Journal of Dairy Research (1978), 45, 259–265

Effect of incubation temperature on the development of lactic acid bacteria and their phages

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(*Received 20 May 1977*)

Summary. Thirty-one strains of mesophilic and thermophilic lactic acid bacteria and their respective phages were tested for their minimum, optimum and maximum multiplication temperatures. Culture growth was strongly influenced by temperature during the first few hours of incubation, but less so after 24 h. Most of the phages showed the same pattern of development as their hosts, but one phage lysing a thermophilic lactobacillus and 3 phages lysing mesophilic streptococci proved temperature-sensitive, having a lower maximum temperature than that of their hosts. One phage was unusual in that its minimum development temperature was 7 °C above that of its host. Differences in temperature sensitivity were insufficient to reduce risk of phage infection by temperature control in industrial processes.

Certain bacteriophages are unable to multiply at temperatures at which their host bacteria still grow actively. This characteristic is widely used for studies of microbial genetics and especially those concerned with phages of certain strains of *Escherichia coli* (Epstein *et al.* 1963; Spiegelmann *et al.* 1968) and *Bacillus subtilis* (Hemphill & Whiteley, 1975).

A similar phenomenon may sometimes be observed with the lactic acid bacteria. For instance, certain wild-type bacteriophages of *Streptococcus cremoris* do not develop at 37 °C although their host grows well at this temperature (Hunter, 1943; Zehren & Whitehead, 1954). Temperature-sensitive phages of both *Leuconostoc mesenteroïdes* (Kaneko, Iwano & Kitahara, 1955) and *Lactobacillus casei* (Murata, 1971) have also been isolated. The latter is not replicated above 40 °C although its host, which has an optimum growth temperature of 37–41 °C, grows well at 43–44 °C.

Recently Keogh (1973), in a study on the latent period and burst size of 19 phages tested at 30 and 37 °C found that for 6 of them, no phage was produced at 37 °C while a burst size of 2–50 was observed at 30 °C.

The aim of our study was to improve our knowledge of lactic acid bacteria and their phages and to see whether production losses caused by phages in the dairy industry could be prevented by careful choice of incubation temperatures.

Table 1. *Origin of the strains tested*

Organisms	Strains	Obtained from*
Streptococcus cremoris	C13, BK5, Z8, P2, E8, TR, H2	Pearce, NZDRI
	Z8(2), HP, ML1, C11	Keogh, CSIRO
Str. lactis	11, Lille, Sa1, 264, 274, 280	Sozzi, LINOR
	ML3	Galloway, WSAC
Str. diacetylactis	26-2	Sandine, OSU
Str. thermophilus	S265, L12, S133	Sozzi, LINOR
	440	Accolas, INRA
	19987	Kiuru-Tybeck, ATCC
Lactobacillus bulgaricus	448, 449	Accolas, INRA
L. helveticus	L112	Sozzi, LINOR
	450	Accolas, INRA
L. lactis	A, F1	Sozzi, LINOR
	15808	Kiuru-Tybeck, ATCC

* NZDRI, New Zealand Dairy Research Institute; CSIRO, Commonwealth Scientific and Industrial Research Organisation, Australia; LINOR, Laboratoire Industriel Nestlé, Orbe, Switzerland; WSAC, West of Scotland Agricultural College, Dept. of Dairy Technology; OSU, Oregon State University, Dept. of Microbiology, U.S.A.; INRA, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; ATCC, American Type Culture Collection.

MATERIALS AND METHODS

Phages and host bacteria. These are listed in Table 1. The taxonomic name *Str. lactis* subsp. *diacetylactis* will hereafter be abbreviated to *Str. diacetylactis*.

Maintenance of cultures. The strains and their phages were transferred and stored using the routine method described previously (Sozzi, 1972).

Culture media. The following 2 media were used: MRS broth (de Man, Rogosa & Sharpe, 1960) for the lactobacilli; Hogg & Jago's (1970) medium for the streptococci.

Temperature gradient. The temperature gradients were obtained by means of an apparatus of our own construction, 'Graditherm', based on Oppenheimer's principle (Oppenheimer & Drost-Hansen, 1960). It provides a linear gradient of 25 temperatures for up to 6 different samples (i.e. 150 tubes in all), with a stability better than 0·2 °C/24 h and ± 0·15 °C fluctuations (after a 4-h equilibration time).

A gradient from 7 to 55 °C (ΔT = 2 °C) for mesophiles and from 20 to 56 °C (ΔT = 1·5 °C) for thermophiles was used.

Experimental technique. The tubes containing the medium (10 ml) were placed in the Graditherm at least 4 h before inoculation to allow temperature to stabilize. Each tube was inoculated (1%) with a 6-h culture of the test strain and cultured in the same medium, at 30 °C for mesophiles and 40 °C for thermophiles. Two series of tubes were inoculated for each strain, the first with the bacterial strain only, the second with the strain and its phage. After careful mixing the tubes were incubated in the Graditherm for 6 h. The growth in each tube was then determined by measuring the optical density of the suspension at 540 nm in a Spectronic 20 spectrophotometer (Baush and Lomb, 14625-Rochester N.Y. U.S.A.). In cases of slow growth, this determination was repeated after 24-h incubation.

Phage count. The phages were estimated by counting the plaques obtained by the double layer technique of Sozzi, Maret & Poulin (1976). The samples used for

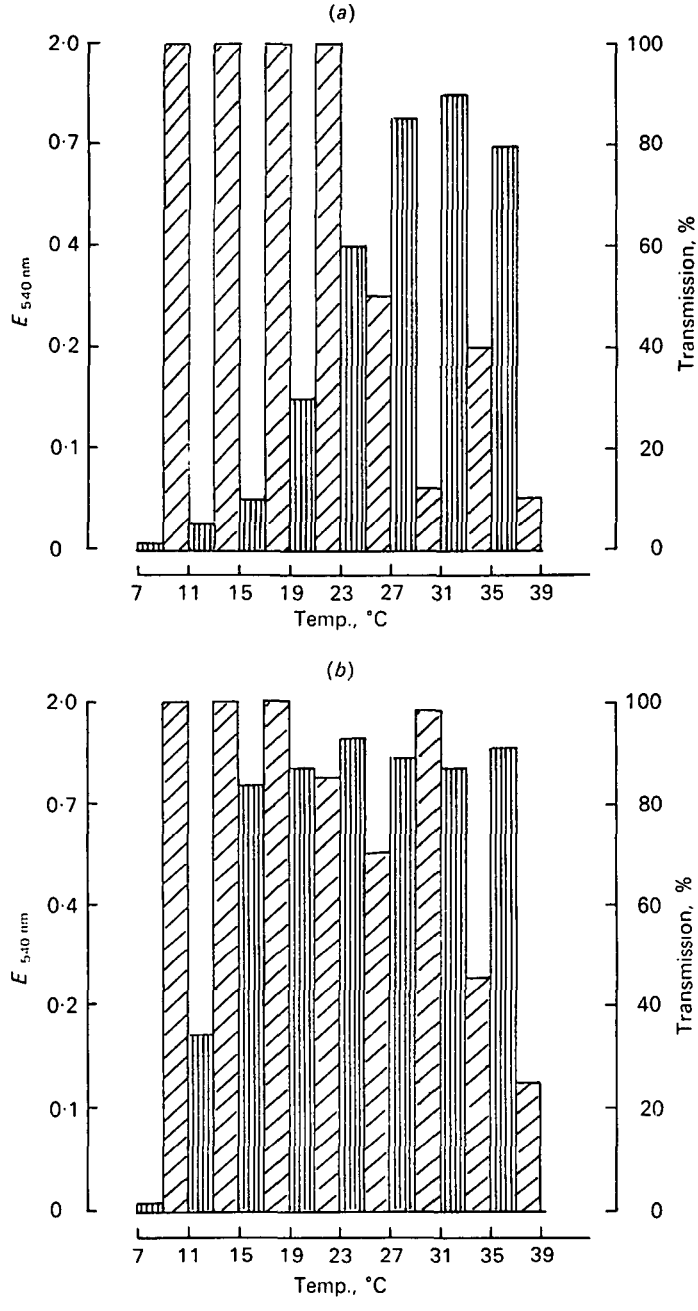

Fig. 1. Effect of incubation temperature on the growth of *Streptococcus cremoris* CSIRO C 11 and its phage. In a temperature gradient: (*a*) 6-h incubation; (*b*) 24-h incubation. ▥, Absorbance 540 nm phage free host; ▨, transmission phage infected host.

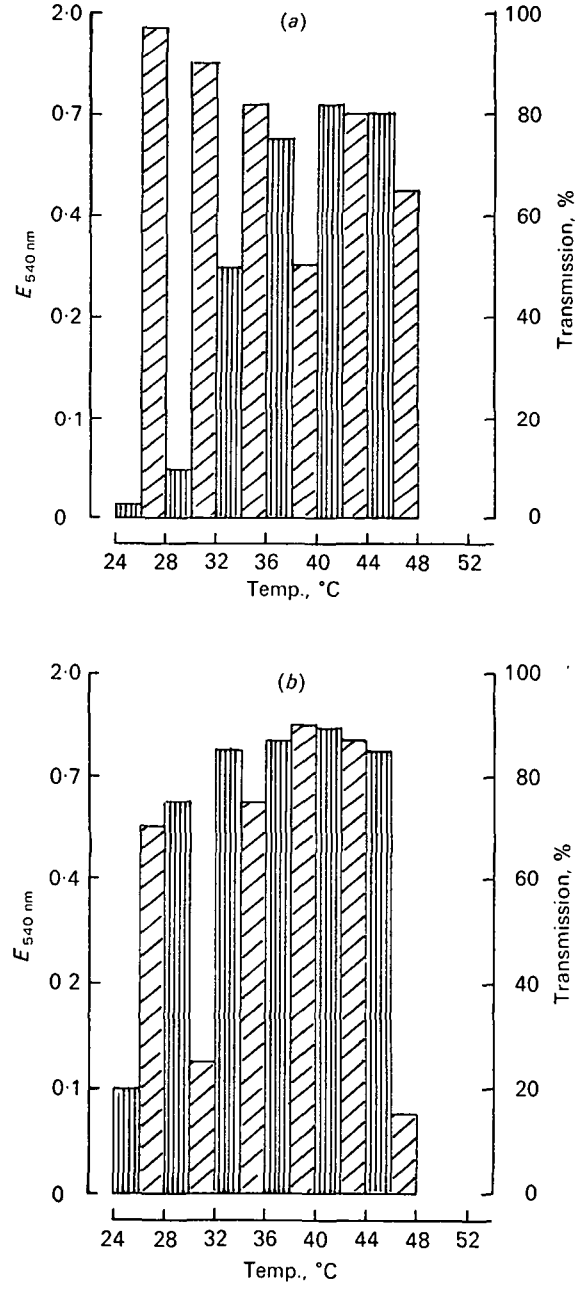

Fig. 2. Effect of incubation temperature on the growth of *Streptococcus thermophilus* ATCC 19987 and its phage. In a temperature gradient: (*a*) 6-h incubation; (*b*) 24-h incubation. ▥ Absorbance 540 nm phage free host; ▨, transmission phage infected host.

Table 2. *Minimum, optimum and maximum growth temperatures for the host bacteria and their bacteriophages*

Taxonomy, no. of strains	No. of phages*	Temp. °C, average values: Bacteria Min.	Bacteria Opt.	Bacteria Max.	Phages Min.	Phages Opt.	Phages Max.	Cases of thermo-sensitivity
Streptococcus cremoris (11)	11	11±2	30±3	35±3	11±2	30±3	35±3	0
Str. lactis (7)	11	9±1	34±1	41±2	9±1	33±2	41±2	2
Str. diacetylactis (1)	1	9	33	37	9	29	33	1
Str. thermophilus (5)	5	21–23	39–41	46–47	21–23	39–40	46–47	0
Lactobacillus bulgaricus (2)	2	29–32	42–43	49–50	29–32	42–43	49–50	0
L. helveticus (2)	2	23–28	44–45	55–50	30–28	38–45	50–50	1
L. lactis (3)	3	26–29	43–45	50–51	26–29	43–45	50–51	0

* Each bacterial strain had 1 specific phage except *Str. lactis* 274, which was the host for 5 phages.

Table 3. *Minimum, optimum and maximum temperatures of multiplication of some temperature-sensitive phages and their hosts*

Host and phage	Temp., °C: Min.	Opt.	Max.
Streptococcus lactis 274	9	33	41
Phage 21	9	33	37
Str. lactis 280	9	33	41
Phage 280	9	33	35
Str. diacetylactis 26-2	9	33	37
Phage 26-2	9	29	33
Lactobacillus helveticus L 112	23	44	55
Phage l 112	30	38	50

estimating the number of phages were withdrawn with those for the first spectrophotometric reading, i.e. after 6 h of incubation.

RESULTS

The effect of temperature on the growth of the microorganisms after 6 and 24 h of incubation is shown in Figs 1 and 2. Bacterial growth was strongly influenced by temperature in the first few hours of incubation (Figs 1*a* and 2*a*). However, after 24 h, this effect diminished considerably and the precise optimum temperature was replaced by a range of temperatures permitting maximum growth (Figs 1*b* and 2*b*).

The multiplication of the phages followed almost the same pattern as that of the host strain. The maximum degree of bacterial lysis is attained after 24 h of incubation, but in some cases the bacterial culture was well clarified after 6 h and dense again after 24 h (Fig. 1, 31–35 °C, Fig. 2, 44–48 °C). This is a secondary culture of the host, no doubt due to the proliferation of phage-resistant mutants. This secondary growth was generally greatest close to the optimum temperature of the host strain.

The minimum, optimum and maximum temperatures for development of phages and their host bacteria are grouped in Table 2. The parallel between the phages and

Table 4. *Behaviour of the phages of mesophilic streptococci incubated for 6 h at different temperatures*

Phages of	Approx. concn (pfu/ml)	No. of phage cultures: Initial	After 6 h incubation at °C: 7	17	27	37	47	55
Streptococcus cremoris (11 strains)	10^8	—	—	—	3	—	—	—
	10^7	—	—	8	6	2	—	—
	10^6	5	4	2	2	1	1	1
	10^5	4	5	1	—	4	5	4
	10^4	2	1	—	—	4	3	3
	10^3	—	1	—	—	—	2	3
Str. lactis and *diacetylactis* (12 strains)	10^8	—	—	—	6	4	—	—
	10^7	—	—	8	5	3	—	—
	10^6	4	5	2	1	4	—	—
	10^5	4	4	2	—	1	10	5
	10^4	4	3	—	—	—	2	6
	10^3	—	—	—	—	—	—	1

(Distribution of the cultures according to their approximate concentration in pfu/ml.)

their hosts is evident and there are very few temperature-sensitive phages. In general, the bacteriophages multiplied whenever the bacteria grew.

Table 3 shows in more detail the differences between the 4 temperature-sensitive phages which we found. All of them differed from their hosts in maximum temperature, but one only in minimum temperature, and two in optimum temperature.

To supplement the photometric measurements, we estimated the rate of phage multiplication after 6 h of incubation at different temperatures using the plaque count method. The results are grouped in Table 4. Each strain was tested for the number of active phages at the time of inoculation, and after 6 h of incubation at 6 different temperatures. The Table shows, for each temperature, the number of cultures containing the concentration of phages (pfu/ml) indicated in the margin.

These results are in agreement with those obtained photometrically, and it is interesting to note that the phages are not killed by exposure at 55 °C for 6 h: their multiplication is blocked, but their numbers are not reduced.

DISCUSSION

In this study we have only used short incubation times – 6 and 24 h – to investigate the effect of temperature on the growth of phages and their host bacteria. Although the results may be of little interest to taxonomists, who generally work with much longer incubation periods, they should be valuable to the food industry, for in any fermentation process the quality of the end-product is greatly dependent on what happens in the first few hours of fermentation. This is illustrated by the temperature profiles which we obtained. Indeed, although quite a wide range of temperatures favour growth after 24 h incubation, after only 6 h incubation there is a precise optimum temperature and deviations from this may cause serious delays in growth. The importance of strictly controlling the optimum temperature for

development of lactic acid bacteria is thus obvious, especially during the milk coagulation process in cheese production.

The knowledge acquired from this study may be valuable for the future preparation of starters and especially mixed cultures. Attention must be paid to the fact that the optimum temperature is very near to the maximum one, above which bacterial growth decreases extremely rapidly. Thus, temperature regulation cannot be used to prevent phage attacks because, with few exceptions, the phages exhibit the same temperature curves as their hosts.

We are grateful to Mr O. Depierraz for his excellent technical assistance and to Miss A. Walker for her help in writing this paper in English.

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Printed in Great Britain