Bacterial Growth Rates above 90°C in Yellowstone Hot Springs

Abstract. *Growth rates of bacteria living in boiling springs have been measured by determining rate of increase in cell numbers on microscope slides immersed in the springs. Distinction between growth and passive attachment was made with ultraviolet radiation. In all cases, slides irradiated at intervals had significantly fewer bacteria than controls. Estimated generation times ranged from 2 to 7 hours, values which are comparable to those of aquatic bacteria living in less extreme environments.*

A question of considerable biological interest is the highest temperature at which life is possible. Observations on life in hot springs have shown that there is a definite upper temperature limit for photosynthetic microorganisms, but that nonphotosynthetic bacteria are present even in superheated springs at temperatures above 90°C (*1*). Although the arrangement of bacterial cells in microcolonies on microscope slides immersed in these springs suggested that growth actually took place at such temperatures, it was important to confirm this indication and attempt to establish growth rates under these extreme conditions. We now present a method for measuring growth rates of bacterial populations *in situ* and demonstrate conclusively that growth does take place at these high temperatures.

All studies were done on hot springs in the Lower Geyser Basin of Yellowstone National Park (*2*). Four of the springs studied are superheated, and the other one has a temperature just below the boiling point (about 92.5°C at the altitude of Yellowstone). Bacteria grow attached to the siliceous walls of the springs, but they are also present in small numbers in the water. Microscope slides immersed in these springs probably provide a substrate for bacterial attachment and growth reasonably similar to the walls of the springs themselves. Slides immersed for 1 or 2 days usually contain dense populations of rod-shaped bacteria and, in some cases, filamentous bacteria. To demonstrate that organisms were growing on the slide surfaces some slides were treated with germicidal ultraviolet radiation at intervals of between one and two generations. Any organisms that had attached to the slide during the interval since the last irradiation would thus be killed and could not produce progeny.

Microscope slides, heated in an oven to oxidize organic matter, were placed in Plastisol-coated racks which were then immersed in the springs at least 1 meter below the water surface. Temperatures were measured with thermistors (Yellow Springs, Inc., model 42-SC) precisely at the position where the slides were immersed. At intervals the racks were raised to the surface, and slides were removed in duplicate and air dried. Slides were never removed from the water during the irradiation process but were transferred from the rack to petri dish lids submerged about 1 cm in the water.

Growth rates for unicellular rod-shaped bacteria were determined by counting the number of cells per microscope field or per microcolony (phase microscopy) and obtaining an average value for the slide. Usually 100 fields or microcolonies were counted on each slide.

Conclusive evidence that growth occurred was obtained, for in every instance the counts on the irradiated slides are much lower than on the controls (Table 1). Counts on the bottoms of irradiated slides are similar to those of unirradiated controls, a result to be expected because germicidal wavelengths of ultraviolet radiation should not pass through the glass.

Growth rates of bacteria in the spring "Pool A" were measured in two separate experiments (Fig. 1). Because rod-

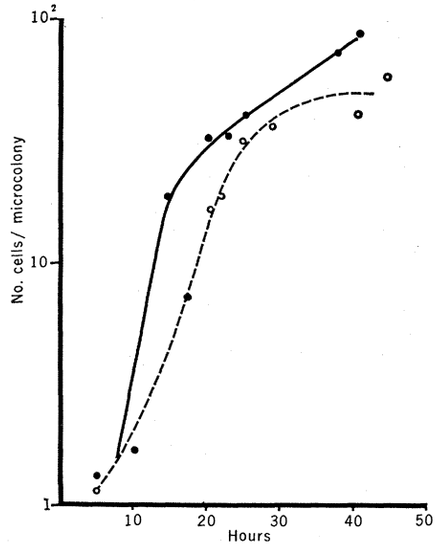

Fig. 1. Growth curves of bacteria in Pool A. Results of two independent experiments.

Table 1. Bacterial counts on irradiated (UV) and unirradiated microscope slides. Irradiated slides were treated eight times at approximately equal intervals during the immersion period.

Treatment	Immersion time (hr)	Average number of cells per field: Top of slide	Average number of cells per field: Bottom of slide
	Geyserino		
UV	74.5	3.6	889
UV	74.5	1.4	431
Control	74.5	948	1385
	Porcupine		
UV	138.75	1.4	149.0
Control	138.75	158.1	118.1
	Stepbrother		
UV	72.0	8.2	136
UV	72.0	1.6	7.4
Control	72.0	42.4	34.0
	Boulder Experiment 1		
UV	74.5	0.56	144
UV	74.5	0.88	120
Control	74.5	250	125
	Experiment 2		
UV	72.0	9.4	889
UV	72.0	1.6	961
Control	72.0	1361	626
	Boulder Effluent Experiment 1*		
UV	12.0	2.62	32.2
Control	12.0	30.7	24.7
	Experiment 2		
UV	12.0	4.0	29.6
UV	12.0	0	26.5
UV	12.0	0	31.3
Control	12.0	32.0	23.2
	Pool A Experiment 1*		
UV	53.0	2.0	32.5
Control	53.0	10.5	15.8
	Experiment 2		
UV	33.0	2.5	39.0
UV	33.0	2.2	39.2
Control	33.0	37.9	44.8

* Counts for Pool A and Boulder Effluent are number of cells per microcolony rather than number of cells per field.

Table 2. Summary of bacterial generation times for various springs.

Experiment	Temperature range* (°C)	*p*H	Generation time (hr)
	Geyserino		
1	93.0–94.5	8.5	4.0
2	92.5–94.5		5.0
	Porcupine		
1	92.8–94.8	8.6	6.0
2	94.2–95.2		6.0
	Stepbrother		
1	91.8–92.5	8.65	5.5
2	91.9–93.0		3.5
	Boulder		
1	90.0–91.8	8.9	7.5
2	90.5–91.5		4.75
3	90.3–91.8		3.0
	Boulder Effluent		
1	78.0–80.5	8.9	2.2†
2	79.0–80.5		2.2†
3	79.8–81.8		2.1†
	Pool A		
1	90.5–92.0	8.1	3.0†
2	89.0–91.0		2.0†

* Temperature ranges listed encompass measurements made during the experiments, which usually extended over 2 to 3 days. Measurements of *p*H were made with a glass electrode at the temperature of the spring. † These values calculated on the basis of rate of increase in number of cells per microcolony. All others are from counts of number of cells per microscope field.

shaped bacteria grow in the form of microcolonies in this spring, data are presented as number of cells per microcolony. Generation times calculated from the exponential phase of growth of these two experiments are 2.5 and 3.0 hours. In other springs, where growth does not occur in discrete microcolonies, results are expressed as numbers of cells per microscope field. Calculated generation times are summarized in Table 2. Growth rates of filamentous bacteria that also occur in some of these springs have been determined, and their generation times are similar to those of unicellular bacteria. Qualitative observations of bacterial growth have also been made in many other alkaline springs in Yellowstone Park, and in every spring bacteria accumulated rapidly upon microscope slides, although rate of accumulation varied from spring to spring. From these results we conclude that not only is bacterial growth occurring at temperatures greater than 90°C, but that the rates are surprisingly rapid.

THOMAS L. BOTT, THOMAS D. BROCK
Department of Microbiology,
Indiana University, Bloomington 47401

References and Notes

1. T. D. Brock, *Science* **158**, 1012 (1967).
2. Boulder Spring is west of Fountain Freight Road in the Fairy Creek area. The other springs are apparently unnamed, and we have given them trivial names for the purposes of this report. Geyserino is a superheated semieruptive spring in the White Creek basin east of Firehole Lake Loop Road. Pool A is in front of a cold-water marsh northeast of Geyserino. Stepbrother is a superheated semieruptive spring in the White Creek valley east of Five Sisters springs. Porcupine Spring is in the Porcupine Hills group and is a superheated spring near drill hole Y-13 of the U.S. Geological Survey.
3. Supported by NSF grants GB-5258 and GB-7815 and AEC contract C00-1804-5. T. Bott is a postdoctoral fellow of the U.S. Public Health Service. T. D. Brock was research career development awardee of the USPHS. We thank I. Yoder and T. Daley for technical assistance.

30 January 1969; revised 25 March 1969

Antigenic Changes in Lymph-Node Cells after Administration of Antiserum to Thymus Cells

Abstract. *Mice of the RIII and C57BL strains were treated with rabbit antiserum to thymus (ATS), and cells of their lymph nodes were analyzed serologically at intervals after treatment. While lymph-node cells of untreated mice were sensitive to the cytotoxic effect of isoantibodies against the theta antigen, lymph-node cells of ATS-treated mice showed a significantly reduced sensitivity. Three days after ATS treatment lymph-node cells of most mice were completely refractory to the cytotoxic effect of theta antibodies. Administration of normal rabbit serum elicited only a slight reduction of the sensitivity of lymph-node cells to the cytotoxic effect of theta antibodies. The results support the hypothesis that ATS treatment selectively affects a population of thymus-dependent circulating lymphocytes.*

The administration of heterologous antiserum to lymphocyte (antilymphocyte serum, ALS) results in a depression of the immunological competence of the adult organism (*1*). The mechanism of action of ALS is still unclear, and several hypotheses have been put forward (*2*). Several lines of evidence indicate that a prerequisite for the activity of ALS is its capacity to bind complement. Pepsin digestion (*3*) or exposure to low *p*H (*4*) not only abrogates the capacity of ALS to bind complement but also prevents its immunosuppresive activity. It therefore seems that at least part of the immunosuppressive effect of ALS may be due to its cytotoxic effect on cells involved in the immune response (*5*). Evidence has been presented that some populations of lymphocytes are eliminated in ALS-treated animals. After ALS treatment, lymph nodes may be depleted of lymphocytes (*6*, *7*), and the population of circulating long-lived lymphocytes decreases significantly (*8*).

In our present study, the effect of rabbit antiserum to thymus cells (ATS) on the theta isoantigenicity of lymph-node cells was analyzed. The theta isoantigen system of the mouse is a non–H-2 antigen system characterized by its high concentration in the thymus and brain (*9*). In the presence of guinea-pig complement antibodies against the theta isoantigens show a significant cytotoxic effect not only on thymus cells but also on lymph-node cells, while there is no such effect on cells resident in the spleen or in Peyer's patches (*9*, *10*). We now report that lymph-node cells of ATS-treated mice transiently lose their sensitivity to the cytotoxic effect of antibodies against theta isoantigens.

ATS was prepared by repeated intraperitoneal injections of RIII thymus cells. The effect of ATS was studied in mice of the inbred RIII/Jem and C57BL/6 strains. These strains contain respectively the theta AKR and theta C3H isoantigens (*11*). Experimental mice received four intraperitoneal injections of 0.25 ml of ATS on alternate days and were killed at intervals after the last injection. Some control mice received four intraperitoneal injections of 0.25 ml of normal rabbit serum (NRS).

Cytotoxic tests were performed with suspensions of cells obtained from axillary and inguinal lymph nodes. A modification (*12*) of the cytotoxic test of Gorer and O'Gorman (*13*) was used. The isoantiserums used were prepared by repeated administration of allogeneic spleen cells. The AKR/J antiserum to C3H was used for detection of the theta C3H antigen, while the C3H antiserum to AKR/J was used for detection of theta AKR antigen. The ATS was unabsorbed, polyvalent, rabbit antiserum to mouse thymus cells and is assumed to contain heterologous antibodies against a variety of mouse antigens. On the other hand, the reagents used employed for the detection of theta isoantigens (that is, the C3H antiserum to AKR/J and the AKR/J antiserum to C3H) were strictly specific for theta isoantigens.

Cytotoxic indices were calculated from the results of the cytotoxic tests as follows (*14*): the difference between the percentages of nonviable experimental and nonviable control cells divided by the percentage of viable control cells, the result then being multiplied by 100. (Figures 1 and 2 show the mean of cytotoxic indices obtained in tests with six to ten individual mice per experimental group. Two days after the last injection of ATS to RIII mice, the lymph nodes of these mice showed a significant reduction in sensitivity to antibodies against the theta AKR antigen as compared to untreated controls (Fig. 1). Three days after the last injection of ATS, the lymph-node cells of all RIII mice tested were highly refractory to the cytotoxic effect of theta AKR antibodies. Lymph-node cells of most mice lost completely their sensi-