Survey of Modifying Enzymes and Plasmids in Amikacin-Resistant Serratia marcescens

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Abstract Forty amikacin-resistant strains of Serratia marcescens isolated from four different hospitals (A, B, C, and D) were examined for modifying enzymes and plasmids. Twenty-one of the isolates produced acetyltransferase that modified amikacin. Eighteen of the 21 acetyltransferase-bearing isolates were from different inpatients in hospital A and the other three were from hospital C. Amikacin resistance was mediated by conjugative plasmid of 24 megadaltons in 15 of the 18 acetyltransferase-bearing isolates of hospital A and by nonconjugative plasmids, derivatives of the 24-megadalton plasmids, in the remaining three isolates of the same hospital. The 24-megadalton plasmid determined aminoglycoside acetyltransferase (6') IV. This plasmid-borne enzyme conferred amikacin resistance on S. marcescens but not on Escherichia coli K12. The frequency of transfer of the 24-megadalton plasmid from the S. marcescens isolate to E. coli K12 by conjugation was approximately $10^{-7}$ (transconjugants/donors) and was 0.1% of that between E. coli strains. In acetyltransferase-bearing isolates from hospital C, the enzyme was mediated by a nonconjugative plasmid in one case and could not be associated with a plasmid in the remaining two cases. Neither enzymes nor plasmids could be associated with amikacin resistance of the isolates of the other two hospitals.

Serratia marcescens is primarily a hospital-acquired pathogen that exhibits resistance to various antibiotics including aminoglycoside antibiotics (AGs) (8, 12, 18, 29). Resistance to gentamicin, tobramycin, dibekacin, and kanamycin is known to be mediated by modifying enzymes encoded by a conjugative R plasmid (7, 8, 13, 14).

During the last 7 years, we continuously surveyed the conjugal transfer of AGs resistance from hospital isolates of S. marcescens to E. coli K12 and obtained the preliminary information that transferable AMK resistance, though rare, was present (15, 17). To confirm the presence of transferable AMK resistance in our isolates, we examined the strains for the modifying enzyme and plasmids by a molecular epidemiological procedure. As a result, it was determined that a conjugative R plasmid mediating AAC(6') IV and some nonconjugative plasmids were associated with AMK resistance.
MATERIALS AND METHODS

Strains. Forty strains of *S. marcescens*, resistant to more than 25 μg of AMK per ml, were used. They were isolated mainly from urinary specimens of different patients in four hospitals, Saiseikai Utsunomiya Hospital (designated as hospital A), Tokyo Medical and Dental University Hospital (hospital B), Teikyo University Hospital (hospital C), and Tokyo University Hospital (hospital D) in 1980. *Escherichia coli* K12 strain W3104 (rif), *E. coli* C (nal), and *S. marcescens* strain #7 were used as recipients of R plasmids in conjugation experiments. *Serratia marcescens* strain #7 is a clinical isolate susceptible to AGs and resistant to nalidixic acid (NA). *Escherichia coli* C (nal) and *E. coli* HB101 (str) were used in experiments on transformation. *Bacillus subtilis* ATCC 6633 was used as a test organism for microbiological assay of AGs-modifying enzyme.

AGs. Kanamycin A (KM), dibekacin (DKB), ribostamycin (RSM), gentamicin (GM), tobramycin (TOB), neomycin B (NM), paromomycin (PRM), and AMK were obtained from commercial sources. Butirosin A (BUT), lividomycin A (LVDM), sisomicin (SISO), HAPA-B, and fortimicin (FTM) were provided by the laboratories of Sankyo Co., Ltd., Japan, Kowa Co., Ltd., Japan, Essex Nippon Co., Ltd., Japan, Toyo Jozo Co., Ltd., Japan, and Kyowa Hakko Kogyo Co., Ltd., Japan, respectively.

Antibiotic susceptibility test. Minimum inhibitory concentrations (MIC) were determined on Mueller Hinton Medium (Difco Laboratories, Detroit, Mich., U.S.A.) by a standard agar dilution method with an inoculum of approximately 5 × 10^4 cells per spot.

Assay for AGs-modifying enzymes. Cell-free extracts prepared by the method of Haas and Dowding (10) were used as enzyme solutions. Microbiological assay was carried out to determine the presence or absence of modifying enzymes in *S. marcescens* isolates, their transconjugants and transformants. Residual antibiotic activity against the test organism was assayed by a disc method after reaction at 30°C for 18 hr in the following reaction mixtures. For acetylation: 0.1 M Tris-maleate pH 6.0, 10 mM MgCl₂, 3 mM 2-mercaptoethanol, 50 μM AMK, 200 μM acetyl-CoA, and cell-free extract from approximately 10^8 cells. For phosphorylation or adenylylation: the same mixture was used except that it contained 1 mM ATP instead of acetyl-CoA and was adjusted to pH 7.0. Substrate specificity was measured by the phosphocellulose paper binding assay described by Haas and Dowding (10) and expressed as the percent [14C]acetyl-group incorporation of each antibiotic relative to that of KM. The reaction was allowed to proceed for 15 min at 30°C in 50 μl of a mixture which contained 0.1 M Tris-maleate pH 6.0, 10 mM MgCl₂, 3 mM 2-mercaptoethanol, 200 μM antibiotic, 135 μM [14C]acetyl-CoA (20.0 μCi/μmol) (The Radio Chemical Centre, Amersham, England) and cell-free extract containing 40 μg of protein. Protein was determined by the method of Lowry et al (20).

Plasmid DNA analysis. Cleared lysates were prepared from stationary phase cultures of *S. marcescens* isolates, their transconjugants and transformants, by the
method described by Colman et al (5) or by Takahashi and Nagano (28). Plasmid DNA in the cleared lysate was concentrated by precipitation in ethanol or in isopropyl alcohol. HindIII (Takara Shuzo Co., Ltd., Kyoto, Japan) digestion was performed at 37°C for 12 hr in the buffer described by Davis et al (6). Plasmid DNA and HindIII digests were analyzed by agarose gel (1.0 and 1.2%, respectively) electrophoresis at 60 to 75 V for 3 to 5 hr in 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3. EcoRI and HindIII double digests of lambda phage DNA (21, 5.1, 5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.3, 1.0, and 0.8 Kb) and plasmid RP4 DNA (35 and 20 Kb) were used as internal molecular size markers.

Conjugal transfer of R plasmid. Overnight cultures of a donor and a recipient were mixed at a ratio of 1:9 (v/v), and then diluted fivefold in prewarmed Antibiotic Medium 3 (Difco) and incubated at either 30°C or 37°C for 3 hr to 18 hr. Transconjugants were selected on heart infusion (HI) agar (Eiken Kagaku Co., Ltd., Tokyo) plates containing RSM (25 μg/ml) or AMK (12.5 μg/ml) for plasmid selection and rifampicin (100 μg/ml) or NA (50 μg/ml) for the counterselection of the donors. Transfer frequency was expressed as the ratio of the number of transconjugants to that of the donors.

Transformation. Transformation of the E. coli strains by plasmid DNA in crude lysates from S. marcescens isolates was carried out by the CaCl₂ method of Cohen et al (4). Transformants were selected on HI agar plates containing AMK (12.5 μg/ml) or RSM (12.5 μg/ml). Clones able to grow on the selection plates were subcultured and tested for susceptibility to AGs, including AMK.

Loss of AMK resistance. AMK-susceptible clones were selected by the replica plating method after incubation of the AMK-resistant isolates in cooked meat broth (Nissui Seiyaku Co., Ltd., Tokyo) at room temperature for 10 months. HI agar plates containing AMK (12.5 μg/ml) were used as replica plates. Clones unable to grow on the replica plates were subcultured from the master plates and loss of resistance was confirmed by determining the MIC.

RESULTS

Susceptibility of S. marcescens Isolates

MIC distribution of several AGs against the 40 AMK-resistant isolates of S. marcescens is shown in Table 1. All of the 40 isolates were resistant to KM, TOB, and DKB (MIC ≥ 12.5 μg/ml). More than 90% were resistant to HAPA-B (MIC ≥ 12.5 μg/ml) and about 68% to SISO (MIC ≥ 12.5 μg/ml). MIC distribution of GM was relatively wide and had two peaks. More than 80% of the isolates were susceptible to GM (MIC ≤ 3.13 μg/ml). FTM was the most active AGs and all of the AMK-resistant isolates were susceptible to this antibiotic (MIC ≤ 3.13 μg/ml). Furthermore, there was no significant difference in MIC distribution of the AGs against the isolates among the four hospitals.

Detection of AGs-Modifying Enzyme

The 40 isolates were examined for AMK-modifying activity. Acetylation of
AMK was detected in 21 isolates (52.5%). Of these 21, 18 were from hospital A and the other three were from hospital C. Acetylation of AMK was not demonstrated in the isolates of the other two hospitals. Phosphorylation or adenylylation was not detected in any of the isolates from any of the hospitals.

Detection of Plasmid DNA

Figure 1 shows the results of agarose gel electrophoresis of crude lysates from the 40 isolates. One or two plasmids, which were expected to be large enough to contain the necessary genes for their own transfer, were observed in a total of 33 isolates.
of the four hospitals. Small plasmids, which migrated faster than chromosomal DNA fragments, were found in at least 18 isolates of hospitals A and C. Plasmid DNA was not detected in seven isolates. All of the acetyltransferase-bearing isolates of hospitals A and C, shown in the brackets in the figure, contained one or more plasmids. Furthermore, it was worthy of note that the 18 acetyltransferase-bearing isolates of hospital A had the same or very similar plasmid profiles.

Transfer of Resistance

None of the 40 S. marcescens donors transferred selected resistance to AMK (12.5 μg/ml) to the E. coli K12 recipient strain. However, the transconjugants, obtained by RSM selection from the 15 acetyltransferase-bearing isolates of hospital A, exhibited lower susceptibility to AMK than the recipient strain. In addition, the RSM-selected transconjugants of hospital A were found to produce acetyltransferase that could modify AMK. Therefore, further characterization of the transconjugants was carried out. Several transconjugants were obtained by RSM selection from the isolates of the other three hospitals; however the transconjugants were the same as the recipient strain in their susceptibility to AMK.

Characterization of Transconjugants

MICs of 12 AGs for the 15 RSM-selected transconjugants of hospital A were determined. As all 15 transconjugants proved identical in their susceptibility to

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum inhibitory concentration (μg/ml)</th>
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<tr>
<td></td>
<td>First transfer</td>
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<tr>
<td></td>
<td>Transconjugant</td>
</tr>
<tr>
<td>Amikacin</td>
<td>6.25</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>100</td>
</tr>
<tr>
<td>Ribostamycin</td>
<td>400</td>
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<tr>
<td>Butirosin A</td>
<td>50</td>
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<tr>
<td>Tobramycin</td>
<td>12.5</td>
</tr>
<tr>
<td>Dibekacin</td>
<td>12.5</td>
</tr>
<tr>
<td>HAPA-B</td>
<td>6.25</td>
</tr>
<tr>
<td>Neomycin B</td>
<td>3.13</td>
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<td>Sisomicin</td>
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<td>Paromomycin</td>
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<td>Lividomycin A</td>
<td>3.13</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≤0.39</td>
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a) The transconjugant strain E601 was obtained by ribostamycin selection in the first transfer experiment where S. marcescens S601 was used as donor strain and E. coli K12 W3104 as recipient strain, and produced acetyltransferase for amikacin.

b) The transconjugant S6017 was obtained in the retransfer experiment where E. coli E601 was used as donor strain and S. marcescens #7 as recipient strain.
12 AGs, the results with the representative strain E601 are shown in Table 2. MICs of AMK, KM, RSM, BUT, DKB, HAPA-B, and NM for E601 were higher than those for the recipient strain W3104, but they were lower than those for the donor strain S601. MIC of AMK for E601 (6.25 μg/ml) was one-eighth of that for S601 (50 μg/ml). Therefore, to determine if this transferable AGs resistance was responsible for the AMK-resistance phenotype of S. marcescens, retransfer of the resistance from E601 to S. marcescens strain #7 was attempted. MICs of 12 AGs for the resulting S. marcescens transconjugant S6017 are also shown in Table 2. The MIC of AMK for S6017 was much the same as that for the parental isolate S601. Resistance to KM, RSM, BUT, TOB, DKB, and HAPA-B was retransferred simultaneously with AMK resistance.

Plasmids in the transconjugants were analyzed by agarose gel electrophoresis. All of the 15 E. coli transconjugants of hospital A, including strain E601, and the S. marcescens transconjugant S6017 were found to contain a single plasmid showing the same migration rate. Profiles of the plasmids of representative strains E601 and S6017 are shown in Fig. 2. On the basis of the data from HindIII digestion analysis, all of the plasmids found in the 15 E. coli transconjugants were assumed to be molecularly identical (Fig. 3). HindIII digestion of the plasmids produced three cleavage fragments, estimated to be 34 Kb, 2.0 Kb, and 1.4 Kb in size. The molecular weight of the plasmid, therefore, was calculated to be approximately 24 megadaltons.

The substrate specificity of the acetyltransferase produced by E601 is shown in Table 3. The substrate specificity of this enzyme was consistent with that of

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Fig. 2. Plasmid DNA profiles of the transconjugant strains E601 and S6017, obtained by 1.0% agarose gel electrophoresis. (A) Plasmid DNA prepared from an amikacin-resistant isolate of S. marcescens designated as S601. (B) Plasmid DNA from an E. coli transconjugant called E601 resulting from the mating of E. coli strain W3104 with S. marcescens strain S601. (C) Chromosomal DNA fragments from the recipient strain W3104. (D) Plasmid DNA of strain E601. (E) Plasmid DNA from a S. marcescens transconjugant, S6017, resulting from the mating of S. marcescens strain #7 with strain E601. (F) Chromosomal DNA fragments of the recipient strain #7. The short arrows indicate the plasmid DNA bands and the long arrows show the position of chromosomal DNA fragments.
AMIKACIN RESISTANCE IN SERRATIA MARCESCENS

Transfer frequency of the plasmid was determined by RSM selection. The frequency of transfer from the S. marcescens isolate S601 to E. coli W3104 was low \((10^{-7})\) and that from E. coli E601 to E. coli C was 1,000 times higher \((10^{-4})\).

**Table 3. Substrate specificity of the acetyltransferase produced by Escherichia coli strain E601**

<table>
<thead>
<tr>
<th></th>
<th>Relative activity ((%))^a</th>
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<th>Relative activity ((%))^a</th>
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<th>Relative activity ((%))^a</th>
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<th>Relative activity ((%))^a</th>
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</thead>
<tbody>
<tr>
<td>KM</td>
<td>100</td>
<td>AMK</td>
<td>66</td>
<td>RSM</td>
<td>60</td>
<td>BUT</td>
<td>71</td>
<td>TOB</td>
<td>105</td>
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</table>

^a Percentage relative to kanamycin A as monitored by phosphocellulose paper binding assay.

^b Abbreviations of antibiotics: KM, kanamycin A; AMK, amikacin; RSM, ribostamycin; BUT, butirosin A; TOB, tobramycin; DKB, dibekacin; SISO, sisomicin; NM, neomycin B; GM, gentamicin; PRM, paromomycin; LVDM, lividomycin A.

the AAC(6')IV described by Wiedemann et al (30).

Transfer frequency of the plasmid was determined by RSM selection. The frequency of transfer from the S. marcescens isolate S601 to E. coli W3104 was low \((10^{-7})\) and that from E. coli E601 to E. coli C was 1,000 times higher \((10^{-4})\).

**Transformation**

Transformation of E. coli C or HB101 by the plasmid DNA prepared from a total of 25 isolates of the four hospitals was investigated. Conjugative AMK-resistant plasmids had not been detected in these 25 isolates. An AMK-resistant transformant of E. coli C, designated #51, was obtained from the crude lysate of one isolate from hospital C. This strain was shown to produce acetyltransferase for AMK. MICs of AMK, KM, RSM, BUT, TOB, DKB, HAPA-B, NM, and SISO for #51 were higher than those for the recipient strain (Table 4). From the resistance profile of strain #51, it was deduced that the acetyltransferase was a member of AAC(6'). On the basis of the data of agarose gel electrophoresis, we propose that
strain #51 acquired the plasmid of more than 50 megadaltons in molecular weight which was derived from the respective *Serratia* isolate (data not shown). This plasmid could not be transferred between *E. coli* strains. The transfer frequency was less than $2 \times 10^{-8}$.

Three RSM-resistant transformants of *E. coli* HB101, obtained from the crude lysates of three isolates from hospital A, were found to contain acetyltransferase for AMK. MIC determination showed that these transformants exhibited the same phenotype for resistance to 12 AGs as the transconjugant E601. The transformants were also resistant to ampicillin, though E601 and the other 14 transconjugants of hospital A were susceptible to several established antibiotics, e.g. ampicillin, chloramphenicol, tetracycline, and streptomycin. Conjugal transfer of the RSM resistance to *E. coli* recipients was not detected in the three transformants. The transfer frequencies ranged from $< 2 \times 10^{-7}$ to $< 8 \times 10^{-8}$. Plasmid analysis showed that all three of the transformants contained a single plasmid that appeared to be a derivative of the conjugative AMK-resistance plasmid of 24 megadaltons. That is to say, *HindIII* digestion of the plasmid DNAs in the three transformants generated three fragments, 40 Kb, 2.0 Kb, and 1.4 Kb (data not shown).

### Curing of AMK Resistance

Curing of AMK resistance was attempted with the 40 isolates. AMK-susceptible clones were not derived from the isolates other than those of hospitals A and C, which carried the conjugative or the nonconjugative AMK-resistance plasmids described above.

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**Table 4. Minimum inhibitory concentrations of 12 aminoglycoside antibiotics for amikacin-resistant transformant #51**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum inhibitory concentrations (µg/ml)</th>
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<tr>
<td></td>
<td>Transformant #51</td>
</tr>
<tr>
<td>Amikacin</td>
<td>50</td>
</tr>
<tr>
<td>Kanamycin A</td>
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<td>Ribostamycin</td>
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<td>Dibekacin</td>
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<tr>
<td>HAPA-B</td>
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<td>Neomycin B</td>
<td>6.25</td>
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<td>Paromomycin</td>
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<td>0.78</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>$\leq 0.39$</td>
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</table>

*a* The transformant strain #51 was obtained by transformation of *E. coli* C with the plasmid DNA prepared from *S. marcescens* S51.
DISCUSSION

It is believed that R plasmids and transposons play important roles in the increase in antibiotic-resistant bacteria. AMK is known to be a useful antibiotic in treatment of Serratia infection but several authors have reported hospital isolates which exhibit resistance to this antibiotic (1, 19, 24). Production of AAC(6')IV is considered to be a major mechanism of AMK resistance in gram-negative bacilli, and conjugative plasmids specifying the enzyme have been found in Pseudomonas aeruginosa (16) as well as in some organisms of the family enterobacteriaceae other than S. marcescens (2, 22, 25). Transposons coding for AAC(6')IV have also been found in P. aeruginosa (21) and E. coli (27). It may therefore be reasonable for a conjugative plasmid determining AAC(6')IV to be responsible for the dissemination of AMK resistance in S. marcescens. However, to our knowledge, no conjugative plasmid encoding AAC(6')IV has so far been demonstrated in S. marcescens. Several authors have reported the presence of AAC(6')IV or related enzymes in AMK-resistant strains of S. marcescens (3, 23, 26, 30); however it remains to be determined if a conjugative plasmid is associated with the enzyme.

In the present study, 40 AMK-resistant isolates of S. marcescens from four hospitals were examined for plasmids and modifying enzymes and it was demonstrated that a conjugative plasmid of 24 megadaltons encoding AAC(6')IV was responsible for the AMK resistance in the majority of isolates from one hospital. The 15 isolates carrying the AMK-resistance plasmid of 24 megadaltons were obtained from separate inpatients of two wards of the hospital during a period of 8 months. This indicates the intrahospital spread of AMK-resistant organisms of S. marcescens by clonal dissemination of a single strain or by transfer dissemination of the plasmid. This may be the first reported case where a conjugative R plasmid is associated with a persistent outbreak of AMK-resistant S. marcescens.

The 24-megadalton plasmid appears to be different in its resistance pattern from the other AAC(6')IV-bearing plasmids in gram negative bacilli which were described previously (16, 22, 25). That is to say, this plasmid does not confer resistance to several other established antibiotics but is only responsible for the resistance owing to AAC(6')IV. On the other hand, the plasmids previously described conferred resistance to streptomycin, tetracycline, chloramphenicol, etc.

During the last 7 years, we surveyed transferable AGs resistance in S. marcescens and determined that transferable AMK resistance is extremely rare in contrast to the other AGs. There are two probable reasons why epidemiological study to detect transferable AMK resistance is difficult. The first is the reduced expression of AMK resistance by AAC(6')IV in E. coli. In our study, the expression of AMK resistance of the 24-megadalton plasmid appeared to be lower in the E. coli K12 strain that was used as a recipient than in the S. marcescens strains. Therefore, the transconjugants were not detected satisfactorily until RSM was used as a selective antibiotic in place of AMK. A similar observation was reported with the plasmid-coded AAC(6'), which determined a high level of resistance to KM, TOB, AMK,
and NM in *P. aeruginosa*, but only a relatively low level to KM in *E. coli* (9). The second reason is the low frequency of transfer of plasmids from *S. marcescens* to *E. coli* K12. The frequency of transfer of the 24-megadalton plasmid from the *S. marcescens* isolate to the *E. coli* K12 strain was very low, only 0.1% of that between *E. coli* strains. Several authors have reported that *E. coli* is not always an efficient recipient of R plasmid from *S. marcescens* in mating experiments (11).

We are most grateful to the members of the clinical microbiology laboratories of The University of Tokyo Hospital, Tokyo Medical and Dental University Hospital, Saiseikai Utsunomiya Hospital, and Teikyo University Hospital for supplying the isolates of *S. marcescens*. We also thank the personnel of the laboratories of Sankyo Co., Ltd., Japan, Kowa Co., Ltd., Japan, Essex Nippon Co., Ltd., Japan, Toyo Jozo Co., Ltd., Japan, and Kyowa Hakko Kogyo Co., Ltd., Japan for providing antibiotics.

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