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Characteristics of Environmental Isolates of *Legionella pneumophila*

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Thirty-eight cultures of *Legionella pneumophila* isolated from surface waters were characterized by their morphological, tinctorial, biochemical, and serological properties and by their ability to produce disease in guinea pigs. Their susceptibility to antimicrobial agents also was tested. When they were compared with clinical isolates, no important differences were found between cultures from the two sources. Sodium hippurate hydrolysis, gelatin liquefaction, pigment formation, and β-lactamase and alkaline phosphatase activity were useful in differentiating the four described species of *Legionella*. Hydrolysis of diacetylfluorescein and the inability to reduce nitrate help to distinguish *Legionella* species from other gram-negative bacterial rods.

Legionella pneumophila is a gram-negative, non-acid-fast, nonsporeforming, motile bacterium with fastidious growth habits. It was first recognized in yolk sacs of embryonated eggs by McDade et al. (17) and shown to be the etiological agent of legionellosis. Weaver (30) first isolated *L. pneumophila* on nonliving media and characterized the bacterium culturally and biochemically. However, only a few cultural and biochemical tests are used in the identification of *L. pneumophila* because most common bacteriological media do not support its growth. We used the methods of Weaver and Feeley (31), with some modifications, to characterize 38 environmental isolates. We also used some additional substrates to test for specific enzyme activities. In addition, we determined the antimicrobial susceptibility and the cellular fatty acid composition of these cultures.

MATERIALS AND METHODS

All 38 cultures used in this study were isolated from large-volume (20-liter) water samples from the littoral zone of lakes or streams predominantly in the southeastern United States (8, 9). The samples were collected by aseptic techniques with a peristaltic pump from depths ranging from the surface to 16 m (8). The sources of these isolates (SRP-2 through SRP-39) and the characteristics of the water samples are given in Table 2 of Fliermans et al. (8). The samples were processed by continuous flow centrifugation to concentrate the microflora in a small volume (40 ml) of water (8).

Three hundred and eighteen samples containing the largest number of *Legionella*-like bacteria, when tested by direct fluorescent antibody staining (3, 18), were injected intraperitoneally into guinea pigs. Forty-seven strains were isolated on charcoal-yeast extract (CYE) agar (6, 7). The 38 cultures reported on here were isolated from 30 of these animals (30 water samples) after either a first or second passage. Each of eight guinea pigs yielded two different serogroup cultures.

For comparison, four clinical cultures (18) of *L. pneumophila* were used. These were Knoxville and OLDA (serogroup 1), Togus 1 (serogroup 2), and Los Angeles 1 (serogroup 4). Bloomington 2, a serogrouped 3 culture isolated from water but geographically unrelated to our environmental cultures, was also included. A group of 19 clinical isolates was used for antimicrobial susceptibility tests. Cultures of *Legionella* species other than *L. pneumophila* that were tested consisted of *Legionella micdadei* (11, 12) (*Legionella pittsburgensis* [24]) strains TATLOCK, HEBA, and the Pittsburgh pneumonia agent, and strains designated Tex-KL, WIGA, and LS-13. WIGA is the type culture of *Legionella bozemanii* (2, 16); Tex-KL is a strain of *Legionella dumoffii* (2, 15); and LS-13 (4) is the type strain of *Legionella gormanii* (21). All of these cultures, except LS-13, were presumably isolated from human infections.

Staining procedures. The Gram stain (Hucker's modification) and the fat stain (Sudan black B) were done on each isolate as reported by Weaver and Feeley (31). Acid-fast stains were performed by the Ziehl-Neelsen procedure. The 38 isolates were serogrouped by direct fluorescent antibody staining of cell suspension smears from 0.85% saline containing 1% formaldehyde (37%). Cell suspensions were prepared from 24- to 48-h cultures on CYE agar slants. The smears were stained with fluorescein isothiocyanate-labeled conjugates for serogroups 1 through 6 of *L. pneumo-*

phila (3, 5, 18, 19). Direct fluorescent antibody examination was done on an Ortholux II microscope by incident illumination from an HBO-200 mercury arc lamp. The filters used were a red-absorbing BG-38 filter, a K 470 edge filter, and the Ploem Pak containing a KP 500 dichroic mirror and a K 510 suppression filter. A 10× ocular was used with both 63× (N.A. 1.30) and 100× (N.A. 1.25) objectives.

Biochemical assays. The oxidase, catalase, and nitrate reduction tests were performed as described by Weaver and Feeley (31). The chromogenic cephalosporin test for the detection of β-lactamase production was performed on cells grown for 24 h on CYE agar as described by Thornsberry and Kirven (29). Liquefaction of gelatin by *L. pneumophila* was tested by inoculating 24-h cultures into 13- by 100-mm tubes containing 3 ml of CYE medium prepared by adding 3% gelatin and omitting the agar. These tubes were incubated at 35°C together with uninoculated control tubes. After several days of incubation, all tubes were refrigerated at 4°C, and the inoculated tubes were compared with the controls for liquefaction of the gelatin.

Tests for ability to hydrolyze sodium hippurate to benzoic acid and glycine were carried out on cells grown on CYE agar slants at 35°C for 24 to 36 h. A 1% solution (0.05 M) of sodium hippurate (Difco Laboratories, Detroit, Mich.) was prepared in sterile distilled water, and 0.4-ml amounts were placed in 13- by 100-mm screw-capped tubes and stored at −20°C. A 3.5% solution of ninhydrin reagent (Pierce Chemical Co., Rockford, Ill.) was prepared in a 1:1 solution of acetone and *n*-butanol and was stored in the dark at room temperature. The test procedure was that described by Hèbert (10). A heavy loopful (3-mm diameter) of growth from each of the CYE agar slant cultures was inoculated into a tube of the hippurate substrate. The turbid suspensions were incubated at 34°C for 18 to 20 h, and 0.2 ml of ninhydrin was then added. The tubes were incubated for an additional 10 min at 35°C and observed for a color reaction within 20 min after removing them from the incubator. Known positive control suspensions of *L. pneumophila* included in the test were prepared from the serogroup 1 cultures designated Philadelphia 1, Knoxville 1, Oak Ridge 1, and Togus 3 and 4 and from Oak Ridge 2, a culture serologically related to serogroup 4. Negative controls consisted of suspensions of cultures of *L. bozemanii*, *L. micdadei*, and *L. dumoffii*. In addition, uninoculated tubes of sodium hippurate and both inoculated and uninoculated tubes of sterile distilled water were subjected to the test conditions.

To observe growth, colony morphology, and pigment formation, cultures were inoculated onto CYE, Feeley-Gorman (F-G) medium (7), blood agar, and Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The method of Baine and Rasheed (1) was modified to test for the production of the brown pigment first described by Weaver and Feeley (31). The modification consisted of suspending 6 g of activated charcoal (Norite SG) in 200 ml of distilled water in a dialysis sac. The sac was placed in 1 liter of melted agar medium (F-G) and autoclaved as suggested by Ristroph et al. (26) for removal of growth inhibitors. The sac of charcoal was aseptically removed from the medium after autoclaving, and, after cooling the medium to 50°C, 400 mg of filter-sterilized L-cysteine-hydrochloride and 250 mg of filter-sterilized ferric pyrophosphate were added. The pH was adjusted to 6.9 ± 0.05 with 1.0 M KOH.

The cellular fatty acid composition of each isolate was obtained by gas-liquid chromatography as described by Moss et al. (22, 23).

Enzyme tests. Appropriate fluorogenic substrates were used to test for the following enzymes. For esterases, we used diacetylfluorescein (Eastman Kodak Co., Rochester, N.Y.), 4-methylumbelliferyl propionate (4-MU propionate) and 4-methylumbelliferyl nonanoate (Koch-Light Laboratories, Buckinghamshire, England). For alkaline phosphatase, disodium-*p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was the substrate.

The esterase substrates were prepared by dissolving diacetylfluorescein, 4-MU propionate, or 4-methylumbelliferyl nonanoate (4-MU nonanoate) in acetone at a concentration of 5 mg/ml. The stock solutions then were diluted $1:10^3$ in distilled water (27). The alkaline phosphate substrate (0.01 M *p*-nitrophenyl phosphate) was dissolved in 0.04 M glycine-NaOH buffer at pH 10.5.

Cell suspensions (no. 3 McFarland) of 24-h cultures grown on CYE agar were made in 0.3 ml of the test substrates. Similar suspensions in distilled water constituted negative controls. To test esterase activity for diacetylfluorescein, the tubes were incubated at 20°C for 5 min; for esterase activity on the other two substrates, the tubes were incubated at 37°C for 30 min. The alkaline phosphatase tests were incubated at 37°C for 180 min. After incubation of the diacetylfluorescein and the 4-MU propionate and 4-MU nonanoate with the cell suspensions, the tubes were examined in the dark with a long-wavelength (365-nm) 4-W ultraviolet lamp. The appearance of the yellow-green color of fluorescein or the deep blue color of 4-methylumbelliferone was evidence of esterase activity. Alkaline phosphatase activity produced the yellow color of *p*-nitrophenol.

For the arylsulfatase enzyme assay, all cultures were grown for 24 h on CYE medium containing 10 mM filter-sterilized tyramine. The methods of Henderson and Milazzo (13) and Kersterks and DeLey (14) were both used for detection of arylsulfatase. In the Henderson and Milazzo procedure (13), cell suspensions (no. 3 McFarland) were made in 0.2 ml of 0.05 M tris(hydroxymethyl)aminomethane-maleic acid buffer (Tris buffer), pH 6.7. Each cell suspension was incubated with an equal amount (0.2 ml) of 2 mM potassium-*p*-nitrophenyl sulfate (Koch-Light Laboratories) in 0.05 M Tris buffer (pH 6.7) at 37°C for 1 h. The reaction was terminated by the addition of 0.8 ml of 0.2 N NaOH. The yellow color of *p*-nitrophenol developed if arylsulfatase was present. In the Kersterks and DeLey (14) procedure, 2.5 mM *p*-nitrophenyl sulfate in 0.5 M sodium acetate-acetic acid (pH 5.8) was used as the substrate solution; the reaction was terminated by adding 0.3 ml of 0.04 M glycine-NaOH buffer (pH 10.5). Two cultures of *Salmonella typhimurium* and one culture each of *Salmonella mississippi* and *Klebsiella pneumoniae* capsular type 30 served as positive controls for the arylsulfatase test.

Antimicrobial susceptibility testing. Thirty-five strains of *L. pneumophila* isolated from environmental sources and 19 strains from clinical sources were tested for in vitro susceptibility to nine antimicrobial agents by the agar dilution method with CYE agar (11, 28). The inoculum was composed of cells from 24-h growth of the cultures on CYE agar. The cells were suspended in sterile distilled water and diluted to contain about 3×10^8 colony-forming units per ml (McFarland no. 1). This inoculum is less than that previously described (11).

RESULTS

The 38 environmental isolates of *L. pneumophila* had the physiological characteristics described by Weaver (30) and by Weaver and Feeley (31). They were slender, gram-negative, non-acid-fast rods having the morphological and staining properties of this species. They did not readily take up safranin in the gram-stain procedure so that it was desirable to counterstain for 1 to 2 min. Freshly isolated cultures generally contained more vacuoles that were stained by Sudan Black B than did stock cultures that had been transferred several times. Like their clinical counterparts, these environmental cultures were fastidious in their growth requirements, failing to grow on blood or Trypticase soy agar. They grew on F-G agar, but the best growth was obtained on CYE agar containing L-cysteine and iron.

All 38 isolates liquefied gelatin and were catalase positive (Table 1). Kovacs oxidase test was negative for 22 isolates and weakly positive for 16 isolates. Some clinical isolates of *L. pneumophila* were oxidase negative when tested under the same conditions. The *L. pneumophila* isolates did not reduce nitrate. All 38 isolates produce β-lactamase when examined by the chromogenic cephalosporin test described by Thornsberry and Kirven (29) (Table 1). The three cultures of *L. micdadei* were β-lactamase negative (Table 2). All of the 38 isolates hydrolyzed hippurate as judged by positive tests with the ninhydrin reagent. All isolates showed some color production after the preliminary 10-min incubation period, and all reactions were complete within the additional 20-min time period (Table 1). Cultures of *L. bozemanii*, *L. gormanii*, *L. micdadei*, and *L. dumoffii* were negative for hippurate hydrolysis, as were controls which consisted of uninoculated substrate and cells suspended in distilled water (Table 2).

The cellular fatty acid composition of each of the 38 isolates was consistent with that reported by Moss and associates for *L. pneumophila* (22, 23).

Direct fluorescent antibody staining was performed on all 38 environmental cultures; 24 (63%) of the isolates belonged to serogroup 1, 1 each (3%) belonged to serogroups 2 and 3, 11 (28%) belonged to serogroup 4, and 1 (3%) belonged to serogroup 6. When the cultures were tested for pigment production on F-G agar without the addition of L-tyrosine, many of them either produced no pigment or very slight pigment. When L-tyrosine was added to the F-G agar, each of the environmental isolates, except SRP-14, produced a brown pigment. The production of this pigment by other cultures of *L. pneumophila*, by *L. bozemanii*, and by *L. dumoffii* was consistent with previous reports (1, 2, 21, 31). *L. micdadei* did not produce the brown pigment even on the tyrosine-enriched F-G medium (Table 2). Brilliant blue autofluorescence of *L. bozemanii*, *L. dumoffii*, and *L. gormanii* was seen when cell suspensions or cultures on solid media were examined by ultraviolet light (Table 2).

The addition of tyramine to CYE agar was essential for the production of arylsulfatase by the four *Salmonella* and *Klebsiella* control cultures; however, neither the *L. pneumophila* clinical and environmental cultures nor *L. bozemanii*, *L. dumoffii*, *L. micdadei*, or *L. gormanii* produced arylsulfatase when grown on this medium (Table 3).

All of the environmental isolates (Table 3), the four clinical cultures of *L. pneumophila*, and the cultures of *L. micdadei* hydrolyzed diacetylfluorescein to give an intensely fluorescent yellow-green product. The same cultures uniformly hydrolyzed the 4-MU propionate and 4-MU nonanoate substrates to give the bright blue fluorescence of 4-methylumbelliferone. *L. bozemanii*, *L. dumoffii*, and *L. gormanii* could not be tested on the two 4-MU substrates because the blue autofluorescence of the cell suspensions masked the action of esterase.

Both environmental and clinical cultures of *L. pneumophila* and all cultures of *L. bozemanii*, *L. gormanii*, and *L. dumoffii* had alkaline phosphatase activity (Table 3). All cultures of *L.*

TABLE 1. *Biochemical characteristics of 38 environmental isolates of Legionella pneumophila*

Test	No. positive	% Positive
Oxidase	16[a]	42
Catalase	38	100
β-Lactamase	38	100
Gelatin liquefaction	38	100
KNO_3 reduction	0	0
Brown pigment production	37	97
Sodium hippurate hydrolysis	38	100

[a] Weakly positive in 10 s.

TABLE 2. *Biochemical characteristics of five species of Legionella*

Cultures	Biochemical tests for: Oxidase	Catalase	β-Lactamase	Gelatin liquefaction	KNO_3 reduction	Brown pigment[a]	Blue fluorescent pigment[b]	Sodium hippurate hydrolysis
L. pneumophila								
Knoxville 1,[c] Togus 1,[c] Bloomington 2,[d] OLDA[c]	+	+	+	+	−	+	−	+
Los Angeles 1[c]	+	+	+	+	−	+	−	−
L. bozemanii								
WIGA	−	+	+	+	−	+	+	−
L. dumoffii								
TEX-KL	−	+	+	+	−	+	+	−
L. micdadei								
HEBA	+	+	−	+21[e]	−	−	−	−
TATLOCK	+	+	−	−21[e]	−	−	−	−
PPA[f]	+	+	−	−21[e]	−	−	−	−
L. gormanii								
LS-13	+	+	+	+	−	+	+	−

[a] Water-soluble pigment.
[b] Autofluorescent cellular pigment.
[c] Clinical isolates from serogroups 1, 2, and 4.
[d] Environmental isolate of serogroup 3.
[e] Indicates delayed or no liquefaction after 21 days.
[f] PPA, Pittsburgh pneumonia agent.

micdadei were negative for alkaline phosphatase (Table 3).

Data from the in vitro antimicrobial susceptibility testing of clinical and environmental strains of *L. pneumophila* are shown in Table 4. Antimicrobial susceptibility data for *L. bozemanii* (WIGA) and for *L. micdadei* were described previously (2, 11), but are included in Table 4 for comparison with *L. pneumophila*. The susceptibility patterns of the environmental and clinical isolates of *L. pneumophila* were the same for the nine antimicrobial agents tested, with the exception that the minimum inhibitory concentrations to ampicillin were slightly lower for the environmental isolates. The *L. bozemanii* strain was more susceptible to the combination sulfamethoxazole-trimethoprim and less susceptible to erythromycin than were the *L. pneumophila* isolates. As reported previously, the *L. micdadei* strain is β-lactamase negative and very susceptible in vitro to β-lactam antibiotics, but less susceptible to erythromycin and chloramphenicol than are *L. pneumophila* isolates (11).

TABLE 3. *Results of enzymatic tests performed on 49 Legionella cultures[a] belonging to five species*

Substrate	Enzyme	Product	% Positive
Diacetylfluorescein	Esterase	Fluorescein	100
4-MU propionate	Esterase	4-MUO[b]	94[c]
4-MU nonanoate	Esterase	4-MUO[b]	94[c]
Disodium-NP[d] phosphate	Alkaline phosphatase	*p*-Nitrophenol	94[e]
Potassium-NP[d] sulfate	Arylsulfatase	*p*-Nitrophenol	0

[a] *L. pneumophila* (39 environmental cultures and 4 clinical cultures), *L. bozemanii* (1), *L. dumoffii* (1), *L. micdadei* (3), and *L. gormanii* (1).
[b] MUO, Methylumbelliferone.
[c] Blue autofluorescence of cells of *L. bozemanii, L. dumoffii,* and *L. gormanii* prevented determination of enzyme activity.
[d] NP, *p*-Nitrophenyl.
[e] *L. micdadei* cultures were negative.

DISCUSSION

We attempted to demonstrate the hemolytic activity of the environmental and clinical isolates of *L. pneumophila* on guinea pig, rabbit, and sheep cells by adding 5% defibrinated blood to complete F-G agar, as described by Baine et al. (1). Both surface and subsurface growth was poor and hemolysis was weak or absent so that it was not possible to characterize the hemolytic reactions. Baine et al. also reported weak and variable hemolytic reactions with lysis occurring mainly around areas of confluent growth.

TABLE 4. *Comparison of the in vitro activity of nine antimicrobial agents on environmental and clinical isolates of L. pneumophila, L. bozemanii, and L. micdadei*[a]

Antimicrobial agent	Minimum inhibitory concn (μg/ml) for:							
	Environmental *L. pneumophila* (35 isolates)			Clinical *L. pneumophila* (19 isolates)			*L. bozemanii*	*L. micdadei*
	GM[b]	Mode	Range	GM	Mode	Range		
Erythromycin	0.39	0.25	0.12–0.5	0.22	0.25	0.06–0.5	4–2[c]	4–2[c]
Rifampin	0.05	0.06	0.03–0.06	0.06	0.06	0.03–0.06	0.06	0.06
Doxycycline	8	8	8	8	8	8	8	2
Cephalothin	13.6	16	8–32	22	32	8–32	8	2
Cefoxitin	0.28	0.25	0.12–0.5	0.38	0.5	0.12–0.5	0.25	0.12
Ampicillin	1.4	1.0	0.5–4	4.2	4	1–16	4	≤0.12
Chloramphenicol	1	1	1	1.2	1	1–2	2	4
Gentamicin	1.8	2	1–2	1.7	2	1–2	2	1
Sulfamethoxazole/ trimethoprim[d]	10.8/0.57	9.5/ 0.5	9.5/0.5–19/1	8.6/0.45	9.5/0.5	4.8/0.25–9.5/0.5	1.2/0.06	9.5/0.5

[a] All cultures were grown on CYE agar.
[b] GM, Geometric mean.
[c] Results on two different strains.
[d] Slash represents a ratio of approximately 19/1 between the concentration of the two drugs.

We were unsuccessful also in clearly demonstrating lecithinase and lipase activity of *L. pneumophila* environmental and clinical isolates on egg yolk in either F-G agar or in a modified Ristroph broth (26) and agar medium. Neither 2% nor 5% hen egg yolk added to the media as substrate for the enzyme tests gave satisfactory results primarily because of poor growth of the cultures. As suggested by Baine et al, the egg yolk appears to inhibit growth of *L. pneumophila.*

It is apparent that adequate definition of both the hemolytic activity of *L. pneumophila* and their enzymic activity on egg yolk phospholipids requires further study and evaluation before these tests can be determined useful for characterization of these bacteria.

On fluorogenic substrates, *L. pneumophila* was shown to contain enzymes that hydrolyze diacetylfluorescein, 4-MU propionate and 4-MU nonanoate yielding fluorescent end products that are easy to detect with a long-wavelength ultraviolet lamp. When 16 miscellaneous gram-negative cultures and one *Staphylococcus* culture were tested with diacetylfluorescein, only one culture of *Proteus rettgeri* and one unidentified culture were able to hydrolyze this substrate. Thus, because all cultures of the five species of *Legionella* tested hydrolyzed diacetylfluorescein, this substrate may help to differentiate *Legionella* from many other gram-negative bacteria. The enzymes necessary for splitting 4-MU propionate and 4-MU nonanoate were found in a variety of gram-negative bacteria which were tested. Therefore, the 4-MU substrates are of no value in differentiating *L. pneumophila* from these bacteria. Also, the enzyme activity of *L. bozemanii* (2), *L. dumoffii* (2), and *L. gormanii* (4, 21) on the 4-MU substrates could not be determined because of the natural bright blue autofluorescence of these species. If, however, future studies show that cellular blue autofluorescence is a consistent characteristic of all strains of these three species, this feature will serve as an excellent marker to differentiate them from *L. pneumophila* and *L. micdadei.* None of the *L. pneumophila* cell suspensions tested autofluoresced.

Because many gram-negative bacteria have alkaline phosphatase activity, tests for this enzyme are not helpful in characterizing the genus *Legionella.* The absence of alkaline phosphatase activity in *L. micdadei,* however, distinguishes this species from the other four *Legionella* species. Tests for diacetylfluorescein esterase appear to be valuable in characterizing *Legionella,* but other enzyme tests should be evaluated for this purpose.

Henderson and Milazzo (13) reported that arylsulfatase activity is induced in *S. typhimurium* by the presence of tyramine. Milazzo and Fitzgerald (20) and Rammler et al. (25) found that arylsulfatase production was repressed by cysteine, thiosulfate, sulfate, and sulfite. Because *L. pneumophila* requires cysteine for growth (6, 7), the addition of tyramine to CYE medium was necessary in testing for arylsulfatase. With L-cysteine in the medium, arylsulfatase was induced in the three *Salmonella* control cultures and one *Klebsiella pneumoniae* control culture by 10 mM tyramine. Arylsulfatase activity was not detected in any *Legionella* culture grown on CYE with tyramine under the same conditions.

Because of variations from standardized conditions the in vitro antimicrobial susceptibility data must be interpreted cautiously, particularly from the standpoint of establishing an optimum therapeutic regimen. The smaller inoculum used in this study resulted in minimal inhibitory concentration endpoints that are about one dilution lower than those previously reported from all species of *Legionella* (11). The *L. pneumophila* strains isolated from the environment have the same antimicrobial patterns as those isolated from clinical cases, but all *L. pneumophila* isolates have a pattern distinctly different from that of *L. bozemanii* and *L. micdadei*. CYE agar provides growth requirements for *Legionella* strains that permit a smaller cell inoculum for susceptibility testing while giving adequate and consistent growth. Thus, a valid comparison can be made of susceptibility profiles among the different species of *Legionella* and also of isolates from different sources.

When 38 environmental cultures of *L. pneumophila* were compared with clinical isolates, no important differences were found in their morphological, physiological, or serological properties or in their susceptibility to antimicrobial agents. Guinea pigs infected with the water strains showed the clinical symptoms associated with infection of these animals by human lung isolates of *L. pneumophila*. Regardless of the serogroup to which they belonged, all 38 water strains reacted positively in the indirect fluorescent antibody test with antibodies in the convalescent serum (1:256 dilution) of a human case of legionellosis proved by isolation of a serogroup 1 culture. The evidence from this and other reports (8, 9) suggests that the etiological agent of human legionellosis resides in natural or artificial aqueous habitats.

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