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Temperature Compensation of Circadian Period Length in Clock Mutants of *Neurospora crassa*[1]

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GEORGE F. GARDNER AND JERRY F. FELDMAN[2]
Thimann Laboratories, University of California at Santa Cruz, Santa Cruz, California 95064

ABSTRACT

Temperature compensation of circadian period length in 12 clock mutants of *Neurospora crassa* has been examined at temperatures between 16 and 34°C. In the wild-type strain, below 30°C (the "breakpoint" temperature), the clock is well-compensated (Q_{10} = 1), while above 30°C, the clock is less well-compensated (Q_{10} = 1.3). For mutants at the *frq* locus, mutations that shorten the circadian period length (*frq-1*, *frq-2*, *frq-4*, and *frq-6*) do not alter this temperature compensation response. In long period *frq* mutants (*frq-3*, *frq-7*, *frq-8*), however, the breakpoint temperature is lowered, and the longer the period length of the mutants the lower the breakpoint temperature. Long period mutants at other loci exhibit other types of alterations in temperature compensation—*e.g.* *chr* is well-compensated even above 30°C, while *prd-3* has a Q_{10} significantly less than 1 below 30°C. *Prd-4*, a short period mutant, has several breakpoint temperatures. Among four double mutants examined, the only unusual interaction between the individual mutations occurred with *chr prd*, which had an unusually low Q_{10} value of 0.86 below 27°C. There was no correlation between circadian period length and growth rate. These strains should be useful tools to test models for the temperature compensation mechanism.

Circadian rhythms are daily oscillations that persist in constant conditions with periodicities close to, but not exactly, 24 h. A striking feature of these rhythms is that their period lengths are nearly constant over a wide range of physiological temperatures, a feature called temperature compensation.

The importance of temperature compensation has been widely discussed since its emphasis by Pittendrigh (16) in 1954, who used it to argue that circadian rhythms were a manifestation of an internal biological clock with a time-keeping function for the organism, and that such a clock must keep accurate time at different ambient temperatures. Mechanistically, such a system could approach temperature independence in the true sense by having its rate determined by physical processes such as diffusion, whose rates are proportional to the absolute temperature (3). Alternatively, the apparent temperature independence could be achieved by a temperature compensation system (2, 10), whose overall temperature independence is the result of the interaction of two or more components, each of which is temperature-dependent.

Several lines of evidence indicate that circadian rhythms are temperature-compensated rather than temperature-independent. (a) For some organisms (*e.g.* *Gonyaulax polyedra*) the clock runs slightly faster at low temperatures than at high ones—the Q_{10} of the rhythm is less than one. An "over-compensation" explanation seems the most plausible for this fact (10). (b) Circadian systems can be phase-shifted, or reset, by temperature steps or temperature pulses (23). This indicates that the clock recognizes temperature differences and adapts to new steady-state temperatures. In this regard, Pavlidis *et al.* (15) have proposed a mathematical model for temperature compensation which also accounts for a large part of the data on phase shifting by temperature steps and pulses. (c) For a number of organisms, such as *Euglena* (2) and *Neurospora* (19), the apparent temperature independence exists only within a limited temperature range, and outside this range the circadian system becomes significantly more temperature-dependent. This suggests that a temperature compensation mechanism works efficiently only within certain physiological or biochemical limits.

In recent years, several laboratories have turned to genetic analysis to help elucidate the molecular mechanisms underlying circadian clocks. Single gene mutants that alter the free-running period length of the rhythm have now been found in four organisms—*Drosophila melanogaster* (12), *Drosophila pseudoobscura* (17), *Chlamydomonas reinhardi* (1), and *Neurospora crassa* (6). It is of interest to determine whether the mutations that alter period length in these strains also affect temperature compensation of period length. In *C. reinhardi*, the mutants have normal temperature compensation (1) while in *D. melanogaster* some small differences in the Q_{10} values have been found (11).

In *N. crassa*, several analyses of the temperature responses of the wild-type (*i.e.* *band*) strain have been carried out. Temperature steps and pulses produce phase shifts similar to those seen in other organisms (8). In addition, the period length of the rhythm is temperature-compensated, but only within a limited temperature range. Sargent *et al.* (19) found that between 18 and 25°C, the *Neurospora* clock has a Q_{10} of 0.95, *i.e.* it is slightly over-compensated, while in the range of 25 to 35°C, the Q_{10} increases to 1.2. This distinction between a low temperature range, in which the clock is well-compensated, and a high temperature range, in which it is less well-compensated, has recently been confirmed (13), although small differences in the "break-point" temperature between the high and low range (30 *versus* 25°C) and the Q_{10} in the high temperature range (1.3 *versus* 1.2) were observed.

The importance of temperature compensation as a distinguishing property of circadian rhythms has gained added interest recently as a result of attempts to explain this phenomenon in several molecular models of clock mechanism (14, 21, 22). The *Neurospora* system appears to be an excellent one to explore this question further because of the difference in temperature compensation at high and low temperatures and because of the wide range of genetic and biochemical tools available for application to this problem. This paper presents an analysis of the temperature compensation properties of 12 previously isolated *Neurospora* clock mutants.

MATERIALS AND METHODS

Strains. All strains used in these experiments carry the *band* (*bd*) gene, which allows clear expression of the circadian conidia-

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[2] To whom reprint requests should be sent.

tion rhythm (19). This gene has no effect on the clock mechanism itself (6). The following clock mutants were previously isolated in this laboratory: *frq-1*, *frq-2*, *frq-3* (6), *frq-4* (7), *frq-6*, *frq-7*, *frq-8*, *chr* (5, 9), *prd-1*, formerly called *frq-5* (4), and *prd-2*, *prd-3*, and *prd-4*, formerly called UV IV-2, UV IV-4, and UV V-7, respectively (5). Table I lists the properties of these mutants.

Culture Conditions. Methods for maintaining stock cultures and for growing cultures to assay the circadian rhythm of conidiation on race tubes were as previously described (6), except that race tubes were kept at the temperature to be tested from the time of their inoculation. In a few experiments, race tubes were inoculated and kept in constant light at 25°C for 24 h, then transferred to another temperature and placed in constant darkness to assay the rhythm. There was no detectable effect of this temperature shift made at the time of transfer from light to dark on either the phase or the period length of the rhythm. All experiments were carried out in an Environmental Growth Chamber in a light-tight room maintained at 25°C. Temperature was monitored continuously inside the chamber with a recording thermometer. Period lengths were determined from at least six replicate race tubes for each strain at each temperature except at 34°C, where at least 12 replicate tubes were used, since banding was less clear at this temperature and more tubes were needed to obtain reliable data. Calculations of the period length of the rhythm were done as previously described (6), except that the positions of the daily growth fronts and conidial bands were obtained with a digitizer (Bit Pad, Summagraphics Corp., Fairfield, Conn.) interfaced with a Northstar Horizon Microcomputer.

RESULTS

Temperature Compensation of the Band (*bd*) Strain. Previous studies measuring the temperature compensation of the period length of the wild-type (*i.e.* *bd*) strain of *Neurospora* have found that there are two temperature ranges in which the Q_{10} values differ from each other. Sargent *et al.* (19) found that the Q_{10} of the rhythm was 0.95 in the range of 18 to 25°C and 1.2 from 25 to 34°C. Nakashima and Feldman (13) obtained similar results but found a Q_{10} close to 1 between 20 and 30°C and 1.3 above 30°C. In this study we have also identified two ranges (Fig. 1), and although the major break appears at 30°C in our study, there is also a smaller change in Q_{10} at about 22°C. It seems likely that the minor differences between studies depend on small differences in culture conditions, and the general character of the responses is quite similar among all three studies.

Temperature Compensation of the *frq* Mutants. There are seven mutants that map to the *frq* locus on linkage group VIIR (6, 7, 9). Four of these have period lengths shorter than wild-type, while three are longer (Table I). Figure 1 shows the period lengths of the short period mutants *frq-1* and *frq-2* from 16 to 34°C and indicates that there is no significant difference in their temperature compensation from the wild-type. *Frq-4* and *frq-6* were indistinguishable from *frq-2*.

Figure 1 also shows that the long period *frq* mutants have a significant alteration in their temperature compensation. In the case of *frq-3*, the pattern of a high and low range is retained, but the temperature at which the change occurs is lowered from 30 to 25°C. In the case of *frq-7*, the change is more dramatic, because there is no detectable region where the Q_{10} is 1—*i.e.* the Q_{10} is approximately 1.3 for the entire temperature range of 18 to 34°C. *Frq-8* was indistinguishable from *frq-7*. Thus, it appears that mutations at the *frq* locus which shorten the period do not change the temperature compensation of the clock, but mutations which lengthen the period also lower the temperature at which the clock transitions from well-compensated to poorly compensated, and the longer the period length of the mutant, the lower this transition temperature.

Temperature Compensation of other Clock Mutants. Five additional mutants, all at different loci, have also been previously isolated and characterized genetically (Table I). The data in Figure 2 indicate that these mutants show a variety of temperature compensation patterns. *Prd-1* and *prd-2* have essentially unaltered patterns, although they both have long circadian periodicities. *Chr* is typically well-compensated below 30°C but is well-compensated above 30°C, unlike wild type. *Prd-3* is also altered, the most obvious change being that the period length increases with increasing temperature ($Q_{10} < 1$) up to 30°C. Unfortunately, banding for this strain above 30°C was not clear enough to obtain reliable period length data. Finally, *prd-4* has an unusual temperature compensation pattern—the Q_{10} changes several times between 18 and 34°C.

Temperature compensation of double mutants. In previous studies (4, 9), we constructed double mutants—*i.e.* strains carrying two

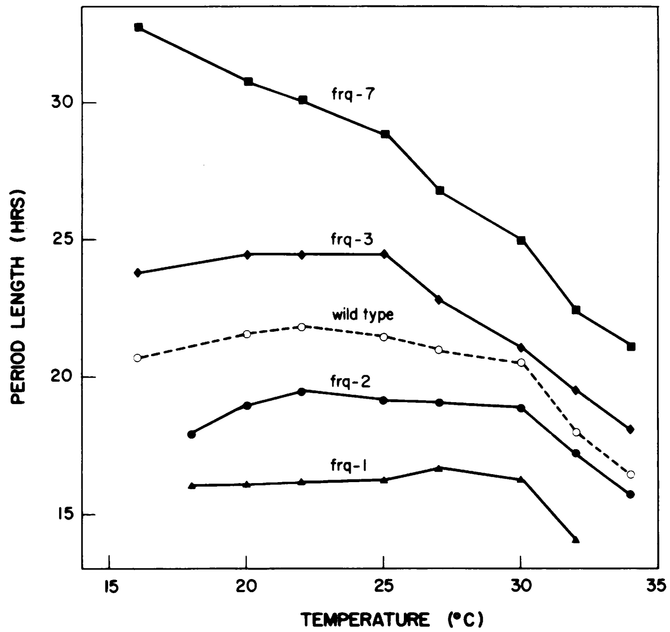

FIG. 1. Period lengths of wild-type and *frq* mutants at different temperatures. The average SD for each strain was as follows: Wild-type, 0.5 h; *frq-1*, 0.4 h; *frq-2*, 0.5 h; *frq-3*, 0.5 h; *frq-7*, 0.6 h.

Table I. *Properties of Circadian Clock Mutants*

Strain[a]	Period Length at 25°C	Linkage Group	Reference
	h		
Mutants at the *frq* locus			
frq-1	16.5	VII R	6
frq-2	19.3	VII R	6
frq-3	24.0	VII R	6
frq-4	19.3	VII R	7
frq-6	19.2	VII R	9
frq-7	29.0	VII R	9
frq-8	29.0	VII R	9
Mutants at other loci			
prd-1	25.8	III C	4
prd-2	25.5	V R	5
prd-3	25.1	I C	5
prd-4	18.0	I R	5
chr	23.5	VI L	9

[a] *Prd-1* was formerly called *frq-5*; *prd-2* was UV IV-2; *prd-3* was UV IV-4; and *prd-4* was UV V-7.

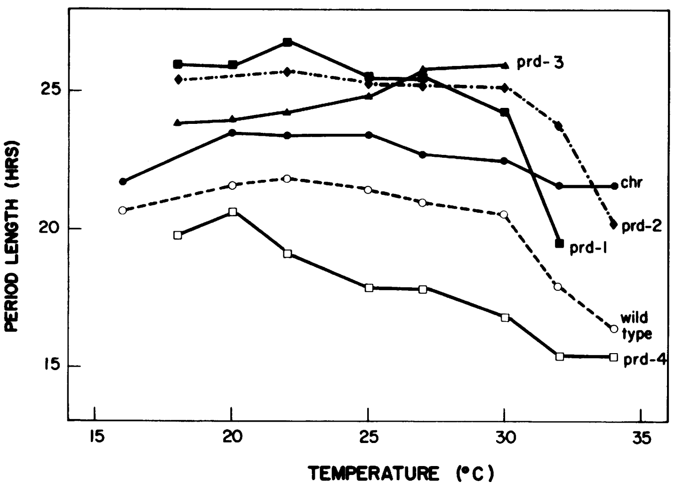

FIG. 2. Period lengths of wild-type and clock mutants not at the *frq* locus at different temperatures. The average SD for each strain was as follows: Wild-type, 0.5 h; *prd-1*, 1.1 h; *prd-2*, 0.4 h; *prd-3*, 0.5 h; *prd-4*, 0.3 h; *chr*, 0.5 h.

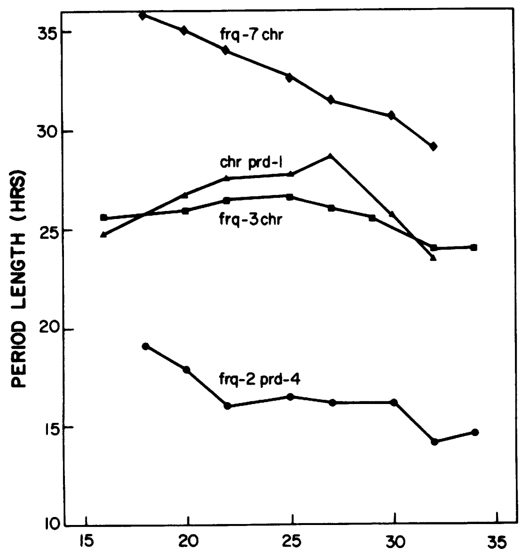

FIG. 3. Period lengths of double mutants at different temperatures. The average SD for each strain was as follows: *frq-7 chr*, 0.7 h; *chr prd-1*, 0.9 h; *frq-3 chr*, 0.6 h; *frq-2 prd-4*, 0.5 h.

mutations—in order to determine whether mutations at different loci interact with each other. In those studies we asked whether the effects of the individual mutations were additive or nonadditive in the multiple mutants. In nearly all cases studied at 25°C, we concluded that the effects were additive or nearly additive and that we had found no convincing evidence of gene interaction among the mutants at the different clock loci. With the finding of differences in temperature compensation among the various mutants, we can now ask a new type of question: In a double mutant, will the temperature response be like one of the mutants, intermediate between the two, or a pattern different from either single mutant?

Figure 3 shows the temperature compensation of four double mutants. *Chr* and *frq-3* each have changes in their temperature compensation (Figs. 1 and 2), and the *chr frq-3* double mutant shows aspects of both abnormalities. It has the *chr* characteristic of being reasonably well compensated above 30°C but also the *frq-3* characteristic of a higher Q_{10} value between 25 and 30°C. With *chr frq-7*, the most dramatic effect is that the *frq-7* characteristic of poor temperature compensation below 30°C is clearly evident in the double mutant. Thus, in these two double mutants, the altered temperature compensation of the two *frq* mutants is also evident in the double mutants, and the nature of the interaction (or lack of interaction) between the *frq* locus and the *chr* locus does not change significantly at different temperatures.

In contrast is the behavior of the *chr prd-1* double mutant, which shows an unusually low Q_{10} value of 0.86 below 27°C, a value not seen in either single mutant. This indicates that the interaction between *chr* and *prd-1* depends on the temperature at which it is studied.

Finally, a double mutant of *frq-2 prd-4* was studied. It shows much of the altered temperature compensation of the *prd-4* single mutant throughout the entire temperature range.

Temperature coefficients of growth rates. It has previously been shown for the wild-type strain that growth rate on race tubes is temperature-dependent and thus differs significantly from the temperature independence of circadian periodicity (19). To determine whether any of the mutations that alter temperature compensation of circadian periodicity affect the temperature coefficient of growth rate, growth rates were calculated from the race tubes used to determine period length.

Figure 4 shows a plot of growth rate *versus* temperature for wild-type, *frq-1*, *frq-3*, and *frq-7*. The wild-type curve is similar to that published previously (19) and all curves are similar to each other. The data for *frq-2*, *frq-4*, *frq-6*, and *frq-8* were also the same as those shown. Thus, the mutations that alter the period length in all the *frq* mutants and that also alter the temperature compensation of the clock in *frq-3*, *frq-7*, and *frq-8* do not alter the temperature coefficient of growth rate.

Figure 5 shows growth rates for the mutants at other loci. The growth curves of *chr* and *prd-4* are the same as wild-type, and while the *prd-3* and *prd-1* mutations reduce growth rates, the temperature coefficient of the growth rate is not changed. On the other hand, the growth rate of *prd-2*, whose clock has an unaltered temperature coefficient, has an unusual response to temperature. Although its response to temperature is normal below 25°C, above that temperature growth rate does not increase—*i.e.* above 25°C

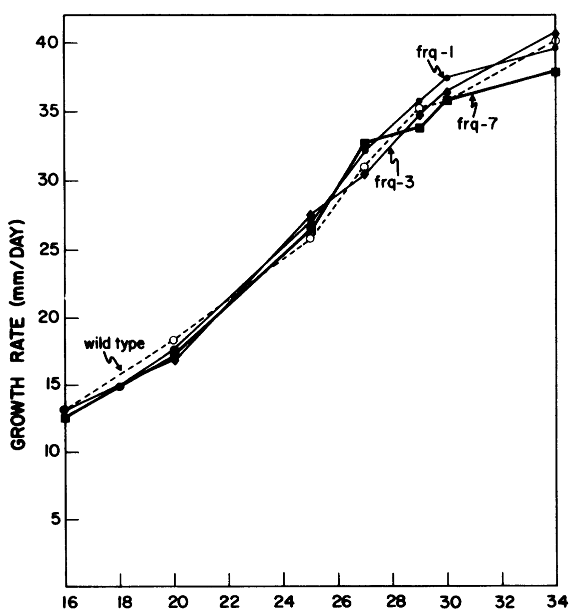

FIG. 4. Growth rates of wild-type and *frq* mutants at different temperatures. The average SD for each strain was as follows: Wild-type, 0.4 mm; *frq-1*, 0.9 mm; *frq-2*, 0.7 mm; *frq-3*, 0.7 mm; *frq-7*, 0.4 mm.

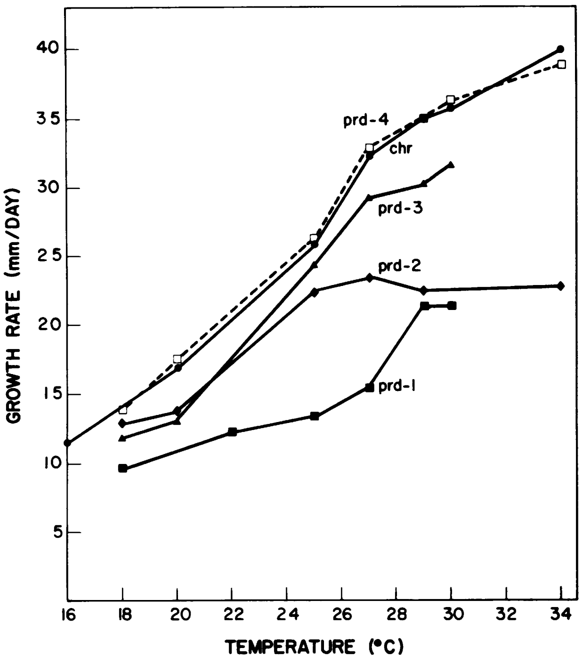

FIG. 5. Growth rates of mutants not at the *frq* locus at different temperatures. The average SD for each strain was as follows: *prd-1*, 1.2 mm; *prd-2*, 1.4 mm; *prd-3*, 1.2 mm; *prd-4*, 0.5 mm; *chr*, 0.6 mm.

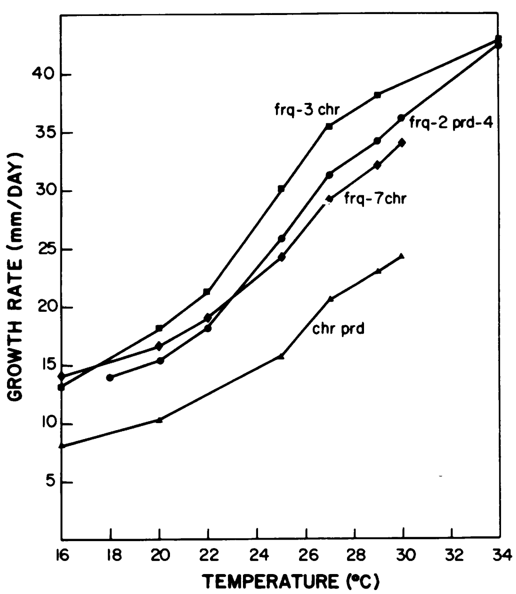

FIG. 6. Growth rates of double mutants at different temperatures. The average SD for each strain was as follows: *frq-3 chr*, 0.8 mm; *frq-2 prd-4*, 0.9 mm; *frq-7 chr*, 0.6 mm; *chr prd-1*, 1.1 mm.

growth rate of *prd-2* is temperature independent.

Finally, growth rate curves for several double mutants are shown in Figure 6. The temperature dependence of the growth rates of each of the single mutants used in these strains is normal (Figs. 4 and 5), and no unusual growth interactions emerged, since the temperature coefficients of growth rate of double mutants are also normal.

DISCUSSION

The most striking result from these studies is the alteration in the temperature compensation of the circadian clock in the long period *frq* mutants. In the wild-type strain below 30°C, the clock is well-compensated and has a Q_{10} of about 1, while above 30°C the clock is less well-compensated and has a Q_{10} of about 1.3. However, in *frq-3* the temperature at which the Q_{10} changes from 1.0 to 1.3 (the "breakpoint" temperature) is lowered from 30 to 25°C. In *frq-7*, the Q_{10} is about 1.3 for the entire temperature range of 18 to 34°C. Thus, *frq-7*, which produces a more extreme alteration of phenotype in one parameter (period length) also produces a more extreme alteration of phenotype in another parameter (temperature compensation). In this regard, one might have expected that the short period *frq* mutants should have a breakpoint higher than 30°C. The lack of symmetry in these results suggests that some factor other than the product of the *frq* locus is involved in the loss of compensation above 30°C. This idea is supported by the behavior of the *chr* mutation (see below).

Our genetic analysis has also led us to suggest that alterations at the *frq* locus might change the quantity of some gene product essential to the clock and thereby change the rate of some reaction (7, 9). This idea is also consistent with the alteration in temperature compensation in *frq-3*, *frq-7*, and *frq-8*. If the temperature compensation system functions efficiently only when the rates of certain biochemical reactions are within certain limits, mutations which alter the rate of one of these reactions might alter the temperature range within which the system is compensated. Since *frq-7* is a more extreme change in period length than *frq-3*, it might cause a greater change in the rate of some reaction than *frq-3* and, as a result, cause a greater change in the breakpoint temperature.

Mutants at other loci show a variety of temperature compensation patterns. For example, in *prd-1* and *prd-2*, the clock has a normal response to temperature, even though both of these mutants have lengthened circadian periodicities. This demonstrates that lengthening the period is not sufficient in itself to alter the temperature compensation mechanism. *Chr* causes the clock to be well compensated even above 30°C and in this respect is complementary to the *frq* mutations. This result is consistent with two other observations indicating that *chr* affects a different component of the clock than that affected by *frq*. First, the *chr* mutation is in a different gene, unlinked to *frq* (9), and second, there seems to be little or no interaction between *chr* and *frq* in the *chr frq-3* and *chr frq-7* double mutants (Fig. 3). Finally, *prd-3* and *prd-4* each have alterations in their temperature compensation which differ from any of the other mutants.

In contrast to the lack of gene interaction in the *chr frq* double mutants is the behavior of *chr prd-1*. The clock in this strain has an unusually low Q_{10} of 0.86 below 27°C. This suggests that the *chr* and *prd* gene products may interact at some level in clock function.

These studies also extend the observations made previously that growth rate and clock periodicity are not coupled. Our results show that (a) some mutations that affect clock periodicity have no effect on growth rate (*frq*, *chr*, *prd-4*), (b) mutations that alter the temperature coefficient of the clock do not alter the temperature coefficient of growth rate (*frq-3*, *frq-7*, *frq-8*, *chr*, *prd-3*, *prd-4*), and (c) one mutation that alters the temperature coefficient of growth rate does not alter the temperature coefficient of the clock (*prd-2*).

These mutants offer a unique opportunity to test a number of ideas about the temperature compensation mechanism of the clock. For example, Pittendrigh and Caldarola (18) suggested that temperature compensation is just one example of a general homeostasis of the clock that is buffered against a variety of factors in the environment. Consistent with this suggestion is our recent finding that in the wild-type strain above 30°C, the temperature at which the clock loses its temperature compensation, it also becomes sensitive to changes in nutritional conditions (13). The mutants with altered temperature compensation offer a much wider range of conditions in which to test this idea further.

In addition, there are a number of biochemical models for temperature compensation (10, 14, 21, 22), including one that suggests that temperature dependent changes in the fatty acid composition of membranes could account for the temperature compensation of circadian clocks (14). Again, these mutants should provide useful tools to test such models.

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