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Filament Formation in *Clostridium acidiurici* Under Conditions of Elevated Temperatures[1]

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ABSTRACT

TERRY, DAVID R. (Brigham Young University, Provo, Utah), ABDUL GAFFAR, AND RICHARD D. SAGERS. Filament formation in *Clostridium acidiurici* under conditions of elevated temperatures. J. Bacteriol. **91**:1625–1634. 1966.—Vegetative cells of *Clostridium acidiurici*, when grown at temperatures up to 42 C, are straight rods varying from 2.5 to 4 μ in length. When grown at 43 C, the cells show a definite tendency to elongate, and, when grown at 44 C, filaments are formed, often exceeding 500 μ in length. Only an occasional cross wall is apparent in the heat-induced long forms, but as the temperature is lowered they readily form cross walls and fragment into short, single cells. Chromatin material is distributed in evenly spaced clusters throughout the length of the filaments. The filaments grown at 44 C are gram-negative, whereas cells grown at 37 C are gram-positive. However, filament formation and gram-negativity apparently are not due to magnesium deficiency, since the gram-negative filaments are formed in concentrations of magnesium ranging from 10^{-6} to 10^{-2} M. The rapid transition from filaments to single cells upon lowering the temperature from 44 to 37 C suggests that the temperature-related repression of the cross wall-forming system is a phenotypic response rather than the selection of specific mutants which produce the observed phenomena.

Filament formation in bacteria may be induced by a variety of environmental factors, such as the presence of antibiotics and other metabolic inhibitors (9), deficiency of essential growth factors including metals (5, 6, 7, 17, 18), and various forms of physical traumata, e.g., radiation (1), and manipulation of the pressure (20) and temperature (8, 12).

In comparison with the amount of work done in relation to the other factors, relatively little work has been done in relation to the effect of temperature on filament formation. Maclean and Munson (12) and Hoffman and Frank (8) reported the induction of filaments in *Escherichia coli* at elevated growth temperatures, and Barker and Beck (2) reported that, when *Clostridium acidiurici* was grown at temperatures above 37 C, the cells tended to elongate and often formed cells 30 times the normal length.

The present studies were undertaken to explore further the phenomenon of filament formation in *C. acidiurici* and to study the properties of the filaments in relation to substrate utilization, distribution of nuclear material, and response to variations in the concentration of metal ions.

MATERIALS AND METHODS

Stock cultures of *C. acidiurici* were maintained at 5 C as spore suspensions after growth at 37 C in a semisolid agar medium containing 0.2% uric acid, 0.05% yeast extract, 0.05% K_2HPO_4, 0.02% magnesium sulfate (4×10^{-4} M), and 0.2% agar. Such spore suspensions have proved viable after several years of storage, and have been inoculated into new medium to give vegetative cultures as needed. Portions of 1 liter of the stock culture medium were prepared by adding the uric acid to 970 ml of hot distilled water to which 13 ml of 1 N NaOH had been added. After the uric acid was completely dissolved, the *p*H was adjusted to 7.6 with phenol red as an external indicator. The rest of the components were then added, and the *p*H was readjusted to 7.6 and enough distilled

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water was added to bring the total volume to 1 liter. The medium was dispensed into screw-cap tubes (20 by 150 mm) and autoclaved, and was then stored under refrigeration until used.

To assure the rapid initiation of growth when the stored medium was to be used, the oxidation-reduction potential was lowered by adding to 12 ml of culture medium 0.3 ml of 1% sodium hydrosulfite buffered in 5% $NaHCO_3$.

The medium used for growth experiments was the same as that described above except that the agar was omitted. The medium was dispensed into sterile tubes (13 by 100 mm) which were sealed with rubber stoppers, and the various growth measurements outlined below were determined.

Determination of growth. Growth responses were indicated as (i) turbidity of the culture, measured at 660 mμ by use of either a Bauch & Lomb Spectronic-20 colorimeter or a Beckman DU spectrophotometer; (ii) the disappearance of uric acid, which has an absorbance maximum at 290 mμ and a molar extinction coefficient of 1.2×10^4 cm^2 per mole; and (iii) the production of ammonia from uric acid. Growth was initiated in groups of 30 tubes, and at designated time intervals three groups of 10 tubes were used for the determination of turbidity, uric acid utilization, and ammonia production. The points shown on the curves for turbidity, uric acid utilization, and ammonia production in the Results section are the average of 10 determinations, and the maximal deviations were within ±5% of the average.

The inoculum cultures were grown at 37 C, and, when the cultures reached a turbidity of 0.20 at 660 mμ, 0.2-ml portions of the inoculum were added to each of the 30 tubes containing the freshly autoclaved medium which had been brought to the temperature of the respective experiments.

Production of ammonia from uric acid was determined titrimetrically on portions of the growth medium after steam distillation from sodium tetraborate. The distillate was collected in 4% boric acid to which bromocresol green and methyl red were added as a double indicator (11), and ammonia was determined by back-titration of the distillate with standard 0.01 N sulfuric acid.

The total cellular nitrogen in the growth tubes (13 by 100 mm) was determined after sedimentation of the cells by centrifugation and washing of the cells twice in normal saline. The washed cells were digested for 4 hr in 1 ml of boiling concentrated sulfuric acid containing 1 mg of selenium oxychloride and 1 mg of copper sulfate as catalysts, and 100 mg of sodium sulfate. The cellular nitrogen, released as ammonia, was then determined by steam distillation as above, with the exception that 50% sodium hydroxide instead of sodium tetraborate was used to neutralize the sulfuric acid and release the ammonia from solution.

Temperature control. A Yellow Springs Thermistemp model 71 temperature controller with a no. 403 probe was used to regulate the temperature of a water bath within ±0.1 C of the designated temperature settings. The water in the bath was stirred constantly.

Determination of magnesium. Magnesium in the medium was determined by the spectrophotometric method suggested by Cuttitta and White (3), with the use of bissalicylideneethylenediamine to chelate the magnesium. The high-purity reagent was prepared by the method of Freeman and White (4).

Nuclear stains. Chromatin bodies in the filaments of *C. acidiurici* were stained by the method of Robinow (14).

Cell wall stains. Cell walls were stained with alcian blue according to the method of Tomcsik and Grace (16).

RESULTS

Growth responses measured as turbidity, utilization of uric acid, and production of ammonia. Correlation of the growth responses of *C. acidiurici*, measured as turbidity, uric acid utilization, and ammonia production, is shown in Fig. 1. Good agreement was obtained among the decrease in optical density at 290 mμ (indicating the utilization of uric acid), the production of ammonia from the substrate, and the measurement of turbidity when the organism was grown at 37 C. Over 98% of the uric acid had been utilized, and over 96% of the theoretical yield of ammonia [4 moles of NH_3 per mole of uric acid, (2)] had been produced within 10 hr after inoculation. In other experiments, as the temperature was raised above 37 C, curves similar to those shown in Fig. 1 for uric acid utilization and ammonia production were obtained, but the turbidity decreased sharply at temperatures above 37 C. The results of such experiments are shown in Fig. 2. Of special significance in this figure is the utilization of essentially all of the uric acid in the medium over the temperature range between 35 and 44 C. At 44.5 C, there was a sudden drop in the uric acid utilization, which correlated with a corresponding decrease in ammonia production and in the growth of the cells. At 45 C, no metabolic activity was detectable as measured by uric acid disappearance or ammonia production. If the turbidity measurements alone were used as a criterion of metabolic activity, decreased substrate utilization and decreased cell yields would also be expected at temperatures between 37 and 44 C, since the turbidity decreased over this range. However, the continued utilization of all of the uric acid at these elevated temperatures would indicate either that the substrate, although being metabolized, was not being converted to cellular material, or that the cells grown at the elevated temperatures had less light-scattering capacity than cells grown at lower temperatures. In exploring these possibilities, the total cellular nitrogen was measured by use of the contents of tubes incubated at 37 and 44 C in which at least 98% of the

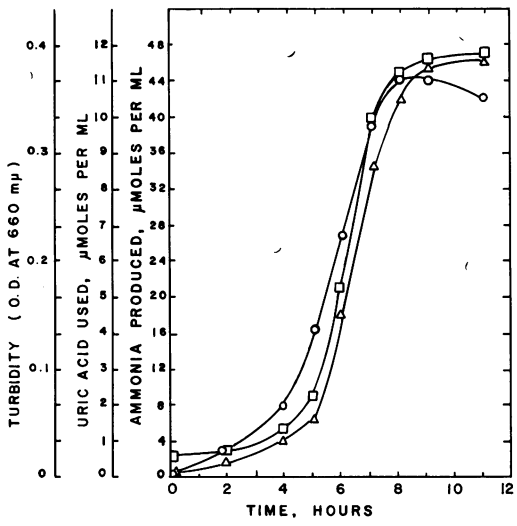

FIG. 1. *Growth responses of Clostridium acidiurici measured as:* ○, *turbidity;* △, *uric acid used; and* □, *ammonia produced.*

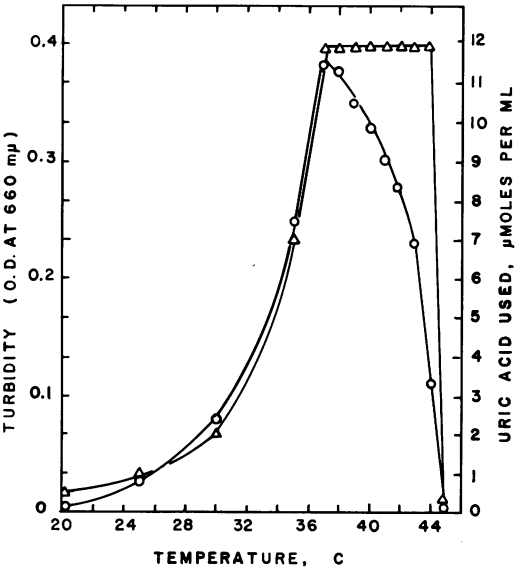

FIG. 2. *Effect of increasing temperature upon the growth responses of Clostridium acidiurici, measured as:* ○, *turbidity; and* △, *uric acid utilization. Determinations were made 10 hr after inoculation of the culture tubes.*

substrate had been utilized. The results of several measurements are shown in Table 1. It was determined that essentially the same amount of cellular nitrogen per growth tube was obtained from cultures at 37 and 44 C, indicating that the substrate was being converted to cellular material

TABLE 1. *Total cellular nitrogen recovered from growth tubes at 37 and 44 C**

Tube no.	Total cellular nitrogen (μmoles of NH_3)	
	From 37 C tubes	From 44 C tubes
1	12.2	11.5
2	12.0	11.5
3	11.4	12.5
4	12.2	11.5
5	12.3	11.8
6	11.8	11.2

* Cells from the growth tubes were washed and digested in a micro-Kjeldahl apparatus as described in Materials and Methods.

and that turbidimetric measurements were not an accurate index of growth at the elevated temperatures.

Morphological changes. Upon microscopic examination of the cells grown over the temperature range between 16 and 44 C, an impressive phenomenon was observed. As shown in Fig. 3, 4, and 5, cells grown at 20, 37, and 42 C, respectively, exhibited little or no difference in morphology, except that at 42 C there was a slight tendency toward elongation. In all cases, however, the cells were single short rods ranging in length from 2.5 to 4 μ. At 43 C, the cells showed a distinct tendency to elongate, but usually did not exceed 20 to 30 μ in length (Fig. 6). However, when the cells were grown at 44 C (Fig. 7), enormously long filaments were produced, often exceeding 500 μ in length. Thus, a very drastic change in the morphology of the cells occurred within the narrow temperature gradient between 42 and 44 C. At 44.5 C, no growth occurred, and filament formation was not observed. The filamentous cells shown in Fig. 6 and 7 could account for the decreased turbidity at the elevated temperatures as shown in Fig. 2, since the light-scattering ability of a suspension of small particles is related to the size of the particles as well as to the number in suspension.

Effect of metals. Inasmuch as filament formation in certain other bacteria has been correlated with a deficiency in magnesium ions (17, 18), experiments to determine the effect of magnesium concentration on the growth of *C. acidiurici* over the temperature range between 37 and 44 C were carried out. For these experiments, the uric acid medium described in Materials and Methods was employed, except that the magnesium was omitted and 10^{-5} M ethylenediaminetetraacetate was added to chelate metal ions which might be present in low concentrations in the medium.

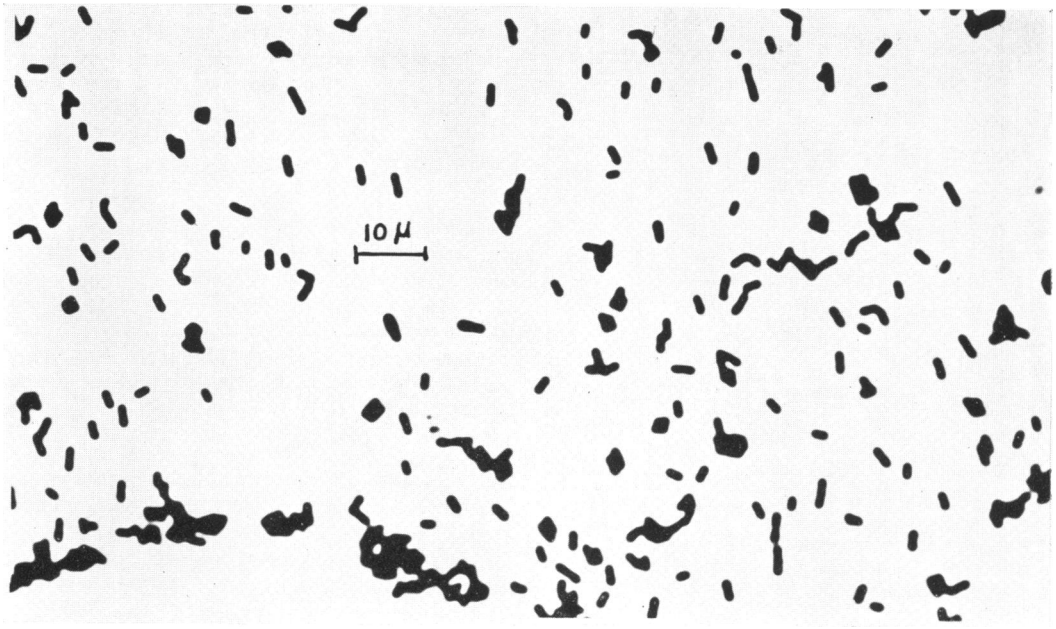

FIG. 3. *Cells of Clostridium acidiurici grown at 20 C. The cells in Fig. 3 through 7 were stained with crystal violet.*

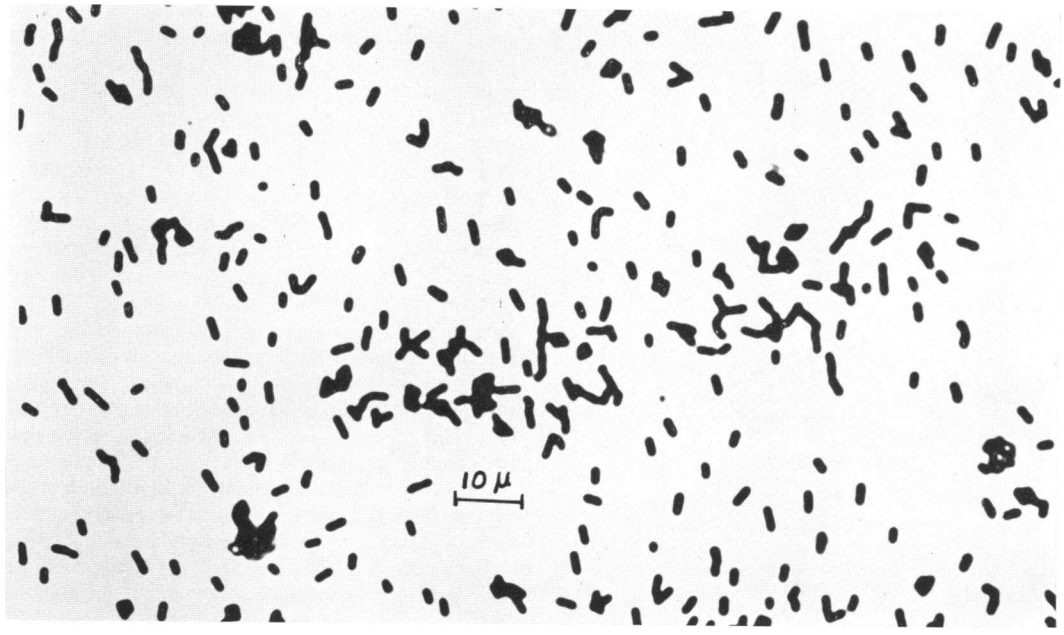

FIG. 4. *Cells grown at 37 C.*

Under these conditions, no growth was observed at any of the temperatures employed. Upon addition of magnesium ions at concentrations between 10^{-6} and 10^{-2} M, still no growth occurred unless ferrous iron at 8×10^{-5} M was added to the medium. After supplementation with iron, however, good growth was achieved at all of the Mg^{++} concentrations, but, even so, addition of

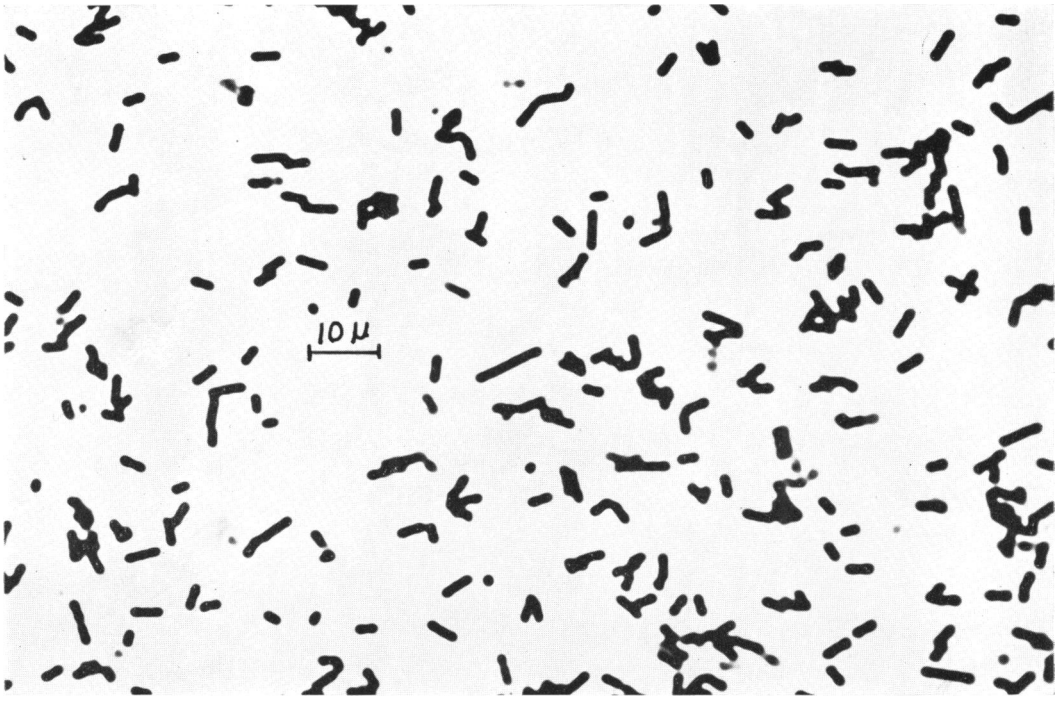

FIG. 5. *Cells grown at 42 C.*

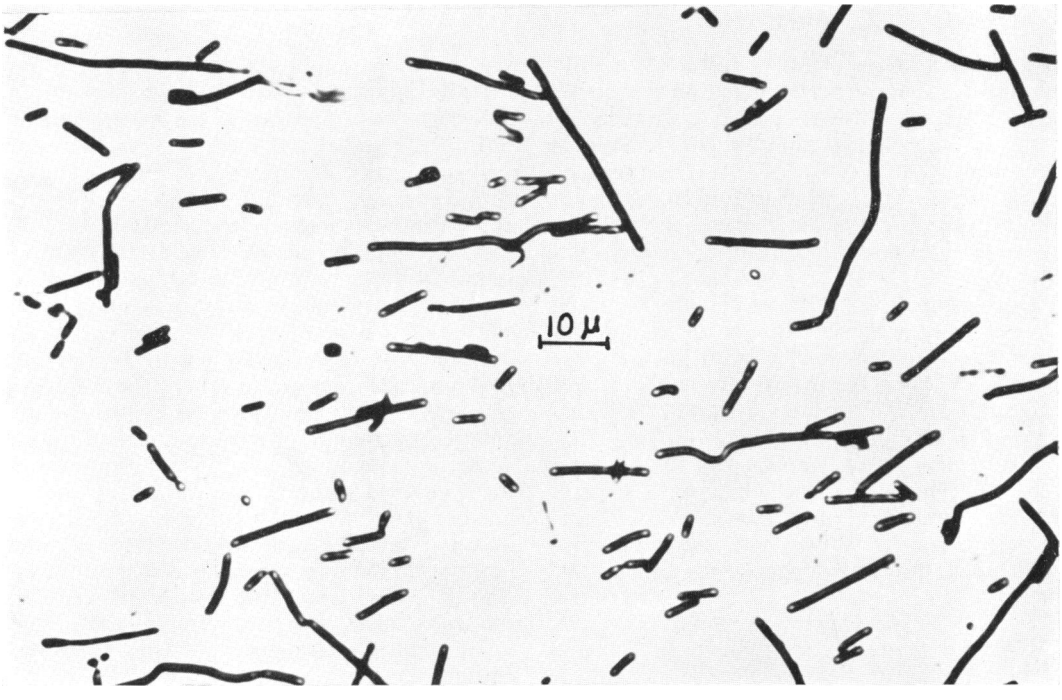

FIG. 6. *Cells grown at 43 C, showing tendency to elongate.*

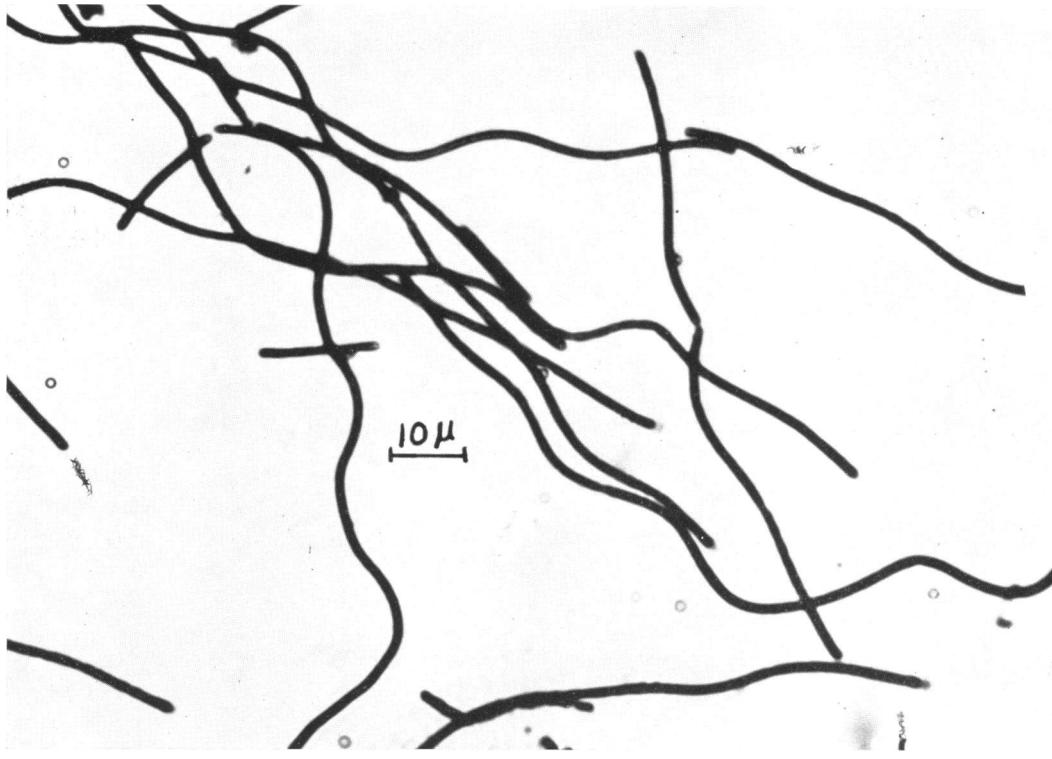

FIG. 7. *Filaments grown at 44 C.*

high concentrations of Mg^{++} (up to 10^{-2} M) did not prevent filament formation at 44 C. Furthermore, when the cells were grown at temperatures below 42 C, filaments could not be induced by decreasing the Mg^{++} or Fe^{++} concentrations. Increasing the iron content to 2×10^{-4} M had no effect on the length of the filaments formed at 44 C or the ability of the organism to grow into filaments.

Gram stain. Gram stains of the filaments showed the organisms to be gram-negative, whereas cells grown at 37 C normally are gram-positive. Varying the magnesium and iron concentrations as indicated above did not alter the gram-negativity of the filaments grown at 44 C.

Distribution of nuclear bodies in the filaments. Smears of the heat-induced filaments prepared for the examination of nuclear material (Fig. 8) revealed that chromatin bodies were uniformly distributed throughout the length of the filaments.

Inability of the filaments to form cross walls. Cross walls were not discernible in the filaments stained by the method used (14) to reveal the nuclear bodies, and, when the filaments were observed by phase-contrast microscopy, only a very occasional cross wall was detectable. When the filaments were stained with alcian blue (Fig. 9), which stains specifically the material in the cell walls, again it was found that the filaments were essentially devoid of cross walls. Only a very occasional "joint" or septum could be seen along the entire length of the filaments.

As mentioned earlier (see Fig. 2), the filaments grown at 44 C were able to utilize completely the uric acid present in the growth tubes. In an effort to determine whether these filaments would continue to elongate when supplied with fresh medium, cells were grown at 44 C until the uric acid was entirely used; then the contents of the culture tube were transferred to an equal volume of fresh medium and growth was determined by the continued utilization of uric acid and by microscopic examination of the cells. It was observed that the cells continued to elongate, resulting in the formation of a distinct myceloid growth. When serial transfers were made to successive tubes of fresh medium, the culture tubes had the appearance of being filled with cotton fibers.

Cross wall formation and fragmentation into single cells at lowered temperatures. Inasmuch as

FIG. 8. *Distribution of chromatin material in filaments grown at 44 C.*

the major effect of growing the cells at 44 C appeared to be the repression of the cross wall-forming system, resulting in the formation of the very long filaments, experiments were performed to determine whether such filaments were capable of forming cross walls and dividing into individual cells when the temperature was lowered. Cultures grown at 44 C until all the uric acid had been utilized were transferred to a bath (37 C), and samples were removed at intervals, stained with alcian blue, and examined microscopically. Within 2 hr after lowering the temperature, the filaments began to divide and form chains of cells (Fig. 10). The chains eventually fragmented into separate cells, and the culture assumed the appearance of one grown at 37 C. In another experiment, the 44 C culture was supplied with fresh medium immediately before being placed in the 37 C bath, and it was observed that division and fragmentation to single cells took place within 30 min and often within 20 min.

Temperature optima. C. acidiurici derives its entire carbon, nitrogen, and energy supply from the degradation of uric acid, and thus the organism must depend upon a relatively restricted metabolic sequence in deriving its energy and biosynthetic intermediates. It seemed possible, therefore, that this organism might be a sensitive indicator of any abrupt changes in the structual properties of water which were reported by Oppenheimer and Drost-Hansen (13) to manifest themselves in biological systems as multiple optima for growth over the temperature range between 0 and 100 C. However, as seen in Fig. 2, no such multiple optima were observed for the growth of *C. acidiurici* under the conditions of these experiments.

DISCUSSION

Morphological changes observed in *C. acidiurici* when the cells are grown at 44 C appear to be due to the specific inhibition of the cross wall-forming system. The cells are able to metabolize uric acid and they form new cellular material at a maximal rate, but they are unable to divide and form single cells of normal length. The repression of cross wall formation in the filaments apparently has little or no tendency to repress other cellular processes required for the growth and continued metabolic activities of the filaments, including the formation of longitudinal walls.

It was significant to determine whether filament formation was related to the concentration

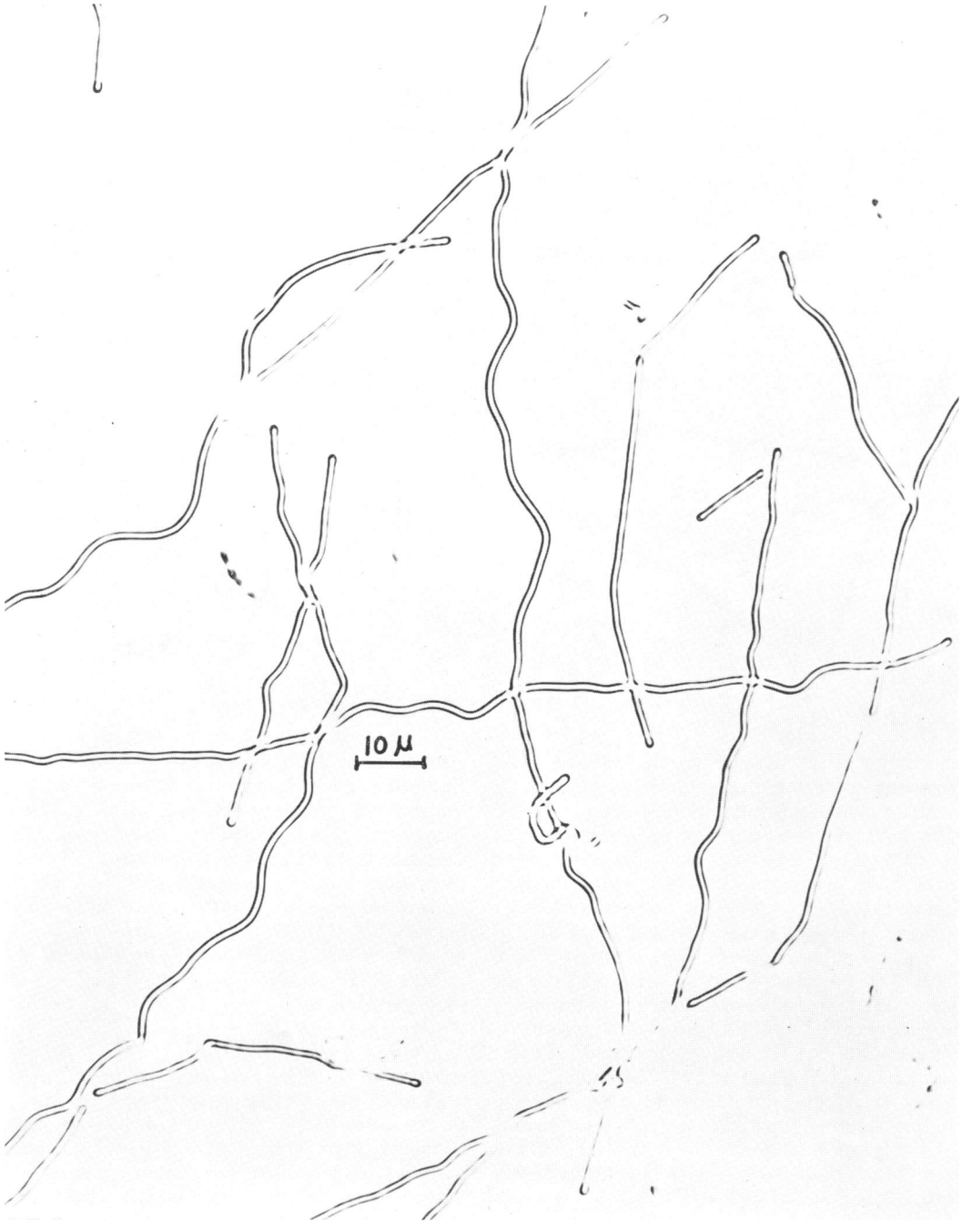

FIG. 9. *Cell wall stain of filaments grown at 44 C; stained with alcian blue.*

of magnesium ions. Webb (17) noted the effect of varying the concentration of the Mg^{++} upon the growth of gram-positive and gram-negative organisms, and noted filament formation at certain concentrations. Shankar and Bard (15) observed filament formation in cultures of *C. perfringens* when this organism was grown in suboptimal levels of magnesium. The possibility

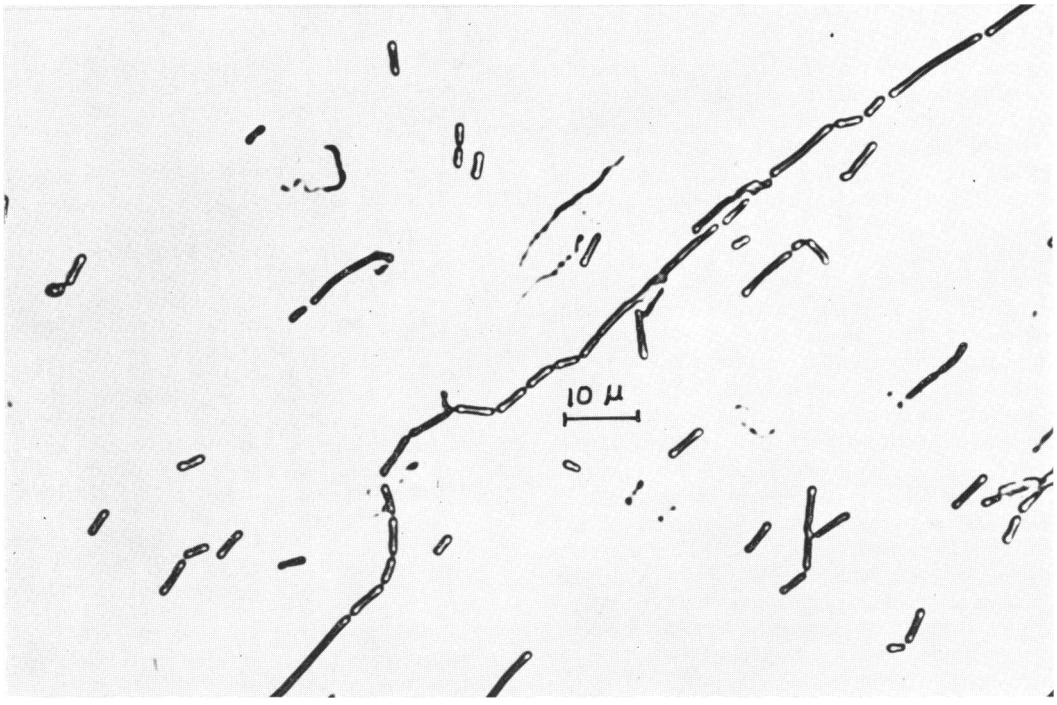

FIG. 10. *Alcian blue cell wall stains of filaments grown at 44 C, then placed at 37 C, showing fragmentation to short, individual cells.*

that filament formation in *C. acidiurici* results simply from deficiency in magnesium may be ruled out, however, since filament formation occurred at all magnesium concentrations between 10^{-6} and 10^{-2} M.

Filaments grown at 44 C were gram-negative, even in the presence of high magnesium concentrations (10^{-2} M), whereas cells grown at 37 C normally are gram-positive. This may indicate either that the cells are unable to incorporate sufficient magnesium at the elevated temperature and hence are incapable of forming the complex of molecules responsible for the gram reaction, or that factors other than the magnesium concentration were altered in the filaments, causing the normally gram-positive nature of the cells to be changed.

A point to be considered is whether filament formation is due to a phenotypic or genotypic response of the cell. A phenotypic response to adverse environmental factors resulting in filament formation may be characterized by the reversion of such filaments to normal cells upon transfer to a more favorable environment (12). A few cytological studies correlating the arrangement of nuclear material with the formation of filaments have been reported in the literature. Robinow (14) mentioned that in filaments of *E. coli* or of *Proteus* induced by certain doses of ionizing radiation, the distribution of the chromatin bodies was "chaotic" compared with the regular arrangement of the chromatin material found in normal cells. Jeener and Jeener (10) obtained poorly nucleated, very long filaments of *Thermobacterium acidophilus* when this organism was grown on a thymidine-deficient medium. Such filaments contained abundant ribonucleic acid but only a few widely spaced deoxyribonucleic acid-containing nuclear bodies. Zamenhof, De Giovanni, and Rich (19) induced the development of viable, poorly nucleated filaments of *E. coli* by cultivating this organism in the presence of a thymine analogue, 5-bromouracil. On the other hand, apparently normal synthesis of nuclear material was noted in filaments of *E. coli* induced either by elevated temperature (8) or by ionizing radiation (1).

In the case of *C. acidiurici*, the filaments are polynucleate, and chromatin bodies appear to be evenly distributed throughout the length of the filaments, with nondifferentiated cytoplasm between them (Fig. 2). The internuclear spaces may represent the locations where septa would be formed if such formation were permitted. It

seems likely that the failure to form cross walls and to divide is a phenotypic response of the organism, caused by inactivation of the cross wall-forming enzymes at the elevated temperature, rather than the interference with the synthesis of nuclear material controlling the synthesis of the enzyme proteins. The rapidity with which the filaments form cross walls upon being transferred to a lower temperature and supplied with fresh medium would support this idea. A key to the solution of this problem would be to demonstrate in vitro the presence of the cross wall-forming enzymes in the filaments grown at 44 C, and to show that the system is functional at temperatures below 42 C, but nonfunctional at 44 C. However, an adequate in vitro assay for the synthesis of cross wall material is not available at present, and so the interpretation of the possible genetic implications of filament formation in *C. acidiurici* must await the development of sufficiently sensitive methods for detecting specifically cross wall material and its synthesis. Cytological evidence, showing the presence of either regularly arranged or poorly arranged nuclear bodies, may not be a safe criterion for the translation of proposed genetic information into enzyme functions.

ACKNOWLEDGMENTS

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