Correlation between penicillin-binding protein 2 mutations and carbapenem resistance in Escherichia coli

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It is well known that carbapenem-resistant mutations in penicillin-binding proteins (PBPs) are not observed in most Gram-negative bacteria under either clinical or experimental conditions. To understand the mechanisms involved in carbapenem resistance, this study constructed a mutS- and tolC-deficient Escherichia coli strain, which was expected to have elevated mutation frequencies and to lack drug efflux. Using this mutant, carbapenem-resistant strains with target mutations were successfully and efficiently isolated. The mutations T547I/A, M574I and G601D were identified in the PBP2 gene. Meropenem (MEPM)-resistant strains with the PBP2 T547I mutation showed fourfold increased resistance to 1-β-methyl-substituted carbapenems, such as doripenem, MEPM and biapenem, but not to non-substituted carbapenems such as imipenem and panipenem and other β-lactams. In addition, resistance resulting from the G601D mutation was limited to MEPM, whilst the M574I mutation conferred resistance to MEPM, imipenem and panipenem. This is the first report, to the best of our knowledge, that E. coli also has a carbapenem-resistance mechanism as a result of PBP2 mutations, and it provides insight into the resistance profiles of PBP2 mutations to carbapenems with and without the 1-β-methyl group.

INTRODUCTION

Carbapenems have the broadest spectrum of activity in the β-lactam class and show in vitro bactericidal activity against both Gram-positive and Gram-negative bacteria. Among the carbapenems, new-generation carbapenems such as meropenem (MEPM), biapenem (BIPM) and doripenem (DRPM) have the methyl group at position 1, in contrast with the old-generation carbapenems such as imipenem (IPM) and panipenem (PAPM), which do not (Papp-Wallace et al., 2011). This methyl group prevents the compounds from degradation by dehydropeptidase-1 (Perry & Ibbotson, 2002; Tsuji et al., 1998).

All carbapenems and other β-lactams exhibit bactericidal activity based on the inhibition of penicillin-binding proteins (PBPs) (Zhanel et al., 2007). β-Lactams bind to PBPs and inhibit cell-wall synthesis of bacteria by disturbing the completion of transpeptidation of peptidoglycan strands. Each β-lactam shows specific affinities with respective PBPs. For example, in Escherichia coli, these carbapenems bind preferentially to PBP2 (Davies et al., 2008; Jones et al., 2004; Livermore et al., 2003; Yang et al., 1995).

It is reported that Gram-negative bacteria gain resistance to antibiotics mainly by acquiring mutations in drug targets, by overexpression of efflux pumps or by producing enzymes that inactivate the antibiotics. In fact, high resistance to quinolones is known to be caused by a combination of all these mechanisms. In contrast, in the case of β-lactams, mutation of the target (PBPs) is less common in Gram-negative bacteria except for a few species such as Haemophilus influenzae and Neisseria gonorrhoeae (Zapun et al., 2008).

The Dam-dependent mismatch repair system (MMR) is the most important DNA repair mechanism in bacteria. In E. coli, the mismatch repair pathway is initiated by the MutS, MutL and MutH proteins (Horst et al., 1999). MutS

Abbreviations: AMP, ampicillin; AZT, aztreonam; BIPM, biapenem; CAZ, ceftazidime; CPFX, ciprofloxacin; CXM, cefloxime; DRPM, doripenem; IPM, imipenem; LFX, levofloxacin; MEPM, meropenem; MMR, mismatch repair system; PAPM, panipenem; PBP, penicillin-binding protein; PIPC, piperacillin; QTDR, quinolone-resistance determining region.
recognizes mismatched base pairs as well as insertions or deletions of one to four nucleotides. MutL forms a complex with MutS that activates the MutH endonuclease. Inactivation of the MMR is known to increase the mutation rate (Chopra et al., 2003). Indeed, MMR-deficient, multidrug-resistant Gram-negative bacteria have been reported in clinical situations (Boe et al., 2000; Denamur et al., 2002; Maciá et al., 2005).

The tolC gene encodes a multifunctional outer-membrane channel that efflux protein toxins and antibacterial drugs as part of an efflux pump, AcrAB-ToIC, in E. coli (Koronakis et al., 2004). Previous studies have shown that E. coli strains lacking tolC are highly susceptible to multiple antibiotics compared with those lacking acrAB (Sulavik et al., 2001; Zgurskaya et al., 2011). Therefore, we speculated that disruption of the tolC gene could produce a resistant phenotype that is not mediated by drug efflux.

In this study, we generated a mutS- and tolC-deficient E. coli strain with elevated mutation frequencies and with no efflux resistance pathways against β-lactams including carbapenems. Using this strain, we identified PBP2 mutations that conferred resistance in carbapenems, strongly suggesting that this strain is a powerful tool for inducing mutations in the targets of carbapenems and other types of antibiotic. To our knowledge, this is the first report of a carbapenem-resistant E. coli strain with mutations in the target molecule. In addition, we showed a correlation between the susceptibility of PBP2 mutants and the structure of the carbapenem.

**METHODS**

**Strains, media and plasmids.** Efflux pump-deficient E. coli MG1655 (ΔtolC) (Masuda & Church, 2002) was used as the host strain for the mutS deletion in this study. DH5α competent cells (Toyobo) were used for the amplification of plasmids. These strains were grown at 37°C in Luria–Bertani broth (LBB), Luria–Bertani agar (LBA), Mueller–Hinton broth, cation-adjusted Mueller–Hinton II broth and Mueller–Hinton agar (MHA). All reagents were purchased from Becton Dickinson. When required, ampicillin (AMP; Sigma-Aldrich) at 100 µg ml⁻¹ and kanamycin (Nacalai Tesque) at 5, 25 or 50 µg ml⁻¹ were added to the medium to select transformants. Plasmids pKD13 and pKD46 (Datsenko & Wanner, 2000) were provided by Yale University (CT, USA). pKD13 is a template plasmid containing a kanamycin resistance gene and pKD46 is an arabinose-inducible λ-red recombinase expression plasmid.

**Antibiotics and susceptibility tests.** Ciprofloxacin (CPFX), levofloxacin (LVFX), PAPM and BIPM were synthesized at Daichi Sankyo. DRPM, MEMP and IPM were extracted from commercial preparations (DRPM, Finibax, Shionogi; MEMP, Sumitomo Pharmaceuticals; IPM; Tienam, Banyu Pharmaceutical). Penicillin V (Sigma-Aldrich), piperacillin (PIPC; Sigma-Aldrich), AMP, cefuroxime (CXM; Wako Pure Chemical Industries), cefazidime (CAZ; Sigma-Aldrich) and aztreonam (AIZ; Sigma-Aldrich) were purchased. The compounds were dissolved in sterilized water. MICs were determined from three independent experiments, according to a standard method (NCCLS, 2003). Susceptible, intermediate and resistant breakpoints of IPM, MEMP and DRPM were ≤1, 2 and ≥4 µg ml⁻¹, respectively.

**Construction of a mutS-deficient ΔtolC strain (ΔmutSΔtolC).** The protocol used to produce the mutant strain is illustrated in Fig. 1. In brief, a one-step deletion of the E. coli mutS gene was performed using λ-red recombinase (Datsenko & Wanner, 2000). The mutS-coding region was replaced with a kanamycin resistance gene from pKD46 by homologous recombination via the recombinase, which was produced from pKD46 in the presence of arabinose. The DNA fragment for recombination was prepared as follows: PCR using AccuPrime Pfx DNA Polymerase (Life Technologies) was carried out with pKD46 as a template and the two primers, Ec mutS KO Fcat/kan (H1) (5'-AAAAACCATCACCCCCCATTTAATACGGAAGCG-GACATAAACCCTATTGGTACGGGAGCTGCTTCT-3') and Ec mutS KO Rkan (H2) (5'-AGTTGTGTTAATTTCCGGATAGCGAAAAGCATATCGGGAAATTTAATTCGAGGATCGTGGACC-3'). The underlining indicates the sequences 60 bp upstream and 429 bp downstream of the kanamycin resistance gene in pKD13, respectively. The sequences were upstream (H1) and downstream (H2) of the mutS gene, respectively. Amplified products were purified and treated with DpnI to degrade the template. Fifty microlitres of ΔtolC competent cells was mixed with 60 ng pKD46 in a 0.1 cm Gene Pulser cuvette (Bio-Rad); this mixture was then electroporated using a pulse controller (Bio-Rad) at 25 μF, 2.5 kV and 200 Ω. After the pulse, 1.0 ml LBB was added and the cells were incubated at 30°C for 1 h. The cells were then plated on an LBA plate containing AMP and were grown at 30°C for 24 h. Each colony obtained was grown in LBB containing 0.08% arabinose and AMP at 37°C until an optical density at 600 nm of 0.6 was reached, and competent cells of the ΔtolCpKD46 strain were prepared as described above. Subsequently, the purified PCR product was transformed into the ΔtolCpKD46 strain and transformants were selected by culturing on an LBA plate containing 5 µg kanamycin ml⁻¹. Deletion of mutS was confirmed by PCR using PrimeSTAR MAX Premix (Takara Bio) with Ec mutS F1 primer (5'-CCCGGAACGACATCAAGAACTC-3') and Ec mutS R1 primer (5'-GGTGATAGCATCGATGTTTACC-3') (p1 and p2 in Fig. 1, respectively).

**Selection of antibiotic-resistant mutant and determination of resistant frequency.** The ΔtolC and ΔmutSΔtolC strains were grown in LBB at 37°C overnight and the cultures were then concentrated 10- and 100-fold by centrifugation. Aliquots of 40 µl of the bacterial suspension were spread onto MHA plates containing multiples of the MICs for CPFX, IPM and MEMP, or no antibiotic (the MICs of the ΔtolC and ΔmutSΔtolC strains were 0.004, 0.03 and 0.25 µg ml⁻¹, respectively). The plates containing antibiotics were incubated at 35°C for 48 h, whilst the antibiotic-free plates were incubated at 35°C for 24 h. The frequency of resistance was calculated as the ratio of the number of resistant colonies at 48 h to the number of cells plated.

**PCR amplification of the quinolone-resistance determining region (QRDR) of gyrA, mrdA (ppb2) and ftsI (ppb3), and DNA sequence analysis.** The presence of mutations in the QRDRs of the gyrA genes in CPFX-resistant clones was investigated by sequence analysis of the gyrA QRDR amplified by PCR using PrimeSTAR MAX Premix (Takara Bio), ECGA1 primer (5'-ATGAGCCGACCTTGCGAAGAGAATTTACAC-3') and ECGA4 primer (5'-AAGAACCGTGTC-AACCAGCGTTG-3'). The presence of mutations in the mrdA and ftsI genes in IPM- and MEMP-resistant mutants was also investigated. The mrdA and ftsI genes were amplified by PCR using PrimeSTAR MAX Premix with primers Ec mrdA F1 (5'-CTGCGAGAAGTTGCGT-3') and Ec mrdA R1 (5'-GATCGCCGATTGCGTGGAC-3') for mrdA and primers Ec ftsI F1 (5'-CTGCGAGAAGTTGCGTGGAC-3') and Ec ftsI R1 (5'-AGTTGTCGCGATTGCGTGGAC-3') for ftsI. DNA sequencing of the PCR products was carried out with an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies) in accordance with the manufacturer’s instructions.
RESULTS

Generation of a mutS-deficient ΔtolC strain (ΔmutSΔtolC)

As the first step in investigating the effect of target mutations on carbapenem resistance in E. coli, we obtained a strain (ΔmutSΔtolC) lacking tolC, the multifunctional outer-membrane channel, and mutS, a component of the most important DNA repair mechanisms. As described in Methods, the mutS gene was deleted in a ΔtolC strain (Masuda & Church, 2002) (Fig. 1a). We confirmed the deletion of mutS in the clones obtained by PCR, by detecting 2.8 kb mutS-specific and 1.6 kb kanamycin resistance gene products (Fig. 1b).

Frequency of resistance in the ΔmutSΔtolC strain

It has been reported that the frequency of CPFX-/rifampicin-resistant strains is higher in E. coli mutS-deficient strains than in the wild-type (Chopra et al., 2003; Morero et al., 2011). Therefore, we tested the frequency of resistance of the ΔmutSΔtolC strain against CPFX. Table 1 shows the results for the ΔtolC and ΔmutSΔtolC strains. The ΔmutSΔtolC strain had higher frequencies of CPFX resistance at 2×, 4×, 8× and 16× MIC than the ΔtolC strain. These results indicated that the mutS gene has a significant effect on CPFX resistance. We then analysed the QRDR region in the DNA gyrase A subunit, GyrA, which is known to be the primary target of CPFX resistance. As expected, the frequencies of gyrA mutations in the CPFX-resistant ΔmutSΔtolC strain were higher than in the ΔtolC strain (Table 1).

Next, we measured resistance frequencies to carbapenems using IPM and MEPM (Table 2). The ΔmutSΔtolC strain had higher frequencies of IPM resistance at 2× and 4× MIC, and of MEPM resistance at 2× MIC than the ΔtolC strain, as is the case for CPFX. As it is known that IPM binds preferentially to PBP2, and MEPM binds most strongly to PBP2 followed by PBP3, we analysed the sequences of the full-length PBP2 and PBP3. Interestingly, almost all MEPM-resistant clones had mutations in the pBP2 gene and no mutation was found in the pBP3 gene (Table 2). In addition, the frequencies of pBP2 mutation in the MEPM-resistant ΔmutSΔtolC strain were also higher than those in the ΔtolC strain (Table 2). In the case of IPM, one PBP2 mutant was obtained from the ΔmutSΔtolC strain (Table 2).

Correlation between PBP2 mutation and antibiotic resistance against carbapenems and other β-lactams

Next, we measured the MICs of carbapenems and β-lactams for the PBP2 mutants obtained in this study (Table 3). The fold changes in the MICs for the PBP2 mutants of the ΔmutSΔtolC strain are illustrated graphically in Fig. 2. Both MEPM-resistant mutants (MR1 and MR2) with a T547I mutation in PBP2 showed more than a fourfold increase in resistance to DRPM, MEPM and BIPM, but not to IPM or PAPM (Fig. 2). However, the mutation at

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**Results**

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### Table 1. Frequency of selection and mutation analysis of CPFX-resistant mutants in *E. coli ΔtolC* and Δ*mutSΔtolC* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg ml⁻¹)</th>
<th>Clone no.</th>
<th>Frequency of selection of resistant mutants</th>
<th>Total no. analysed mutants</th>
<th>Target mutation/total mutants</th>
<th>No. strains with amino acid mutations in the Gyra QRDR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2×MIC</td>
<td>4×MIC</td>
<td>8×MIC</td>
<td>16×MIC</td>
</tr>
<tr>
<td>ΔtolC</td>
<td>0.004</td>
<td>1</td>
<td>2.0×10⁻⁷</td>
<td>1.4×10⁻⁷</td>
<td>&lt;2.3×10⁻⁹</td>
<td>&lt;2.3×10⁻⁹</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>2.0×10⁻⁶</td>
<td>1.4×10⁻⁷</td>
<td>3.4×10⁻⁸</td>
<td>&lt;3.4×10⁻⁹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>6.7×10⁻⁶</td>
<td>1.2×10⁻⁷</td>
<td>&lt;3.0×10⁻⁹</td>
<td>&lt;3.0×10⁻⁹</td>
</tr>
<tr>
<td>ΔmutSΔtolC</td>
<td>0.004</td>
<td>1</td>
<td>4.8×10⁻⁶</td>
<td>2.8×10⁻⁷</td>
<td>3.4×10⁻⁷</td>
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<td>2</td>
<td>NC</td>
<td>3.6×10⁻⁶</td>
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<td>3</td>
<td>NC</td>
<td>4.1×10⁻⁶</td>
<td>4.9×10⁻⁷</td>
<td>1.1×10⁻⁷</td>
</tr>
</tbody>
</table>

NC: Not countable.
Table 2. Frequency of selection and mutation analysis of IPM- and MEPM-resistant mutants in *E. coli ΔtolC* and *ΔmutSΔtolC* strains

NC, Not countable; NT, not tested.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain</th>
<th>MIC (µg ml⁻¹)</th>
<th>Clone no.</th>
<th>Frequency of selection of resistant mutants</th>
<th>Total no. analysed mutants</th>
<th>Target mutation/total mutants</th>
<th>No. strains with amino acid mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2×MIC</td>
<td>4×MIC</td>
<td>8×MIC</td>
<td>16×MIC</td>
<td>T547A</td>
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<tr>
<td>MEPM</td>
<td>ΔtolC</td>
<td>0.03</td>
<td>1</td>
<td>$&lt;9.1 \times 10^{-10}$</td>
<td>$&lt;9.1 \times 10^{-10}$</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ΔmutSΔtolC</td>
<td>0.03</td>
<td>2</td>
<td>$&lt;1.3 \times 10^{-9}$</td>
<td>$&lt;1.3 \times 10^{-9}$</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>$&lt;1.3 \times 10^{-9}$</td>
<td>$&lt;1.3 \times 10^{-9}$</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>IPM</td>
<td>ΔtolC</td>
<td>0.25</td>
<td>1</td>
<td>$5.3 \times 10^{-6}$</td>
<td>$&lt;2.8 \times 10^{-9}$</td>
<td>$&lt;2.8 \times 10^{-9}$</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>ΔmutSΔtolC</td>
<td>0.25</td>
<td>2</td>
<td>$1.0 \times 10^{-5}$</td>
<td>$&lt;3.4 \times 10^{-9}$</td>
<td>$&lt;3.4 \times 10^{-9}$</td>
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<td>NT</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>2</td>
<td>NC</td>
<td>$2.3 \times 10^{-7}$</td>
<td>$&lt;3.1 \times 10^{-9}$</td>
<td>$&lt;3.1 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>NC</td>
<td>$7.9 \times 10^{-8}$</td>
<td>$&lt;3.6 \times 10^{-9}$</td>
<td>$&lt;3.6 \times 10^{-9}$</td>
</tr>
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</table>
appears to be a powerful tool to investigate the correlation between target mutations and the resistance profile of the mutants without the influence of drug efflux. For example, this strategy will be applicable to the discovery of targets of new antibacterial compounds.

In *E. coli*, MMR is initiated by the MutS, MutL and MutH proteins. MutS recognizes mismatched base pairs, as well as insertions or deletions of one to four nucleotides. Interestingly, in the \( \Delta \text{mutS} \Delta \text{tolC} \) strain, we observed only two kinds of mutation based on the mismatched base pairs A–G and C–T, even though several groups have reported that recognition potencies of MutS against A–G and C–T substitutions were weaker than those for other mismatched base pairs (Horst *et al.*, 1999). Our results implied that any mutations can be obtained using the \( \Delta \text{mutS} \Delta \text{tolC} \) strain, regardless of the preference for mismatch mutations of MutS.

By using the \( \Delta \text{mutS} \Delta \text{tolC} \) strain, we isolated, for the first time, four mutants with single mutations of T547I, T547A, M574I and G601D in PBP2 in *E. coli*. The penicillin-binding domain of PBP2 is a monofunctional transpeptidase involved in peptidoglycan metabolism. This domain harbours three specific catalytic motifs: SXXK, (S/Y)XN and (K/H)(S/T)G. Position 547 is next to the third catalytic (K/H)(S/T)G motif. Several mutations close to this motif in PBPs are known to be associated with resistance to β-lactams in other bacterial species. For example, the T547A substitution is analogous to the PBP2x T550A mutation, which contributes to resistance in *Streptococcus pneumoniae*, although it is reported that there is no analogous

### Table 3. MICs for the PBP2 mutants

ATCC 25922 is the *E. coli* control strain and MG1655 is the parental strain.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ATCC 25922</th>
<th>MG1655</th>
<th>ΔtolC</th>
<th>ΔmutSΔtolC</th>
<th>MR1 (T547I)</th>
<th>MR2 (T547A)</th>
<th>MR3 (T547A)</th>
<th>MR7 (T547A)</th>
<th>MR9 (G601D)</th>
<th>IRE35 (M574I)</th>
</tr>
</thead>
<tbody>
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<td>LVFX</td>
<td>0.015</td>
<td>0.03</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
<td>0.008</td>
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<tr>
<td>IPM</td>
<td>0.12</td>
<td>0.12</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>PAPM</td>
<td>0.12</td>
<td>0.12</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MEPM</td>
<td>0.015</td>
<td>0.015</td>
<td>0.03</td>
<td>0.03</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>BIPM</td>
<td>0.03</td>
<td>0.06</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.12</td>
<td>0.25</td>
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</tr>
<tr>
<td>DRPM</td>
<td>0.03</td>
<td>0.03</td>
<td>0.06</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin V</td>
<td>&gt;64</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
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<td>16</td>
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</tr>
<tr>
<td>PIPC</td>
<td>8</td>
<td>4</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
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### Fig. 2. Fold changes in MICs for PBP2 mutants compared with those for the ΔmutSΔtolC strain.
T547 mutation in *H. influenzae* or *N. gonorrhoeae* (Asahi *et al*., 1999). In contrast, there are no such reports for the other two substitutions, M574I and G601D. Thus, these mutations will provide new clues to understanding the interaction and resistance mechanisms between carbapenems and PBP2. Our study also revealed PBP2 to be the primary target of IPM and MEPM, as suggested by a PBP-binding study in *E. coli* (Livermore *et al*., 2003).

Using PBP2 mutants, we examined the susceptibility of these strains against various β-lactams including carbapenems. We found that only the efficacies of 1-β-methyl carbapenems were affected by the substitutions of T547I and T547A (Table 3, Fig. 2). The methyl group is reported to give resistance to degradation by dehydropeptidase-1; however, these results are the first data showing that the group has an influence on the resistance resulting from PBP2 mutations. According to the co-crystal structure of PBP2x from *S. pneumoniae* and BIM, the side chain of T550 forms hydrogen bonds with the oxygen atoms at the third position in the ring of BIPM (Yamada *et al*., 2008). In *E. coli*, dependency on this hydrogen bond might be strong for 1-β-methyl carbapenems, because the susceptibilities to PBP2 mutants were decreased. In addition, the PBP2 T547I/A mutants did not show resistance to many other β-lactams. By the same token, the analogous T550 substitution in PBP2x confers resistance to cephalosporins but not to penicillin (Grebe & Hakenbeck, 1996). These data indicate that the correlation between PBP2 mutation and the degree of susceptibility to β-lactams is different among bacteria, although more studies with other bacteria will be necessary to draw a firm conclusion.

This substitution in PBP2x was identified in laboratory as well as in clinical isolates in *S. pneumoniae* (Asahi *et al*., 1999; Grebe & Hakenbeck, 1996). Although there are no reports related to the T547I/A mutation in *E. coli* in clinical isolates, hypermutators have been reported in several bacteria. Therefore, a breakout of PBP2 mutants might arise as a consequence of overuse of 1-β-methyl carbapenems. Taken together, 1-β-methyl carbapenems such as IPM and PAPM could have a low risk for the occurrence of PBP2 mutants. The antibacterial activity against these mutations should be considered in the step of derivatization.

In this study, generating a mutS and tolC double-mutant *E. coli* strain allowed us to demonstrate the existence of resistant PBP2 mutations against carbapenems for the first time. We also showed a correlation between the sensitivity to PBP2 mutants and the structure of carbapenems. Our study strongly suggests that this double-mutant strain is a powerful tool for primary target research using several antibacterial compounds. We also believe that this study could provide a clue to explaining why there are no clinical isolates with PBP mutations in a number of bacteria.

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**REFERENCES**


