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A Continuous Culture Study of an Obligately Psychrophilic *Pseudomonas* Species*

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Summary. The optimum temperatures for growth and respiration of an obligately psychrophilic *Pseudomonas* spec. were 14° C and 23° C, respectively. The maximum temperature for growth was between 19 and 20° C. When cells were grown in a chemostat with lactate as the growth-limiting substrate at a specific growth rate of 0.05 hr^{-1} over a temperature range of 5—19° C, it was found that RNA concentration was lowest at 14° C. At lower temperatures the cells compensated the decrease of reaction rates by increasing the concentration of RNA and of respiratory enzymes. A temperature raise above 14° C also increased cellular RNA, which probably counteracted an impairment of protein synthesis. Above 18° C the RNA increase ceased, resulting in a rapid decrease of protein synthesis, until between 19 and 20° C growth ceased entirely. Cells grown at 14° C showed a linear increase of RNA content and Q_{O_2} values with growth rate, when this was varied from 0.025 to the maximum value of 0.2 hr^{-1}.

Most psychrophilic microorganisms isolated thus far grow at temperatures down to 0° C or even below, but have an optimum temperature for growth above 20° C. Bacteria with growth optima below that temperature were isolated by Eimhjellen (Hagen *et al.*, 1964) and Morita and Haight (1964). The fact that only a limited number of such obligate psychrophiles has been isolated until recently is probably not due to their rarity, but to their thermal death at temperatures above 20° C. One would expect that organisms with this property might commonly occur in environments which permanently have a low temperature. It is thus not surprising that Morita (1966) reports that obligate psychrophiles occur commonly in ocean waters, since 95% of the oceans (by volume) is colder than 5° C.

Harder and Veldkamp (1966) similarly found that obligately psychrophilic bacteria could easily be isolated from North Sea water, when precautions were taken to avoid exposure of samples to temperatures above 20° C. One of these isolates, a *Pseudomonas* strain, was grown in a chemostat which permitted its study under steady state

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conditions and which offered moreover the unique possibility of growing the organism at a constant growth rate over a range of different temperatures.

Materials and Methods

Organism. The *Pseudomonas* strain was isolated from an aerobic enrichment culture inoculated with a plankton sample obtained from the North Sea. The medium is described below. During enrichment and isolation the temperature was kept at 1° C. The optimum temperatures for growth and respiration were 14° C and 23° C, respectively. The maximum temperature for growth was between 19 und 20° C. Details of isolation and characteristics of the organism will be reported elsewhere (HARDER and VELDKAMP, 1967).

Growth Medium. The growth medium used in all experiments had the following percentage composition (w/v): NaCl 2.5, $(NH_4)_2SO_4$ 0.1, $MgCl_2 \cdot 6H_2O$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.02, K_2HPO_4 0.02, KCl 0.02, $CaCl_2$ anh. $2\,10^{-3}$, tris(hydroxymethyl)aminomethane 0.1, and Na-lactate 0.25. One ml of a trace element mixture (HARDER and VELDKAMP, 1967) and 0.3 ml of a solution containing 250 mg $FeCl_3 \cdot 6H_2O$/l were added to 1 l of medium. The water used was deionized and the pH was adjusted to 8.0 before sterilizing the medium at 118° C; lactate was sterilized separately.

Synthetic Seawater. This term refers to the above medium when lacking Na-lactate and $(NH_4)_2SO_4$.

Growth Conditions in Chemostat. A three fermenter assembly was used (Model 40—302, Virtis Company, Inc., Gardiner, New York, U.S.A.); the working volume was 2.5 l. Agitation was fixed at 400 r.p.m. and aeration at 2.5 l air/min. A constant pH of 8.0 was maintained by means of an automatic titrator (TTT 1; Radiometer, Copenhagen, Denmark) using a heat sterilizable combined glass- and calomel electrode (Type 465-35; Ingold, Zürich, Switzerland). Temperature fluctuations of the cultures did not exceed 0.2° C. Sterile medium was added with a D.C.L. Micropump (Series II; Distillers Co. Ltd., Epsom, England), and the culture volume was maintained constant by an internally placed overflow tube which also served as air outlet. Silicone antifoam (Rhodorsil; Rhone-Poulenc, Paris, France; diluted 1:40 in deionized water) was added at a constant rate of 1.5 ml/hr.

Determination of Q_{O_2}. 50 ml volumes were collected from the chemostat, centrifuged (6000 g, 40 min, 2° C) and washed twice with 50 ml of precooled synthetic seawater. The washed cells were resuspended and the suspension was adjusted to a density of about 1 mg dry weight/ml. Q_{O_2} values were determined by the conventional manometric technique at 14° C. A correction was applied for endogenous respiration. The values thus obtained are referred to as Q_{O_2} 14°.

Determination of Maximum Specific Growth Rates. Maximum specific growth rates (μ_{max}) at different temperatures in the above medium were determined in batch culture, using modified Kluyver flasks. The sulphite oxidation value (COOPER *et al.*, 1944) for the aeration applied was 140 mM O_2/l/hr. Growth was measured as increase of O.D. at 430 mμ.

Dry Weight Determination. 40 ml culture volumes were centrifuged (12500 g, 20 min, 0° C) and cells were washed twice with ice-cold synthetic seawater. Pellets were washed into weighing flasks with 1—2 ml deionized water, and dried for 3 hrs at 120° C. A correction for salt in the adhering water was applied as follows. Samples containing equal numbers of cells were washed with synthetic seawater containing different NaCl concentrations. Extrapolation of the dry weight-salt concentration curve to zero salt concentration provided the correction factor.

Fractionation of Cell Components and Analytical Procedures. 200 ml volumes were collected from the chemostat, centrifuged (12500 g, 20 min, 0° C), and sediments were washed with ice-cold synthetic seawater. The sediments were resuspended in 100 ml synthetic seawater, dispensed in 8 ml quantities in 15 ml tubes, an centrifuged (6000 g, 40 min, 2° C). These sediments were extracted with 2.0 ml ice-cold 0.2 N $HClO_4$ at 0° C for 20 min and insoluble material was collected by centrifugation (6000 g, 20 min, 2° C). After decantation, 2.0 ml 1 N KOH was added to two tubes and protein was determined after incubation at 37° C for 1 hr. In sediments of three other tubes, RNA and DNA were separated by a modified Schmidt-Thannhauser procedure (MUNRO and FLECK, 1966) and determined in the fractions obtained. Protein was determined by the biuret method (GORNALL *et al.*, 1949); bovine serum albumin (Poviet Products, Amsterdam) was used as a standard. RNA was determined by the orcinol method (MUNRO and FLECK, 1966); yeast RNA prepared from baker's yeast (CRESTFIELD *et al.*, 1955) was used as a standard. DNA was determined by the method of BURTON (1956) with calf thymus DNA (Na-salt; the British Drughouses Ltd.) as a standard. Lactate was determined by the microdiffusion method of RYAN (1958).

Results

Effect of Temperature on Cells Grown at Constant Growth Rate. The organism was grown at a dilution rate of 0.05 hr^{-1} (doubling time 13.8 hr) over the temperature range 5—19° C; the growth-limiting factor was lactate. Results are shown in Fig. 1.

The difference between the actual growth rate (μ) in the chemostat and the maximal possible growth rate (μ_{max}) was calculated for different temperatures. These figures provided the μ-μ_{max} curve.

Two Q_{O_2} curves are given, one referring to Q_{O_2} 14°, and the other to Q_{O_2} values at growth temperature. The latter values were calculated as follows. Q_{O_2} values at different temperatures were determined of cells grown at 14° C in batch culture. An Arrhenius plot of these data provided the correction factors with which Q_{O_2} 14° values of chemostat grown cells were multiplied to obtain the actual-Q_{O_2} values. A decrease of temperature below 14° C resulted in a marked increase of Q_{O_2} 14° and cellular RNA content. Raising the temperature above the optimum also resulted in an increase of cellular RNA; between 18° C and 19° C, however, this increase suddenly ceased. A further temperature elevation to 20° C rendered protein synthesis impossible and the culture then was washed out. Preliminary experiments indicated that the RNA increase above 14° C was mainly due to r-RNA, whereas that observed below 14° C was due to both r-RNA and s-RNA. In contrast to the changes in cellular RNA content, the concentration of DNA varied only slightly; a small increase was noticed at temperatures above optimum for growth. The protein content of the cells and the actual-Q_{O_2} values increased towards the temperature extremes, whereas the molar growth yields concomitantly decreased. The lactate concentration in the chemostat was less than 10 μg/ml throughout the temperature range 5—19° C.

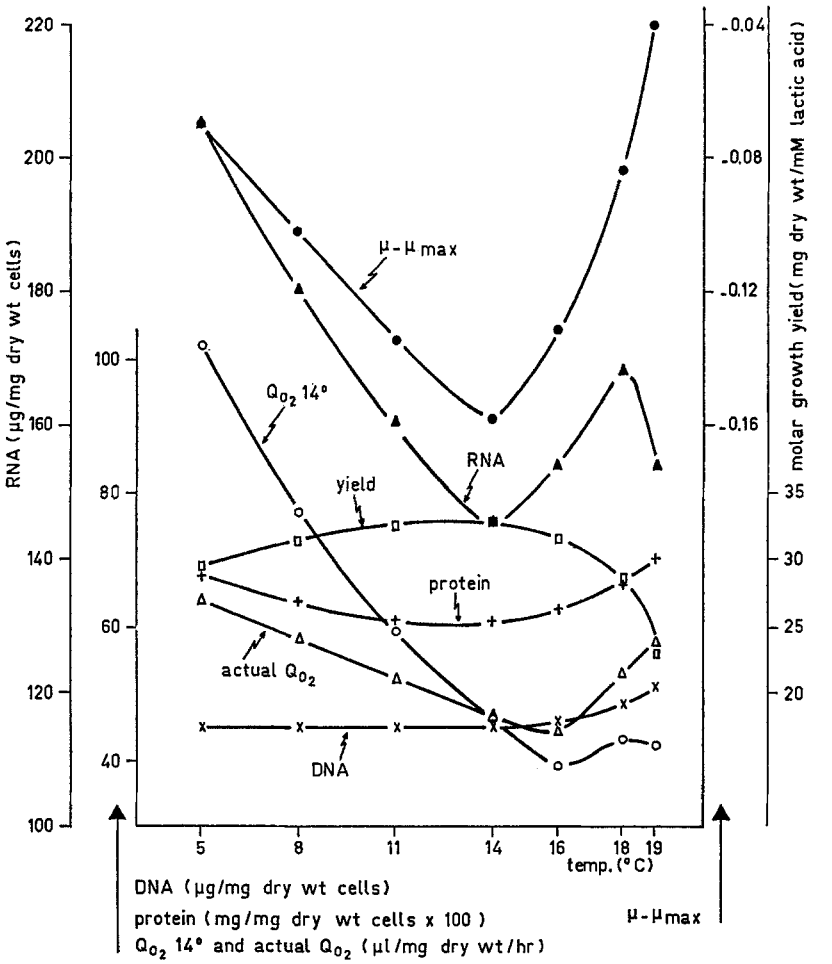

Fig. 1. Effect of temperature on lactate-limited *Pseudomonas* spec., grown at a dilution rate of 0.05 hr^{-1}

Effect of Growth Rate on the Properties of Cells Grown at Constant Temperature. One would expect that growth at constant temperature and increasing growth rates requires a cellular response similar to that needed for growth at a constant rate and progressively decreasing temperatures. For this reason the experiment shown in Fig. 2 was carried out.

According to the above expectation, Fig. 2 shows a marked increase of Q_{O_2} 14° and of RNA concentration with increasing growth rates. This was accompanied by a slight decrease of cellular DNA.

The shapes of the curves representing bacterial- and lactate concentration are in agreement with theoretical prediction (HERBERT *et al.*,

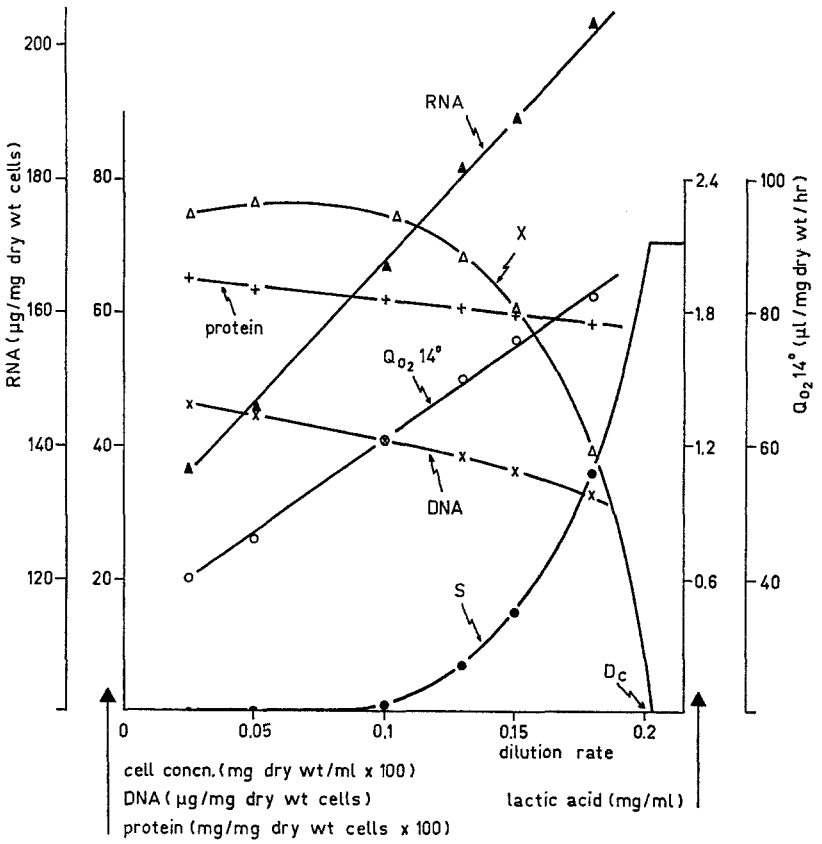

Fig. 2. Effect of growth rate on properties of *Pseudomonas* cells grown at 14° C; X bacterial concentration; S lactate concentration in culture, expressed as lactic acid; D_c critical dilution rate

1956). The critical dilution rate at 14° C was 0.2 hr^{-1}, a value similar to that found in batch culture for μ_{max} at that temperature (0.208 hr^{-1}; doubling time 3.3 hr). Since the bacterial concentration remained approximately constant at low dilution rates, the maintenance requirement apparently was very low at 14° C.

Discussion

The results shown in Fig. 1 indicate that different phenomena dominate cellular activities below and above the optimum temperature. When the temperature is decreased in a culture growing under steady state conditions at 14° C, the cells have to compensate the temperature-induced decrease of metabolic reaction rates. A decrease of temperature disturbs the steady state, in which growth rate (μ) equals dilution rate

(D); μ becomes smaller than D, and if this were a permanent change, the culture would be washed out. However, the cells appear to be able to offset the temperature effect by manufacturing RNA and enzymes involved in energy generation in greater quantity. The limit of this compensating mechanism is obviously set by that temperature at which the growth rate is no longer submaximal. The increase in concentration of respiratory enzymes as found by Q_{O_2} determinations at 14° C was, however, more than a mere compensation for slow-down of reaction rates. Fig. 1 shows that the actual-Q_{O_2} values increased towards the temperature extremes. The concomitant decrease in molar growth yields indicates that the energy needed per unit weight per unit time to maintain a certain growth rate increased with increasing deviation of growth temperature from the optimum.

Aerobacter aerogenes cells, grown in the chemostat at a fixed dilution rate (0.2 hr^{-1}) showed about 30% increase in ribosome content when the temperature was lowered from 35° C to 25° C. The increase was independent of the nature of the growth-limiting substrate (TEMPEST and HUNTER, 1965). These results are in accordance with those reported above, though it should be emphasized that comparison of the macromolecular composition of cells cultivated at temperatures below and above the optimum for growth should be considered with care. At temperatures above the optimum, a response to thermal damage may be involved, as will be indicated below. The response to temperature decrease below optimum showed the same tendency as that observed at constant temperature when the growth rate was progressively increased (Fig. 2). The differences in cellular response in both types of experiments were of a quantitative nature; the temperature decrease caused an almost logarithmic increase of RNA and Q_{O_2} 14°, whereas a linear increase was observed with increasing growth rate. The latter observation is in agreement with results obtained with *A. aerogenes*, in which Q_{O_2} (HERBERT, 1958) and cellular RNA content (TEMPEST *et al.*, 1965) were also found to be a function of growth rate.

As shown in Fig. 1, an increase of temperature above the optimum also resulted in an increase in RNA content of the cells. This obviously must be a response to a phenomenon entirely different from that observed on the other side of the temperature optimum. It seems likely that the RNA increase above 14° C must be considered as an effort to compensate an impairment in protein synthesis; for as soon as thermal damage prevented a further increase in RNA concentration, the growth rate decreased dramatically with a small increase of temperature. Since the optimum temperature for respiration was 23° C, it was obviously not denaturation of respiratory enzymes which caused growth to cease at 20° C. At 18—19° C, 260 mμ absorbing material leaked out of the cells;

the amounts were, however, so small that increased permeability to this material does not offer an explanation for the decrease in protein synthesis either. Enrichment of the growth medium with complex substrates as yeast extract or peptone (up to concentrations of 0.5%) did not allow growth above 20° C in batch culture. Cultures which were exposed to progressively increasing temperatures above maximum for growth, showed a progressive increase of the thermal death rate (cf. HARDER and VELDKAMP, 1967). A 50 ml suspension containing $2 \cdot 10^9$ viable cells per ml of synthetic seawater was completely killed when exposed for 6 hrs to 30° C.

It is not yet clear what advantage it could be to an organism to be an obligate psychrophile. Available evidence, though scant, indicates that it is not the slope of the Arrhenius curves (ln μ—1/T), but the temperature range at which it is straight, which is different for obligate and facultative psychrophilic bacteria (HARDER and VELDKAMP, 1967). If it turns out that the obligate psychrophiles as a general rule grow faster at the lower temperature extreme, then the inability to grow at temperatures above 20° C might be the price they pay for the advantage they have in the very cold natural environments.

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