clinical isolates was inhibited in TSB of lot A.

3.3. *Cell growth of other species of bacteria on agar plates containing TSB of lot A*

We compared the cell growth of 11 strains (9 species) of bacteria on agar media prepared with TSB of lot A or lot B. *S. epidermidis* ATCC12228 formed colonies on agar plates prepared with TSB of lot B, it did not form colonies on agar plates prepared with TSB of lot A (Figure 1C). On the other hand, cell growth of *Bacillus subtilis* 168 strain, *Lactococcus lactis* MG1363 strain, *Lactococcus lactis* 11/19-B1 strain, *Enterococcus mundtii* EM1s strain, *Klebsiella pneumoniae* ATCC10031 strain, *Salmonella enterica* ATCC14028s strain, *Serratia marcescens* 2170 strain, *Pseudomonas aeruginosa* PAO1 strain, *Escherichia coli* JM109 strain, and *E. coli* O-157 Sakai strain did not differ between agar media prepared with TSB of lot A or lot B (Figure

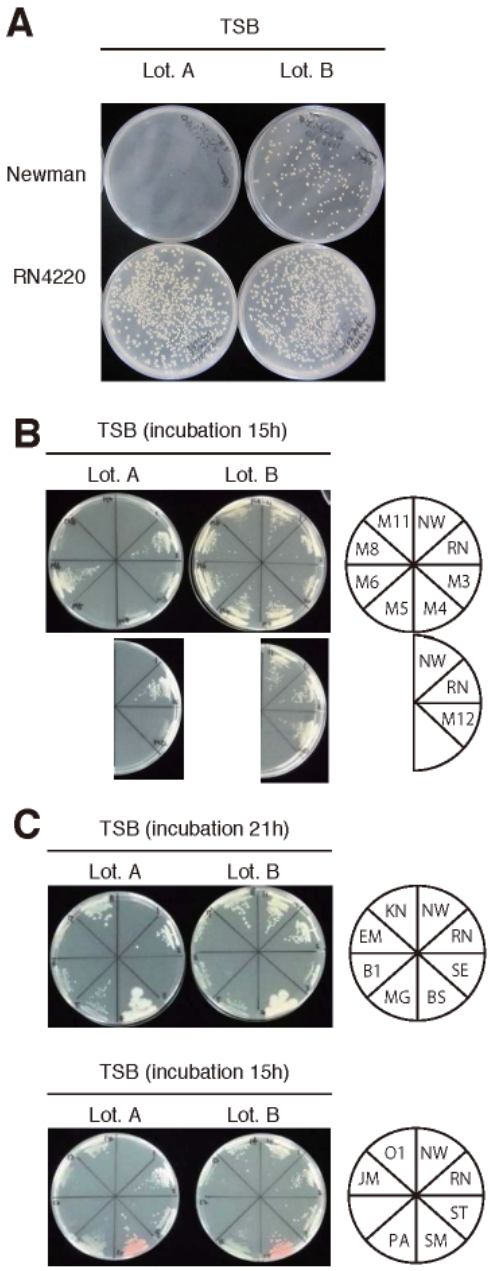

Figure 1. Cell growth inhibition of the Newman strain and MRSA strains on agar plates prepared with TSB of lot A. **(A)** Colony formation of the *S. aureus* Newman strain and the RN4220 strain on agar plate prepared with TSB of lot A or lot B was examined. The Newman strain and RN4220 strain were cultured overnight at 37°C in liquid medium prepared with TSB of lot B, the bacterial suspensions were diluted 1 × 10^6-fold, and 100-µL aliquots were spread on the agar plates, followed by incubation at 37°C for 24 h. **(B)** Growth of the *S. aureus* Newman strain, the RN4220 strain, and several MRSA strains. Fully grown bacterial cultures were diluted 1 × 10^2-fold with saline, and streaked by a loop on agar plates prepared with TSB of lot A and lot B followed by incubation at 37°C for 15 h. Strains are shown on the right side of the photographs. NW, Newman; RN, RN4220; M3, MRSA3; M4, MRSA4; M5, MRSA5; M6, MRSA6; M8, MRSA8; M11, MRSA11; M12, MRSA12. **(C)** Growth of various bacterial strains on agar plates prepared with TSB of lot A or lot B. Bacteria were cultured overnight at 37°C or at 30°C (MG1363 strain and 11/19-B1 strain), and streaked on agar plates by a loop. Plates were incubated at 37°C. Bacterial strains are shown on the right side of the photographs. NW, Newman; RN, RN4220; SE, ATCC12228; BS, 168; MG, MG1363; B1, 11/19-B1; EM, EM1s; KN, ATCC10031; ST, ATCC14028s; SM, 2170; PA, PAO1; JM, JM109; O1, O-157 Sakai.

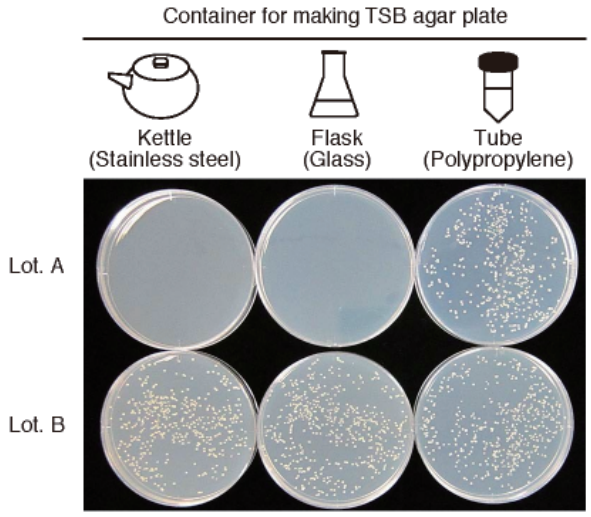

Figure 2. Effect of containers used to autoclave TSB-agar on cell growth of the Newman strain. The Newman strain was cultured at 37°C overnight in a liquid medium prepared with TSB of lot B. The culture was diluted 1 × 10^6-fold, spread on agar plates prepared with TSB of lot A or lot B autoclaved in different containers, and incubated at 37°C for 18 h.

1C). These findings suggest that TSB of lot A contained compounds that specifically inhibit the cell growth of certain strains of *S. aureus* and *S. epidermidis*.

3.4. *Effect of different containers used for autoclaving agar on the growth inhibition of Newman strain by TSB lot A*

Growth inhibition of the Newman strain by TSB of lot A was observed when the agar was autoclaved in a stainless steel container (kettle) or a glass container (conical flask), but not when it was autoclaved in polypropylene tubes (Figure 2). We considered the possibility that substances that eluted from the surface of the polypropylene tubes inactivated the compounds that inhibited the cell growth of the Newman strain. To test this, we autoclaved water for medium preparation in polypropylene tubes, and then prepared agar plates with TSB of lot A using water from the glass container. We examined whether the Newman strain would grow on the agar plates. The Newman strain did not form colonies on the agar plates (Figure 3B, plate 2), thereby excluding the possibility that substances that eluted from polypropylene during the autoclaving inhibited the cell growth inhibiting activity of TSB of lot A.

We then examined the possibility that the compound responsible for cell growth inhibition in TSB of lot A was absorbed on the surface of the polypropylene tubes during autoclaving. To test this, we compared the cell growth of Newman strain on agar plates prepared with TSB of lot A with or without pieces of polypropylene tubes in a glass container. The Newman strain did not form colonies on the agar plates prepared by autoclaving without the addition of pieces of polypropylene tubes (Figure 3B, plate 3). In contrast,

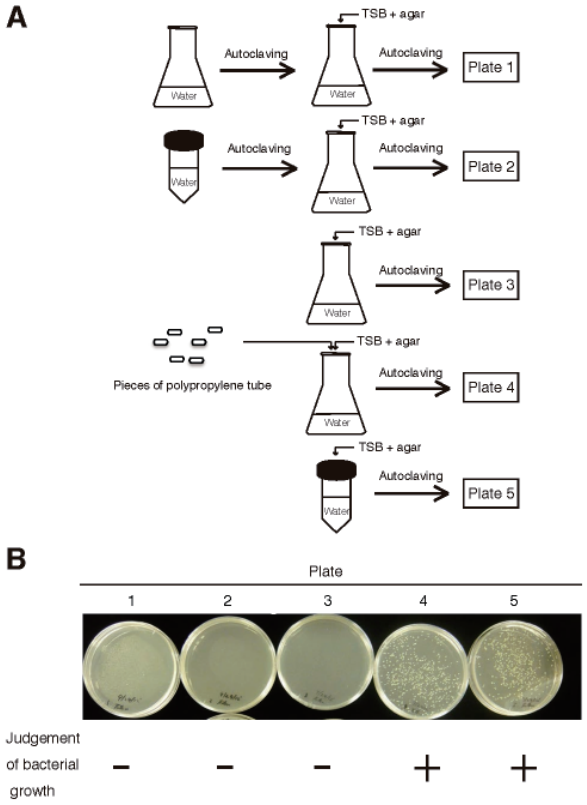

Figure 3. Effects of different preparations of TSB (lot A)-agar on growth of the Newman strain. TSB (lot A)-agar was autoclaved in glass containers (plates 1-4) or in a polypropylene tube (plate 5). **(A)** Schematic protocols for the preparation of TSB (lot A)–agar are shown. **(B)** Plate 1, Water was autoclaved in a glass flask, followed by repeat autoclaving with TSB (lot A) and agar in a glass flask. Plate 2, Water was autoclaved in a polypropylene tube, followed by autoclaving in a glass flask. Plate 3, TSB (lot A) and agar in water were autoclaved in a glass flask (control). Plate 4, TSB (lot A) and agar in water were autoclaved in a glass flask with pieces of polypropylene. Plate 5, TSB (lot A) and agar in water were autoclaved in a polypropylene tube. After gelation of the agar medium in Petri dishes, overnight cultures of the Newman strain were diluted 1×10^6-fold, and 100-µL aliquots were spread on agar plates, followed by incubation at 37°C for 18 h.

the Newman strain formed colonies on agar plates prepared by autoclaving with the addition of pieces of polypropylene tubes (Figure 3B, plate 4). These findings suggest that the compound responsible for cell growth inhibition of the Newman strain in TSB of lot A binds the surface of polypropylene tubes during autoclaving.

3.5. *Isolation of the polypropylene-binding compounds from TSB of lot A that inhibit cell growth of the Newman strain*

Cell growth inhibition of the Newman strain by TSB of lot A was observed not only on agar plates but also in a liquid medium in glass test tubes (Figure 4, left). The cell growth inhibitory effect against the Newman strain in the liquid medium with TSB of lot A was not observed, however, in polypropylene tubes (Figure 4, right). This finding suggests that the cell growth

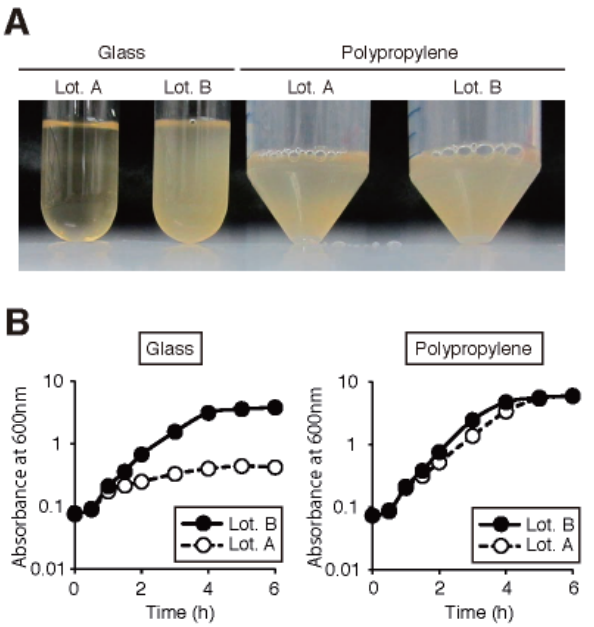

Figure 4. Effect of culture tubes on growth of the Newman strain in a liquid medium prepared with TSB of lot A. The TSB liquid medium (5 mL) from lot A or lot B was added to glass containers or polypropylene tubes. Aliquots (50 µL) of overnight culture of the *S. aureus* Newman strain were added and incubated at 37°C. Turbidity (absorbance at 600 nm) was measured. **(A)** Culture solutions after 3.5 h. **(B)** Left, culture in a glass container; Right, culture in a polypropylene tube. ○ , a TSB liquid medium of lot A. ●, a TSB liquid medium of lot B.

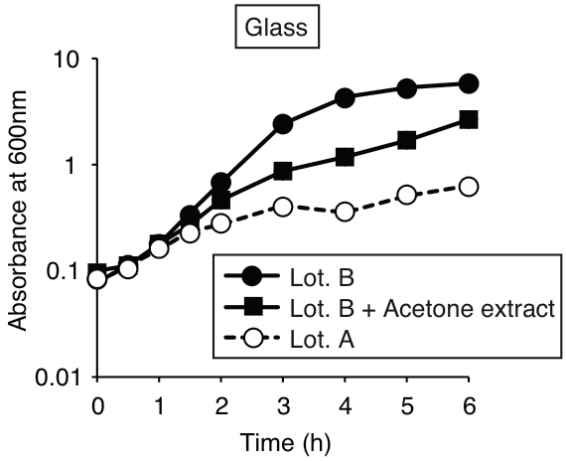

Figure 5. Inhibitory effect of polypropylene-binding substances from TSB of lot A on cell growth of the Newman strain. TSB medium from lot A was incubated overnight at room temperature in a polypropylene tube, followed by extraction with acetone. The acetone extract was added to the liquid medium of TSB (lot B). Fully grown cultures of the Newman strain were added, and incubated at 37°C. Aliquots were sampled and turbidity (absorbance at 600 nm) was measured. ○ , TSB of lot A; ■, TSB of lot B with the acetone extract; ●, TSB of lot B.

inhibitor present in TSB of lot A binds polypropylene not only under autoclave conditions, but also under a cultivation temperature of 37°C.

We attempted to isolate the polypropylene-binding compounds inhibiting the cell growth of the Newman

strain from TSB of lot A. The liquid medium of TSB of lot A prepared by autoclaving in a glass bottle was transferred to a polypropylene tube, and allowed to stand at room temperature. Then, the TSB liquid medium was decanted, and material adsorbed on the surface of the tube was extracted with acetone. Acetone was removed by evaporation, and a liquid medium prepared with TSB of lot B was added, followed by incubation of the Newman strain. Consequently, the medium containing the acetone extract fraction inhibited the cell growth of the Newman strain (Figure 5). This finding suggests that the compounds that inhibit the cell growth of the Newman strain are present in TSB of lot A, and that this compound binds to polypropylene.

4. Discussion

In the present study, we demonstrated that TSB of lot A contained compounds that inhibit the cell growth of the *S. aureus* Newman strain and a majority of MRSA strains. TSB contains tryptone and a soy extract (*3*). Compounds in TSB of lot A that inhibit the cell growth of the Newman strain probably originated from one of these natural products. Therefore, the amount of the cell growth inhibitory compounds in TSB probably varies greatly between different lots of TSB. Proper management of product lots in laboratories is crucial, especially given the importance of *S. aureus* as a clinical pathogen.

Although compounds in TSB of lot A severely inhibited cell growth of the Newman strain and a majority of MRSA strains, they did not inhibit cell growth of the RN4220 strain or several other MRSA strains. Therefore, the cell growth inhibiting effect of the compounds is specific to particular strains of *S. aureus*. Because the whole genome sequences of Newman and RN4220 have been determined (*4,5*) by comparative genomic analysis and recombinant technology of genes by phage transduction, genetic approaches will be applicable for dissecting the mechanism of selective cell growth inhibition against the Newman strain. The capacity of cell growth inhibiting compounds to absorb to polypropylene resins may allow us to purify and determine the structure of the compounds. Additional genetic and biochemical studies are necessary to further elucidate the compounds responsible for the inhibition of cell growth.

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