

1 The Role of TEM-1 β -lactamase in the Predominance of Ampicillin-Sulbactam-Nonsusceptible

2 *Escherichia coli* in Japan

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18 **ABSTRACT (243 words)**

19 We investigated the epidemiology and resistance mechanisms of
20 ampicillin-sulbactam-nonsusceptible *Escherichia coli*, focusing on the role of TEM-1 β -lactamase.
21 We collected all non-duplicate *E. coli* clinical isolates at 10 Japanese hospitals during December
22 2014 and examined their antimicrobial susceptibility, β -lactamases, TEM-1 transferability, TEM-1
23 β -lactamase activity, outer membrane protein profile, membrane permeability, and clonal genotypes.
24 Among the 329 isolates, 95 were ampicillin-sulbactam nonsusceptible. Of these
25 ampicillin-sulbactam-nonsusceptible isolates, β -lactamases conferring resistance to sulbactam such
26 as AmpC were present in 33%. Hyperproduction of sulbactam-susceptible β -lactamases, TEMs with
27 a strong promoter, were rare (5%). The remaining 59 isolates (62%) had only sulbactam-susceptible
28 β -lactamases, including TEM-1 with a wild-type promoter ($n = 28$), CTX-Ms ($n = 13$), or both ($n =$
29 17). All 45 transconjugants from 96 donors with TEM-1 had higher ampicillin-sulbactam MICs (4-96
30 mg/l) than the recipient (2 mg/L). In donors with only TEM-1, TEM-1 activity correlated with the
31 50% inhibitory concentration of sulbactam and ampicillin-sulbactam MICs. The decreased
32 membrane permeability of sulbactam was associated with an increased ampicillin-sulbactam MIC.
33 The reduced permeability was partly attributable to deficient outer membrane proteins, which were
34 observed in 57% of the ampicillin-sulbactam-nonsusceptible isolates with only TEM-1 and a
35 wild-type promoter. Sequence type 131 was the most common clonal type (52%). TEM-1 with a
36 wild-type promoter primarily contribute to ampicillin-sulbactam nonsusceptibility in *E. coli* with

37 partial support of other mechanisms such as the reduced permeability. Conjugative TEM-1 and
38 clonal spread of ST131 may contribute to the prevalence of Japanese
39 ampicillin-sulbactam-nonsusceptible isolates.

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42 INTRODUCTION

43 *Escherichia coli* causes extraintestinal infections, including urinary tract infections,
44 intra-abdominal infections, and bacteremia. Ampicillin-sulbactam (SAM) and
45 amoxicillin-clavulanate (AMC) have wide spectra of activity against Gram-positive, Gram-negative,
46 and anaerobic organisms. In Japan, SAM is commonly used in daily clinical practice, whereas
47 intravenous AMC is not available. However, a decreasing rate of susceptibility to SAM among *E.*
48 *coli* threatens its continued clinical use (1).

49 The TEM-1 β -lactamase belongs to group 2b in the Bush-Jacoby classification scheme and
50 is inhibited by β -lactamase inhibitors, such as sulbactam and clavulanate (2). Therefore, *E. coli*
51 isolates with TEM-1 are usually susceptible to SAM and AMC. However, hyperproduction of
52 TEM-1 overcomes the inhibitory effects of sulbactam and clavulanate (3) and has been reported to be
53 a common resistance mechanism against SAM and AMC in *E. coli* (4, 5). A strong promoter, such as
54 the Pa/Pb promoter can contribute to TEM-1 hyperproduction (6).

55 Other mechanisms that have been described include plasmid-mediated AmpC β -lactamase
56 (p-AmpC), hyperproduction of the chromosomal AmpC β -lactamase (c-AmpC), OXA β -lactamase,
57 and inhibitor-resistant TEM (IRT) β -lactamase (7). Deficiency of outer membrane proteins (OMPs),
58 such as OmpF and OmpC, has been indicated to contribute to increases in the MIC for isolates with
59 TEM-1 (3). However, the prevalence and contribution of OMP deficiency in SAM- or
60 AMC-nonsusceptible *E. coli* remain unknown.

61 In addition to horizontal gene transfer of *bla*_{TEM-1} which is usually associated with a
62 transposon (8) and is present on a plasmid (9), the dissemination of SAM-resistant *E. coli* is
63 attributable to *E. coli* sequence type 131 (ST131) which has spread worldwide and is recognized as a
64 main driver of fluoroquinolone resistance and ESBL producers (10, 11). ST131 frequently harbors
65 *bla*_{TEM-1} (10). ST131 consists of 3 different clades (A, B, and C). In clade C, subclades C1 and C2
66 are associated with fluoroquinolone resistance. Subclade C2 and a specific lineage within C1,
67 C1-M27, are associated with ESBLs (12, 13). The ST131 clade responsible for the spread of SAM
68 resistance is not known.

69 To understand the mechanisms and epidemiology of SAM resistance and the increase in
70 SAM-nonsusceptible *E. coli*, we investigated β -lactamase genes, the transferability of *bla*_{TEM-1}, the
71 TEM-1 activity, OMPs, membrane permeability, and clonal genotypes among clinical *E. coli* isolates
72 in Japan.

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74 RESULTS AND DISCUSSION

75 We investigated a total of 329 clinical *E. coli* isolates that were consecutively collected by a
76 Japanese multicenter surveillance program (14). Of these isolates, 95 isolates (29%) were
77 nonsusceptible (intermediate, 60 isolates; resistant, 35 isolates) to SAM, and 61 isolates (19%) were
78 nonsusceptible to AMC. The prevalence of SAM-nonsusceptible isolates is consistent with the values
79 reported in previous studies from the Asia-Pacific region (30%) (15) and the United States (31%) (4).

80 **Epidemiology of β -lactamase genes.** The comparison of β -lactamase genes between the
81 SAM-nonsusceptible and SAM-susceptible isolates revealed a high prevalence of the acquired
82 β -lactamase gene (91% and 28%, respectively; $p < 0.01$), especially *bla*_{TEM-1} with a P3 promoter,
83 which weakly promotes *bla*_{TEM-1} expression, in SAM-nonsusceptible isolates (Table 1). This high
84 prevalence of *bla*_{TEM-1} with a P3 promoter is consistent with that in previous reports (4).
85 Hyperproduction of sulbactam-susceptible β -lactamases (group A in Table S1), namely, TEMs with
86 the Pa/Pb promoter, were rare (5%).

87 Twelve isolates (13%), including the 9 isolates lacking any acquired β -lactamase genes,
88 were defined as c-AmpC hyperproducers with promoter mutations (Table S2), and their prevalence is
89 similar to that in a Spanish study (5). The rarity of IRT (1%) and the relatively low frequencies of
90 p-AmpC and *bla*_{OXA-1} (6% and 13%, respectively) in SAM-nonsusceptible isolates are consistent
91 with those in previous studies (4, 16). These sulbactam-resistant β -lactamases together accounted for
92 one-third of the SAM-nonsusceptible isolates in our study (group B in Table S1).

93 The β -lactamases that were not considered to confer SAM nonsusceptibility as a single
94 resistance mechanism included *bla*_{TEM-1} with a P3 promoter (29%), the combination of *bla*_{TEM-1} and
95 *bla*_{CTX-M} (18%), and *bla*_{CTX-M} only (14%) (group C in Table S1). The ubiquitous presence of the
96 acquired β -lactamases or c-AmpC and the high frequency of *bla*_{TEM-1} in the SAM-nonsusceptible
97 isolates support the notion that β -lactamases, especially *bla*_{TEM-1} with a P3 promoter, are important
98 contributors to SAM nonsusceptibility.

99 **Epidemiology of deficient OMPs in SAM-nonsusceptible isolates.** We investigated the
100 OMP profiles of SAM-nonsusceptible isolates by sodium dodecyl sulphate polyacrylamide gel
101 electrophoresis (SDS-PAGE). Representative OMP profiles in SDS-PAGE are shown in Figure S1.
102 We identified 38 isolates (40%) with deficient OMPs (deficient OmpF: 35 isolates, deficient OmpC:
103 3 isolates, both: 3 isolates), and their sequence profiles of deficient OMPs are shown in Table S3.
104 Among 28 isolates with only *bla*_{TEM-1} with a P3 promoter, more than a half of the isolates (57%) had
105 deficient OMPs (Table S1).

106 **Transferability of *bla*_{TEM-1} by conjugation.** A conjugation assay was performed to assess
107 *bla*_{TEM-1}-transferability and the influence of *bla*_{TEM-1} on SAM nonsusceptibility in all 96
108 *bla*_{TEM-1}-positive isolates. We obtained 45 *bla*_{TEM-1}-positive transconjugants, one of which harbored
109 *bla*_{TEM-1} and *bla*_{CTX-M} (Table S4). Plasmid replicon typing revealed that incompatibility group (Inc) F
110 accounted for 89% of the transconjugants with *bla*_{TEM-1} (Table S5).

111 **The SAM MIC for donor-transconjugant pairs harboring TEM-1 with or without**
112 **other β -lactamases genes.** The SAM MICs for the recipient *E. coli* J53 (2 mg/l) were elevated
113 through acquisition of conjugative *bla*_{TEM-1} in all transconjugants (median 12 mg/L; range, 4-96
114 mg/l) (Figure S2), suggesting the potential role of TEM-1 in SAM nonsusceptibility.

115 Higher MICs for donors with the combination of *bla*_{TEM-1} and *bla*_{CTX-M} than those for their
116 transconjugants harboring only *bla*_{TEM-1} (Figure S2) and a higher frequency of this combination in
117 SAM-nonsusceptible isolates than SAM-susceptible isolates (19% and 2%, respectively, $p < 0.01$)

118 (Table 1) suggest that the combination of multiple β -lactamases, each of which alone could be
119 inhibited by sulbactam (17), may increase the MIC of SAM.

120 **The SAM MIC and β -lactamase activity of donor-transconjugant pairs harboring only**

121 **TEM-1.** Of the 45 donor-transconjugant pairs, we selected the 32 pairs with only *bla*_{TEM-1} to further
122 investigate the role of TEM-1. Acquisition of conjugative *bla*_{TEM-1} in transconjugants did not always
123 increase the SAM MIC as observed for donors (Spearman's rank correlation [r_s] = 0.34, p = 0.06)
124 (Figure 1A).

125 We measured the β -lactamase activity of the 32 pairs (Figure 1B and 1C). A previous study
126 defined AMC-resistant *Enterobacteriaceae* with only *bla*_{TEM-1} whose β -lactamase activity was at
127 least two-fold higher than that of ATCC35218, a reference *E. coli* strain harboring *bla*_{TEM-1}, as
128 TEM-1 hyperproducers and found that 8 of 10 isolates were TEM-1 hyperproducers (18). Among the
129 32 donors, this definition identified 2 TEM-1 hyperproducers (6%), which showed high SAM MICs
130 (64 and 128 mg/l). One of them possessed TEM-1 with the Pa/Pb promoter which showed the
131 highest β -lactamase activity and the highest MIC for both the donor and its transconjugant. Although
132 the definition of TEM-1 hyperproducers is dependent on the cut-off value of β -lactamase activity, the
133 frequency of TEM-1 hyperproducers may be low in SAM-nonsusceptible isolates in our study as
134 opposed to a previous study suggesting a predominance of TEM-1 hyperproducers among
135 SAM-nonsusceptible isolates (4).

136 The positive correlation between the β -lactamase activity of TEM-1 with a P3 promoter and

137 the SAM MICs (donors, $r_s = 0.59$, $p < 0.01$; transconjugants, $r_s = 0.82$, $p < 0.01$) is consistent with a
138 study analyzing TEM-1-producing *E. coli* clinical isolates (19), in which some isolates did not
139 conform to a quantitative relationship between β -lactamase activity and the AMC MIC. Likewise, in
140 our study, donors likely showed a smaller correlation coefficient between β -lactamase activity and
141 the SAM MICs than transconjugants ($p = 0.07$, Fisher's z transformation), reflecting diverse host cell
142 characteristics among donors which can affect SAM MICs in contrast to the same host cells (J53)
143 among transconjugants. This difference in host characteristics may also explain the discrepancy in
144 the SAM MIC between donors and transconjugants mentioned above (Figure 1A). The very low
145 activity (<0.5 nmol/min/ml) of a basal level of the c-AmpC β -lactamase of J53 and the correlation of
146 the β -lactamase activity between donors and transconjugants, except for 2 isolates (Figure 1D),
147 implies that the β -lactamase activity of donors primarily represented the activity of TEM-1.
148 Therefore, in addition to TEM-1 activity, which showed a positive relationship with the SAM MIC,
149 host factors other than β -lactamase may contribute to increased SAM MICs.

150 **OMPs of donors with only TEM-1.** SDS-PAGE of the 32 donors identified 17 donors with
151 deficient OmpF and 7 donors with deficient OmpC (Figure 1A). The ratio of the MICs for donors to
152 those for transconjugants was higher in isolates with deficient OmpF than that in isolates with
153 wild-type OmpF, whereas this ratio was not associated with the OmpC profile ($p = 0.03$ and $p = 0.12$,
154 respectively, Mann-Whitney test), implying that donors with deficient OmpF are likely to have a
155 higher SAM MIC than their transconjugants regardless of TEM-1 activity. Therefore, deficient

156 OmpF may be one of the host factors influencing the SAM MIC.

157 **The concentration of sulbactam to inhibit TEM-1 for determination of membrane**
158 **permeability.** To investigate membrane permeability in isolates with non-hyperproducing TEM-1 for
159 which the SAM MIC can be easily influenced by host factors, we analyzed the permeability index
160 (20). To determine the permeability index, we quantified the 50% inhibitory concentrations (IC₅₀) of
161 sulbactam, where TEM-1 activity is reduced by half, for lysates (lysate-IC₅₀) and intact cells
162 (cell-IC₅₀). After excluding the 2 TEM-1 hyperproducers, we selected 19 isolates from the remaining
163 30 donors representing three groups according to their correlations between TEM-1 activity and the
164 SAM MICs (Figure S3) in addition to ATCC35218.

165 The lysate-IC₅₀ and cell-IC₅₀ correlated with the TEM-1 activity of lysates without
166 sulbactam ($r_s = 0.85$, $p < 0.01$ and $r_s = 0.57$, $p < 0.01$, respectively) (Figure 2A and 2B), which
167 confirmed the quantitative relationship between TEM-1 activity and the sulbactam concentration. In
168 contrast to sulbactam's direct inhibition of the released TEM-1 in the suspension of sonicated lysates,
169 permeation of sulbactam into the periplasm is required for inhibition of TEM-1 in intact cells; thus,
170 the varying membrane permeability (e.g., OMPs and efflux pumps) among clinical isolates may
171 affect the cell-IC₅₀. This hypothesis is supported by the correlation observed between the cell-IC₅₀,
172 but not the lysate-IC₅₀, and the SAM-MIC measured using intact cells ($r_s = 0.75$, $p < 0.01$ and $r_s =$
173 0.31 , $p = 0.18$, respectively) (Figure 2C and 2D).

174 **Membrane permeability and OMPs.** For the selected 19 isolates and ATCC35218, we

175 determined the permeability index of sulbactam as the ratio of the cell-IC₅₀ to the lysate-IC₅₀ (20).
176 Isolates with deficient OmpF (10 isolates) had a higher permeability index, reflecting greater
177 penetration difficulty compared to those with wild-type OmpF ($p = 0.03$), although no such
178 association with the permeability index was observed in isolates with deficient OmpC (5 isolates) (p
179 $= 0.18$) (Figure 3A and 3B). Reguera et al. reported that an OmpF-deficient laboratory strain with a
180 *bla*_{TEM-1}-containing plasmid had a higher amoxicillin-sulbactam MIC and AMC MIC than a strain
181 with wild-type OmpF with the same plasmid (3). Our results support their hypothesis that sulbactam
182 penetrates bacterial cells via OmpF. β -lactams, including ampicillin, have also been shown to diffuse
183 principally through OmpF (21). As the permeability index should be determined by the combined
184 effect of the influx and efflux of sulbactam, the impact of the efflux pump remains to be elucidated.
185 In summary, deficient OmpF may play some part in the reduced membrane permeability of
186 sulbactam.

187 **The impact of the permeability index on the relationship between TEM-1 activity and**
188 **the SAM MIC.** We assessed whether the permeability index can explain the discrepancy between
189 TEM-1 activity and the SAM MIC. Figure 1B was re-drawn with the permeability data of 19
190 selected isolates and ATCC35218 (Figure 4A). Isolates with a high SAM MIC compared with
191 TEM-1 activity (isolates above the regression line) tended to have high permeability indices. To
192 clarify these relationships, the ratio of the SAM MIC to TEM-1 activity was calculated (Figure 4B),
193 which was found to correlate with the permeability index. The MIC for one *bla*_{TEM-1}-deficient isolate

194 obtained by plasmid curation experiments suggests that the SAM MIC for the host cells, which may
195 be partially influenced by membrane permeability, may have a basal impact on the SAM MIC for
196 isolates with TEM-1 (Table S6). Therefore, the membrane permeability of sulbactam contributes to
197 an increase in the SAM MIC in addition to TEM-1 activity. However, the relatively small correlation
198 coefficient ($r_s = 0.59$, $p < 0.01$) leads us to suspect the presence of other host factors, including the
199 interaction between ampicillin and the cell wall and the membrane permeability of ampicillin.

200 **Sequence types and the characteristics of isolates.** We previously analyzed the lineage of
201 the study isolates by CH typing using *fumC* and *fimH* alleles and ST131-specific PCR (14). *bla*_{TEM-1}
202 was more common in the ST131 isolates than in the non-ST131 isolates (44% and 22%, respectively,
203 $p < 0.01$) as previously reported (10) and was associated with SAM nonsusceptibility in the ST131
204 isolates (SAM-nonsusceptible isolates, 57%; SAM-susceptible isolates, 33%, $p = 0.01$). ST131 was
205 also associated with TEM-1 with CTX-M and OXA-1 in SAM-nonsusceptible isolates (Table S1).
206 Using the results of multiplex PCR in our previous study (22), we further classified ST131 into
207 clades, each of which showed different characteristics (Table 2). C2 showed an association with
208 SAM nonsusceptibility, and OXA-1 was the most common β -lactamase in SAM-nonsusceptible
209 isolates of this subclade, followed by TEM-1 with CTX-M.

210 Our conjugation assay demonstrated that 47% of *bla*_{TEM-1}, which was mostly on the IncF
211 plasmid, could transfer. Although the conjugative *bla*_{TEM-1} was commonly present in ST131 (26
212 isolates, 58%), it was distributed variably in ST131 clades (C1-non-M27, 12 isolates; C2, 5 isolates;

213 A, 4 isolates) as well as non-ST131 clones (7 CH types other than ST131), indicating horizontal
214 transfer by plasmid or other mobile genetic elements such as the transposons (8).

215 Some potential limitations exist in this study. We examined OMPs by SDS-PAGE and the
216 permeability index, but we did not perform gene-editing of OMPs; thus, the role of OMPs requires
217 further clarification. Other host characteristics, such as efflux pumps or the permeability of
218 ampicillin, were not evaluated. We did not confirm the links between specific plasmids of
219 transconjugants and *bla*_{TEM-1}. We plan to identify whole nucleotide sequences of these plasmids to
220 clarify such links. The short duration of isolate collection may reflect a potential selection bias. To
221 our knowledge, this is the first study to comprehensively analyze the roles of TEM-1 in the SAM
222 MIC by *bla*_{TEM-1} epidemiology, TEM-1 activity, the inhibitory effect and membrane permeability of
223 sulbactam, and OMP profiles. We believe that this research provides insight into the resistance
224 mechanisms of SAM-nonsusceptibility.

225 **Conclusions.** In Japan, SAM-nonsusceptible *E. coli* is prevalent and threatens the optimal
226 use of SAM. TEM-1, the most common β -lactamase, alone or in combination with decreased
227 membrane permeability, overcame the inhibitory effect of sulbactam and contributed to an increase
228 in the SAM MIC. The impaired permeability of sulbactam was associated with deficient OmpF,
229 which was observed in more than half of SAM-nonsusceptible isolates with only TEM-1. Other
230 possible mechanisms include CTX-M in combination with *bla*_{TEM-1} and sulbactam-resistant
231 β -lactamases (c-AmpC, *bla*_{CMY-2}, and *bla*_{OXA-1}). The increase in SAM-nonsusceptible *E. coli* is

232 associated with conjugative plasmids with *bla*_{TEM-1} and/or ST131 pandemic clone carrying the above
233 mentioned β -lactamases. We must continue to monitor the abundance of β -lactamases and clones that
234 contribute to resistance.

235

236 MATERIALS AND METHODS

237 In total, 329 non-duplicate clinical *E. coli* isolates consecutively collected at 10 hospitals
238 from 5 prefectures (5 hospitals in Kyoto, 2 hospitals in Osaka, and 1 hospital each in Aichi, Hyogo,
239 and Shiga) in Japan during December 2014 (14) were included. The most common source of the
240 isolates was the urinary tract (214 isolates, 65%), followed by blood culture (39 isolates),
241 intraabdominal sites (19 isolates), the female genital tract (18 isolates), the skin and subcutaneous
242 tissue or pus (17 isolates), the respiratory tract (15 isolates) and others (7 isolates). Antimicrobial
243 susceptibility to SAM and AMC was evaluated by microdilution at the reference laboratory using a
244 Dry Plate Eiken (Eiken, Tokyo, Japan) following the CLSI standards (23). *E. coli* isolates with an
245 MIC $>8/4$ mg/L at a fixed ratio of 2:1 were defined as SAM nonsusceptible regardless of the
246 susceptibilities of the other antimicrobials. The Ethics Committee of the Kyoto University Graduate
247 School and Faculty of Medicine approved this study (E-2103) and waived the requirement to obtain
248 informed consent from each patient.

249 **β -lactamase identification.** Bacterial DNA was isolated using a QIAamp DNA Mini kit
250 (Qiagen, Hilden, Germany). The presence of β -lactamase genes was defined by PCR amplification

251 and sequencing of the *bla*_{TEM} (24), *bla*_{SHV} (25), *bla*_{CTX-M} (26), *bla*_{GES} (27), *bla*_{PER} (27), *bla*_{VEB} (27),
252 and *bla*_{OXA} (28, 29) genes and the six main groups of p-AmpC-type genes (30). C-AmpC promoter
253 mutations were also examined by PCR and sequencing (31). C-AmpC hyperproducers were defined
254 as SAM-nonsusceptible isolates with mutations in the promoter and/or attenuator region and a
255 positive phenotypic test by the ceftiofur-cloxacillin disk method (32).

256 **Conjugation.** The transferability of *bla*_{TEM-1} was assayed by conjugation. Conjugation
257 experiments were performed by the liquid-mating method using azide-resistant *E. coli* J53 as the
258 recipient and all the *bla*_{TEM-1}-harboring clinical isolates, including *bla*_{CTX-M} or *bla*_{CMY-2}-co-harboring
259 isolates, as the donors, regardless of SAM susceptibility. Transconjugants selected on trypticase-soy
260 agar (TSA) containing azide (50 mg/l) and ampicillin (50 mg/l) were further differentiated from
261 donors by phylogenetic grouping (33). The transfer of *bla*_{TEM-1} and other β -lactamase genes from
262 donors was confirmed by PCR. The SAM susceptibility of the transconjugant and its donor was
263 tested by the Etest (Sysmex bioMérieux, Tokyo, Japan). Plasmid replicon typing of transconjugants
264 was performed as described previously (34, 35).

265 **β -lactamase activity assay.** We measured the β -lactamase activity of the donors and
266 transconjugants with only *bla*_{TEM-1}. Cells were harvested from mid-growth Luria-Bertani broth
267 cultures (5 ml) by centrifugation and resuspended in sodium phosphate buffer (0.1 M, pH 7) at an
268 optical density (OD) 600 of 0.3. One milliliter of cell suspension was sonicated to release
269 β -lactamase (six bursts of 10 seconds on ice). ATCC35218 was used to determine the appropriate

270 duration of sonication to completely release β -lactamase (Figure S4). β -lactamase activity was
271 measured spectrophotometrically against 100 μ M nitrocefin at 492 nm with the MTP-310 microplate
272 reader (Corona Electric, Ibaraki, Japan). All assays were performed in triplicate at room temperature.
273 As a negative control, an equivalent amount of buffer rather than cell lysate was added. ATCC35218
274 was used as a positive control.

275 **Assay of the IC₅₀ and permeability index.** The cell suspension described above was
276 divided into 2 portions (1 ml each). The first aliquot which was directly used to determine the IC₅₀
277 for TEM-1 in intact cells (cell-IC₅₀) was incubated with sulbactam (6 to 8 different concentrations) at
278 37°C for 120 minutes as described previously (36). The cell suspension was washed twice. After
279 sonication, the uninhibited β -lactamase activity was measured against nitrocefin. The second aliquot
280 was sonicated to first disrupt the cells to determine the IC₅₀ for released TEM-1 (lysate-IC₅₀). The
281 lysate was incubated with sulbactam for 20 minutes. Then, β -lactamase activity was measured. A
282 4-parameter logistic model was used to determine the IC₅₀ values of intact cells and lysates. The
283 permeability index was defined as the ratio of cell-IC₅₀ to lysate-IC₅₀ values (20). We used the drc
284 package in R for the analysis (37).

285 **OMP analysis.** The coding sequences of *ompF* and *ompC* amplified as previously described
286 (38) were compared with the reference strain *E. coli* K-12 (GenBank CP001637.1). OmpF and
287 OmpC were identified by SDS-PAGE as described previously (39). Briefly, a membrane fraction was
288 extracted from a cell suspension (7 ml) at an OD₆₀₀ of 0.5 with sodium N-lauroyl sarcosinate. Then,

289 the extracted protein was analyzed by SDS-PAGE. *E. coli* ATCC 25922 (wild-type) was used as a
290 control.

291 **Clonal group identification.** The genetic relationship between the *E. coli* isolates was
292 determined by CH typing using *fumC* and *fimH* alleles as in our previous study (40). The ST131
293 status was also determined by PCR-based detection of ST131-specific single nucleotide
294 polymorphisms in *gyrB* and *mdh* (41). To confirm the major ST131 clades, multiplex PCR was
295 performed in our previous study (22).

296 **Statistical analysis.** Categorical variables were compared using Fisher's exact test.
297 Continuous variables were compared using the Mann-Whitney U test. The relationships between the
298 continuous variables were represented by Spearman's rank correlation, r_s . The correlation
299 coefficients were compared using Fisher's z transformation. The results were considered significant
300 at an alpha level of 0.05. We conducted our statistical analysis using Stata, version 13 (StataCorp,
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- 445
- 446

447 **TABLE 1 β -lactamases and promoter types of bla_{TEMs} detected in SAM-nonsusceptible and**
 448 **SAM-susceptible isolates**

Types of β -lactamase and Bush and Jacoby classification ^a	SAM susceptibility		P-value
	Nonsusceptible ^b (n = 95)	Susceptible (n = 234)	
<i>bla</i> _{TEM} promoter			
<i>bla</i> _{TEM}			
<i>bla</i> _{TEM-1}	53 ^d (56%)	43 ^e (18%)	<0.01
P3 promoter	50 (53%)	41 (18%)	<0.01
Pa/Pb promoter	3 (3%)	0	0.02
IS26 insertion	0	2 ^f (1%)	1.00
<i>bla</i> _{TEM-135}	2 ^g (2%)	0	0.08
Pa/Pb promoter	2 (2%)	0	0.08
<i>bla</i> _{TEM-34}	1 (1%)	0	0.29
P3 promoter	1 (1%)	0	0.29
<i>bla</i> _{CTX-M}			
<i>bla</i> _{CTX-M-27}	5 (5%)	20 (9%)	0.37
<i>bla</i> _{CTX-M-15}	18 ^h (19%)	2 (1%)	<0.01
<i>bla</i> _{CTX-M-14}	14 ⁱ (15%)	3 (1%)	<0.01
other <i>bla</i> _{CTX-M} ^c	8 (8%)	2 (1%)	<0.01

<i>bla</i> _{OXA-1}	2d	12 (13%)	0	<0.01
<i>bla</i> _{CMY-2}	1	6 (6%)	0	<0.01
<i>bla</i> _{SHV-1}	2b	1 (1%)	0	0.29
c-AmpC hyperproduction	1	12 (13%)	NT	NT

449 NT, not tested.

450 ^a β -lactamase inhibitors inhibit group 2b and 2be β -lactamases but not group 1, 2br, and 2d
451 β -lactamases.

452 ^b All the SAM-nonsusceptible isolates produced acquired β -lactamases or c-AmpC.

453 ^c Other *bla*_{CTX-M} included *bla*_{CTX-M-2}, n = 4; *bla*_{CTX-M-1}, n = 1; *bla*_{CTX-M-3}, n = 1; *bla*_{CTX-M-27-like}, n =
454 1; and *bla*_{CTX-M-55}, n = 1 in SAM-nonsusceptible isolates and *bla*_{CTX-M-24}, n = 1; and *bla*_{CTX-M-55}, n = 1
455 in SAM-susceptible isolates.

456 ^d Seventeen isolates harbored *bla*_{CTX-Ms}, 2 isolates harbored *bla*_{CMY-2}, and 1 isolate harbored
457 *bla*_{CTX-M-15} and *bla*_{OXA-1}.

458 ^e Four isolates harbored *bla*_{CTX-Ms}.

459 ^f -35 box of the *bla*_{TEM-1} promoter was truncated by IS26.

460 ^g One isolate harbored *bla*_{CTX-M}.

461 ^h Nine isolates harbored *bla*_{OXA-1}, and 1 isolate harbored *bla*_{CTX-M-14} and *bla*_{OXA-1}.

462 ⁱ One isolate harbored *bla*_{OXA-1}.

463 **TABLE 2 The ST131 clades of the SAM-nonsusceptible and SAM-susceptible isolates**

Characteristics	A	B	C				Other than A, B, and C
			C1		C2		
			C1-non-M27	C1-M27			
SAM susceptibility							
Susceptible (n = 56)	3 (5%)	6 (11%)	22 (39%)	19 ^a (34%)	5 ^a (9%)	1 (2%)	
Nonsusceptible (n = 49)	6 (12%)	4 (8%)	18 (37%)	5 ^a (10%)	15 ^a (31%)	1 (2%)	
Major β-lactamases (group) in SAM-nonsusceptible isolates							
Hyperproduction of sulbactam-susceptible β-lactamases							
(A)							
TEM-1 with a Pa/Pb promoter (n = 3)	2 (67%)	0 (0%)	1 (33%)	0 (0%)	0 (0%)	0 (0%)	

Sulbactam-resistant β -lactamases (B)						
c-AmpC (n = 1)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)
OXA-1 (n = 10)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (100%)	0 (0%)
CMY-2 (n = 2)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
TEM-34 (inhibitor-resistant TEM) (n = 1)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Other β -lactamases (C)						
TEM-1 with a P3 promoter (n = 11)	3 (27%)	1 (9%)	6 (55%)	0 (0%)	0 (0%)	1 (9%)
TEM-1 and CTX-M (n = 13)	0 (0%)	0 (0%)	7 (54%)	1 (8%)	5 (38%)	0 (0%)
CTX-M (n = 8)	1 (13%)	1 (13%)	3 (38%)	3 (38%)	0 (0%)	0 (0%)

464 ^a P-value <0.05 compared with SAM-nonsusceptible and SAM-susceptible isolates (Fisher's exact test).

465 **FIGURE LEGENDS**

466 **Figure 1. TEM-1 activity and SAM MIC for 32 donor-transconjugant pairs with only TEM-1**
467 **and ATCC35218.**

468 (A) The SAM MICs (shown by the ampicillin MICs) for donors and transconjugants. The regression
469 line was calculated from all the donors and their transconjugants with TEM-1. When the SAM MICs
470 for the donor and its transconjugant are equal, the corresponding dot should be on the line $y = x$
471 (dash line). The TEM-1 activities and SAM MICs for (B) donors and (C) their transconjugants. The
472 regression lines were calculated from donors or transconjugants with TEM-1 with a P3 promoter. (D)
473 TEM-1 activities of donors and their transconjugants. The regression line was calculated from all the
474 transconjugants and their donors with TEM-1. When the TEM-1 activities of the donor and its
475 transconjugant are equal, the corresponding dot should be on the line $y = x$ (dash line). In two donors
476 (arrow), MICs and TEM-1 activities were two-times higher than those of their transconjugants (SAM
477 MIC, 24 vs. 8 mg/l and 64 vs. 8 mg/l; TEM-1 activity, 17.7 vs. 7.2 nmol/min/ml and 37.0 vs 10.4
478 nmol/min/ml), and their cefoxitin-cloxacillin disk test results were negative. Spearman's rank
479 correlation is indicated by r_s . A transconjugant of ATCC35218 could not be obtained.

480
481 **Figure 2. The 50% inhibitory concentration of sulbactam and its relationships with TEM-1**
482 **activity and the SAM MIC for 19 clinical isolates and ATCC35218.**

483 TEM-1 activity and the 50% inhibitory concentration (IC_{50}) of sulbactam for (A) sonicated lysates
484 (lysate- IC_{50}) and (B) intact cells (cell- IC_{50}) harboring only *bla*_{TEM-1} with a P3 promoter. (C)
485 Lysate- IC_{50} and (D) cell- IC_{50} compared with the SAM MIC. The ampicillin MICs are shown.
486 Spearman's rank correlation is indicated by r_s .

487

488 **Figure 3. Outer membrane proteins and the permeability index of sulbactam for 19 clinical**
489 **isolates and ATCC35218.**

490 The permeability indices of sulbactam for clinical isolates and ATCC35218 (arrow) with (A) OmpF
491 and (B) OmpC profiles in SDS-PAGE. A higher permeability index corresponds to less sulbactam
492 penetration of the outer membrane. The median permeability index is indicated by the horizontal line.
493 The statistical analysis was performed with the Mann-Whitney U test.

494

495 **Figure 4. Relationships between the permeability index and TEM-1 activity or the SAM MIC**
496 **in 19 clinical isolates and ATCC35218.**

497 (A) The TEM-1 activities and SAM MICs (shown by the ampicillin MICs). Each plot is labeled with
498 the permeability index. A higher permeability index corresponds to less sulbactam penetration of the
499 outer membrane. (B) The permeability indices and ratio of the SAM MIC to TEM-1 activity. A high
500 ratio implies a relatively high SAM MIC for TEM-1 activity. Spearman's rank correlation is
501 indicated by r_s .

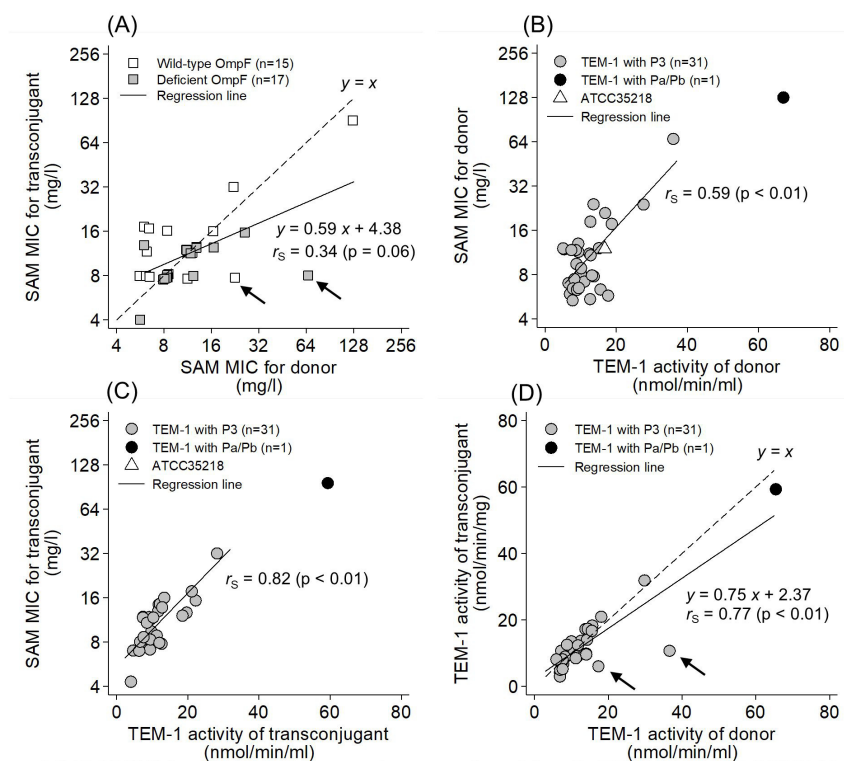


Figure 1. TEM-1 activity and SAM MIC for 32 donor-transconjugant pairs with only TEM-1 and ATCC35218.

(A) The SAM MICs (shown by the ampicillin MICs) for donors and transconjugants. The regression line was calculated from all the donors and their transconjugants with TEM-1. When the SAM MICs for the donor and its transconjugant are equal, the corresponding dot should be on the line $y = x$ (dash line). The TEM-1 activities and SAM MICs for (B) donors and (C) their transconjugants. The regression lines were calculated from donors or transconjugants with TEM-1 with a P3 promoter. (D) TEM-1 activities of donors and their transconjugants. The regression line was calculated from all the transconjugants and their donors with TEM-1. When the TEM-1 activities of the donor and its transconjugant are equal, the corresponding dot should be on the line $y = x$ (dash line). In two donors (arrow), MICs and TEM-1 activities were two-times higher than those of their transconjugants (SAM MIC, 24 vs. 8 mg/l; TEM-1 activity, 17.7 vs. 7.2 nmol/min/ml and 37.0 vs 10.4 nmol/min/ml), and their cefoxitin-cloxacillin disk test results were negative. Spearman's rank correlation is indicated by r_s . A transconjugant of ATCC35218 could not be obtained.

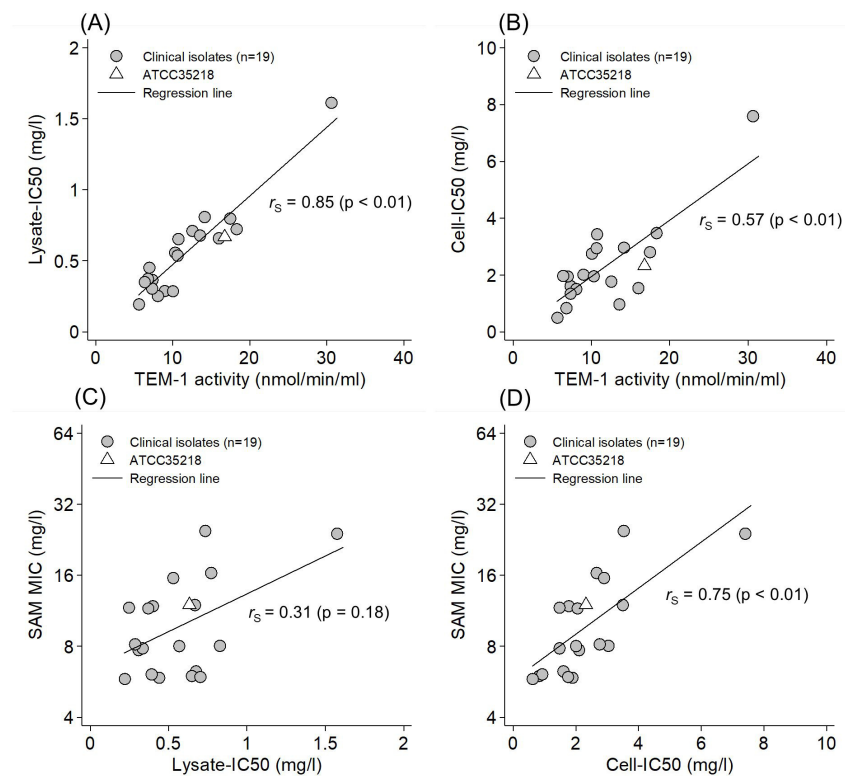


Figure 2. The 50% inhibitory concentration of sulbactam and its relationships with TEM-1 activity and the SAM MIC for 19 clinical isolates and ATCC35218.

TEM-1 activity and the 50% inhibitory concentration (IC₅₀) of sulbactam for (A) sonicated lysates (lysate-IC₅₀) and (B) intact cells (cell-IC₅₀) harboring only *bla*_{TEM-1} with a P3 promoter. (C) Lysate-IC₅₀ and (D) cell-IC₅₀ compared with the SAM MIC. The ampicillin MICs are shown. Spearman's rank correlation is indicated by r_s .

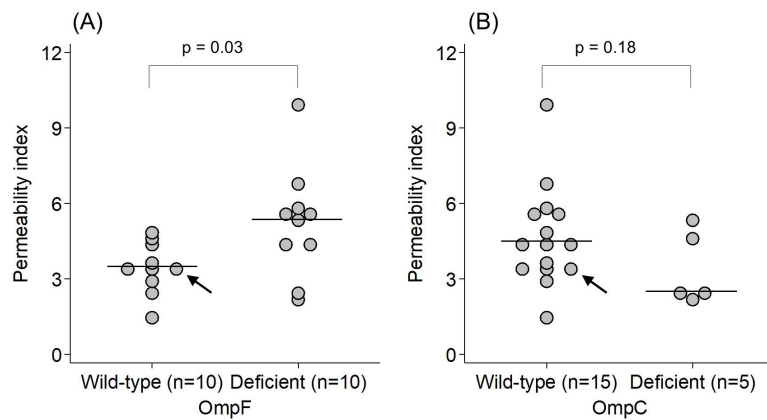


Figure 3. Outer membrane proteins and the permeability index of sulbactam for 19 clinical isolates and ATCC35218.

The permeability indices of sulbactam for clinical isolates and ATCC35218 (arrow) with (A) OmpF and (B) OmpC profiles in SDS-PAGE. A higher permeability index corresponds to less sulbactam penetration of the outer membrane. The median permeability index is indicated by the horizontal line. The statistical analysis was performed with the Mann-Whitney U test.

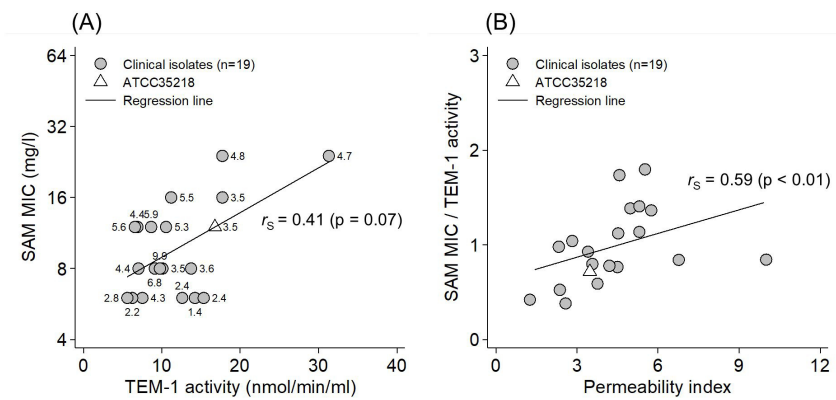


Figure 4. Relationships between the permeability index and TEM-1 activity or the SAM MIC in 19 clinical isolates and ATCC35218.

(A) The TEM-1 activities and SAM MICs (shown by the ampicillin MICs). Each plot is labeled with the permeability index. A higher permeability index corresponds to less sulbactam penetration of the outer membrane. (B) The permeability indices and ratio of the SAM MIC to TEM-1 activity. A high ratio implies a relatively high SAM MIC for TEM-1 activity. Spearman's rank correlation is indicated by r_s .