A Study of the Va-1 Group of Pseudomonads and its Relationship to *Pseudomonas pickettii*

By M. J. PICKETT* AND J. R. GREENWOOD

Department of Microbiology, University of California, Los Angeles, California 90024, U.S.A.

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The Va-1 group of denitrifying pseudomonads was characterized and compared with *Pseudomonas pickettii* (Va-2). They share many features but differ in their production of acid from cellobiose, lactose and maltose and in their denitrification (gas from nitrate) at 35 °C. DNA–DNA hybridizations between a strain of Va-1 and the type strain of *P. pickettii* disclosed 84 % homology and hence indicated that Va-1 is a biovar of this species. Since both are potential human pathogens, features are presented that distinguish these and other phenotypically similar species that are recovered from clinical specimens.

INTRODUCTION

Among the groups of non-fermentative Gram-negative rods reported by King (1964), one, group Va, was non-pigmented, oxidase-positive and denitrifying (gas from nitrate). Weaver *et al.* (1972) reported that strains of the Va group are monotrichous, and that they acidify lactose and maltose but not mannitol, starch and sucrose. Tatum *et al.* (1974) recognized two biotypes within the Va group, Va-1 and Va-2, only the former acidifying lactose and maltose. Independently, Pickett & Pedersen (1970*a*) examined 22 strains of weakly saccharolytic pseudomonads recovered from clinical specimens and erroneously concluded that they represented a second biotype of *Pseudomonas pseudoalcaligenes* (Stanier *et al.*, 1966). Several of Pickett & Pedersen's strains were later examined by Ralston *et al.* (1973), whose data, covering nutritional properties and DNA base composition and hybridization, indicated that these strains were not closely related to *P. pseudoalcaligenes* but rather represented a distinct species for which they proposed the name *Pseudomonas pickettii*. Briefly, thereafter, Tatum *et al.* (1974) and Riley & Weaver (1975) tentatively equated *P. pickettii* with their Va-2. King *et al.* (1979), from a cluster analysis of *P. pickettii*, Va-1 and Va-2, concluded that they are closely related. Thus, the similarity of Va-1 and *P. pickettii* is established but the taxonomic position of the former has not been fully clarified. Hence, we report here our observations on this group, compare it with similar non-fermentative bacilli, particularly strains of *P. pickettii*, and record DNA–DNA hybridization of these.

METHODS

Strains. These were accumulated over a period of 12 years. Most were recovered from clinical specimens in the hospital of the University of California at Los Angeles, U.S.A., although some were received from hospitals throughout the U.S.A. Sources of the Va-1 strains are given in Table 1. Stock cultures were maintained as stabs in a motility-nitrate (MN) medium containing (g l⁻¹): Heart Infusion Broth (Difco), 5; Tryptose (Difco), 10; agar (Difco), 3; glucose, 0.5; KNO₃, 1; sterilization was by autoclaving (121 °C, 10 min). Stock cultures were maintained at 4 °C and transferred fortnightly. Incubation of these and all other cultures was at 35 °C unless otherwise indicated.

Morphology and growth. Colonial morphology was recorded from cultures grown aerobically for 48 h on blood agar plates [Difco Blood Agar Base with 5 % (v/v) defibrinated sheep blood]. Microscopic morphology was recorded from Gram-stains (Hucker) prepared from 24 h cultures on blood agar plates, and from flagellar tains prepared according to Clark (1976). Growth on MacConkey agar (Difco) was determined according

Table 1. *Source of Va-1 strains*

Strain	Source* (all in U.S.A.)	Strain	Source* (all in U.S.A.)
K-276	UCCL	K-1510	UCCL, hospital environment (water)
K-300	UCCL	K-1515	UCCL, hospital environment (water)
K-638	UCCL, tracheal aspirate	K-1532	OVMC, blood specimen
K-792	Los Angeles, from a cosmetic	K-1559	UCCL, hospital environment (water)
K-992	Duarte, California, clinical specimen	K-1560	Denver, Colorado, blood specimen
K-994	Duarte, California, clinical specimen	K-1576	OCHD, aspirate, knee wound
K-1303	OVMC	K-1577	OCHD, pericardial fluid
K-1466	Hillcrest Hospital, Petaluma, California	K-1645	OCHD, infected eye
K-1469	Denver, Colorado, hospital environment	K-1646	OCHD, hospital environment (water)
K-1502	UCCL, hospital environment (water)	K-1647	OCHD, hospital environment (water)
K-1503	UCCL, hospital environment (water)		

* UCCL, UCLA clinical laboratories, Los Angeles; OVMC, Olive View Medical Center, Sylmar, California; OCHD, Orange County (California) Health Department.

to Tatum *et al.* (1974), and at 42 °C by lightly inoculating a tube of nitrate broth (Vera & Dumoff, 1974) and incubating for 48 h. Evidence of motility was observed as spreading growth in both MN and OF (Tatum *et al.*, 1974) media.

Biochemical tests. Acid production from carbohydrates and related compounds was determined in the OF basal medium of Tatum *et al.* (1974), on the 'oxidative low peptone' (OLP) medium of Greenwood & Pickett (1978) and by the 'buffered single substrate' (BSS) procedures of Pickett & Pedersen (1970*a, b*). Alkali production from amides and organic salts was determined by OLP and BSS procedures. Commercial BSS tablets (Key Scientific Products, Los Angeles, U.S.A.) were used according to the manufacturer's directions to test for: arginine dihydrolase, β -galactosidase (the ONPG test), and indole production; hydrolysis of hippurate; oxidation of gluconate to 2-ketogluconate, tryptophan to indolepyruvic acid, and phenylalanine to phenylpyruvic acid; and decarboxylation of lysine and ornithine. Oxidase and catalase were tested according to Greenwood & Pickett (1979) with cell paste obtained from 24 h blood agar plates. Formation of fluorescein was detected in King's medium B (Vera & Dumoff, 1974) supplemented with 0.2 % (w/v) KNO₃, and in fluorescence-lactose-denitrification (FLN) medium containing (g l⁻¹): Proteose Peptone no. 3 (Difco), 10; agar (Difco), 15; lactose, 20; glucose, 0.5; sodium acetate. 3H₂O, 0.5; phenol red, 0.02; MgSO₄. 7H₂O, 1.5; K₂HPO₄, 1.5; KNO₃, 2; NaNO₂, 0.5. This medium was dispensed in 4 ml amounts into 13 × 100 mm tubes, sterilized by autoclaving (121 °C, 10 min), and solidified to provide slants and butts of approximately equal length. The FLN and MN media used here were modified slightly from those previously reported (Pickett & Pedersen, 1970*a*). Hydrolysis of aesculin was detected both with the medium of Vera & Dumoff (1974) and by disappearance of fluorescence in Kligler iron agar medium supplemented with 0.002 % (w/v) aesculin. Formation of deoxyribonuclease was determined on DNase Test Agar (Difco), of amylase, on the starch agar medium of Vera & Dumoff (1974) and of gelatinase, on Trypticase Soy Agar (BBL) plates supplemented with 3 % (w/v) gelatin. Hydrolysis of polysorbate was determined on the following medium (g l⁻¹): Peptone (Difco), 10; agar (Difco), 15; Tween 80 (Sigma), 10; NaCl, 5; CaCl₂. H₂O, 0.1. Reduction of nitrate to nitrite was detected in MN and nitrate broth media, and of nitrate to nitrogen, in FLN, MN, nitrate broth and supplemented King's medium B. Unless otherwise noted, the last two media were incubated at room temperature (22 ± 2 °C). Digestion of casein was detected in Skim Milk Broth (Difco), and of serum, on Loeffler's serum slants. Susceptibility to polymyxin B was determined by placing a 300 unit disc (Pfizer) on an inoculated blood agar plate; a zone of complete inhibition of more than 1 mm (radius) was scored as positive.

Tatum *et al.* (1974) and Oberhofer *et al.* (1977) reported that the Va group is urease-positive, whereas others (Pickett & Pedersen, 1970*b*; Riley & Weaver, 1975; King & Phillips, 1978) reported strain variation within the group. We therefore examined the effect of basal media and size of inocula on the outcome of this test. Stuart *et al.* (1945), in their studies on *Proteus* species, used three urea media which differed in respect to concentration of buffer (Table 5), namely their 'usual' (double-strength Sorensen's phosphate, 133.3 mM), '0.1 buffered medium' (13.33 mM) and '0.01 buffered medium' (1.333 mM). They proposed use of the last medium, which is that of Vera & Dumoff (1974), for rapid urease tests. The urease medium of Key Scientific Products contains 0.1 % (w/v) KH₂PO₄ (7.4 mM). Christensen and OLP media contain phosphate (14.7 and 7.6 mM, respectively) and also 0.1 % (w/v) each of glucose and organic nitrogen. All of these media contain phenol red as indicator. Each of the buffered substrate media was dispensed in 1 ml amounts in 13 × 100 mm tubes and inoculated with 0.1 ml of heavy bacterial suspension.

DNA base composition and hybridizations. The methods were those of Greenwood & Pickett (1980).

RESULTS

Colonies of the Va-1 strains were 0.1 to 0.5 mm in diameter at 24 h (average for 17 strains, 0.25 mm) and enlarged to 0.6 to 1.2 mm at 48 h (average, 0.91 mm). Colonies of Va-2 strains were slightly larger: 0.3 to 0.8 mm at 24 h (average for 13 strains, 0.45 mm) and enlarged to 0.8 to 1.4 mm at 48 h (average, 1.2 mm). The colonies of both biovars at 24 h were transparent to translucent, non-haemolytic, smooth, convex, entire and butyrous, became translucent to slightly opaque at 48 h and were not pigmented. Microscopically (see also Tatum *et al.*, 1974), all strains were slender, Gram-negative, monotrichous rods. Also, as noted by Tatum *et al.* (1974), viability was poor. In our hands, stock cultures of Va-1 maintained at either 4 or 20 °C frequently died in less than 3 weeks.

Other features of both biovars are presented in Table 2. Only four of the 79 features listed are qualitatively and significantly different for the two biovars, namely, denitrification at 35 °C and acid production from cellobiose, lactose and maltose. Tables 3 and 4 present additional data concerning the effect of temperature and media on overt production of gas from nitrate.

Table 5 presents results obtained with different media and inocula in tests for hydrolysis of urea. Table 6 presents data obtained from caesium chloride density gradient centrifugation and DNA-DNA hybridization. Table 7 compares *P. pickettii* with those other species which, in our experience, are most likely to be confused with it.

DISCUSSION

The DNA base composition and hybridization data of Table 6 suggest that both Va-1 and Va-2 are biovars of *Pseudomonas pickettii*. This suggestion is supported by the cluster analysis of King *et al.* (1979) and by the hybridization data of Ralston *et al.* (1973). Ralston *et al.* (1973) reported that the DNA base composition of strain K-300 of biovar Va-1, like that of type strain K-288 (ATCC 27511) of *P. pickettii*, was 64 mol % G + C, and that the DNAs of these two strains showed 90 % binding at 71 °C. They also reported that, in assimilation experiments, strain K-300 was similar to the strains of Va-2 but differed in respect of five of the 133 substrates tested (caproate, histamine, L-histidine, L-phenylalanine and valerate).

Tatum *et al.* (1974) obtained more than two patterns for oxidation of carbohydrates with their strains of group Va but we did not. The discrepancy, we believe, arises from differences in sensitivity between their methods and ours. We, too, found that one out of 17 strains of Va-1 failed to produce acid from lactose in OF medium, a second strain was maltose-negative in this medium, a third was cellobiose-negative in BSS medium, and a fourth was maltose-negative in both BSS and OF media. However, on OLP medium all strains of Va-1 produced acid from all three carbohydrates; no strain of Va-2 produced acid from these carbohydrates with any of the three basal media.

Our results for Va-1 (Table 2) differ from those of Gilardi (1978) in tests for β -galactosidase (ONPG test) and oxidation of phenylalanine. However, our positive tests were both weak and delayed. We do not, then, judge these to be significant discrepancies.

The data of Table 5 indicate that the concentration of buffer, presence or absence of glucose and size of inoculum may significantly influence the outcome of urease tests on bacteria that are saccharolytic but only weakly urease-positive. All of the strains recorded in this table were urease-positive but none made alkaline the strongly buffered medium of Stuart *et al.* (1945). With Christensen medium, even when heavily inoculated, only six strains of Va-1 and two strains of Va-2 gave strong (red slant) positive results; the other 12 strains gave only weak (pink slant) positive results during 8 d incubation.

Since strains of the Va-2 biovar did not denitrify at 35 °C, we determined whether cultures nominally incubated at this temperature would show gas production when they were shifted for different periods to a lower temperature. The data of Table 3 indicate that when relatively

Table 2. *Features of the Va group of pseudomonads*

Feature*	Va-1			<i>P. pickettii</i> (Va-2)		
	No. tested	No. positive	% positive	No. tested	No. positive	% positive
Acid from:						
Adonitol	5	0	0	22	0	0
Amygdalin	5	0	0	5	0	0
D-Arabinose	5	0	0	5	0	0
L-Arabinose	17	17	100	36	36	100
Arabitol	5	0	0	5	0	0
Arbutin	5	0	0	7	0	0
Cellobiose	17	17	100	22	0	0
Dulcitol	5	0	0	9	0	0
Ethanol	15	0	0	16	0	0
Fructose	15	15	100	22	22	100
Galactose	7	7	100	22	22	100
Glucose	17	17	100	22	22	100
Inositol	10	0	0	22	0	0
Inulin	6	0	0	7	0	0
Lactose	19	19	100	36	0	0
Maltose	17	17	100	32	0	0
Mannitol	17	0	0	36	0	0
Mannose	7	7	100	22	22	100
Melezitose	9	0	0	5	0	0
Melibiose	10	0	0	23	0	0
Raffinose	8	0	0	8	0	0
Rhamnose	12	0	0	8	0	0
Ribose	7	2	29	8	0	0
Salicin	8	0	0	8	0	0
Sorbitol	10	0	0	23	0	0
Starch	10	0	0	9	0	0
Sucrose	17	0	0	8	0	0
Trehalose	10	0	0	23	0	0
Xylose	17	17	100	23	23	100
Alkali from:						
Acetamide	13	0	0	35	0	0
Acetate	8	8	100	23	23	100
Allantoin	14	14	100	22	22	100
Asparagine	10	10	100	22	22	100
n-Butyramide	10	9	90	22	14	64
Isobutyrate	9	9	100	23	23	100
n-Butyrate	9	9	100	22	5	23
Citrate, Simmons	14	14	100	11	11	100
Formamide	10	0	0	21	0	0
Formate	9	9	100	23	23	100
Glutamine	10	10	100	23	23	100
Hexanoate	8	0	0	23	0	0
Malonamide	8	8	100	23	23	100
Malonate	17	17	100	23	23	100
Mucate	9	9	100	18	18	100
Nicotinamide	15	0	0	22	0	0
Propionamide	11	11	100	22	7	32
Propionate	9	9	100	23	22	96
Saccharate	9	9	100	23	22	96
Succinamide	10	10	100	23	23	100
Tartrate	16	15	94	23	23	100
Valeramide	10	10	100	24	14	58
Valerate	9	9	100	21	19	90
Arginine dihydrolase	5	0	0	22	0	0
Catalase	16	16	100	11	11	100
DNAase	9	0	0	13	0	0

Table 2. (cont)

Feature*	Va-1			<i>P. pickettii</i> (Va-2)		
	No. tested	No. positive	% positive	No. tested	No. positive	% positive
Fluorescence	17	0	0	22	0	0
Gluconate	14	0	0	36	0	0
Growth at 42 °C	15	9	60	22	6	27
Growth on MacConkey agar	15	15	100	22	22	100
Hydrolysis of:						
Aesculin	15	0	0	8	0	0
Hippurate	15	15	100	10	10	100
Starch	5	0	0	22	0	0
Tween 80	11	11	100	5	5	100
Urea	16	16	100	14	14	100
Indole	14	0	0	24	0	0
Indolepyruvic acid	13	5	38	5	0	0
Lysine decarboxylase	14	0	0	23	0	0
Motility	17	17	100	23	23	100
Nitrate → gas, 35 °C	15	14	93	13	0	0
Nitrate → gas, 30 °C	15	14	93	13	13	100
Nitrate → nitrite	14	14	100	23	23	100
Ornithine decarboxylase	9	0	0	12	0	0
ONPG	9	9	100	15	0	0
Oxidase	16	16	100	23	23	100
Phenylpyruvic acid	8	8	100	5	0	0
Polymyxin B, susceptible	5	0	0	5	0	0
Proteolysis:						
Casein	12	4	33	22	0	0
Gelatin	9	4	44	22	10	45
Serum	5	0	0	23	0	0

* All tests were at 35 °C unless otherwise indicated.

Table 3. Effect of temperature on production of gas by *P. pickettii* in nitrate broth medium

Tube no.	Incubation temp./time			Gas production*		
	Initial, at 35 °C for (h):	Secondary, at RT† for (h):	Final, at (°C):	Va-1 strain K-638	Va-2 strain K-230	Va-2 strain K-288
1	16	0	35	+	—	—
2	16	2	35	+	—	—
3	16	4	35	+	—	+
4	16	8	35	+	—	+
5	16	24	35	+	+	+
6	24	2	35	+	—	—
7	24	4	35	+	—	+
8	24	8	35	+	+	+
9	24	24	35	+	+	+
10	48	24	35	+	—	—
11	48		RT†	+	—	—

* +, Gas produced within 4 d; —, no gas produced within 20 d.

† RT, Room temperature (22 ± 2 °C).

young (16 to 24 h) cultures are shifted from 35 °C to room temperature for as little as 4 h, denitrification may occur. However, older (48 h or more at 35 °C) cultures did not produce gas in nitrate broth when transferred to the lower temperature. Similar results were obtained with cultures shifted from 35 °C to 30 °C. We suggest that these data may in part explain the different results reported by different authors in respect of denitrification by strains of *P. pickettii*.

Table 4. *Detection of denitrification* by P. pickettii: effect of medium and temperature*

Incubation temp. (°C)	Basal medium†	Va-1 (9 strains)						Va-2 (13 strains)					
		Cumulative % positive at day:						Cumulative % positive at day:					
		1	2	4	6	8	10	1	2	4	6	8	10
35	MN	0	0	33	33	33	33	0	0	0	0	0	0
35	FLN	11	33	100	100	100	100	0	0	0	0	0	0
35	Peptone	0	22	78	100	100	100	0	0	0	0	0	0
35	KBN	11	78	100	100	100	100	0	0	0	0	0	0
30	MN	0	0	44	100	100	100	8	23	62	100	100	100
30	FLN	33	100	100	100	100	100	0	54	100	100	100	100
30	Peptone	44	78	89	100	100	100	46	100	100	100	100	100
30	KBN	11	33	100	100	100	100	0	23	100	100	100	100
RT‡	MN	0	0	11	33	78	78	0	0	8	38	77	77
RT	FLN	0	0	67	78	78	78	0	0	62	92	100	100
RT	Peptone	0	22	78	100	100	100	0	31	100	100	100	100
RT	KBN	11	33	100	100	100	100	0	23	100	100	100	100

* Overt production of gas from nitrate.

† MN, Motility-nitrate medium; FLN, fluorescence-lactose-denitrification medium; Peptone, 1 % (w/v) peptone with 0.1 % (w/v) KNO₃; KBN, King's medium B supplemented with 0.2 % (w/v) KNO₃.

‡ RT, Room temperature (22 ± 2 °C).

Table 5. *Comparison of media for urease tests* (at 35 °C)*

Medium	Va-1 (10 strains)				Va-2 (10 strains)			
	Cumulative % positive at day:				Cumulative % positive at day:			
	1	2	4	6	1	2	4	6
Buffered substrate								
Stuart <i>et al.</i> †								
133.3 mm	0	0	0	0	0	0	0	0
13.33 mm	100	100	100	100	20	60	100	100
1.333 mm	100	100	100	100	100	100	100	100
Key	100	100	100	100	20	40	60	100
Christensen‡								
Light inoculum	50	60	60	60	20	20	20	20
Heavy inoculum	100	100	100	100	100	100	100	100
OLP‡	100	100	100	100	100	100	100	100

* With *Proteus mirabilis*, all tests were strongly positive within 24 h.† See Methods for details of phosphate buffers used by Stuart *et al.* (1945).‡ About 2 mm³ of cell paste in a 3 mm loop was used as 'heavy' inoculum; the same loop, without flaming, provided the 'light' inoculum. A 'light' inoculum was also used for OLP medium.Table 6. *Guanine plus cytosine content and relative binding ratios (RBR) of DNA from strain K-638 of biovar Va-1*

Biovar	Strain	Mol % G + C	RBR
Va-1	K-638	62.9 ± 0.4	100
Va-1	K-792	63.3 ± 0.3	74
Va-2	ATCC 27511/K-288	64*	84
CDC M-5	CDC D1674	47	0
CDC DF-2	CDC A3626	61	0

* Datum from Ralston *et al.* (1973).

Table 7. Features that distinguish Va from similar non-fermentative bacilli*

	'Achromobacter'†			Pseudomonas						
	<i>xylosoxidans</i>	Vd-1	Vd-2	<i>pickettii</i> Va-1	<i>pickettii</i> Va-2	<i>aeruginosa</i>	<i>fluorescens</i>	<i>putida</i>	<i>stutzeri</i>	HK-2
Flagella‡	Per	Per	Per	Pol	Pol	Pol	Pol	Pol	Pol	Pol
Polymyxin B, susceptible	100	100	100	0	0	97	96	99	100	0
Acid from:										
Ethanol	100	ND	100	0	0	92	56	86	100	5
Lactose	0	0	0	100	0	0	47	21	0	100
Maltose	0	0	100	100	0	4	94	44	100	100
Mannitol	0	0	100	0	0	69	94	24	67	0
Sucrose	0	0	100	0	0	0	65	8	0	100
Alkali from:										
Acetamide	92	0	0	0	0	100	0	4	0	0
Malonate	20	0	0	100	100	100	94	56	100	0
Tartrate	0	20	0	93	100	0	0	43	0	16
Arginine dihydrolase	0	25	0	0	0	99	99	98	2	0
Fluorescence:										
20–30 °C	0	0	0	0	0	91	100	100	0	0
35–37 °C	0	0	0	0	0	94	47	60	0	0
Gluconate	96	0	0	0	0	92	96	87	0	0
Hydrolysis of:										
Aesculin	0	0	100	0	0	0	0	0	0	95
Starch	0	0	0	0	0	1	5	7	93	95
Tween 80	0	0	0	100	100	69	54	1	100	48
Nitrate → gas, 20–30 °C	77	100	100	93	100	58	6	0	100	0
Nitrate → nitrite	98	100	100	100	100	40	14	0	100	0
Urea, Christensen	0	100	100	100	100	72	42	51	16	100

Numbers indicate the percentage of strains positive; ND, no data.

* Data from Table 2, Oberhofer *et al.* (1977), G. L. Gilardi (personal communication, 1979), and our unpublished studies.

† The genus *Achromobacter*, though not recognized in the 8th edition of *Bergey's Manual of Determinative Bacteriology*, is used here in the sense of Tatum *et al.* (1974).

‡ Per, Peritrichous; Pol, polarly flagellated.

In the identification of non-fermentative Gram-negative rods, differentiation among '*Achromobacter*' species, *P. pickettii* and the *Pseudomonas fluorescens*–*P. putida* complex can present a problem, particularly if incubation is at 35 to 37 °C since the last group frequently fails to show fluorescence at this temperature. For this last group, sensitivity to polymyxin B, production of alkali from arginine and formation of fluorescein at room temperature are a critical constellation of features. Although we are aware of the uncertain future of the genus *Achromobacter* (see Hendrie *et al.*, 1974), we use the name here since many clinical microbiologists feel that this would be a useful genus in which to include peritrichously flagellated oxidative organisms. For *Achromobacter* species, possession of peritrichous flagella is the one most useful feature. When this information is not available, failure to hydrolyse Tween 80 is particularly useful. Other useful features are reduction of nitrate to nitrite or gas, a negative arginine test and susceptibility to polymyxin B. Among all non-fermentative bacilli, *P. pickettii* is uniquely characterized by the following features: resistance to polymyxin B; acid from glucose but never from ethanol, mannitol and sucrose; alkali from malonate and urea; and gas from nitrate (20 to 30 °C).

As with many non-fermentative bacilli, there are few firm data concerning the aetiological significance of Va-1 in human disease. Most of our strains appeared to represent normal flora of the hospital environment. However, both K-1576 and K-1577 (Table 1) were

recovered from active disease processes and the latter strain was recovered in pure culture. Tentatively, then, we conclude that Va-1, like several other non-fermentative species (Pedersen *et al.*, 1970), is occasionally aetiologically significant in human disease.

Since completion of this paper a further five strains of Va-1 have been isolated from clinical specimens.

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