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The obligate alkaliphile *Bacillus clarkii* K24-1U retains extruded protons at the beginning of respiration

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Abstract Alkaliphiles grow under alkaline conditions that might be disadvantageous for the transmembrane pH gradient (ΔpH, outside acidic). In this study, the behaviors of extruded protons by the respiration of obligate alkaliphilic *Bacillus clarkii* K24-1U were investigated by comparison with those of neutralophilic *Bacillus subtilis* IAM 1026. Although whole-cell suspensions of both *Bacillus* species consumed oxygen immediately after the addition of air, there were lag times before the suspensions were acidified. Under alkaline conditions, the lag time for *B. clarkii* significantly increased, whereas that for *B. subtilis* decreased. In the presence of valinomycin or ETH-157, which disrupts the membrane electrical potential (Δψ), the cell suspensions of both *Bacillus* species acidified immediately after the addition of air. Artificial electroneutral antiporters (nigericin and monensin) that eliminate the ΔpH exhibited no significant effect on the lag times of the two *Bacillus* species except that monensin increased the lag times of *B. clarkii*. The inhibition of ATPase and the Na^+ channel also exhibited little effects on the lag times. The increased lag time for *B. clarkii* may represent the Δψ-dependent proton retention on the outer surface of the cytoplasmic membrane to generate a sufficient ΔpH under alkaline conditions.

Keywords Alkaliphiles · *Bacillus clarkii* · Membrane electrical potential · Proton efflux · *Bacillus subtilis*

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Introduction

Alkaliphiles exhibit optimal growth at a pH above 9 (Horikoshi 1999). Under alkaline conditions, alkaliphiles maintain an intracellular environment that is more acidic (pH below 9) than the exterior environment to maintain the functions of their intracellular proteins (Hase et al. 2001; Krulwich et al. 1998, 1996; Padan et al. 2005; Sturr et al. 1994; Yumoto 2002). The more acidic cytoplasm is disadvantageous for the formation of the proton (H^+) motive force (Δp). According to Mitchell's chemiosmotic theory (Mitchell 1961), ATP synthesis depends on the Δp (Belevich and Verkhovsky 2008; Branden et al. 2006; Kitada and Horikoshi 1992; Krulwich 1986) generated by the transmembrane pH gradient (ΔpH; acid out) and the membrane electrical potential (Δψ; positive out). The acidic inside is the opposite of the ΔpH that constitutes the Δp. However, alkaliphiles utilize the ΔpH for ATP synthesis by H^+-translocating ATPase (Hicks and Krulwich 1990; Hoffmann and Dimroth 1991), and they hardly increase their Δψ above the level sufficient for ATP synthesis (Guffanti et al. 1984). A central question is how alkaliphiles maintain their ΔpH under alkaline conditions for ATP synthesis. Since the ΔpH is generated by the extrusion of protons by the respiration system, an investi-

gation of the behavior of respiratory-extruded protons is a key to understanding the physiology of alkaliphiles.

Bacillus clarkii K24-1U is an obligate alkaliphile which shows no growth at pH7. The specific growth rate of *B. clarkii* at pH10 (μ_{max}=0.33) is larger than that (μ_{max}=0.26) at pH7 of its counterpart, neutralophilic *Bacillus subtilis* IAM 1026 (Ogami et al. 2009). A comparison of the behavior of respiratory-extruded protons from the two *Bacillus* species would reveal the alkaline adaptation mechanisms.

In this study, behaviors of the respiratory-extruded protons from *B. clarkii* and *B. subtilis* were investigated. At the beginning of respiration, there were lag times until the extruded H^+ entered the bulk water phase. The lag times were disrupted upon the addition of valinomycin and *N*,*N*′-dibenzyl-*N*,*N*′-diphenyl-1,2-phenylenedioxydiacetamide (ETH-157), which reduced the $\Delta\psi$ to zero (Simon and Carafoli 1979). The current experimental report is the first that suggests $\Delta\psi$-dependent H^+ retention on the surface of the cytoplasmic membrane of an alkaliphile under alkaline conditions.

Materials and methods

Organisms and growth conditions

B. clarkii K24-1U was isolated from soil in Yuubari, Hokkaido, Japan (Ogami et al. 2009). *B. subtilis* IAM 1026 was obtained from the IAM culture collections of the University of Tokyo. *B. clarkii* and *B. subtilis* were cultured in a peptone-yeast extract-alkaline medium containing 0.8% polypeptone, 0.1% yeast extract, and 0.1% K_2HPO_4 supplemented with 100 mM buffer solution of sodium phosphate (pH7 and 8) or sodium carbonate/sodium bicarbonate (final concentration: 18 mM sodium bicarbonate and 82 mM sodium carbonate for pH9; 65 mM sodium bicarbonate and 35 mM sodium carbonate for pH10) with reciprocal shaking (120 rpm) at 37°C. The cell growth was monitored by measuring the absorbance at 660 nm. In the log phase at 37°C, the doubling time of *B. clarkii* was 30 min at pH values of 9 and 10, and 100 min at pH8. No growth was observed at pH7 (data not shown). In all experiments, cells in the log phase (OD_{660}=1) were used. The whole cells were pelleted (2,840×*g*, 16 min, 4°C) and washed with solution A, consisting of 0.15 M NaCl, 0.11 M KCl, and 0.3 M sucrose, which was added for cell protection as previously described (Sone et al. 1999). The cells were suspended in the same solution (0.23 g wet weight ml^{-1}), and the cell suspension was kept on ice and used in assays within 10 h. The protein concentration of the cell solution was determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard. Valinomycin, monensin, ETH-157, EIPA, and nifedipine were purchased from Sigma-Aldrich (Bellefonte, PA). All other chemicals were of reagent grade and were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise stated. All experiments were performed more than three times.

Measurement of H^+ efflux

The H^+ efflux was monitored by observing the pH change in the bulk phase at 37°C with an F-52 pH meter (Horiba, Kyoto, Japan) as described previously (Yaginuma et al. 1997), with minor modifications. The cell suspension (0.23 g wet weight ml^{-1} of solution A) was diluted 15-fold with a solution consisting of 250 μM buffer [MOPS (pH7), HEPES (pH8), or glycine (pH9 or 10)] and 140 mM KCl. The pH of the solution was readjusted with a small volume of 0.5 N NaOH to the desired pH. The solution was magnetically stirred and kept under anaerobic conditions with the air over the cell suspension (i.e., in the head space) displaced by flowing water-saturated argon gas for 20 min, and the reaction was initiated by opening the vessel to air. The pH of the reaction mixture was monitored every 2.5 s. The lag time was defined as the time until the pH decrease exceeded 0.02 pH units for more than 5 s following the addition of air. This pH decrease (0.02) was more than 5 times the standard deviations of the pH values of the reaction mixture during anaerobic incubation. For the reference of the pH decrease at pH values of 7, 8, 9, and 10, 30 μl of 0.3 mM HCl was added to 3 ml of the reaction mixture. Valinomycin (1.5 μM), ETH-157 (10 μM), monensin (10 μM), nigericin (10 μM), amiloride (1 mM), EIPA (100 μM), or nifedipine (100 μM) was added to the reaction mixture before anaerobic incubation for 20 min. Representative lag times are reported as the mean±one standard deviation.

Measurement of H^+/O ratio and oxygen consumption rate

For the H^+/O ratio assay, the pH decrease induced by the oxygen pulse of the cell suspension was measured in a closed vessel at 37°C, as previously described (Minohara et al. 2002; Sone and Fujiwara 1991). The reaction mixture was the same as that used to study the H^+ efflux except for the addition of 1.5 μM valinomycin and 50 mM potassium thiocyanate (KSCN). The number of protons was calculated from the decrease in the pH, and the reaction solution added to 5 μl of 10 mM HCl was used as the standard.

Oxygen consumption was assessed by measuring the oxygen concentration with a galvanic oxygen electrode, MD-1000 (Iijima Electronics, Tokyo, Japan). The reaction was initiated by adding the whole cells to an air-saturated solution (total volume: 1.8 ml) consisting of 25 mM buffer

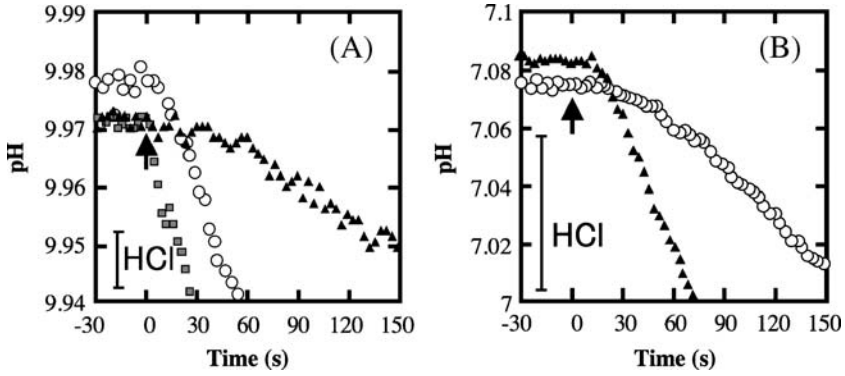

Fig. 1 Typical traces of pH decreases caused by proton effluxes of *B. clarkii* and *B. subtilis*. Changes in pH of a solution containing *B. clarkii* (*closed triangle*) and *B. subtilis* (*open circle*) cells after the addition of air were monitored at pH values of 10 (**a**) and 7 (**b**). The cell suspension of *B. clarkii* was also monitored in the presence of valinomycin (*gray square*). *Arrows* indicate the time (0 s) when the vessel under anaerobic conditions was opened to air. *Vertical bars* in the figures indicate the pH decrease after the addition of HCl (final concentration of 3 μM)

[MOPS (pH7), HEPES (pH8), or glycine (pH9 or 10)] and 140 mM KCl in a closed vessel at 37°C. The number of cells in the reaction mixture was optimized to obtain a linear slope of the electrode response (O_2 consumption/time). The solubility of the oxygen in the 140 mM KCl solution was taken to be 0.40 μg-atom ml^{-1} (Yaginuma et al. 1997).

Inhibition of ATP synthase

ATP synthase was inhibited by incubating the cells (10 mg ml^{-1}) in solution A with 50 μM dicyclohexylcarbodiimide (DCCD) for 30 h at 0°C, as previously reported (Michel and Oesterhelt 1980). The inhibition was confirmed by observing the rate of ATP accumulation in the cells in the reaction mixture of the H^+ efflux assay. The intracellular ATP concentration was determined using the CellTiter-Glo™ Luminescent Cell Viability Assay kit (Promega Corp., Madison, WI), prepared according to the manufacturer's instructions.

Results and discussion

Respiration-dependent acidification

Protons that entered the bulk water phase were monitored by measuring the pH decreases in the suspensions of the whole cells of *B. clarkii* and *B. subtilis*. Figure 1 shows typical trends of the pH decrease. Under anaerobic conditions, the pH values of the reaction mixtures were kept constant, indicating that the pH decrease was mainly caused by respiration-dependent H^+ extrusion in the conditions of this study. The cell solution of *B. clarkii* acidified with a lag time after the addition of air was much longer than that of *B. subtilis* at pH10 (Fig. 1A). On the other hand, *B. clarkii* showed a shorter lag time than *B. subtilis* showed at pH7 (Fig. 1B). Table 1 contains a summary of the calculated lag times of both *Bacillus* species at pH values of 7, 8, 9, and 10. The lag times for *B. clarkii* increased as the pH of the cell suspensions increased. In contrast, the lag times of *B. subtilis* showed no significant increase in the pH range of 7–10. The increased lag times for *B. clarkii* under alkaline conditions probably facilitated the adaptation to alkaline conditions.

Effects of Δψ

In the presence of valinomycin, the pH of the cell suspensions decreased immediately after the addition of air. Figure 1A shows the typical trends of the pH decrease of the cell suspension of *B. clarkii* in the presence of valinomycin at pH 10. This immediate acidification of the cell suspension shows that the respiratory-extruded protons entered the bulk water phase as soon as air was added in the absence of Δψ. Moreover, these results suggest that the respiration system extruded H^+ immediately after the addition of air even in the presence of Δψ. During the lag times, the extruded H^+ was preserved on the surface of the cytoplasmic membrane, perhaps due to the presence of the Δψ. The H^+ accumulation

Table 1 Proton efflux lag times at various pH levels

	Lag time (s)			
	pH7	pH8	pH9	pH10
B. clarkii	18±5.9	23±3.6	66±7.2	120±6.0
B. subtilis	37±4.3	33±2.3	39±3.4	21±6.0

Lag times of both *Bacillus* species were monitored in 140 mM KCl. Lag times are reported as mean±standard deviation

would generate a ΔpH sufficient for ATP synthesis. Our experimental report is the first to show the Δψ-dependent retention of respiratory-extruded H^+.

The accumulation of Δψ-dependent H^+ on the surface of the cytoplasmic membrane has been suggested by Kell and Hitchens (1982). The hypothetical proton well predicts H^+ accumulation due to the Δψ in H^+-conducting crevices on the exterior of the cytoplasm membrane (Mulkidjanian 2006). Based on electrostatic calculations, it was predicted that the membrane surface would separate from the bulk water phase by Δψ, an electrostatic barrier (Cherepanov et al. 2003; Mulkidjanian et al. 2006). These hypotheses explain the H^+ accumulation on the cytoplasmic membrane to generate a ΔpH sufficient to synthesize ATP under alkaline conditions. Based on the above-mentioned results and hypotheses, the lag time is likely a period in which the extruded H^+ is accumulated on the surface of the cytoplasmic membrane at the beginning of respiration. In the initial phase of the lag time, the values of the Δψ are

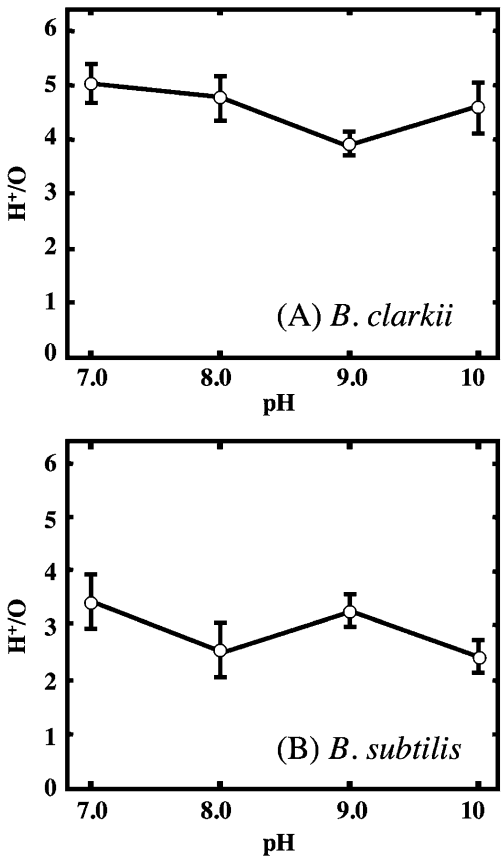

Fig. 2 H^+/O ratios. Changes in the pH of the cell suspensions of (**a**) *B. clarkii* and (**b**) *B. subtilis* on the oxygen pulse were calculated. The cell suspension in the presence of 1.5 μM valinomycin and 50 mM KSCN was kept under anaerobic conditions with water-saturated argon gas for 15 min, and the reaction was started by adding air-saturated 140 mM KCl (25 μl) in a closed vessel. *Error bars* indicate one standard deviation

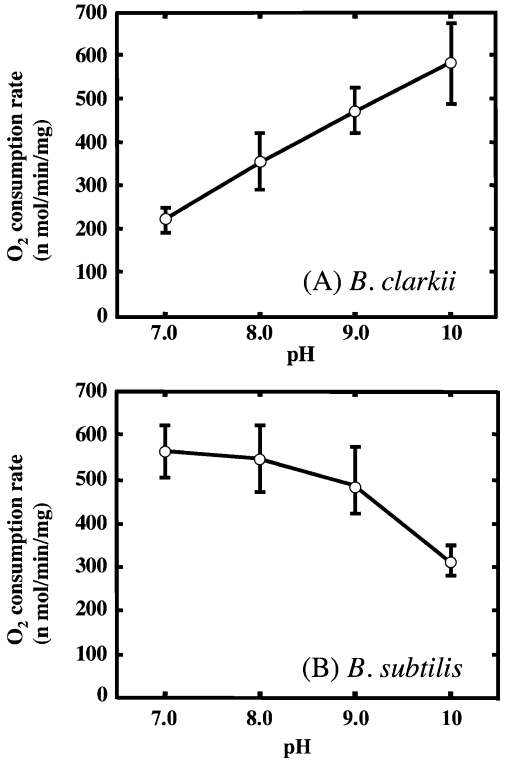

Fig. 3 Oxygen consumption rates of cells. The reaction was initiated by adding (**a**) *B. clarkii* cells and (**b**) *B. subtilis* cells to an air-saturated 140 mM KCl solution at 37°C. The oxygen consumption rate was calculated as the nanomoles of O_2 consumed per minute per gram of protein. *Error bars* indicate one standard deviation

low, presumably because of the low amount of extruded H^+. It is plausible that the low Δψ is sufficient to retain a small amount of H^+ at the beginning of the respiration.

Measurement of H^+/O ratio and oxygen consumption rate

The rates of the respiratory H^+ extrusion of *B. clarkii* and *B. subtilis* were assessed by determining the H^+/O ratios and the oxygen consumption rates. The H^+/O ratios of bacterial cells have been measured to evaluate the efficiency of the respiratory chain (Minohara et al. 2002; Jones et al. 1975). Figure 2 shows the H^+/O ratios of *B. clarkii* and *B. subtilis*

Table 2 Effects of ionophores on lag time

Species	Lag time (s)				
	None	Valinomycin	ETH-157	Monensin	Nigericin
B. clarkii (pH10)	120±6.0	9.6±2.6	40±2.8	230±23	110±9.6
B. subtilis (pH7)	37±4.3	12±3.3	20±5.4	41±2.2	45±4.0

Lag times of *B. clarkii* at pH10 and *B. subtilis* at pH7 were monitored in 140 mM KCl in the absence and presence of valinomycin (1.5 μM), ETH-157 (10 μM), monensin (10 μM), or nigericin (10 μM)

Table 3 Effects of inhibitors on lag time

	Lag time (s)					
	None (30h, 0 °C)[a]	DCCD[b]	None	Amiloride	EIPA	Nifedipine
B. clarkii (pH10)	120±9.0	120±7.5	120±6.0	130±11	160±17	170±11
B. subtilis (pH7)	75±3.9	70±3.8	37±4.3	28±2.9	32±3.1	32±2.5

Lag times of *B. clarkii* at pH10 and *B. subtilis* at pH7 were monitored in 140 mM KCl

[a] Negative control for DCCD-treated cells

[b] Cells were incubated for 30 h on ice in the presence of 50 μM DCCD

[c] Amiloride (1 mM), EIPA (100 μM), or nifedipine (100 μM) was added before the 20-min incubation under anaerobic conditions

at pH values of 7, 8, 9, and 10 in the presence of valinomycin and KSCN to provide a typical membrane-permeant thiocyanate ion and accelerate the translocation of K^+ (Blok et al. 1974). Immediate proton extrusions concomitant with the introduction of air were observed. The pH values of the cell suspensions showed no significant effect on the efficiency of the respiration-dependent H^+ extrusion. Figure 3 shows the oxygen consumption rates of the two *Bacillus* species. *B. clarkii* and *B. subtilis* showed higher oxygen consumption rates at pH values of 10 and 7, respectively, where they showed longer lag times. These facts suggest that the lag times were not caused simply by a decrease in the rate of the respiration-dependent H^+ extrusion. Both *Bacillus* species consumed oxygen immediately upon the addition of air at all pH levels examined (7, 8, 9, and 10) (data not shown). These results are consistent with the assumption that H^+ is extruded by the respiratory system immediately after the addition of air.

Effect of monensin and nigericin

The lag times of *B. clarkii* and *B. subtilis* are only slightly affected by electroneutral ionophores, such as nigericin (K^+/H^+ exchange ionophore; Graven et al. 1966) and monensin (Na^+/H^+ exchange ionophore; Pressman 1976), except for the increased lag times for monensin-treated *B. clarkii* cells, as described below. These electroneutral ionophores eliminate the ΔpH without affecting the Δψ. Therefore, the ΔpH contributes little to the lag times.

The lag time for *B. clarkii* increased approximately two-fold with the addition of 10 μM monensin (Table 2). The specific effect of monensin on the lag times of *B. clarkii* may represent the facilitated re-entry routes for Na^+, such as Na^+-driven flagellar motors (Krulwich et al. 2001), voltage-gated Na^+ channels (Kleyman and Cragoe 1988), and Na^+-coupled solute symporters (Peddie et al. 2000) in alkaliphiles. These re-entry routes for Na^+ are important for alkaliphilic *Bacillus* species that utilize Na^+ for pH homeostasis by a sodium (Na^+)/H^+ antiporter (Krulwich et al. 2001, 1997; Ito et al. 2004).

Effects of inhibitors

The increased lag times for monensin-treated cells of *B. clarkii* but not for the nigericin-treated cells suggest that the control of the movement of Na^+ across the cytoplasmic membrane affects the length of the lag time. There are various inhibitors of these Na^+ channels. Amiloride is an inhibitor of several Na^+ channels in membranes (Kleyman and Cragoe 1988) and is often used at high concentrations such as 1 mM (Atsumi et al. 1992; Avetisyan et al. 1991; Sugiyama et al. 1988). An amiloride analogue, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), is used at concentrations lower than those of amiloride (Vigne et al. 1984, 1983) and inhibits the Na^+-driven flagellar motors of alkaliphilic *B. clausii* (Terahara et al. 2008) and *B. pseudofirmus* (Simon and Carafoli 1979). Nifedipine is a calcium channel blocker that inhibits the Na_vBP channel, a voltage-gated Na^+ channel (Kleyman and Cragoe 1988; Fujinami et al. 2007). Table 3 shows the effects of these inhibitors on the lag times of *B. clarkii* and *B. subtilis.* EIPA and nifedipine slightly increased the lag times of *B. clarkii*, while amiloride had little effect. Table 4 shows the effects of these inhibitors on the lag times of *B. clarkii* in the presence of monensin, where the lag times of *B. clarkii* further increased following the addition of EIPA or nifedipine. Because the Na^+ influx reduced the Δψ, its inhibition may

Table 4 Effects of inhibitors and valinomycin on lag time in presence of monensin

	Lag time (s)				
	None	Valinomycin	Amiloride	EIPA	Nifedipine
B. clarkii (pH10)[a]	230±23	23±1.2	250±10	350±26	330±22

[a] Lag times of *B. clarkii* cells in the presence of monensin (10 μM) in 140 mM KCl at pH10 were monitored

Valinomycin (1.5 μM), amiloride (1 mM), EIPA (100 μM), or nifedipine (100 μM) was added before the 20-min incubation under anaerobic conditions

have accelerated the H^+ influx leading to the increased lag time. Valinomycin decreased the lag times of *B. clarkii* in the presence of monensin, as was also the case in the absence of monensin.

The extruded H^+ re-enters the cytoplasm via the H^+-translocating ATPase (Padan et al. 2005). It is possible that this re-entrance of H^+ increases the lag times. To investigate the effects of re-entrance on lag times, ATPase was inhibited by incubation with 50 μM DCCD for 30 h at 0 °C. The ATP accumulation rates of the DCCD-treated cells of both *Bacillus* species were less than 5% of those of the untreated cells (data not shown). Table 3 shows the effects of DCCD on the lag times. DCCD had no significant effect on the lag times of either *Bacillus* species, although the lag times of *B. subtilis* increased following incubation on ice for 30 h (Table 3).

Conclusions

At the beginning of respiration, the respiratory-extruded protons are retained and they are not detected immediately in the bulk water phase. The increased lag time for *B. clarkii* under alkaline conditions suggests that lag time is involved in alkaline adaptation. During lag time, the respiratory-extruded protons are retained owing to $\Delta\psi$ that is formed across the cytoplasmic membrane. The retention of the protons on the outer surface of the cytoplasmic membrane will generate a sufficient ΔpH to drive cellular bioenergetic pathways, such as ATP synthesis. Under alkaline conditions, *B. clarkii* may increase lag time, because it requires more protons to overcome the disadvantage to generate the ΔpH.

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