Kinetics of Colony Formation by Sulfate-Reducing Bacteria

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Abstract The colony formation of sulfate-reducing bacteria, *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis*, on agar plates well fitted the theoretical curve of the FOR model proposed by Hattori (1985). The estimates of λ for both strains dropped from 5 to 1 day⁻¹ with cultivation in a liquid medium or starvation in a buffer solution, while that of t_r was not affected so much. The estimates of λ and t_r for *D. desulfuricans* were markedly affected by incubation temperature and lactate concentration in the agar medium. Both factors also affected the t_r value for *D. orientis*, however only incubation temperature affected the λ value for the latter organism.

Key words colony formation, first order reaction kinetics, sulfate-reducing bacteria, *Desulfovibrio desulfuricans*, *Desulfotomaculum orientis*

Introduction

Appearance of bacterial colonies on agar plates is considered to follow the first order reaction (FOR) kinetics by Hattori (1985):

$$N_t = N_\infty [1 - \exp\{-\lambda(t - t_r)\}] \quad (t \geq t_r)$$

where N_t is the number of colonies at time t , N_∞ is the expected number of colonies at the infinite incubation time, λ is the rate of the appearance of colonies, and t_r is the retardation time. Recently, Fukui and Fukuhara (1987) studied colony forming curves (CFCs) of sulfate-reducing bacteria (SRB). Effects of culture conditions and physiological factors on the parameters of the model for SRB have not been clarified yet.

The aim of the present study is to examine the effects of incubation temperature, substrate concentration and culture age or starvation of the inocula on the values of the parameters of the FOR model for SRB *Desulfovibrio desulfuricans* and *Desulfotomaculum orientis*.

Materials and Methods

Organisms and cultivation

Two strains of SRB, *D. desulfuricans* (IFO 13692) and *D. orientis* (ATCC 19365), were used in this study. The growth medium was the *C* medium of Butlin *et al.* (1949). The *C* medium contained (g · l⁻¹) K₂HPO₄ (0.5), NH₄Cl (1), Na₂SO₄ (1), CaCl₂ · 2H₂O (0.1), MgSO₄ · 7H₂O (2), Na-lactate (3.5), FeSO₄ · 7H₂O (0.2), and yeast extract (Difco, U.S.A.) (1) (pH, 7.2). The autoclaved medium was supplemented with a separately autoclaved reducing agent (sodium thioglycolate, final 0.045%).

SRB strains were precultured on the *C* medium (15 ml medium bottles) for 4 days at 30°C. An aliquot of the culture (ca. 100 µl) was inoculated into the modified *C* medium filled in 15 ml medium bottles. The medium was modified by reducing the amount of FeSO₄ · 7H₂O to trace. The bottles were sealed with screw caps and incubated at 30°C in the dark. After appropriate incubation times, the cultures were used for the experiments described below.

Colony count of SRB

Plate counts were obtained by the anaerobic petri dish method of Wakao and Furusaka (1972) using five replicate plates except the experiments described in Figure 1 which were carried out using twenty plates. The medium used was the *C* medium containing agar ($15 \text{ g} \cdot \text{l}^{-1}$), except for the experiment to examine the effect of lactate concentration on colony formation; the lactate concentrations were 3.5 (the original concentration in the *C* medium), 0.35, 0.035, 0.0035 and $0.00035 \text{ g} \cdot \text{l}^{-1}$. The culture was inoculated after appropriate dilution with sterilized distilled water. Numbers of colonies were enumerated by the naked eye at appropriate time intervals during incubation at 30°C . To examine the effect of incubation temperature on colony formation, plates were incubated at 37, 30.5, 25, and 20°C .

Parameters of the FOR model, N_∞ , λ and t_r , were estimated by the method of Ishikuri *et al.* (1984).

Starvation of SRB

Cells cultivated for 2 days as described above were harvested by centrifugation at 6000 rpm and 4°C for 25 min and washed twice with anaerobic phosphate buffer (KH_2PO_4 , $0.11 \text{ g} \cdot \text{l}^{-1}$; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $0.64 \text{ g} \cdot \text{l}^{-1}$; pH, 7.0) while flushing N_2 gas. The cell pellets were then resuspended in 10 ml of anaerobic phosphate buffer under N_2 gas. A half ml of these cell

suspensions was mixed with 50 ml of the salts solution (*IG*) of Ingvorsen and Jørgensen (1984) in a 120 ml serum vial and starved at 30°C under N_2 gas. The *IG* solution contained ($\text{g} \cdot \text{l}^{-1}$) KH_2PO_4 (0.11), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (0.64), NaCl (1.0), KCl (0.5), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.6), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.2), and NH_4Cl (0.25) (pH, 7.0). At appropriate time intervals during starvation, starved cells were inoculated to plates for the plate counts.

Results

Colony formation by SRB on multiple plates

Figure 1 shows the changes in the mean number of colonies on twenty parallel plates inoculated with *D. desulfuricans* and *D. orientis* which were obtained from cultures at early stationary phase. Colony formation of both strains well fitted with the theoretical curves of the FOR model. The values of N_∞ , λ and t_r were, respectively, 205, 3.60 day^{-1} and 53.3 hr for *D. desulfuricans*, and 207, 2.83 day^{-1} and 62.3 hr for *D. orientis*. The variance, χ^2 and significant probability (degree of freedom, 19) of N_∞ was, respectively, 204.2, 18.9 and 0.462 for *D. desulfuricans*, and 346.8, 31.8 and 0.630 for *D. orientis*.

Effect of incubation temperature on colony formation

Incubation temperature affected the parameters for both strains (Table 1). The values of N_∞ and λ decreased with decreasing temperature in the

Figure 1 Colony forming curves of *Desulfovibrio desulfuricans* (A) and *Desulfotomaculum orientis* (B). Solid lines indicate the theoretical curves based on the FOR model. The estimate of N_∞ was arbitrarily taken as 100. Parameters of each curve are shown in text. Black circles were used for estimation of parameters of the FOR model and white ones were not used.

Table 1. Effect of temperature on values of parameters of the FOR model

Strain	Temperature (°C)	N_{∞}	λ (day ⁻¹)	t_r (hr)
<i>Desulfovibrio desulfuricans</i>	37	255	5.21	39.9
	30.5	247	3.43	56.2
	25	227	2.28	84.7
	20	196	1.39	130
<i>Desulfotomaculum orientis</i>	37	398	5.79	35.6
	30.5	427	3.73	48.2
	25	399	2.94	70.1
	20	340	1.64	118

Figure 2. Effects of the concentration of lactate in the medium on the value of t_r (A) and λ (B) for *Desulfovibrio desulfuricans* (●) and *Desulfotomaculum orientis* (○).

range from 37 to 20°C. Arrhenius relationships were recognized between $\log 1/t_r$ and $1/T$, where T is the absolute temperature. The slopes for both strains were similar.

Effect of lactate concentration on colony formation

The size of colonies of both strains after four-week incubation became drastically smaller when the concentration of lactate, a sole source of energy and carbon, decreased from 0.35 to 0.035 $\text{g} \cdot \text{l}^{-1}$, and then gradually decreased with decrease in the lactate concentration. The lactate concentration affected also the value of t_r for both strains (Fig. 2-A). The value of t_r increased with decrease in the lactate concentration and reached to larger than

100 hr at 0.0035 $\text{g} \cdot \text{l}^{-1}$. The value of λ for *D. desulfuricans* dropped as the lactate concentration decreased (Fig. 2-B), while the concentration did not affect the value of λ for *D. orientis*.

Effect of culture age on colony formation

Table 2 shows the effect of culture age of inocula on three parameters for both strains. As the growth phase proceeded from the exponential phase (1 to 2 days) to the stationary one (3 to 7 days), the value of λ for both strains drastically dropped from 5 to 2 day⁻¹ and then gradually decreased thereafter. The value of λ for *D. orientis* became smaller than 1 day⁻¹ after only 19 days, while in the case of *D. desulfuricans* the value became smaller than 1 day⁻¹ after 252 days at

Table 2. Relationship between culture age and parameters of the FOR model

Strain	Culture age of inocula (day)	N_{∞} (ml ⁻¹)	λ (day ⁻¹)	t_r (hr)
<i>Desulfovibrio desulfuricans</i>	1	3.85×10^8	4.25	51.7
	2	3.02×10^8	4.66	55.7
	3	1.10×10^8	2.77	54.5
	7	1.95×10^8	1.77	55.7
	17	2.98×10^5	2.63	53.0
	47	1.88×10^5	1.84	50.6
	99	1.28×10^5	2.52	55.7
	117	2.93×10^4	1.61	51.4
	171	4.11×10^5	1.67	54.7
	252	3.10×10^5	0.64	68.9
<i>Desulfotomaculum orientis</i>	1	8.79×10^7	4.60	55.4
	2	9.41×10^7	5.47	58.6
	4	1.26×10^8	4.41	58.0
	7	7.68×10^7	2.24	59.1
	16	3.62×10^4	1.00	76.8
	19	2.03×10^4	0.66	75.0
	29	1.36×10^4	0.57	75.5

last. The value of t_r was about 50–60 hr before 252 days for *D. desulfuricans* and 16 days for *D. orientis* and then became 10–20 hr longer.

Effect of starvation on colony formation

The changes in the parameters during starvation are shown in Figure 3. N_{∞} for both strains showed exponential decreases. The values of λ for both strains drastically dropped for only 6 hr of starvation. On the other hand, the values of t_r for both strains increased from 55 to 80 hr at 47 hr and then decreased gradually to 60 hr.

Discussion

The CFCs of *D. desulfuricans* and *D. orientis* well fitted with the FOR model, like those of *Escherichia coli* and *Agromonas* sp. (Hattori, 1985). Thus it is possible to estimate the value of N_{∞} for both strains by counting N at appropriate time intervals during incubation. Fisher *et al.* (1922) found that under ideal conditions the num-

ber of bacterial colonies from soils on multiplicate plates would vary in the same manner as samples from a Poisson series. Ishikuri and Hattori (1987) also showed that N_{∞} for *Agromonas* sp. on multiplicate plates distributed as a Poisson series. The distributions of N_{∞} on twenty replicate plates for *D. desulfuricans* and *D. orientis* agreed with their results, as evidenced by the value of significant probability. Therefore, the mean values of N_{∞} for SRB can be considered as a reliable measure of a viable population density.

The parameter t_r is the time lag for colony formation of a population. The value of t_r for both strains of SRB became longer with older culture age or longer starvation time (Table 2 and Fig. 3). In the cases of *Agromonas* sp. and *E. coli*, the generation time was similar irrespective of culture age after the initiation of growth when the cells from various culture age were inoculated, except the lag phase (Mochizuki and Hattori, 1986; 1987). Our results suggest that long cultivation in a liquid medium or starvation retarded

Figure 3. Effects of starvation on the value of the FOR model for *Desulfovibrio desulfuricans* (●) and *Desulfotomaculum orientis* (○).

growth initiation of original cells of SRB on a plate.

Decrease of lactate concentration resulted in the increase of the value of t_r , which is partly due to the reduction of their growth rates.

The value of λ for *D. desulfuricans* was markedly affected by two factors, temperature and lactate concentration in the medium, but only temperature affected the value of λ for *D. orientis*. The value of λ for both strains dropped when the cells proceeded from exponential phase to stationary one and for a short period of starvation. In the same experiment of starvation, the ratio of respiring (formazan-forming) but not growing cells to total cells increased with starvation time (Fukui and Takii, 1989). Thus, as supposed by Hattori (1985), the parameter λ for SRB may reflect physiological states of original cell populations inoculated to agar plates. To evaluate this relationship, it is necessary to relate the value to biochemical parameters, such as adenylate energy

charge.

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