

Sequence-Based Typing of *adeB* as a Potential Tool To Identify Intraspecific Groups among Clinical Strains of Multidrug-Resistant *Acinetobacter baumannii*

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Sequence analysis of an 850-bp fragment internal to the aspecific drug efflux gene *adeB* revealed 11 sequence types (STs) among a collection of 50 multidrug-resistant *Acinetobacter baumannii* (MDRAB) strains, including members of pan-European clones I, II, and III. The delineation of STs conformed with the intraspecific grouping of these strains previously determined by different DNA fingerprinting methods. Larger strain collections need to be screened to further explore the potential of sequence-based *adeB* typing as a universal tool for the monitoring of MDRAB clones.

Acinetobacter baumannii is one of the most frequently isolated nonfermentative gram-negative species from critically ill and immunocompromised patients among intensive care units patients worldwide. Strains of this opportunistic pathogen can be involved in a range of nosocomial infections, such as ventilator-associated pneumonia, bloodstream infections, and meningitis (1), and are acquiring resistance to multiple antibiotics at an increasing rate (4, 9). *A. baumannii* infections can occur as sporadic cases associated with single genotypes but can also give rise to epidemic outbreaks caused by genotypically highly related isolates, such as those belonging to the pan-European (pE) multidrug-resistant *A. baumannii* (MDRAB) clones I, II, and III (3, 18). Thus far, the intraspecific differentiation of MDRAB has mainly relied on the single or combined use of DNA fingerprinting methods, including ribotyping, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments, and repetitive DNA element PCR (rep-PCR) (2, 3, 7, 8, 13, 18). Apart from differences in genotypic resolution, labor intensity, and reproducibility, all these techniques share the major drawback that interlaboratory comparison remains problematic because fingerprinting data are generally not portable. DNA sequence-based methods, on the other hand, facilitate unambiguous and global comparisons of the findings between different laboratories and are expected to outcompete banding pattern-based methods in long-term epidemiological studies (17). As opposed to other human pathogens, however, the search for genes that can be used in single or multilocus sequence typing (MLST; <http://www.mlst.net>) at the intraspecific or strain level is ongoing for *A. baumannii*. In previous papers (7, 8, 15), we reported that members of pan-European MDRAB clones I, II, and III as well as a number of genotypically unrelated MDRAB strains all shared the aspecific drug

efflux gene *adeB*, together with various combinations of genes that specifically confer resistance to aminoglycosides (AGs) and tetracyclines. The *adeB* gene makes up part of the *adeABC* gene cluster, which encodes a resistance-nodulation-cell division-type three-component efflux system that was recently described for *A. baumannii* strain BM4454 (10). In this strain, *adeABC* conferred low-level resistance to several aminoglycosides and reduced susceptibilities to various other antimicrobial agents. Triggered by the observation that the *adeB* gene was present in a set of genotypically related and unrelated MDRAB strains from different geographical origins and time periods, the current study set out to investigate whether *adeB* is a suitable locus for sequence-based identification of intraspecific groups among MDRAB strains.

For the purpose of this study, 50 well-documented MDRAB strains mostly from European hospitals were selected from previous studies in which they were typed by one or multiple DNA fingerprinting methods and in which it was shown that they contained the *adeB* gene (Table 1). This selection included members of pE clones I, II, and III but also clinical strains from the Czech Republic previously allocated to three ribotypes, five genotypically unrelated clinical strains, and veterinary isolate LMG 22458. Most of the MDRAB selected strains were resistant to one or multiple aminoglycosides (Table 1), to several fluoroquinolones (18), and to tetracycline (7, 8). Susceptibility to aminoglycosides was determined by the disk diffusion method with BBL Mueller-Hinton II agar (Becton Dickinson and Company), according to the recommendations of CLSI (formerly NCCLS) (12), by using the following disks (Oxoid Ltd., Basingstoke, United Kingdom): kanamycin (30 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), and netilmicin (30 µg). Total genomic DNA was prepared by using a protocol based on the method of Pitcher and co-workers (16). For *adeB* detection, a common PCR core mix (total volume, 50 µl) was used that consisted of 1× PCR buffer (Applied Biosystems, Warrington, United Kingdom), deoxynucleoside triphosphates (dNTPs; Applied Biosystems) at a concentration of 200 µM of each dNTP, 1 U of *AmpliTaq*

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TABLE 1. Typing and AG resistotype data for 50 multidrug-resistant *A. baumannii* strains^a

Original strain no.	LMG deposit no.	Geographical origin	Yr of isolation	Formerly typed as:	Typing method(s) used [reference(s)]	adeB ST	AG resistotype
UZG S91 01483		Gent, B	1991	pE clone I	(GTG) ₅ -PCR (8)	I	K-G ^a
RUH 3247	LMG 22453	Leuven, B	1990	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G-N-T ^b
NIPH 470		Č. Budějovice, CR	1997	pE clone I	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	I	K-N-A ^b
NIPH 7	LMG 22454	Prague, CR	1991	pE clone I	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	I	K-G-A ^b
NIPH 290		Příbram, CR	1994	pE clone I	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	I	K-G-N-T-A ^b
NIPH 1605		Sedláčany, CR	2001	pE clone I	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	I	K-G-T ^b
RUH 436	LMG 10539	Utrecht, NL	1984	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
RUH 2037		Venlo, NL	1986	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
RUH 875	LMG 10543	Dordrecht, NL	1984	pE clone I ^c	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G-T ^b
RUH 510		Nijmegen, NL	1984	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
RUH 3242	LMG 10523	Basilidon, UK	1989	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
RUH 3239		London, UK	1980s	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
RUH 3282		Salford, UK	1990	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G-N-A ^b
RUH 3238		Sheffield, UK	1987	pE clone I	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	I	K-G ^b
HPA A2166		Hospital A, UK	2000	pE clone I	(GTG) ₅ -PCR (8)	I	K-G ^b
HPA A0661	LMG 22456	Hospital J, UK	2000	pE clone I	(GTG) ₅ -PCR (8)	I	K-G-A ^a
DGK 4982	LMG 22458	Gent, B	1998	pE clone II	(GTG) ₅ -PCR (8)	II	K-G ^a
UZG POR 03034		Gent, B	Unknown	pE clone II	(GTG) ₅ -PCR (8)	II	K ^a
NIPH 330		Tábor, CR	1994	pE clone II	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	II	K-G ^b
NIPH 455		Jihlava, CR	1996	pE clone II	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	II	K-G-A ^b
NIPH 1469		Prague, CR	2001	pE clone II	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	II	K-A ^b
NIPH 24	LMG 22457	Prague, CR	1991	pE clone II	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	II	K-G ^b
RUH 3422		Odense, DK	1984	pE clone II	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	II	K ^b
BM4454		Paris, F	1999	pE clone II	(GTG) ₅ -PCR (8)	II	— ^a
RUH 134	LMG 10541	Rotterdam, NL	1982	pE clone II ^c	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	II	K-G ^b
RUH 3240		Newcastle, UK	1989	pE clone II	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	II	G-N ^b
RUH 3245		Salisbury, UK	1989	pE clone II	AFLP (3, 13), ribotyping (3, 13), (GTG) ₅ -PCR (8)	II	G-N ^b
HPA A2161		Hospital A, UK	2000	pE clone II	(GTG) ₅ -PCR (8)	II	K-G-N-A ^a
HPA A0372	LMG 22460	Hospital H, UK	2000	pE clone II-like	(GTG) ₅ -PCR (8)	II	K-G-N-A ^a
UZG S91 00973		Gent, B	1991	pE clone III	(GTG) ₅ -PCR (7)	III	K-G-A ^a
UZG S91 02796	LMG 22452	Gent, B	1991	pE clone III	(GTG) ₅ -PCR (7)	III	K-G-A ^a
UZG F93 03448	LMG 22863	Gent, B	1993	pE clone III	(GTG) ₅ -PCR (7)	III	K-G-A ^a
06A201		Lille, F	1997	pE clone III	AFLP (14, 18), ribotyping (14), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
04C048		Paris, F	1997	pE clone III	AFLP (14, 18), ribotyping (14), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
10C070		Genova, I	1998	pE clone III	AFLP (14, 18), ribotyping (14), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
12A133		Utrecht, NL	1997	pE clone III	AFLP (14, 18), ribotyping (14), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
18D047		Barcelona, S	1997	pE clone III	AFLP (18), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
17C085		Madrid, S	1998	pE clone III	AFLP (18), (GTG) ₅ -PCR (7)	III	K-G-T-A ^b
16D083		Sevilla, S	1997	pE clone III	AFLP (18), (GTG) ₅ -PCR (7)	III	— ^a
NIPH 1717		Prague, CR	2001	Ribotype R2-6	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	IV	K-G-T-A ^b
NIPH 301	LMG 22459	Slany, CR	1994	Ribotype R2-7	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	IV	K-G-N ^b
NIPH 335		Tábor, CR	1994	Ribotype R21-16	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	V	K-G-T ^b
NIPH 1445		Pízeň, CR	2000	Ribotype R21-16	AFLP (13), ribotyping (13)	V	K-G-T ^b
NIPH 1497		Prague, CR	2001	Ribotype R23-19	AFLP (13), ribotyping (13)	VI	K-G-N-T ^b
NIPH 1683		Prague, CR	2001	Ribotype R23-19	AFLP (13), ribotyping (13)	VI	K-G ^b
HPA A1100		Hospital G, UK	2000	Ungrouped	(GTG) ₅ -PCR (8)	VII	K-A ^a
HPA A1392	LMG 22461	Hospital I, UK	2000	Ungrouped	(GTG) ₅ -PCR (8)	VIII	— ^a
AC 658		São Paulo, BR	1997	Ungrouped	(GTG) ₅ -PCR (8)	IX	K-G-A-N ^a
NIPH 1734		Mladá Boleslav, CR	2001	Ribotype R24-20	AFLP (13), ribotyping (13)	X	K-G-N-T-A ^b
NIPH 47		Prague, CR	1991	Ribotype R8-1	AFLP (13), ribotyping (13), (GTG) ₅ -PCR (8)	XI	K-G-T ^b

^a Data from this study.^b Data from reference 14.^c pE clone reference strain proposed by Dijkshoorn et al. (3).^d Abbreviations: B, Belgium; BR, Brazil; CR, Czech Republic; DK, Denmark; F, France; I, Italy; NL, The Netherlands; S, Spain; DGK, Faculteit Diergeneeskunde, Ghent University, Ghent, Belgium; HPA, Health Protection Agency, London, United Kingdom; LMG, BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium; NIPH, Collection of A. Nemec, National Institute of Public Health, Prague, Czech Republic; RUH, Collection of L. Dijkshoorn, Leiden University Medical Center, Leiden, The Netherlands; UZG, Universitair Ziekenhuis Gent, Ghent, Belgium; —, no resistance to the five AGs tested.

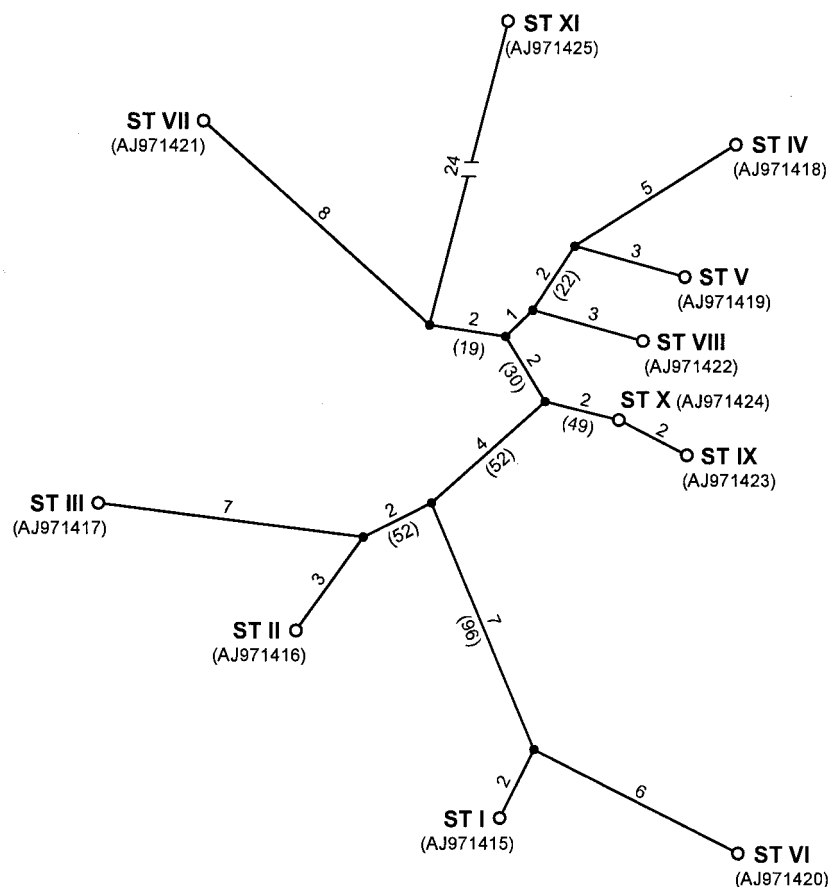


FIG. 1. Unrooted maximum-parsimony tree of multiple aligned partial *adeB* sequences representing 11 STs, indicated by capital Roman letters I to XI followed by the EMBL accession number in parentheses. The number of base conversions over the tree is indicated along the phylogenetic distance lines, and bootstrap percentages are indicated in parentheses for analysis of 100 replicates.

DNA polymerase (Applied Biosystems), and 20 pmol of each primer (Sigma-Genosys Ltd., Cambridgeshire, United Kingdom). A 50-ng portion of intact total DNA was used as the PCR template. Detection and partial sequence analysis of *adeB* were performed with the previously published PCR primer pair O3 (5'-GTATGAATTGATGCTGC-3') and O4 (5'-CACTCGTAGCCAATACC-3') that targets a 979-bp fragment internal to this gene in *A. baumannii* strain BM4454, which was used as a positive control in PCR (10). PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) by using the following temperature program: initial denaturation at 94°C for 5 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Partial sequencing of *adeB* positions 1635 to 2484 was performed by using the BigDye Terminator (version 3.1) ready reaction cycle sequencing kit on an ABI Prism 3100 genetic analyzer (Applied Biosystems). As a control, the sequence of the internal *adeB* fragment of *A. baumannii* strain BM4454 (EMBL accession no. AF370885) (10) was redetermined. Sequence alignments and comparisons were performed by using BioNumerics software (version 3.5; Applied Maths, St.-Martens Latem, Belgium) and the BioEditor program (5).

In the present study, the previously designed primer pair

O3-O4 (10) was used for sequence analysis of an internal region of the *adeB* gene in order to investigate whether this gene contains polymorphic sites that are potentially useful for sequence-based identification of intraspecific groups in MDRAB. As a result of this partial sequencing approach, 11 different *adeB* sequence types (STs; STs I to XI) could be defined (Fig. 1) among a collection of 50 MDRAB strains representing six previously delineated intraspecific groups and five genotypically unrelated strains. By definition, an *adeB* gene was considered to represent a distinct ST when its 850-bp partial sequence differed in at least one position from all other sequences. STs that were shared by two or more strains (i.e., STs I to VI) all displayed a complete internal sequence identity, whereas the number of base conversions between individual STs ranged from 2 to 45 (Fig. 1). Although the majority of these conversions were found to represent silent mutations, a number of substitutions resulted in amino acid sequence polymorphisms (Table 2). The polymorphic sites at positions 551, 584, 606, and 730 may be of diagnostic value for discrimination of pE clones I, II, and III; but clearly, more strains of these intraspecific MDRAB groups need to be investigated to verify this finding. The partially redetermined nucleotide sequence of the *adeB* gene of strain BM4454 (10) differed from the original sequence (EMBL accession no. AF370885) at two positions,

TABLE 2. Polymorphic amino acid positions for 11 *adeB* STs in *A. baumannii*

<i>adeB</i> ST	Amino acid at the following polymorphic amino acid position ^a :							
	551	584	606	642	645	660	730	768
I	A	T	A	D	S	P	L	A
II	T	T	T	D	S	P	F	A
III	A	I	T	D	S	P	L	A
IV	A	T	T	A	T	P	L	A
V	A	T	T	A	T	Q	L	A
VI	A	T	T	D	S	P	L	A
VII	A	T	T	D	T	P	L	T
VIII	A	T	T	A	T	P	L	A
IX	A	T	T	A	T	P	L	A
X	A	T	T	A	T	P	L	A
XI	A	T	T	D	T	P	L	A

^a Aligned nucleotide sequences of partial *adeB* fragments (positions 1635 to 2484 according to the numbering of the *adeB* sequence with EMBL accession no. AF370885) were translated into amino acid sequences by using a polymorphism statistics tool in Bionumerics. Polymorphic amino acid positions were identified with the BioEdit sequence alignment editor.

i.e., positions 1974 and 2295, where in both cases the sequence representing ST II (EMBL accession no. AJ971416) contained an A instead of a G.

The delineation of the 11 *adeB* STs completely agreed with the intraspecific diversity among the 50 MDRAB strains previously assessed by DNA fingerprinting methods such as ribotyping, AFLP analysis, PFGE, and/or rep-PCR. The most remarkable finding was that all members of pE clones I ($n = 16$), II ($n = 13$), and III ($n = 10$) belonged to the same *adeB* ST, i.e., STs I, II, and III, respectively (Table 1). The strains of pE clones I, II, and III included for this study were selected in a way that they represented hospital units from four to six different European countries and that they were isolated at different time points during the past 20 years. The fact that members of a given pE clone with different geographical and/or temporal histories all shared the same *adeB* ST thus indicates that sequence-based typing of this gene may be a potential tool for the quick identification of new members of these widespread clones. As evidenced by the data obtained with the Czech MDRAB strains previously allocated to HindIII-HincII ribotyping groups R21-16 and R23-19 and, accordingly, also grouped in two AFLP clusters (13) (Table 1), partial *adeB* sequencing may also have the potential to identify less widespread intraspecific MDRAB groups. The two representative strains of both groups displayed an identical *adeB* ST; and the two corresponding STs, STs V and VI, appeared to be unique and clearly distinct from STs I to III (Fig. 1). Interestingly, strains NIPH 1717 and NIPH 301, which were previously assigned to the highly related ribotypes R2-6 and R2-7 and that grouped in the same AFLP cluster (13), were also joined together by *adeB* typing as members of ST IV (Table 1). The finding that the five genotypically unrelated MDRAB strains from the Czech Republic, the United Kingdom, and Brazil (Table 1) each exhibited a unique *adeB* ST may indicate that these strains represent distinct intraspecific lineages in *A. baumannii* (Fig. 1); but further evidence, e.g., evidence based on MLST, is awaited to substantiate this. The fact that strains AC 658 and NIPH 1734, which represent the closely related *adeB* STs IX and X (Fig. 1), respectively, also displayed highly sim-

ilar but not identical (GTG)₅-PCR band patterns (G. Huys, unpublished data) again illustrates the good overall agreement found between the *adeB* sequence-based grouping and the intraspecific grouping based on DNA fingerprinting. On the other hand, our sequencing data also indicate that the 850-bp region of the *adeB* gene analyzed in this study is probably not variable enough to discriminate MDRAB strains at the sub-clonal level. For example, clone II strains NIPH 1469 and RUH 3240 shared a ribotype (ribotype R4-2) slightly different from the other clone II ribotypes and grouped in a distinct AFLP subcluster of clone II (13), and yet, both strains belonged to ST II (Table 1). Possibly, sequencing of longer or multiple fragments of *adeB* or other components of the *adeABC* gene cluster may increase the resolution of the method.

At present, little is known about the distribution and evolutionary history of the *adeB* gene in *A. baumannii*. So far, the gene has mainly been detected in *A. baumannii* outbreak strains (6, 7, 8) that exhibit resistance to multiple drugs, usually including one or more AGs. Recently, it was found that *adeB* can be up-regulated in MDRAB (6) and that it can also occur in drug-susceptible *A. baumannii* strains (15), in which the gene may be cryptic due to the regulatory control by the two-component AdeRS system (11). Because of this stringent control, it is difficult to predict the presence of *adeB* in *A. baumannii* based on the AG resistance profile. In addition, many European MDRAB strains are known to carry one or several specific AG resistance genes (14) that can potentially reinforce the AG resistance spectrum encoded by *adeB*. This effect is highly pronounced for pE clones I and II, in which the AG resistotypes differed significantly among *adeB*-positive strains (Table 1). In the course of a PCR-based screening survey for *adeB* in 32 environmental *A. baumannii* isolates of aquatic and terrestrial origins, it was found that none of the strains investigated harbored the efflux gene (Huys, unpublished). Provided that the use of additional (degenerated) primer sets confirm these findings, this suggests that the gene is probably not omnipresent in *A. baumannii* and was acquired by a number of strains from an exogenous source at some point in time. For this reason, it is expected that *adeB* typing will primarily be useful for clinical isolates of this species.

Based on partial sequencing analysis, the present study has demonstrated that the delineation of *adeB* STs among genotypically related and unrelated MDRAB strains corroborated extremely well the genotypic clustering of these strains previously obtained with various banding pattern-based methods. However, it is clear that more extended collections of clinical and environmental isolates need to be investigated in order to obtain better insight into the distribution of the *adeB* gene among *A. baumannii* strains and to further validate the use of *adeB* typing for the rapid identification of known intraspecific groups or the delineation of new intraspecific lineages in this species. When it is integrated in a dynamic Internet-based platform, sequence-based typing of *adeB* could prove to be a useful tool for worldwide monitoring of MDRAB clones.

Nucleotide sequence accession numbers. A selection of the sequences representing the 11 *adeB* sequence types reported in this study have been submitted to EMBL under accession numbers AJ971415 to AJ971425 (Fig. 1).

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