Curr Microbiol (2008) 57:542–546
DOI 10.1007/s00284-008-9239-0

Antibacterial Effects of Green Tea Polyphenols on Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*

Yun-Seok Cho · Neal L. Schiller · Kye-Heon Oh

Received: 7 August 2007 / Accepted: 24 July 2008 / Published online: 10 September 2008

Abstract The antibacterial effects of tea polyphenols (TPP) extracted from Korean green tea (*Camellia sinensis*) against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated. Characterization of the minimal inhibitory concentration (MIC) of oxacillin for 30 *S. aureus* strains isolated from patients treated with oxacillin identified 13 strains with an oxacillin MIC $\geq$ 4 μg/mL as methicillin-resistant *Staphylococcus aureus* (MRSA) (range: 8 to 512 μg/mL), while 17 strains were methicillin-susceptible *Staphylococcus aureus* (MSSA) (range: 0.25–0.5 μg/mL). The MICs of TPP ranged from 50 to 180 μg/mL for both the MSSA and the MRSA strains. The MICs of oxacillin for each of the 13 MRSA strains were reduced between 8- and 128-fold when these strains were coincubated with sub-MIC ($\leq$0.5$\times$ MIC) levels of TPP, demonstrating that the combination of TPP plus oxacillin was synergistic for all of the clinical MRSA isolates. Two-dimensional polyacrylamide gel electrophoresis identified 14 extracellular proteins of MRSA-13 down-regulated and 3 proteins up-regulated by exposure to TPP. These studies demonstrate that TPP can differentially stimulate the expression of various proteins in these bacteria and synergize the bactericidal activity of oxacillin for MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an increasingly important pathogen in both hospital and community settings [15]. Although vancomycin is generally the first choice for treatment of MRSA infections, vancomycin-resistant *S. aureus* has been reported in several countries, including the United States [20]. Therefore, alternative chemotherapeutic agents are needed to be developed and employed to control MRSA.

Previous studies have reported that green tea polyphenols (TPP), collectively termed catechins including epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG), have bactericidal activity against various Gram-positive and Gram-negative bacteria [3, 13, 23]. Recently, EGCG was reported to act synergistically with various β-lactam antibiotics against MRSA [10–12, 21, 22]. However, there are few or no data available on proteomic analysis of MRSA exposed to TPP.

Proteomic analyses have increased our understanding of chemical-mediated stress proteins in bacteria such as *Acinetobacter* [8], *Burkholderia* [5], *Pseudomonas* [4, 17], and *Escherichia* [16]. Recently, we investigated the antibacterial effects of TPP on *E. coli* as well as proteomic changes induced by exposure to TPP [6]. In the present study, we examined the antibacterial and synergistic activities of TPP on 13 clinical isolates of MRSA, and comparative proteomic analysis was performed to elucidate the proteins expressed in MRSA exposed to TPP.

Materials and Methods

Bacterial Strains and Culture Conditions Thirty clinical isolates of *S. aureus* were obtained from Soonchunhyang University Hospital (Chung-Nam, Korea). ATCC 29213, a methicillin-susceptible *S. aureus* (MSSA) strain, and ATCC 43300, a MRSA strain, were used as controls.

Y.-S. Cho · K.-H. Oh (✉)
Department of Biotechnology, Soonchunhyang University, P.O. Box 97, Asan, Chung-Nam 336-600, Republic of Korea
e-mail: kyeheon@sch.ac.kr

N. L. Schiller · K.-H. Oh
Division of Biomedical Sciences, University of California, Riverside, CA 92521, USA

Cultures were normally grown for 18–20 h at 37°C in either tryptic soy broth (TSB; Difco, Detroit, MI, USA) or Muller-Hinton (MH) broth (Difco).

Antibiotics and TPP Oxacillin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TPP (purity, > 97%) extracted from the leaf of *Camellia sinensis* L. were obtained from COSIS Co., Ltd. (Chung-Nam, Korea). Stock dilutions were prepared in 0.15 mM H_3PO_4 to avoid oxidation. HPLC chromatographic analysis (data not shown) of the catechins contained in TPP identified five main compounds: ~50% EGCG, ~24% ECG, ~16% GCG, ~2% EC, and ~1% EGC.

Determination of the Minimal Inhibitory Concentration (MIC) of Oxacillin and TPP The MIC of oxacillin, TPP, or combined oxacillin-TPP for clinical isolates of *S. aureus* was determined by the agar dilution method in MH agar plates, supplemented with 2% NaCl for susceptibility tests for oxacillin and combined oxacillin-TPP. All of the cultures were resuspended in 0.9% saline to a density equivalent to a 0.5 McFarland standard and then diluted 1:10 in sterile MH broth. MH agar plates containing two-fold serial dilutions of antibacterial agents were inoculated with the final suspensions using a multichannel inoculator (Eppendorf Co., Hamburg, Germany) which delivered approximately 10^4 cfu per spot and incubated at 37°C for 18–20 h. The MIC was determined as the lowest drug concentration that inhibited growth, as recommended by the Clinical and Laboratory Standards Institute [7].

Time-Kill Assays Log-phase cultures of MRSA were harvested, washed with phosphate buffer, and resuspended in 4 mL of MH broth with 2% NaCl to a final concentration of ~5×10^6 cfu/mL, in the presence or absence of oxacillin, TPP, or a combination of oxacillin and TPP. Viable cells were counted every 4 h for 0–24 h.

Two-Dimensional Gel Electrophoresis (2-DE) Cultures of MRSA-13 grown overnight in TSB medium in the presence or absence of 60 μg/mL TPP (0.5× MIC) were centrifuged at 8,500 *g* for 30 min at 4°C to remove intact cells. Proteins in the culture supernatants were precipitated by the addition of 100% trichloroacetic acid to a final concentration of 10%. After overnight incubation at 4°C, the precipitate was centrifuged at 10,000 *g* for 30 min at 4°C and washed with ice-cold 100% acetone [1, 2]. 2-DE was performed according to previously described methods [9]. Isoelectric focusing (IEF) was performed with the Ettan IPG phor System (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's guidelines. Proteins were dissolved in 350 μL of rehydration buffer (8 M urea, 2% CHAPS [w/v], and 0.5% IPG buffer, pH 3–10). Focusing was performed in seven steps (300 V for 30 min, 500 V for 30 min, 1000 V for 1 h, 3000 V for 1.5 h, 5000 V for 1.5 h, gradient 8000 V for 2 h, and, finally, 8000 V for 1 h). The second dimension was run on a 12% SDS-polyacrylamide gel using a PROTEAN II xi electrophoresis kit (Bio-Rad, Hercules, CA, USA).

In-Gel Digestion and MALDI-TOF/MS Protein spots were excised from silver-stained 2-DE gels, and peptides digested with trypsin according to previously described methods [9, 14]. Digested peptides were redissolved using 0.1% trifluoroacetic acid (TFA). To reduce chemical background noise for MALDI-TOF, sample peptides were desalted using Zip-tip C_{18} pipette tips (Millipore, Bedford, MA, USA). Peptides were eluted onto a MALDI plate using a CHCA matrix solution (10 mg/mL CHCA in 0.5% TFA/50% acetonitrile, 1:1). All mass spectra were acquired in the reflection mode using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Afterward, proteins were identified from MALDI fingerprint data using MASCOT (http://www.matrixscience.com) against the NCBI database [19].

Results and Discussion

MICs of Oxacillin and TPP Thirty strains of *S. aureus* were isolated from patients treated with oxacillin, and their MICs for oxacillin were determined. Strains were considered methicillin-susceptible if they had an oxacillin MIC < 4 μg/mL, while strains with an MIC ≥ 4 μg/mL were identified as methicillin-resistant. Seventeen isolates were methicillin-susceptible (oxacillin MIC range: 0.25–0.5 μg/mL) (data not shown), while 13 isolates (see Fig. 1) were MRSA, with MICs ranging from 8 to 512 μg/mL. The MIC of TPP for each of these 30 strains ranged from 50 to 180 μg/mL, with similar values obtained for MRSA and MSSA strains (data not shown). EGCG, the main constituent (~50%) in our TPP preparation, is likely to be most responsible for TPP's antibacterial activity against *S. aureus*, since previous studies have suggested that EGCG has bactericidal activity [18].

The synergistic effect of combining TPP with oxacillin for each MRSA strain is shown in Fig. 1. The combination of TPP with oxacillin dramatically lowered the MIC of oxacillin for each isolate. Using TPP concentrations ≤0.5× MIC in combination with oxacillin (see Fig. 1), the MIC for oxacillin dropped 8-fold for two MRSA strains (3 and 6), 16-fold for three strains (12, 44, and 52), 32-fold for three strains (9, 17, and 35), 64 fold for three strains (13, 29, and 31), and 128-fold for two strains (19 and 78). These results demonstrate that TPP was synergistic with oxacillin for each of the 13 MRSA strains tested.

Time-Kill Assays The bactericidal effect of the combined use of oxacillin and TPP for each MRSA strain was evaluated using time-kill curves, and the results shown with MRSA-13 (Fig. 2) are representative. Among the 13 clinically isolated MRSA strains, MRSA-13 showed an

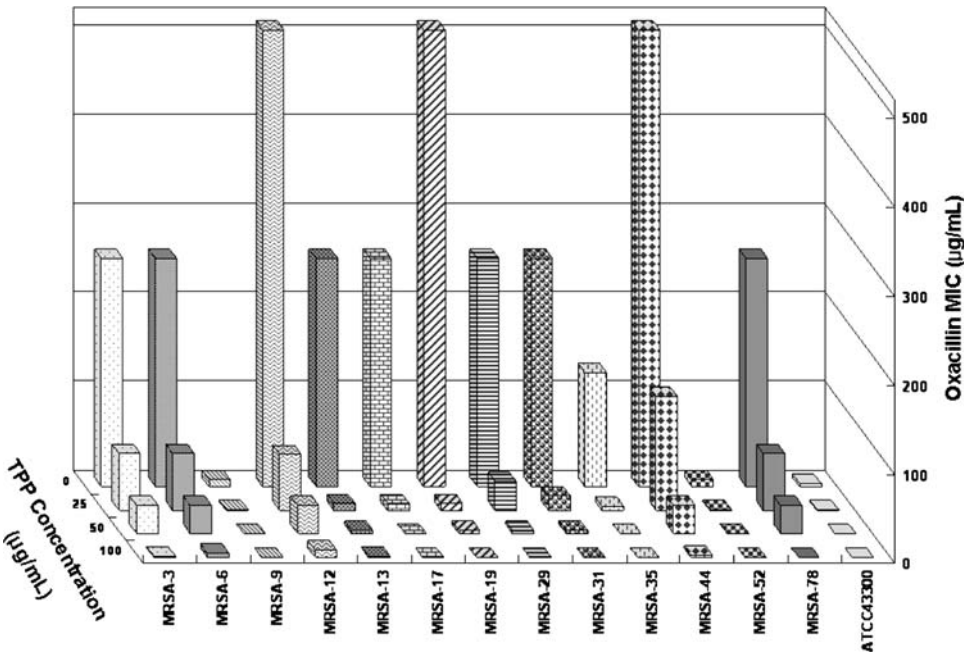

Fig. 1 Synergistic effect of TPP on the oxacillin MICs of 13 clinical isolates of MRSA and ATCC 43300

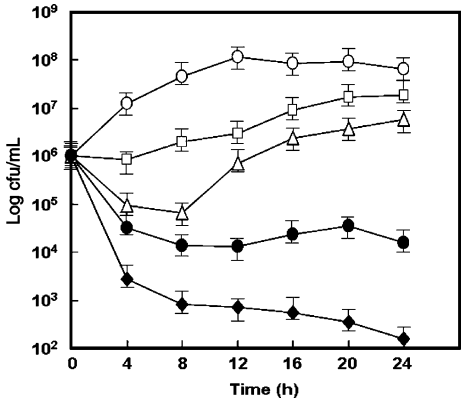

Fig. 2 Time-kill curves for MRSA-13: ○, no addition; □, 0.5× MIC of oxacillin (256 μg/mL); △, 0.5× MIC of TPP (60 μg/mL); ●, 0.25× MIC of oxacillin (64 μg/mL) + 0.25× MIC of TPP (30 μg/mL); ◆, 0.25× MIC of oxacillin (64 μg/mL) + 0.5× MIC of TPP (60 μg/mL). Data shown represent the mean ± SD based on triplicate studies

excellent synergic effect by decreasing the oxacillin MIC when combined with a low concentration of TPP (≤25 μg/mL). In the absence of oxacillin or TPP, MRSA-13 grew well. In the presence of 0.5× MIC of oxacillin (128 μg/mL), growth was initially delayed but reached comparable levels after 24 h, whereas in the presence of 0.5× MIC of TPP (60 μg/mL), there was an initial drop in colony forming units for the first 8 h, followed by growth in bacterial numbers which approached control values at 24 h. The synergistic effect of TPP was observed using 0.25× MIC of TPP (30 μg/mL) and 0.25× MIC of oxacillin (64 μg/mL), which resulted in a drop from 10^6 to $\sim 10^4$ cfu/mL. When 0.5× MIC of TPP (60 μg/mL) was added to 0.25× MIC of oxacillin (64 μg/mL), almost all of the bacteria were killed within 24 h of incubation. Similar results were obtained with each of the clinical MRSA strains (data not shown).

Hu et al. reported that the combination of a β-lactam and EGCG showed potent synergy against MRSA [10, 11]. Zhao et al. reported that EGCG synergizes the activity of β-lactam against MRSA because of interference with the integrity of the cell wall through direct binding to peptidoglycan [24]. On the other hand, Ikigai et al. reported that catechins primarily act on and damage bacterial membranes [13]. In a recent study, we reported that TPP-treated *E. coli* had unique changes in saturated and unsaturated fatty acids in their cell membranes, further supporting the impact of TPP on membranes [6]. These studies illustrate that TPP may have multiple mechanisms of antibacterial activity, which can synergize the bactericidal activity of various antibiotics.

Changes in Extracellular Proteins in MRSA Exposed to TPP As shown in Fig. 2, while 0.5× MIC of TPP was not by itself bactericidal, this amount of TPP did significantly reduce the amount of oxacillin needed to kill this strain. To examine the effect of 0.5× MIC on MRSA-13, this strain was grown overnight in TSB medium in the presence or absence of 60 μg/mL TPP (0.5× MIC). The ability of TPP to either induce or suppress the expression of various extracellular proteins was determined by examining the resulting culture supernatants using 2-DE and IEF from pH 3 to pH 10 (see Fig. 3). Seventeen proteins were successfully identified and characterized by MALDI-TOF/MS as reported in Table 1.

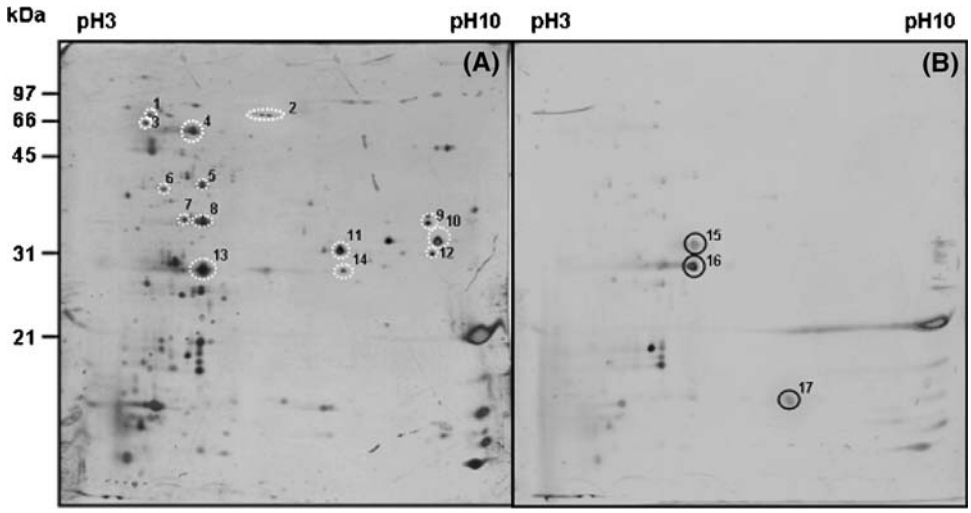

Fig. 3 Two-dimensional electrophoresis pattern of extracellular proteins of MRSA-13 grown in TSB without TPP (**a**) or in the presence of 0.5× MIC (60 μg/mL) of TPP (**b**). Numbers associated with MALDI-MS identify spots listed in Table 1

Three proteins were up-regulated by exposure to TPP. Serine protease (SspA), which plays an important role in the growth and survival of *S. aureus*, was significantly increased in MRSA-13 exposed to TPP. Both peptidoglycan hydrolase (LytM), involved in degradation of the peptidoglycan, and immunodominant antigen A (IsaA), associated with active bacterial growth, were increased in lower amounts.

On the other hand, 14 proteins expressed under normal physiological conditions were markedly decreased or not expressed when exposed to TPP. These down-regulated proteins included the chaperone proteins (DnaK and GroEL) and other proteins (e.g., surface protein, capsular polysaccharide synthesis enzyme Cap5G, leukocidin subunit precursor, α-hemolysin precursor [Hla], *β*-hemolysin [Hlb], and exotoxin15 [Set15]) connected with cellular pathogenicity mechanisms (Table 1). All these proteins were dramatically decreased in MRSA exposed to TPP. These results are reminiscent of those reported by Bernardo et al. [2], who found that sub-MIC levels of linezolid reduced the expression of various *S. aureus* virulence factors. While the synergistic mechanism of TPP for enhanced killing of MRSA by oxacillin is still unclear, these data demonstrate that sub-MIC exposure to TPP dramatically affects the expression of several important proteins in MRSA.

Table 1 Extracellular proteins identified by MALDI-TOF/MS fingerprinting

Spot no.	Putative annotation	Score	GenBank accession no.	Sequence coverage (%)	TPP change[a]
1	DnaK protein	78	BAB57742.1	39	↓↓
2	Similar to autolysin precursor (Aly)	97	BAB00017.4	33	↓↓
3	GroEL protein	73	BAB58191.1	17	↓↓
4	Surface protein	93	BAB56596.1	26	↓↓
5	Capsular polysaccharide synthesis enzyme Cap5G	95	BAB56317.1	18	↓↓
6	Enolase (Eno)	64	BAB56938.1	21	↓↓
7	Fructose-bisphosphate aldolase homologue	77	BAB58768.1	27	↓↓
8	Translation elongationfactor TS	65	BAB57419.1	31	↓↓
9	Leukocidin subunit precursor	75	BAB58166.1	38	↓↓
10	α-Hemolysin precursor (Hla)	140	BAB57325.1	54	↓↓
11	*β*-Hemolysin	101	BAB56257.1	27	↓↓
12	Glycerophosphoryl diester-phosphodiesterase (GlpQ)	89	BAB57121.1	32	↓↓
13	Secretory antigen SsaA homology	133	BAB56827	20	↓↓
14	Exotoxin15 (Set15)	105	BAB56595.1	42	↓↓
15	Peptidoglycan hydrolase (LytM)	128	BAB56438.1	18	↑
16	Serine protease (SspA)	87	BAB57210.1	28	↑↑
17	Immunodominant antigen A (IsaA)	92	BAB58731.1	24	↑

[a] ↓↓, strong down-regulation by tea polyphenols (TPP); ↑, weak up-regulation by TPP; ↑↑, strong up-regulation by TPP

Although green tea is a natural product which is quite popular to drink, this and other studies demonstrate that tea extracts have potent antibacterial effects in vivo and in vitro against a wide range of pathogenic bacteria, even antibiotic-resistant bacteria. Further studies to elucidate how sub-MIC levels of TPP synergize the activity of antibiotics on pathogenic bacteria are in progress. Ultimately, the use of TPP in synergistic combination with antibiotics may become an effective alternative treatment strategy for antibiotic resistant bacteria, including MRSA.

Acknowledgment This research was accomplished while K. H. Oh was on sabbatical leave (2006–2007) from Soonchunhyang University.

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