Application of the Growth Inhibition Test to *Spiroplasma* Taxonomy

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A total of 33 triply cloned *Spiroplasma* strains were studied by the conventional growth inhibition test on M1A or M1D agar plates. Sera were prepared to 17 of the isolates, representing five major groups and the four subgroups (subgroups I-1, I-2, I-3 and I-4) of the *Spiroplasma citri* complex. Zone sizes varied with the titer and the growth rate of the spiroplasma cultures used as antigens. Test results were standardized by recording zone widths that developed on plates inoculated with cultures containing about 10^5 colony-forming units per ml. Zone widths in homologous tests ranged from 5 to 19 mm, but were usually at least 10 mm for all but the fastest growing spiroplasmas. Nonspecific zones 1 to 4 mm in width were observed rarely in heterologous crosses between major serogroups, but were characteristic of certain individual strain-antibody combinations and were not observed when other strains of the group or subgroup were examined. Heterologous reactions resulting in zones 2 to 15 mm wide among the four subgroups of the *S. citri* complex (group I) were observed; such partial reactions were characteristic of all strains within each subgroup. Deoxyribonucleic acid hybridization experiments have shown that major *Spiroplasma* groups are distinct and can be considered to be putative species. Therefore, growth inhibition serology apparently functions in the *Spiroplasmataceae* as a test applicable for differentiation at the species level, as it does in the *Mycoplasmataceae*.

At its meeting in Glasgow in 1976, the Subcommittee on Taxonomy of *Mollicutes* initiated (43) an ad hoc working group to delineate characters suitable for differentiating and classifying strains of the genus *Spiroplasma*. Some of the work of this team, especially that concerning one-dimensional gel electrophoresis (14) and two-dimensional gel electrophoresis (31, 32), has been published. Work involving the spiroplasma genome, including an analysis of the guanine-plus-cytosine contents of isolates and deoxyribonucleic acid (DNA)-DNA homologies, has also been completed (3, 4, 25). Since the 1976 meeting, spiroplasma isolates have accumulated rapidly, and the need for adequate serological methods for the identification and classification of these isolates is more pressing today than ever.

The growth inhibition (GI) test has been employed regularly for the classification of mycoplasmas at the species level (13, 58). This test has also been applied, with limited scope, to differentiation of strains of the genus *Spiroplasma* (6, 15-19, 36, 39, 41, 44-49, 51, 54). The GI test and modifications of it are a result of the original observations of Nicol and Edward (33)

and Edward and Fitzgerald (21) that antisera, when incorporated into the growth media of mycoplasmas, inhibited not only the growth of the mycoplasma against which the serum was directed but also that of closely related strains. This concept was adapted by Huijman-Evers and Ruys in 1956 (22) and modified in detail by Clyde (13). In Clyde's modified procedure, serum-saturated disks were placed on the surfaces of inoculated agar plates. Today, the disk modification of the GI test is the most commonly employed serological method for separation of *Mycoplasma* species (42).

In this study, we examined 33 triply cloned *Spiroplasma* strains and sera prepared to 17 of the strains to assess the resolution provided by application of the disk GI test to questions of *Spiroplasma* taxonomy.

MATERIALS AND METHODS

***Spiroplasma* strains and culture conditions.** *Spiroplasma* strains (Table 1) were obtained from various sources. Many of the strains chosen were used in the deformation and metabolism inhibition tests previously reported (55, 57). Classical strains (subgroup I-1) of *Spiroplasma citri* (strains R8A2 [= Maroc] and C189) were taken from freeze-dried samples held as vouch-

TABLE 1. *Spiroplasma* strains studied

Group	Scientific or common name	Isolate	ATCC no. ^a	Source
I-1	<i>S. citri</i>	Maroc (= R8A2)	27556	Citrus (Morocco)
		C189	27665	Citrus (California)
		Israel		Citrus (Israel)
		Cactus	29594	<i>Opuntia</i> (?)
		Lettuce	29747	Lettuce (?)
		Scaph		<i>Scaphytopius</i> leafhoppers (California)
		SCC		<i>Circulifer</i> leafhoppers (California)
		Algeria		Citrus (Algeria)
		L-163		Citrus (California)
		Iran		Citrus (Iran)
I-2	Honey bee spiroplasma	BC-3	33219	Honey bee (Maryland)
		AS 576	29416	Honey bee (Maryland)
		KC-3		Honey bee (Hawaii)
		G-1		Flower (Florida)
		G-13		Honey bee (Florida)
		L-V RV		Flower (Maryland)
I-3	Corn stunt spiroplasma	E275	29320	Maize (<i>Drosophila</i>)
		Miss E		Maize (Mississippi)
		B655	33289	Maize (Jamaica)
		I-747	29051	Maize (laboratory)
I-4	277F spiroplasma	277F	29761	Rabbit tick (Montana)
III	<i>S. floricola</i>	23-6	29989	Flowers (Maryland)
		OBMG	33221	Flowers (Maryland)
		BNR1	33220	Flowers (Maryland)
IV	"Flower" spiroplasmas	SR 3	33095	Flowers (Maryland)
		L-89		Spittlebug (Corsica)
		PPS1	33450	Flowers (Florida)
		B-13		Honey bee (Morocco)
		F-1		Flowers (France)
		F-2		Flowers (France)
		F-25		Flowers (Corsica)
V	<i>S. mirum</i>	SMCA	29335	Rabbit tick (Georgia)
		GT-48	29334	Rabbit tick (Georgia)
		TP-2	33503	Rabbit tick (Maryland)

^a ATCC, American Type Culture Collection, Rockville, Md.

TABLE 2. Reciprocal GI tests with antigens and antisera of group I strains

Antiserum		Antigen									
Subgroup	Strain	Subgroup I-1 (citrus stubborn spiroplasma)									
		Maroc	C189	Israel	Cactus	Lettuce	Scaph	SCC	Algeria	L-163	Iran
I-1	Maroc	16 ^a	15	7	16	16	13	16	11	13	15
	C189	12	12	10	13	12	7	14	9	11	14
	Israel	14	11	7	12	12	12	13	11	14	18
	Cactus	17	17	9	11	17	14	15	10	12	20
	Lettuce	16	17	11	16	17	15	16	10	13	15
I-2	BC-3	12	12	10	10	11	13	15	10	13	12
I-3	E275	12	11	5	10	10	7	11	6	6	12
	Miss E	11	11	5	11	10	9	11	7	10	16
I-4	277F	3	3	1	2	3	0	0	0	0	0

^a Width (in millimeters) of zone of inhibition, measured from the edge of the disk.

ers from an earlier study (37). Eight other subgroup I-1 strains were utilized. The triply cloned Israel strain was derived from a strain that was furnished by M. Daniels et al. from their studies (29) and that had been triply cloned and freeze-dried in the Beltsville laboratory. "Cactus" and "lettuce" spiroplasmas, which were claimed to have been isolated from *Opuntia tuna* subsp. *monstrosa* (F. Kondo, A. H. McIntosh, S. B. Padhi, and K. Maramorosch, Proc. Soc. Gen. Microbiol. 3:154, 1976) and commercial lettuce (8), respectively, were derived from ATCC 29594 and ATCC 29747 cultures. The Scaph, SCC, Algeria, and Iran strains were obtained from the collection of P. H. M. Saglio; strain L-163 was obtained from A. Granett. These five strains were triply cloned in Beltsville. The BC-3 and KC-3 strains of honey bee spiroplasma (subgroup I-2) were obtained from lines studied (10, 11) by T. B. Clark. The AS 576, G-1, G-13, and BW strains of subgroup I-2 were obtained from R. E. Davis. The E275 and Miss E strains of subgroup I-3 corn stunt spiroplasma were derived from lines maintained in previous studies (24, 56). The B655 isolate (Jamaica) of corn stunt spiroplasma (20) was triply cloned and preserved in the Beltsville laboratory after receipt as an uncloned isolate from S. Eden-Green. The single subgroup I-4 strain, 277F spiroplasma (5, 34, 39) from W. Burgdorfer, was triply cloned and preserved at the Bethesda laboratory. The type strain (strain 23-6) of *Spiroplasma floricola* (group III) (18) was furnished by R. E. Davis. The OBMG and BNR1 strains of *S. floricola* were furnished by T. B. Clark from his studies (11). Seven representatives of group IV spiroplasmas were used. The SR 3 strain was obtained from R. E. Davis, and the PPS1 strain was obtained from R. E. McCoy. The L-89, B-13, F-1, F-2, and F-25 strains were derived from field isolates obtained in the Mediterranean region (50), but were later studied (44) and preserved in the Bethesda laboratory. The SMCA and GT-48 strains of *Spiroplasma mirum* (48) (group V) were from the Bethesda laboratory (47, 49), whereas the TP-2 strain (41) was cloned and preserved in the Beltsville laboratory.

Group V strains were maintained in and plated onto SP-4 medium (47). All other *Spiroplasma* isolates were maintained in and plated onto M1D medium; in this

variation of M1A medium (24), the fresh yeast extract component was replaced by sterile water.

Disk GI method. Liquid and solid media were prepared as previously reported. Cultures growing in SP-4 or M1D medium were diluted in 10-fold steps. Four dilutions were selected that were expected to optimize the chance of including at least one dilution that yielded about 10^5 colony-forming units per ml. In many cases, cultures were examined by dark-field microscopy, and preliminary counts of numbers of helical organisms per field were used to estimate the probable numbers of viable spiroplasma cells in the cultures (53). Samples (0.1 ml) of each of the four dilutions were plated onto 15-cm plates. A bent glass stirring rod was used to distribute the inoculum evenly on the plates. The inoculated plates were then allowed to stand for about 30 min at room temperature in a laminar flow hood. The antiserum-saturated disks were then placed on the surface of the agar at the center of the dried inoculated plates. The disks were saturated beforehand with 0.025 ml of antiserum dispensed with a calibrated micropipette. After preparation, the disks were held at 4°C and could be stored for as long as several months (40).

Most plates were incubated at 30°C, but plates inoculated with group V or subgroup I-4 spiroplasmas were incubated at 37°C, and plates inoculated with *S. citri* were held at 32°C. Plates inoculated with spiroplasmas from subgroups I-2 and I-4, and groups III and IV were incubated aerobically. Plates inoculated with other spiroplasmas were incubated in a mixture containing 95% N₂ and 5% CO₂.

Zones were measured after appropriate incubation times, which varied with each *Spiroplasma* strain. For example, group III and group IV strains formed colonies rapidly, and the reactions of these strains could be read within 7 days. On the other hand, group V strains and the B655 strain of subgroup I-3 grew much more slowly, and these strains were incubated for at least 3 weeks before reading. Colony morphology varied with each strain. Strains that grew poorly tended to form discrete colonies, whereas strains that grew rapidly during the logarithmic growth phase, producing short helical cells with only a few turns, tended to form diffuse colonies with indistinct boundaries.

TABLE 2—Continued

Antigen										
Subgroup I-2 (honey bee spiroplasma)						Subgroup I-3 (corn stunt spiroplasma)				Subgroup I-4 (277F spiroplasma)
BC-3	AS 576	KC-3	G-1	G-13	BW	E275	Miss E	I-747	B655	
10	6	7	9	6	5	9	8	13	15	1
8	5	6	6	5	6	8	9	8	14	1
9	6	7	7	5	5	13	4	12	13	0
9	6	8	8	7	7	8	9	16	13	1
9	7	9	9	7	6	8	7	13	15	1
12	7	9	8	9	8	12	12	20	18	2
4	5	2	3	2	0	18	18	21	19	9
4	4	5	5	4	4	16	16	20	20	8
0	0	0	0	0	0	3	3	8	3	14

TABLE 3. Reciprocal GI tests with antigens and antisera of group III, IV, and V spiroplasmas

Antiserum		Antigen												
Group	Strain	Group III (<i>S. floricola</i>)			Group IV							Group V (<i>S. mirum</i>)		
		23-6	BNR1	OBMG	SR 3	L-89	PPS1	B-13	F-1	F-2	F-25	SMCA	GT-48	TP-2
III	BNR1	5 ^a	5	5	0	0	0	0	0	0	0	0	0	0
	OBMG	6	6	6	0	0	0	0	0	0	0	0	0	0
IV	SR 3	0	0	0	5	4	3	5	6	4	6	0	0	0
	PPS1	0	0	0	5	5	6	5	7	5	7	0	0	0
	L-89	0	0	0	7	6	5	5	9	4	6	0	0	0
V	SMCA	0	0	0	0	0	0	0	0	0	0	13	15	15
	GT-48	0	0	0	0	0	0	0	0	0	0	13	17	12
	TP-2	0	0	0	0	0	0	0	0	0	0	9	17	10

^a Width (in millimeters) of zone of inhibition, measured from the edge of the disk.

TABLE 4. GI tests with antisera to group III, IV, and V spiroplasmas tested against group I antigens

Antiserum		Antigen												
Subgroup	Strain	Group III (<i>S. floricola</i>)			Group IV							Group V (<i>S. mirum</i>)		
		23-6	OBMG	BNR1	SR 3	L-89	PPS1	B-13	F-1	F-2	F-25	SMCA	GT-48	TP-2
I-1	Maroc	0 ^a	0	0	0	0	0	0	0	0	0	0	0	0
	C189	0	0	0	0	0	0	0	0	0	0	0	0	0
	Israel	0	0	0	0	0	0	0	0	0	0	0	0	0
	Cactus	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lettuce	0	0	0	0	0	0	0	0	0	0	0	0	0
I-2	BC-3	0	0	0	0	0	0	0	0	0	0	0	0	0
I-3	E275	0	0	0	0	1.5	0	0	0	0	0	0	0	0
	Miss E	0	0	0	0	0	0	0	0	0	0	0	0	0
I-4	277F	0	0	0	0	0	0	0	0	0	0	0	0	0

^a Width (in millimeters) of zone of inhibition, measured from the edge of the disk.

TABLE 5. GI tests with group I antigens tested against antisera of group III, IV, and V spiroplasmas

Antiserum		Antigen									
Group	Strain	Subgroup I-1 (citrus stubborn spiroplasma)									
		Maroc	C189	Israel	Cactus	Lettuce	Scaph	SCC	Algeria	L-163	Iran
III	BNR1	0 ^a	0	0	0	0	0	0	0	0	0
	OBMG	0	0	0	0	0	0	0	0	0	0
IV	SR 3	0	0	0	0	0	0	0	0	0	2
	PPS1	0	0	0	0	0	0	0	0	0	2
	L-89	0	0	0	0	0	0	0	0	0	0
V	SMCA	0	0	0	0	0	0	4	0	4	1
	GT-48	0	0	0	0	0	2	0	0	0	0
	TP-2	0	0	0	0	0	0	0	0	0	0

^a Width (in millimeters) of zone of inhibition, measured from the edge of the disk.

RESULTS

Although colonial morphology varied, homologous sera invariably produced discrete zones of inhibition. Homologous zones (Tables 2 and 3) varied in width from 5 or 6 to 18 mm, depending on the strain, and, to a lesser extent, on the potency of the specific antiserum. For example, most group III and IV strains grew very rapidly, with colonies apparent within 3 days; such fast growth corresponds to doubling times of about 2 h in liquid media (R. F. Whitcomb and M. E. Coan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, G18 p. 79). With these strains, homologous GI zones were only 5 to 6 mm in diameter. On the other hand, homologous zones up to 18 mm wide were obtained with spiroplasma strains that grew especially slowly, such as all group V strains and the B⁷5 strain of subgroup I-3.

None of the strains of the group I (*S. citri*) complex crossed significantly with strains of any other group (Tables 4 and 5). On the other hand, discrete zones, which were smaller than their homologous counterparts, were consistently observed in heterologous crosses within the group I complex (Table 2). It was not surprising to find such less intense heterologous crossing, because the members of this group have been shown to possess varying degrees of interrelatedness as determined by DNA-DNA homology (3, 4, 7, 25, 26, 28, 35) and polyacrylamide gel electrophoresis (14, 31, 32). Heterogeneity in intragroup crossing within group IV was less apparent (Table 3).

The heterologous reactions within the group I complex followed the general patterns that were expected from results involving DNA-DNA homology. Mean zone widths were computed from the reaction grids within each serological subgroup of group I and were used as rough estimates of homologous serological cross-reactivities. For example, the 50 cross-reactions of *S. citri* strains versus *S. citri* antisera gave a mean

zone width of 13.3 mm. These means could then be used to construct a table of relative cross-reactivity (Table 6). It is clear from a comparison of the data from GI zones and DNA-DNA homology data (3, 4, 7, 25, 26, 28, 35) that to a large extent, GI patterns parallel patterns in DNA-DNA homology among the subgroups.

Zones were rarely observed in heterologous crosses between groups (Tables 3 through 5). Although such zones could be obtained repeatedly with individual serum-strain combinations, they were not characteristic of all strains within each group and hence can be considered to be nonspecific.

DISCUSSION

In the genus *Mycoplasma*, cross-reactivity in the GI test (expressed as zones greater than 4 mm wide) is construed as evidence that two isolates are members of the same species. The applicability of this principle to spiroplasmas has recently been questioned (16), especially in reference to the four subgroups of the group I complex. However, the honey bee (subgroup I-2) and corn stunt (subgroup I-3) spiroplasmas show high levels of DNA-DNA homology, as measured by various methods (3, 4, 25, 26). In light of data from studies of the spiroplasma genome, the Subcommittee on Taxonomy of *Mollicutes* recommended at the meeting in Custer, South Dakota, in 1980 (manuscript in preparation) that species designations be postponed for members of group I isolates. Actually, the degree of DNA-DNA homology (21%) between the 277F spiroplasma (subgroup I-4) and *S. citri* (2) is considerably less than values that have conventionally been considered to be required for species distinction (23). If no intergrading members of group I existed, a case for recognition of subgroup I-4 as a species might be reasonable. Evidence of such intergrading first came when it was revealed that subgroups I-3 and I-4 showed significant crossing in deforma-

TABLE 5—Continued

Antigen										
Subgroup I-2 (honey bee spiroplasma)						Subgroup I-3 (corn stunt spiroplasma)				Subgroup I-4 (277F spiroplasma)
BC-3	AS 576	KC-3	G-1	G-13	BW	E275	Miss E	I-747	B655	
0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0
0	0	0	2	2	3	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	2	0	1
0	0	0	0	0	0	0	0	0	0	0

^a Width (in millimeters) of zone of inhibition, measured from the edge of the disk.

TABLE 6. Relative zone widths obtained with reciprocal GI tests with group I strains^a

Anti-serum	Antigen			
	Subgroup I-1	Subgroup I-2	Subgroup I-3	Subgroup I-4
I-1	1.00 ^b	0.79	0.57	0.06
I-2	0.89	1.00	0.79	0.14
I-3	0.68	0.40	1.00	0.61
I-4	0.09	0.00	0.17	1.00

^a Mean zone widths ($\bar{W}_z$) were calculated from the data in Table 2. Because the mean zone width was strongly influenced by spiroplasma growth rate, relative widths (W_r) were calculated as follows: $W_r = (\bar{W}_z \text{ for subgroup } s_x \text{ antigen versus subgroup } s_y \text{ antiserum}) / (\bar{W}_z \text{ for subgroup } s_x \text{ antigen versus subgroup } s_x \text{ antiserum})$. Boxhead = s_x .

^b Relative zone width.

tion and metabolism inhibition tests (55). Recent studies have provided further evidence for intergrading in the complex. For example, isolation of the LB-12 strain from the green leaf bug (27), isolation of the N525 strain from *Cocos* in Jamaica (19), and isolation of a cluster of flower strains (M52, M55, M58) from fall flowers in Maryland (52) have revealed the existence of other serovars that may be recognized as new subgroups. Discovery of new insect or flower strains could result in a myriad of subgroups or, even worse, the possible lack of any clear grouping (51). If further isolations continue to blur the distinctions among group I strains, the subgroup concept itself could be open to serious challenge. Extensive analyses of DNA-DNA homologies of some *Acholeplasma* species have shown that there is a broad cline of strains whose DNA-DNA homologies intergrade imperceptibly (G. S. Aulakh, E. B. Stephens, and M. F. Barile, personal communication) over a wide range. In a sense, such large clusters are reminiscent of "superspecies." *S. citri* may occupy a similar position.

Concepts of spiroplasma groupings (17, 55) have been formalized in two proposals. In one of these schemes (17), three serogroups and three subgroups were proposed and later supported by molecular genetic studies (26). A second proposal (25), based in part on serological groupings (55) and also on DNA-DNA hybridization data, analyses of guanine-plus-cytosine content, and two-dimensional electrophoresis data, defined five major groups and four subgroups. The latter scheme, because it is more inclusive in its analysis of divergent *Spiroplasma* strains and fully incorporates data on the genome, has now come into general acceptance. Our study demonstrates that the two spiroplasma groupings are consistent. Ten strains considered to represent the classical serovar of *S. citri* (subgroup I-1 of

Junca et al. and subgroup I-A of Davis et al.) reacted similarly in their GI patterns. The BC-3 isolate of the honey bee spiroplasma (10, 55) reacted similarly to the AS 576 honey bee strain of Davis et al. (R. E. Davis, J. F. Worley, T. B. Clark, and M. Moseley, Proc. Am. Phytopathol. Soc. 3:304, 1976) and four other strains from bees and flowers. Thus, subgroup I-2 of Junca et al. and subgroup I-B of Davis et al. are homologous. Similarly, the I-747, E275, and Miss E strains form a cluster that represents subgroup I-3 of Junca et al. and subgroup I-C of Davis et al. Neither subgroup I-4 (spiroplasma 277F) nor group II of Junca et al. (*Drosophila* sex ratio spiroplasma) was studied by Davis et al. Group III of Junca et al., represented by the OBMG and BNR1 strains, according to our GI tests, is similar or identical to the 23-6 strain designated (18) as the type strain of *S. floricola* (serogroup II of Davis et al.). A similar close relationship or identity is indicated between serogroup III of Davis et al. and group IV of Junca et al., since the SR 3 strain from flowers in Connecticut (15), the PPS1 strain from flowers in Florida (30), and five strains from flowers and insects in the Mediterranean region (L-89, B-13, F-1, F-2, and F-25) (50) appear to be closely related. However, analysis of this group (44) by metabolism inhibition has demonstrated considerable heterogeneity. Group V of Junca et al., representing *S. mirum*, was not studied by Davis et al. A recent report of a new spiroplasma from *Ixodes pacificus* ticks (45) established a candidate sixth major group, pending results from studies of its genome. Discovery of other spiroplasmas from arthropods (12) with diverse host relationships makes it certain that the grouping scheme will require frequent revisions and updates (2) to maintain its utility as a working system. Because classification in this grouping scheme requires not only serological data but also genomic data, provision has been made for establishment of provisional serogroups and serological subgroups (51). For example, the *Ixodes* spiroplasma has been designated as (VI).

Zones in nonspecific crosses were only rarely wider than 2 to 4 mm. The factors that cause such zones are unclear. Such crosses, even though reproducible within batches of prepared disks, occurred only with certain combinations of strains and antisera. Of course, the occasional occurrence of nonspecific zones underscores the importance of using adequate controls and the value of examining several strains of any given organism in the assessment of its serological reactions.

A high general level of agreement between GI tests and other refined techniques for elucidation of spiroplasma interrelationships is evident. For example, results from molecular genetic

(guanine-plus-cytosine determinations and DNA-DNA hybridization) and protein (two-dimensional polyacrylamide gel electrophoresis) analyses are in good accord with results obtained by GI tests. GI tests probably reflect affinity of antisera for membrane proteins (R. F. Whitcomb, J. G. Tully, and H. Wróblewski, in preparation). The membrane systems of spiroplasmas appear to be complex and, in the type species, involve at least 29 protein molecules, as resolved by one-dimensional polyacrylamide gel electrophoresis (59). This complexity forms a basis for a graded response between organisms that share some, but not nearly all, of their antigenic determinants. Other serological tests, such as the enzyme-linked immunosorbent assay (1, 9, 38), may prove to be superior for elucidation of partial relationships. Particularly in comparison with the metabolism inhibition test (55), the GI test is relatively insensitive and does not dramatically reflect minor crossing. Probably for this reason the GI test in *Mycoplasma* and, apparently from our data, in *Spiroplasma*, functions appropriately at the species level. The disk GI test is therefore an appropriate serological test in assessing the species taxonomy of new candidate *Spiroplasma* strains.

REPRINT REQUESTS

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