

Nonequilibrium growth of *Klebsiella ozaenae* on agar plates

Ishwar Das* & Anuj Kumar

Department of Chemistry, University of Gorakhpur, Gorakhpur 273 009, India

and

U K Singh

Department of Microbiology, B.R.D. Medical College, Gorakhpur 273 013, India

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Morphology and growth kinetics of the bacteria *Klebsiella ozaenae* on agar surface have been investigated. Bacterial colonies grow two dimensionally with random branches identical to nonequilibrium growth processes in non-living systems and generate self organization. The colony pattern is analysed and fractal dimensional is found to be 1.61 which is in excellent agreement with the expected value of the diffusion limited aggregation (DLA) model. Both phenotypic and genotypic adaptation are observed under different experimental conditions. Influence of certain vitamins, disinfectant, antibiotics and the microorganism *Bacillus subtilis* on the growth behaviour of *K. ozaenae* has been investigated.

Pattern formation processes are important in almost all areas of science and technology. For several years, fractal^{1,2}, densed branched morphology³, rhythmic⁴, spiral⁵⁻⁷ and other patterns have attracted considerable attention. Remarkably similar patterns are observed in electrodeposition⁸, dielectric breakdown⁹, viscous fingering¹⁰, dissolution patterns and dendritic growth¹¹. The concept of symmetry breaking, the generation of stable, spatial patterns through the interplay of reaction dynamics and diffusion processes have applications not only to the problem of pattern formation in embryology but also to neurobiology¹². Pattern formation in biological systems is believed to be much more complicated than that in chemical and physical systems. The study of bacterial colony growth is one of the fascinating examples of pattern formation. *Serratia marcescens* exerts fractal morphologies in the process of spreading growth on agar surface¹³. Colony of *Proteus vulgaris* was observed to possess concentric rings¹⁴. *Bacillus subtilis* have been shown to grow with various morphologies under different experimental conditions^{15,16}.

The model proposed by Ben Jacob¹⁶ incorporates random walkers which communicate with each other by means of chemotactic 'feedback'. Each microorganism may excrete and consume cAMP and move according to its concentration gradient. Spots, strips and rings were formed by motile cells of *E. coli*¹⁷ grown in a thin layer of agar agar. In the present investigation, we report

some new results on the development of morphology of colony of *K. ozaenae* on agar surface, in the nutrient medium containing peptone, agar agar, sodium chloride and beef extract. Its dependence on peptone, beef extract concentrations, hardness of medium, vitamin B complex, ascorbic acid and hydrogen peroxide, have also been studied. Influence of growth characteristics in co-culture of *K. ozaenae* with a gram positive *B. subtilis* has been investigated. Growth kinetics have also been studied under various experimental conditions. The colony pattern was analysed to calculate its fractal dimension.

Materials and Methods

Nutrient agar (Qualigens), nutrient broth (Span diagnostics), beef extract (Qualigens), sodium chloride (LR, BDH), H₂O₂ (Loba Chemicals LR), oxytetracyclin (Pfizer India), lactose (SD fine Chem), bromothymol blue (SD fine Chem), vitamin B complex (LR, IDPL), ascorbic acid (LR, IDPL) were used as such.

Procedure

One bacterial colony was inoculated into 2 ml sterilised nutrient broth and incubated for 3 h. Two loopful of the culture broth having bacteria in log phase were inoculated at the centre of culture plate and incubated in a REMI (India) BOD incubator maintained at 37.5 ± 0.1°C. Composition of nutrient medium was altered by varying

the concentration of one of the constituents at a time while other constituent concentrations were kept constant. After pouring 20 ml of nutrient medium into disposable sterilised petridishes (85 mm diameter) they were kept at room temperature for solidification. Moist surface of culture plates were dried in an incubator for about half an hour. Influence of vitamin C, vitamin B complex, H_2O_2 , oxytetracyclin and the organism *Bacillus subtilis* on growth pattern were studied by adding, known amount of additives separately to the medium before pouring into the petridish. *Bacillus subtilis* was inoculated alongwith *K. ozaenae* on lactose-bromothymol blue agar plate. In order to check the reproducibility, three plates were simultaneously prepared. The growth kinetics was studied by measuring the colony diameter as a function of time with a travelling microscope accurate to ± 0.001 cm. Measurements were made in three directions, mean values are plotted and mean deviations are indicated in Figs 1, 3.

Computation of fractal dimension

A typical bacterial colony (Fig. 2) was photographed and scanned with the help of a HP Scan Jet 4C Scanner attached with a computer (HP, PCAT 486 Model Vectra VL 2). The fractal dimension of the picture was determined by box counting method on the computer screen. The relation between the total number of boxes $N(r)$ inside a circle of radius r should be a power law with noninteger exponent D as $N(r) \sim r^D$ where the thickness of the branches is considered as zero dimensional⁹. Thus, the log-log plot of $N(r)$ versus r was made. The fractal dimension D is

given by the slope of the best fitted straight line using least squares method.

Results and Discussion

Bacterial colony grow two dimensionally on the agar surface. It is an example of nutrient diffusion controlled growth process similar to diffusion controlled growth of non-living systems. At macrolevel the nature of growth is a result of phenotypic and genotypic adaptation. The growth of bacterial colonies resemble those observed in non-living systems but shows additional level of complexity. A typical growth pattern of gram negative non-motile organism *K. ozaenae* on agar surface is shown in Fig. 2. The diffusion limited aggregation theory (DLA) assumes that microbial growth could be thought of primarily as a consequence of nutrient diffusion and reproduction. Analysis of the colony yields the fractal dimension (D)=1.61, in excellent agreement with that of 2d DLA clusters (D =1.66). $\log N$ versus $\log r$ plot yields a good linear regression line with the correlation coefficient 0.99. The reproduction rate determines the overall growth rate. Bacteria perform a random like movement within a well defined envelope and depend on the peptone and beef extract concentrations. Peptone is an intermediate product of hydrolysis of protein and function of beef extract in the medium is to supply substances that stimulate bacterial activity and cause accelerated growth of microorganism.

Effect of peptone

At low and moderate peptone level, bacterial colonies show Eden-like colony patterns while

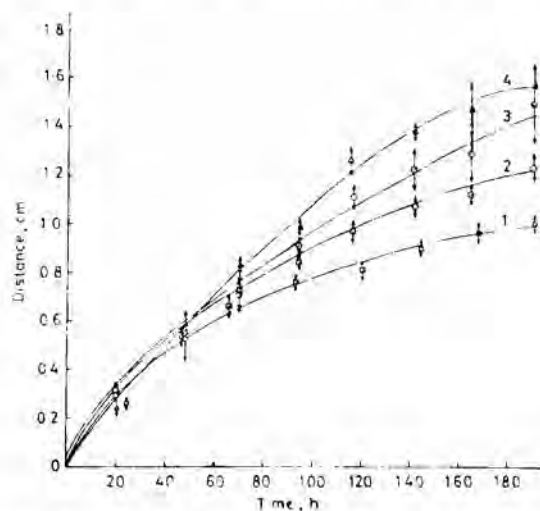


Fig. 1—Growth of *K. ozaenae* colony as a function of time for different peptone level. Curves 1 to 4 correspond to peptone concentration 1, 4, 5 and 7 g/l respectively

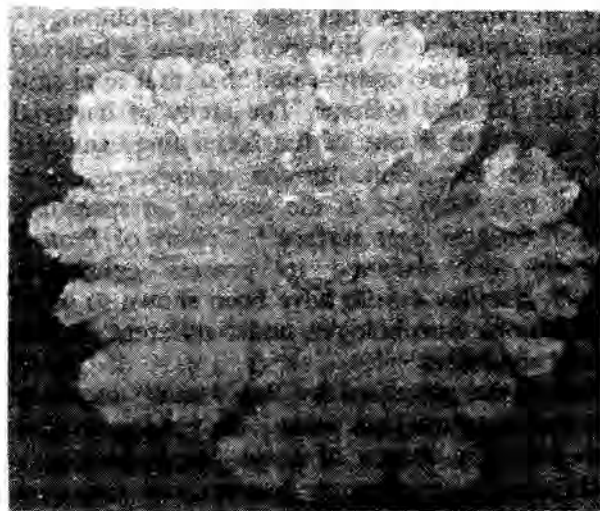


Fig. 2—Pattern of *K. ozaenae* colony on agar substrate (15 g/l) containing beef extract 4.2 g/l, peptone 5 g/l and sodium chloride 5 g/l, observed after 15 days

Table 1—Values of slope (m) intercept (C) and correlation coefficient (R) used in equation $d^2 = mt + C$ for growth of *Klebsiella ozaenae* at various peptone concentrations (temp. = $37.5 \pm 0.1^\circ\text{C}$)

[Peptone] g/l	Slope (m) cm^2/h	Intercept (C) cm^2	Correlation coefficient (R)
1	0.005	-0.0109	0.99
4	0.008	-0.086	0.99
5	0.012	-0.257	0.99
7	0.015	-0.0326	0.99

simple spreading smooth colony patterns were observed at very high peptone level (20 g/l and above) indicating that branches are fused together to form a compact pattern at such a high peptone level. The extent d of spreading of colony as a function of time for different peptone level are plotted in Fig. 1. The rate of spreading is not uniform throughout. Initially, the rate is fast, then it spreads at a relatively slow rate because the rate of multiplication is not constant for indefinite period due to exhaustion of nutrient, accumulation of toxic metabolic waste products and owing to the fact that many of the cells die. Further the rate increases with increase in peptone level. At a low peptone concentration, bacterial density is low, collisions are less and rate would be slow. The process is diffusion controlled as indicated by equation $d^2 = mt + C$ for peptone level 1-7 g/l where m and C are slope and intercept respectively (Table 1).

Effect of beef extract

Beef extract concentration in the medium was varied in the range 0-20% while concentration of other constituents (peptone, agar agar, and sodium chloride) were kept constant. The medium which did not contain beef extract, the growth rate was extremely slow and a facet like growth pattern was observed. With 5% beef extract the growth front breaks and branched structures were found. While at a relatively high beef extract concentration, branches are diffused and become more compact. The extent d of spreading colony as a function of time (t) for different beef extract level are plotted in Fig. 3. The growth rate was optimum at normal conditions (10.7%, curve 1). Perusal of curve 4 for 5% beef extract shows complex behaviour. The rate increases in the region AB, becomes almost stationary in the region BC and again increases beyond C. It indicates that at low concentration the multiplication does

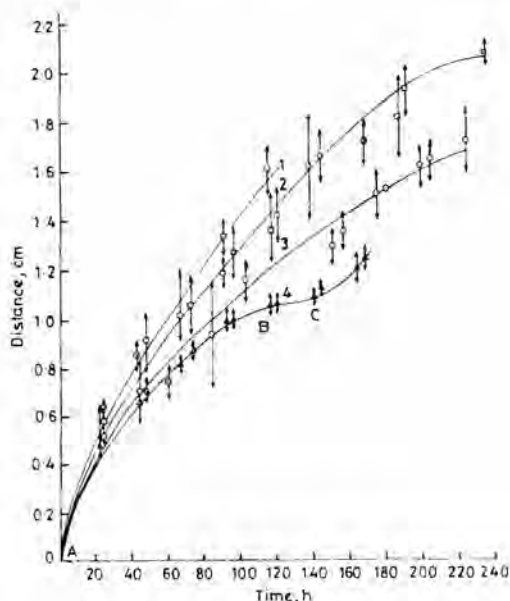


Fig. 3—Growth of *K. ozaenae* colony as a function of time for different beef extract level. Curves 1 to 4 correspond to beef extract concentration 10.7%, 15%, 20% and 5% respectively at $37.5 \pm 0.1^\circ\text{C}$

not take place in a regular manner. It may be due to feedback inhibition in which product of reaction inhibits its own formation. At higher beef extract concentration the data obey the simple equation $d^2 = mt + C$ where d , m and C have their usual significance.

Effect of agar agar

Agar agar provides compactness to the medium, thereby allowing separate colony formation which thus affects growth kinetics and morphology. For the movement of bacterial cells on the surface of agar plate, the average pore size of gel network should be smaller than the size of the bacteria, otherwise they perform cell division locally. An interesting observation of present investigation is the appearance of burst at low agar concentration. For a given peptone and beef extract level the patterns become more remified and compact as the hardness increased. It is a sharp and localized transition, not observed in non-living systems so far. It arises from a mutation which improves the adaptation and become the dominant genotypic irreversible adaptation.

Effect of additives

Experiments were also performed to study the effect of vitamin B complex (1% v/v) and ascorbic acid (0.5 g/l) on growth behaviour. Both of the vitamins showed stimulatory effect. On the other hand, oxytetracyclin (1% v/v) and ascorbic

acid (10 g/l) completely inhibited the growth of microorganism. Hydrogen peroxide which produces nascent oxygen also inhibited the growth of microorganism when H_2O_2 exceeding the lethal dose (3% v/v) was added to the medium.

Influence of Bacillus subtilis

Influence of co-culture of *K. ozaenae* with a gram positive *B. subtilis* has been studied on lactose bromothymol blue agar plate. *K. ozaenae* and *B. subtilis* were also inoculated separately on the same plate. The plate was incubated aerobically and observed after 4 days. Organisms vary considerably in their ability to ferment various carbohydrates. Such information is of great value in the identification of organisms. Due to lactose fermentation *K. ozaenae* developed yellow colouration with a change in colony morphology amounting to more spread out growth with irregular margin in co-culture as compared to isolated culture. Whereas, in the co-culture, colony morphology of *B. subtilis* was unaffected but the growth rate of *B. subtilis* was accelerated in the presence of *K. ozaenae* to some extent as compared to isolated culture on the same plate. It appears that the metabolic end product of fermentation of lactose by *K. ozaenae* is supportive for the accelerated growth of *B. subtilis* as lactose is not fermented by *B. subtilis*. However, some metabolites which could not be identified by us due to limitation in our laboratory may be responsible for improvement in the growth character of *K. ozaenae* when the two cultures were inoculated at the same spot.

The experiments of *Klebsiella ozaenae* and *Bacillus subtilis* co-culture behaviour was extended to study the interaction between *K. ozaenae*-*K. ozaenae*, *B. subtilis*-*B. subtilis* and -*K. ozaenae*-*B. subtilis* where the organisms were inoculated at two points separated by 10 mm on a culture plate. After incubation it was found that *K. ozaenae* colony growth rate was too slow to contact each other even after 4 days whereas in the case of *K. ozaenae*-*B. subtilis*, fusion of the two colonies was seen. In *B. subtilis*-*B. subtilis* ex-

periment, colonies repelled each other but did not fuse together. This result suggests that *K. ozaenae*-*B. subtilis* give mutualistic support to each other not only in co-culture but also in adjacent culture, which may be due to extracellular signals produced by them.

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