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Properties of alkaliphilic halophiles

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SUMMARY

Microorganisms capable of growth under highly alkaline conditions have been isolated from natural habitats. The isolation process utilized pH levels of 9.7 to 11.0, salts of 4.0 to 5.0%, temperatures of 45–50°C, and anaerobic conditions. Four isolates are described as gram-positive, spore-forming, motile, catalase-positive rods. This indicates that they are in the genus *Bacillus*. The culture characteristics of these and two other isolates were evaluated. All six strains tolerated up to 11.0% salts in the growth medium. The purpose of this study was to evaluate these microorganisms for their potential use in combination with an alkaline flood for enhanced oil recovery. Useful products in in situ microbial-enhanced oil recovery include gases, surfactants, polymers and acids. The best gas producer was *Bacillus* strain ACP-1 which evolved an average of 2.11 ml gas per ml medium in 96 h. *Bacillus* strain ACP-1 reduced the surface tension of the growth medium from 50.3 mN/m to an average of 44.1 mN/m. Another aspect of this research project included investigation of the movement of these microbes through a porous medium. Various synthetic surfactants were found to improve movement through a porous system, while other surfactants improved gas production.

INTRODUCTION

Alkaliphiles are microorganisms with the capacity to grow at high levels of pH (9.0–11.0). Isolates that are capable of growing at pH 11.0 or higher may be considered extreme alkaliphiles. Most alkaline-resistant, bacterial isolates can also tolerate high concentrations of salt. Thus, they can be referred to as alkaliphilic halophiles.

Alkaliphilic or halophilic microorganisms have received limited attention in the literature as compared to other types of bacteria. The major contributions to the study of alkaliphiles have been summarized in the monograph by Horokoshi and Akiba [10]. A great deal of work prior to their publication involved the study of enzymes produced by alkaliphiles. Horokoshi [7] provides some important work on isolation and characterization of an alkaline protease, alkaline amylase [8] and an alkaline pectinase [9]. More recent work pertinent to isolation and analysis of alkaliphilic microorganisms includes that of Nakamura and Horikoshi [16] and Ikura and Horikoshi [11]. The most common genus of alkaliphiles is the genus *Bacillus* [6,17].

Most research on alkaliphiles, their growth characteristics, enzymes, and products has resulted from

the interest in their unique ability to tolerate extreme physical conditions. However, there are many applied industrial problems which could utilize microbes of this type. Alkaliphilic halophiles may be very useful in microbial-enhanced oil recovery (MEOR). By conventional methods, only 20–30% of the petroleum can be recovered from an oil reservoir. The other 70–80% remains trapped within the porous reservoir mineral which is usually sandstone and/or dolomite. Some microbiologists are working to find bacterial types that are useful in producing bulk products either at the surface or in situ for economic recovery of crude oil. A more established chemical method in enhanced oil recovery (EOR) is the use of the alkaline waterflood. However, these oil recovery systems need improvement. The pH of an alkaline waterflood is usually above 10.0. Besides caustic, sodium orthosilicate is often added to give alkaline conditions. Publications of interest relating to alkaline flooding include Van Poollen [19], Burdyn et al. [1] and Chang [2]. Some important literature on the use of microorganisms for oil recovery include Clark et al. [3], Grula et al. [5], Knapp et al. [15] and Finnerty and Singer [4].

The purpose of this investigation was to isolate and characterize bacteria which are alkaline resistant and which produce products that enhance oil recovery. Alkaline tolerance would allow their use in conjunction with an alkaline flood. The microbes developed must also be able to tolerate the reservoir's physical conditions of high pressure and temperature. Thermotolerance ($< 65°C$) is necessary, as temperatures increase 1°C with every 100 ft of depth. Also important is tolerance to the high salt concentrations (4–13%) often found in oil fields. In addition, for application in in situ programs, the isolates should be facultative anaerobes in order to survive in an oil reservoir where oxygen availability is quite limited.

Six strains of *Bacillus* were isolated that were capable of tolerating the physical, chemical conditions existing in an oil reservoir during or following an alkaline flood. These isolates were evaluated for their product-forming capabilities. Microbial products that are important in removing crude oil from a reservoir include gases, surfactants, polysaccharides and, for dolomite-carbonate reservoirs, possibly organic acids. This study focussed on production of gases and surfactants by the alkaliphilic halophiles.

In in situ MEOR, one must understand the movement and/or plugging properties of the microorganisms to be used. *Bacillus* strain ACP-1 was analyzed for its plugging effects upon injection into sand columns. In addition, various surfactants were used to pretreat these columns in an attempt to reduce microbial plugging. An effective surfactant may be used to improve the distribution of microbes in a reservoir. Surfactants were also tested for their effect on growth and product formation by the microbes. Pertinent literature relating to microbial plugging and movement through porous media include Jenneman et al. [14], Updegraff [18], Jang et al. [13] and Jack et al. [12].

MATERIALS AND METHODS

Isolation of strains

An enrichment medium was developed for the purpose of isolating anaerobic, thermotolerant, alkaliphilic halophiles. The original medium was similar to that recommended by Horikoshi and Akiba [10]. Various changes were instituted during the isolation process mostly to correct the problem of precipitation of salts. The final medium used was: 2.0% sucrose/0.5% yeast extract/0.5% peptone/2.5% NaCl/1.0% Na_2CO_3/0.5% KCl/0.5% sodium citrate/0.3% $NaNO_3$/0.2% K_2HPO_4/0.05% cysteine HCl/0.02% $MgCl_2$-$7H_2O$/1 ppm $MnCl_2$-$7H_2O$ (v/v). These components were separated into three flasks, one containing the Na_2CO_3, one containing all other salts, and another containing the remaining components. The flask containing the salts was acidified to pH 4.0 with 2 N HCl to prevent precipitation of salts. After sterilization, the flasks were cooled and combined. Except where noted, the final pH was 9.7–9.8. All pH determinations were completed with a Sargent-Welch pH meter.

Two source materials were utilized to isolate al-

kaliphilic halophiles. One source was a core from an oil field in Pecos, TX. The other source was a collection of sediment samples from a body of standing water on a salt flat 80 miles east of El Paso, TX. Small amounts of powdered core and salt flat sediment were added to sterile 125 ml flasks. The flasks were filled as high as possible with the isolation medium. In the original isolations, pH levels of 10.0–11.0 and greater were used and some precipitation of salts was observed. These flasks were plugged with No. 5 stoppers fitted with syringe needles. The head space was never greater than 5 mm and the syringe needles allowed release of any pressure from accumulation of metabolic gases. With the presence of a poising agent, cysteine-HCl, and by limiting the oxygen transfer to the system, facultative anaerobic conditions were achieved. The incubation temperature was 45°C.

Initially, it was difficult to isolate stable microorganisms that could be consistently subcultured. For this reason, the isolation medium was eventually modified to give a lower pH (9.7–9.8) with virtually no precipitates. Several stable cultures were isolated and lyophilized. However, the only isolates that grew from these lyophiles were ASF-2, ASF-4 (salt flat isolates), ACP-1 and ACP-2 (core isolates, Pecos). These four isolates were studied along with two other cultures, CR-9 and D2-1, that were isolated as commensals during growth of the algae *Spirulina maxima* under alkaline conditions (>pH 10.0). Cellular morphology, Gram reaction, spore formation and motility were determined along with maximum pH, salt concentration and temperature for growth. In these and all subsequent experiments, the modified isolation medium was used as the standard culture medium unless specified otherwise. The standard growth conditions were anaerobic at 45°C. All inoculations were 5% (v/v) and the growth period was 48 h.

The alkaliphilic halophiles were grown anaerobically in 125 ml Erylenmeyer flasks and analyzed for biosurfactant production on the basal medium at pH levels of 9.0, 9.5 and 10.0. Adjustments of pH were made with 1 M HCl or 1 M NaOH. Surface tension of cultures and supernatant samples were determined at room temperature with a Fisher Autotensiomat which uses the Du Nuoy ring method. Removal of cells to obtain supernatant samples and biomass measurements involved centrifugation for 15 min at 10000 × *g*.

Gas production

The alkaliphilic halophiles were tested for gas production on the standard basal medium with and without various surfactants added. In each case, the isolates were grown in two classical Durham fermentation tubes. One tube had a capacity of approximately 28 ml, the other approximately 36 ml. The tubes were fitted with stoppers and syringe needles. Cumulative gas production was determined by periodically checking the tubes, marking them at the level of gas production, inverting them to remove the gas bubble, and allowing for further growth and gas production. Each mark on the tubes was accompanied by a notation of the growth time. At the end of 96 h, the volume required to fill the tubes to each mark was recorded and cumulative gas production was calculated.

Porosity and permeability

Cells of isolate ACP-1 were analyzed for their ability to pass through a porous system. The quartz sand columns consisted of PVC piping cut in lengths of 27.2 cm and 4.1 cm in diameter. The columns were closed at one end with a No. 9 rubber stopper fitted with a glass tubing port. Five layers of wire screen were fixed to the inside end of the stopper to confine the sand to the column. The column was filled with clean sand (all <0.35 mm in diameter) and agitated for 3 min to allow settling. The other end of the column was fitted with another stopper, port and wire screen barrier. The glass tubing ports were fitted with pieces of rubber tubing and clamps to allow control of flow through the column. Each completed column was weighed for dry weight.

To determine the permeability for the columns, a 5% NaCl flood was passed through each packed column. This was accomplished by placing the injection fluid in a small vessel at a level of 58 cm above the top of the column. The vessel was connected to the bottom of the column by Tygon tub-

ing. The clamps were opened, and the flow of brine was controlled by using a specific gravity head pressure as the column was flooded. When brine emerged at the top of the column, it was channeled into a graduated cylinder. Times were recorded for every 50 ml collected until 300 ml of effluent were recovered. The flow rate in ml/s was converted into permeability by using Darcy's formula:

$$K = \frac{Q}{\frac{\Delta h}{L} \times A}$$

where K is permeability, Q is the flow rate, Δh is the loss in head pressure, L is the length of the column, and A is the cross-sectional area of the column.

The column was then clamped off and weighed for wet weight. The increase in weight is a measure of the pore volume or void space of the sand column. The pore volume divided by the total column volume (314 ml) gave the percent porosity. At this point, the columns were utilized for additional evaluation: 200 ml of brine (control) or 0.5% surfactant in brine. A cell suspension was then injected into the end of the Tygon tubing where it connects to the bottom of the columns. The cells were injected into the columns prior to injection of additional brine. Flow rates were determined again and percent plugging as measured by the decrease in permeability was calculated.

RESULTS AND DISCUSSION

Table 1 lists the general characteristics of the six alkaliphilic halophiles. Microscopic examination of these isolates revealed that all were motile and rod shaped. The core and salt flat isolates were Gram positive, spore formers. These isolates, which are also catalase positive, can be classified in the genus *Bacillus*. The algal commensals were gram-negative, non-spore formers. The isolates were studied for growth in duplicate test-tubes at pH levels from 5.0 to 11.5. Isolates ACP-1, ACP-2 and ASF-4 grew at all levels up to and including pH 10.0. Isolates D2-1 and CR-9 grew at all pH levels from 6.5 to 10.5. Isolate ASF-2 could only grow as high as pH 9.7–9.8. The isolates were tested for growth in duplicate culture tubes containing test media with total salts ranging from 5.0 to 17.0%. The bacteria growing in conjunction with *Spirulina* were more salt tolerant than the other cultures. However, all of the cultures tolerated far more salt than the amount used in the isolation medium. The cultures were also tested in duplicate test-tubes on the standard test medium for growth at 19, 36, 45, 53 and 65°C. Only the algal commensals grew at 19°C. The data in Table 1 indicate that the algal commensals were far more tolerant to physical, chemical changes in growth conditions than the other alkaliphiles. In contrast, the core and salt flat bacterial isolates possessed several interesting properties of value when coupled to oil recovery processes. The

Table 1

General characteristics of isolates of *Bacillus*

ACP, core isolates, Pecos, TX; ASF, salt flat isolates, southern New Mexico; D2-1 and CR-9, Commensals with *Spirulina maxima*. Note: ACP + ASF isolates are strong producers of gas, slime and organic acids.

Isolate	Cell morphology	Gram reaction	Spores	Motility	Max pH	Max % salt conc. (v/v)	Max temp (°C)
ACP-1	rods	+	+	+	10.0	12.5	45
ACP-2	rods	+	+	+	10.0	12.5	45
ASF-2	rods	+	+	+	9.8	12.5	53
ASF-4	rods	+	+	+	10.0	11.0	45
D2-1	rods	–	–	+	10.5	15.5	45
CR-9	rods	–	–	+	10.5	15.5	45

ability to form spores is quite important as spores move more easily through reservoir mineral than the vegetative cells. Of course at some point, germination of spores, formation of vegetative cells and product synthesis must follow. Visual examination of these isolates in liquid media indicated that they produce copious quantities of gas and polysaccharide slime. In addition, these strains produce organic acids (low final pH) which could be very useful in oil recovery from limestone reservoirs. The acids can react with carbonate in these reservoirs, resulting in the release of carbon dioxide.

Five of the six alkaliphilic halophiles were tested for reduction of surface tension of the standard medium adjusted to three levels of pH 9.0, 9.5 and 10.0. Samples of spent culture media were centrifuged and surface tension was determined for each supernatant sample. Spent medium and supernatant surface tension data are shown in Table 2 along with final pH and biomass values. Of the cultures, isolates ASF-4, ACP-1, and D2-1 reduced the surface tension the most. Isolate ACP-1 appears to be the most productive of the three, especially at pH 9.0 and 9.5. None of the five alkaliphilic cultures are regarded as good surfactant producers. Removing the cells from these samples usually had a critical effect on the surface tension. Dramatic increases in surface tension resulted when cells were removed from broth for ASF-4 at pH 9.0, ACP-1 at pH 9.0 and 9.5, and ACP-2 at pH 9.0. This is associated with the surface-active effect of the cells per se. Isolates ASF-2, ACP-1, and ACP-2 had difficulty growing at pH 10.0. Interestingly, any culture that grew at pH 10.0 did not reduce the pH of the medium significantly. The salt flat and core isolates consistently reduced the pH to 5.7–6.3 when the initial pH was 9.0 or 9.5.

During the initial isolation process, it was noted that all core and salt flat isolates appeared to produce large amounts of gas. Thus, the six alkaliphilic halophiles were tested for cumulative gas production over a 96-h growth period in two classical Durham fermentation tubes. This preliminary study

Table 2

Biosurfactant production from alkaliphilic halophiles

Growth period was 72 h. γ_S, surface tension between liquid and air; γ_S of water = 72.7 mN/m; γ_S of medium = 50.3 mN/m. The study involved duplicate flasks.

Isolate	Initial pH	Whole Broth (γ_S mN/m)	Supernatant (γ_S mN/m)	Final pH	Biomass (g/l)
	9.0	55.5	58.9	5.9	0.65
ASF-2	9.5	53.7	56.8	6.0	0.95
	10.0	–	–	–	–
	9.0	41.6	51.8	6.3	1.04
ASF-4	9.5	45.5	48.6	5.9	0.65
	10.0	47.8	50.7	9.6	1.09
	9.0	42.7	52.0	6.2	1.00
ACP-1	9.5	42.7	50.5	6.0	0.63
	10.0*	49.5	51.5	9.7	1.13
	9.0	46.8	57.2	5.7	0.95
ACP-2	9.5	48.5	54.1	6.2	0.50
	10.0*	51.8	55.6	9.6	1.07
	9.0	46.6	48.0	8.7	1.10
D2-1	9.5	45.3	46.7	9.1	1.18
	10.0	43.1	46.4	9.6	1.20

* Only one duplicate grew.

showed that the algal commensals were very poor gas producers., Of the core and salt flat isolates, ASF-2 was the most productive, evolving an average of 52.6 ml of gas. ACP-1 was also productive, evolving an average of 44.0 ml of gas. Another study indicated that carbon dioxide is the most abundant gas produced. Since isolate ACP-1 is both a good gas producer and a moderate surfactant producer, this microbe and isolate ASF-2 were selected for further gas production studies.

Notably, synthetic surfactants are utilized to recover crude oil from petroleum reservoirs in what are known as miscible oil floods. In addition, this area is regarded as important, since certain surfactants have potential for improving penetration of microorganisms into the oil reservoir. For these reasons, isolates ACP-1 and ASF-2 were tested for growth and gas production on the standard test medium with selected surfactants added. Each surfactant was tested at 0.5% concentration in two Durham fermentation tubes with an incubation period of 96 h. The surfactants were added to the growth medium prior to sterilization. None of them changed the pH of the medium. Isolate ASF-2 was tested on six surfactants with a range of hydrophilic-lipophilic balances (HLB) from 4.7 to 24.0. Table 3 provides data on cumulative gas production by this microbe on the test medium (control) and using six surfactants. The most notable effect on this isolate was the total inhibition of growth by Span 20, HLB 8.6. All other surfactants, with the exception of P75 (HLB 16.5), caused partial inhibition of gas production.

Table 4 shows data on cumulative gas production by isolate ACP-1 on the standard test medium as a control and the same medium to which seven surfactants were added. The HLB range of these surfactants was 4.7–29.0. This isolate was also inhibited by 0.5% Span 20 (HLB 8.6). However, far more important was the effect of adding 0.5% P104 (HLB 13.0) to the medium. This surfactant caused an increase in gas production of over 44%. The surfactants F87 (HLB 24.0) and Span 60 (HLB 4.7) also increased gas production. In the presence of 0.5% P104, isolate ACP-1 produced over 15% more gas than isolate ASF-2 with 0.5% P75 added

Table 3

Effect of various surfactants on gas production of *Bacillus* strain ASF-2

HLB, hydrophilic-lipophilic balance; TW, Tween; NG, no growth. The study involved duplicate tubes, capacity approx. 32 ml. All surfactant concentrations at 0.5% v/v.

Growth period (h)	Gas production (ml)						
	Control	Span 60 HLB 4.7	Span 20 HLB 8.6	P104 HLB 13.0	TW 80 HLB 15.0	P75 HLB 16.5	F87 HLB 24.0
17	0	0	NG	0	0	0	0
20	0	0	NG	9.1	0	2.6	4.8
23	0	1.6	NG	18.1	0	20.0	13.8
26	0	13.3	NG	23.2	0	39.1	30.7
29	15.2	25.7	NG	25.8	2.2	42.9	34.0
32	33.6	31.4	NG	–	9.0	–	–
38	46.6	34.5	NG	28.5	20.8	49.4	37.1
47	49.0	37.0	NG	30.7	31.6	50.5	38.9
59	50.7	39.0	NG	32.7	35.4	51.5	40.8
72	51.6	41.2	NG	34.7	38.2	52.5	42.4
96	52.6	43.4	NG	37.7	41.1	53.7	45.1
Final pH	6.4	6.3	NG	6.0	6.2	6.3	6.2
Biomass g/l	0.67	0.70	NG	0.67	0.76	0.53	0.72

Table 4

Effect of various surfactants on gas production of *Bacillus* strain ACP-1

HLB, hydrophilic-lipophilic balance; TW, Tween; NG, no growth. The study involved duplicate tubes, capacity approx. 32 ml. All surfactant concentrations at 0.5% v/v.

Growth period (h)	Gas production (ml)							
	Control	Span 60 HLB 4.7	Span 20 HLB 8.6	P104 HLB 13.0	TW 80 HLB 15.0	P75 HLB 16.5	F87 HLB 24.0	F68 HLB 29.0
F68 HLB 29.0								
17	0	0.5	NG	0	0	0	0	0
20	1.5	13.2	NG	0	0	0	1.5	0
23	10.5	25.3	NG	0.1	0	1.1	14.5	0.5
26	21.3	29.6	NG	5.7	0.8	7.6	23.7	2.6
29	26.3	32.1	NG	21.2	2.9	14.9	34.1	11.1
32	30.5	34.1	NG	34.0	9.9	20.4	38.8	18.5
38	32.8	36.8	NG	47.8	19.5	27.0	41.1	22.0
47	35.7	42.5	NG	53.0	25.2	31.3	43.7	25.9
59	37.8	45.2	NG	58.2	28.5	35.0	46.5	29.6
72	41.7	47.3	NG	60.7	31.9	38.4	48.6	33.6
96	44.0	48.9	NG	63.5	35.0	42.0	51.0	40.2
Final pH	5.9	6.2	NG	6.4	6.0	6.0	6.1	6.2
Biomass g/l	1.22	1.54	NG	1.29	1.47	1.54	1.64	1.21

(the second highest gas production observed). This effect was so intriguing that subsequent experiments were designed to investigate some of the important parameters involved in gas production. Several of these studies utilized high pressure gas cylinders which contained 200 ml of culture and were fitted with pressure gauges. Isolate ACP-1 produced 25% greater pressure in a cylinder containing the test medium supplemented with 0.5% P104 than it did in the control cylinder.

The presence of 1.0% sodium carbonate in the media used for gas production studies simulates limestone reservoirs or sandstone reservoirs of the carbonate type. Analysis of the test medium showed that no more than 16 ml of carbon dioxide per 32 ml of medium are evolved upon reduction of the pH to 5.5. This shows that metabolic gases account for at least 74.8% of the gases produced by isolate ACP-1 from the standard medium with P104 added.

Several surfactants were tested for their ability to improve the movement of isolate ACP-1 through a porous system. Eight sand columns were constructed. Their permeabilities to 5% NaCl floods and porosities were determined. Four of the columns were flooded with an additional 200 ml of brine. The other four columns were flooded with 200 ml of a solution of 0.5% F87 in brine. The surfactant F87 is a Pluronic with an HLB of 24.0. Two columns, one control and one treated with the surfactant, received injections of 0.17 g of cells in 20 ml of 1% NaCl. Six columns, three controls and three treated with F87, received injections of 0.36 g of cells in 20 ml of 1% NaCl. Table 5 shows the initial permeabilities and porosities of these eight columns. Also given are the permeabilities of the sand columns after being loaded with cells. The percentage plugging and decreased permeabilities resulting from cell loading are shown in the far right column. These data clearly indicate that by pretreating the columns with the surfactant F87, flow and cellular movement through the columns are im-

Table 5

Movement of *Bacillus* strain ACP-1 through sand columns

IP, initial permeability; PAL, permeability after load; D, permeability in Darcy.

Column	Treatment	Cell load (g/20 ml)	Porosity (%)	ID (D)	PAL (D)	Plugging* (%)
1	5% NaCl Control	0.17	36.2	33.7	2.1	93.8
2	5% NaCl Control	0.36	34.9	33.7	3.2	90.5
3	5% NaCl Control	0.36	34.5	32.6	6.8	79.1
4	5% NaCl Control	0.36	35.9	39.0	12.2	68.7
5	5% NaCl 0.5% F87	0.17	34.9	30.8	13.2	57.1
6	5% NaCl 0.5% F87	0.36	34.6	31.5	9.3	70.5
7	5% NaCl 0.5% F87	0.36	36.6	40.8	15.8	61.3
8	5% NaCl 0.5% F87	0.36	35.7	36.9	17.2	53.4

* Percent decrease in permeability.

proved significantly. Although not all surfactants give beneficial results, F87 certainly improved the flow rate through all sand packs tested. Injection of 0.17 g of cells into columns Nos. 1 and 5 showed that the percentage plugging for the F87-treated column (No. 5) was 36.7% lower than that of the brine flood column (No. 1). Upon injection of 0.36 g of cells, the average decrease in permeability for treated sand columns was 17.7%, lower than that for untreated sand columns. Four other surfactants, P75, P104 (Pluronics), Tween 80 and Tween 20, were tested at 0.5% concentration for improvement of mobility of isolate ACP-1 through sand columns. However, the Pluronic F87 was clearly the most effective. Although the sand columns are much more permeable than reservoir material, the results of this study should encourage more investigative work in this area.

CONCLUSIONS

The major objective of this research has been to investigate alkaliphilic microorganisms for their ability to tolerate some of the typical oil reservoir conditions of high salt concentration, high temperature, and high pressure. In addition, the property of alkaliphilicity was examined to see what level of caustic pH tolerance existed for bacteria that might be used in an alkaline flood for oil recovery. Several strains were isolated that may have potential for use in this area. Another goal was to examine alkaline strains for productive capabilities for enhanced oil recovery. The original screen for a surfactant producer was not very successful. However, several strong gas producers were encountered. The most important finding was that some synthetic surfactants may be used in conjunction with mi-

crobes, not only to increase oil recovery, but also to enhance their gas production and movement through porous systems with reduced porosity. Additional studies should be conducted in this area utilizing more sophisticated porous systems. The research project on alkaliphilic halophiles requires a detailed study of the taxonomy of these microorganisms, as they quite possibly represent new species of the genus *Bacillus*.

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