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# Effect of Growth Temperature on Several Exported Enzyme Activities in the Psychrotrophic Bacterium *Pseudomonas fluorescens*

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**In accordance with previous results, the activity of extracellular proteases from *Pseudomonas fluorescens* MF0 is maximal at a growth temperature of 17.5°C, well below the optimal growth temperature. In addition, the activities of three periplasmic phosphatases display the same growth temperature optimum. Chemostat experiments have shown that it is the growth temperature itself and not the value of the growth rate that regulates these activities. In contrast, a foreign periplasmic phosphatase, expressed under the control of its own promoter, displays a different sensitivity toward temperature. We conclude that in the psychrotrophic strain *P. fluorescens* MF0, growth temperature exerts a specific control upon the activity of certain enzymes. The critical temperature (17.5°C) is within the range of normal growth, suggesting that this control is probably different from a cold shock or heat shock response.**

*Pseudomonas fluorescens* is well known as a major psychrotrophic contaminant of raw milk stored in refrigerated tanks (16). Many studies of this bacterium have been concerned with the production of deleterious extracellular enzymes, such as thermostable proteases (8, 15, 27). Among the numerous observations concerning these enzymes, it has been repeatedly shown that most strains maximally produce proteases at a temperature (15 to 20°C) well below the optimal growth temperature (25 to 30°C) (13, 19, 25). However, no studies have yet dealt with the mechanism of regulation of protease production with regard to temperature.

At this stage two main questions can be raised regarding the elucidation of this mechanism. The first one relates to the specificity of this temperature effect, i.e., whether it is restricted to the production of proteases or extended to the production of other enzymes. To this end, the activities of extracellular proteases as a function of growth temperature were compared with those of several periplasmic phosphatases. The exported enzymes all showed the same regulation by temperature even though they are clearly differentially regulated by other growth conditions. Thus, it was important to determine whether this temperature effect might involve protein export through the cytoplasmic membrane. If so, any foreign exported protein should be submitted to the same effect. The expression of a gene from the mesophilic species *Escherichia coli*, under the control of its own promoter, was studied in *P. fluorescens* at different growth temperatures. In this case, a temperature effect similar to that observed with the native enzymes was not demonstrated.

The second question is whether the temperature itself is the direct cause of the regulation or an indirect effector acting through the growth rate variation; such an indirect effect has indeed been demonstrated for several activities or proteins in mesophilic bacteria (5). To answer this question, the activities of the two acidic phosphatases were assayed in cells grown in a chemostat at two different temperatures and several dilution rates. The results indicate that the observed variations of enzyme activities are not directly related to growth rate but are indeed due to a specific effect of the growth temperature.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *P. fluorescens* A32 (19) was originally isolated from raw milk and has been kindly provided by R. C. McKellar. It was renamed MF0. A strain resistant to rifampin was selected from the wild type and named MF3; it exhibited the same behavior with regard to growth and enzymatic activities. *P. fluorescens* cells were maintained on nutrient agar (Difco) plates at 20°C or deep frozen with 30% glycerol in nutrient broth (Difco).

*E. coli* strains were cultivated in Luria broth supplemented when necessary with the following antibiotics: kanamycin, 30 μg/liter (Sigma); tetracycline, 25 μg/liter (Boehringer); and rifampin, 50 μg/liter (Serva). Strains and plasmids are listed in Table 1.

**Batch cultivation.** Bacterial strains were grown in Erlenmeyer flasks under slow rotatory agitation in a thermostated water bath at the desired temperatures. Different media were used on the basis of the enzymes assayed.

To determine acidic phosphatase activities, the following citrate mineral salts medium was used (in grams per liter): trisodium citrate, 3; $K_2HPO_4$, 10.5; $KH_2PO_4$, 5.4; $SO_4(NH_4)_2$): 1.2; $MgSO_4$, 0.4; $CaCl_2$, 0.15; pH 6.8. Alkaline phosphatase activity was measurable in cells grown in the following phosphate-limited semisynthetic medium (in grams per liter): proteose peptone (Difco), 5; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 23.8; $NH_4Cl$, 1; $MgCl_2$, 0.3; KCl, 1.5; pH 6.8. Protease activity was detected in citrate mineral salts medium supplemented with 10% liquid skim milk as an inducer.

Growth was monitored by turbidity measurements at 580 nm in a Spectronic spectrophotometer (1 optical density unit at 580 nm [$OD_{580}$] = 0.65 mg [dry weight] per ml).

**Continuous cultivation.** Continuous cultures were carried out in a trypsinization flask, with a working volume of 200 ml. They were started as batch cultures for 10 to 12 h, and

TABLE 1. Strains and plasmids

| Strain or plasmid | Genotype or description | Reference or source |
|---|---|---|
| Strains | | |
| HB101 | *hsdS20 recA13 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* | 17 |
| S17-1 | *pro thi hsdM*$^+$ *recA*; chromosomally integrated RP4-2 (Tn*1*::TSR1 Tc::Mu Km::Tn*7*) | 26 |
| MF3 | Rif$^r$ derivative of strain MF0 (A32) | This work |
| MF301 | MF3(pMF1) | This work |
| MF302 | MF3::pGM50 | This work |
| Plasmids | | |
| pTB107 | IncQ Km$^r$ Sm$^r$ | 2 |
| pUC4K | Km$^r$ Ap$^r$ | 29 |
| pGM567 | 4.6-kb *Bam*HI-*Bgl*II insert containing *appA* gene in pUC4K | Pawlak-Letouvet |
| pMF1 | 4.6-kb *Eco*RI-*Eco*RI insert containing *appA* gene in pTB107 | This work |
| pGM50 | R68.45::pPB1176 Km$^r$ Ap$^r$ Tc$^r$ *appA* | 6 |

then sterile medium flow was initiated at the desired rate (with a peristaltic pump [Ismatec]). The culture volume was kept constant by an internally placed overflow tube and a peristaltic pump (Masterflex). Aeration and agitation were performed by injecting air and by using a magnetic stirrer. Temperature was maintained at either 25 or 30°C. Fluctuations did not exceed 0.2°C.

All continuous cultures were run in the citrate mineral salts medium under carbon limitation. Steady-state conditions were achieved after a minimum of five mean residence times had passed.

**Detection of enzyme activities on plates.** The detection of hyperacidic pH 2.5 phosphatase activity was performed as previously described (7) with the synthetic substrate pNPP (Merck). Protease activity was detected on 10% skim milk nutrient agar plates as a clear halo surrounding the clones.

**Enzymatic assays. (i) Determination of phosphatase activity.** Cells were harvested by centrifugation either during exponential phase ($OD_{580}$ 0.45 to 0.55) or during stationary phase for batch cultures or at steady state for continuous cultures. Cell pellets were resuspended in 0.9% NaCl ($OD_{580}$ 1.0 to 1.8). Incubation mixtures contained 50 μl of cell suspension, 50 μl of 0.12 M pNPP, and 500 μl of 50 mM Tris-maleate buffer (pH 5.3 or 6.3). The resuspended intact cells were incubated for 30 min at 30°C. After centrifugation, 1 volume of 0.5 M NaOH was added to 1 volume of the supernatant. $A_{420}$ was measured. Results are expressed in milliunits per $OD_{580}$ unit of the suspension. One unit is defined as the hydrolysis of 1 μmol of pNPP per min at 30°C. For the determination of alkaline phosphatase activity, Tris-maleate buffer was replaced by 500 μl of 50 mM Tris-HCl (pH 8.8); for hyperacidic phosphatase activity, 500 μl of 50 mM potassium hydrogenophthalate buffer, pH 2.5, was used.

**(ii) Determination of protease activity.** Extracellular protease activity was assayed in batch culture supernatants. After centrifugation, culture supernatants were filtered on a 0.45-μm-pore-size membrane to obtain a cell-free fraction. The determination of protease activity was performed at 30°C by the azocasein (Sigma) procedure as previously described (20). One unit was defined as the amount of enzyme that hydrolyzed 1 mg of azocasein in 30 min at 30°C.

**Recombinant plasmid preparations.** Plasmids were prepared from *E. coli* and *P. fluorescens* strains by the alkaline lysis method (3) modified as previously described (11). For subclonings, electroelution from agarose gels was performed as previously described (1). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Boehringer).

**Subcloning of the *appA* gene on a multicopy plasmid.** The periplasmic hyperacidic pH 2.5 phosphatase of *E. coli* is coded by the gene *appA*, situated at 22.5 min on the chromosome (7). This gene has been cloned in vivo and subcloned in the plasmid pBR322 (4). A 4.6-kb *Bam*HI-*Bgl*II fragment containing the *appA* gene with its own promoter was subcloned in plasmid pUC4K (pGM567) and kindly provided by Pawlak-Letouvet. It had been previously shown by Leriche (16a) that pTB107 could be transferred to MF3 at a high frequency and could be stably maintained at all growth temperatures studied. This small multicopy IncQ plasmid was chosen as a cloning vector. A 4.6-kb *Eco*RI-*Eco*RI fragment from pGM567, bearing *appA* and its own promoter, was ligated to pTB107 opened at the unique *Eco*RI site.

After transformation into *E. coli* HB101, one of the Km$^r$ clones showing a pNPP$^+$ phenotype on an agar plate was selected and the plasmid (pMF1) was verified by restriction analysis.

Plasmid pMF1 was mobilized into *P. fluorescens* MF3 by conjugation with a filter mating method. *E. coli* S17-1 (26), which contains the *tra* genes of the broad-host-range plasmid RP4 integrated in its chromosome, was used as the donor strain. Transconjugants were selected on nutrient agar medium containing kanamycin (50 μg/ml) and rifampin (100 μg/ml).

**Integration of gene *appA* in the chromosome of strain MF3.** Leriche (16a) has shown that IncP plasmids are unstable in *P. fluorescens* MF0 and MF3 when the cells are grown at temperatures below 30°C. Plasmid pGM50 (6) is a cointegrate between the IncP plasmid R68-45 and a recombinant pBR322 in which gene *appA* is inserted under the control of the *tet* promoter (4). This plasmid was conjugated into MF3, and stable transconjugants were selected after repeated cultures at 20°C. One of them (strain MF302), which had kept all the phenotypic traits of pGM50 but had no detectable free plasmid, was kept for further study.

## RESULTS

**Effects of growth temperature on the activity of extracellular protease in MF0.** As shown in Fig. 1, protease activity rapidly increased during the early stationary phase in the culture supernatant of MF0 cells grown in mineral salts medium supplemented with 10% skim milk and reached a maximal value at constant biomass. The accumulation of this activity resulted in the degradation of milk and was concomitant with the production of the siderophore pyoverdin.

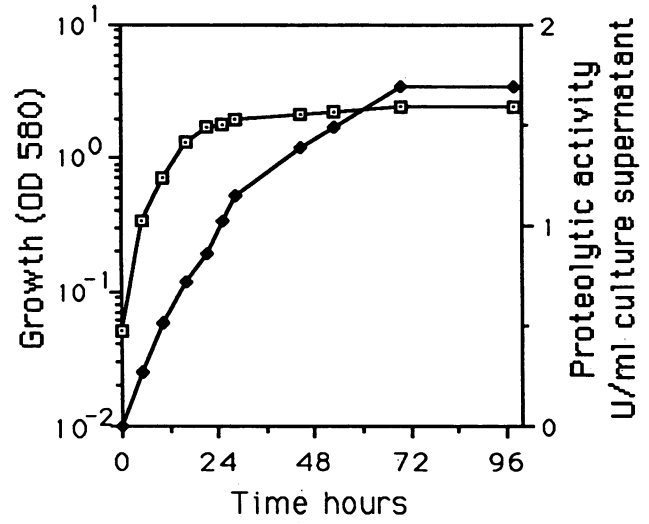


FIG. 1. Relationship between growth and extracellular protease activity in MF0 cells. Cells were batch grown at 17.5°C under slow rotary agitation in citrate mineral salts medium supplemented with 10% skim milk. Growth was estimated by $OD_{580}$ measurements (⊡). Determination of protease activity (◆) was performed by the azocasein method on cell-free fractions obtained after centrifugation and filtration of culture samples.

Similar profiles were obtained at all the temperatures assayed. Thus, the protease activities produced with respect to growth temperature were compared at 30°C by using the maximal activities observed in late stationary phase.

The growth temperature at which protease activity was maximal is 17.5°C, as shown in Fig. 2. However, there was only a twofold difference in activity levels between 17.5 and 8°C or between 17.5 and 30°C.

**Effects of growth temperature on the activity of periplasmic enzyme in MF3 cells.** Whether they are soluble or attached to the outer surface of the inner membrane, most periplasmic phosphatase activities are readily detectable by using pNPP, which freely diffuses into the periplasmic space of intact cells. Therefore, a screening for phosphatase activities was performed on intact cells over a broad pH range: *P. fluorescens* MF3 cells were grown in three different media, i.e., the citrate medium and the proteose peptone medium either complemented or not complemented by 100 mM phosphate.

Cells were sampled during exponential growth or during stationary phase from batch cultures. Results are shown in Fig. 3. Three major pH range activities could be defined: pI,

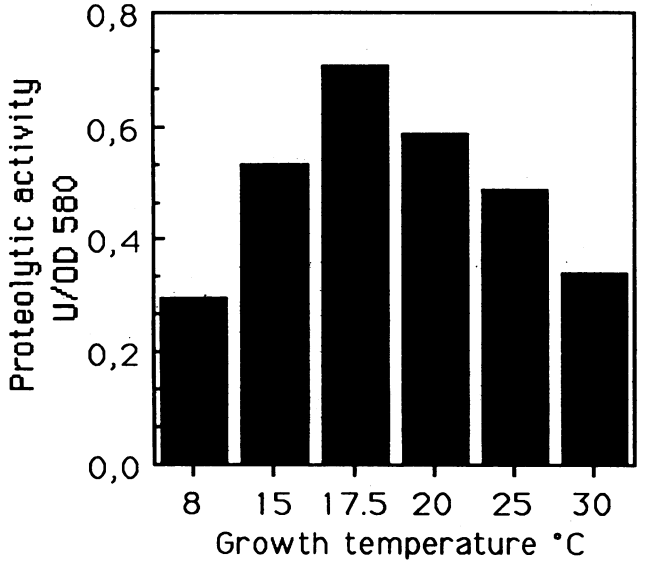


FIG. 2. Effects of growth temperature on protease activity. *P. fluorescens* MF0 was grown at different temperatures as described in the legend to Fig. 1. Maximal protease activities were compared. Results from cell-free fractions of culture samples are expressed in units per $OD_{580}$ unit.

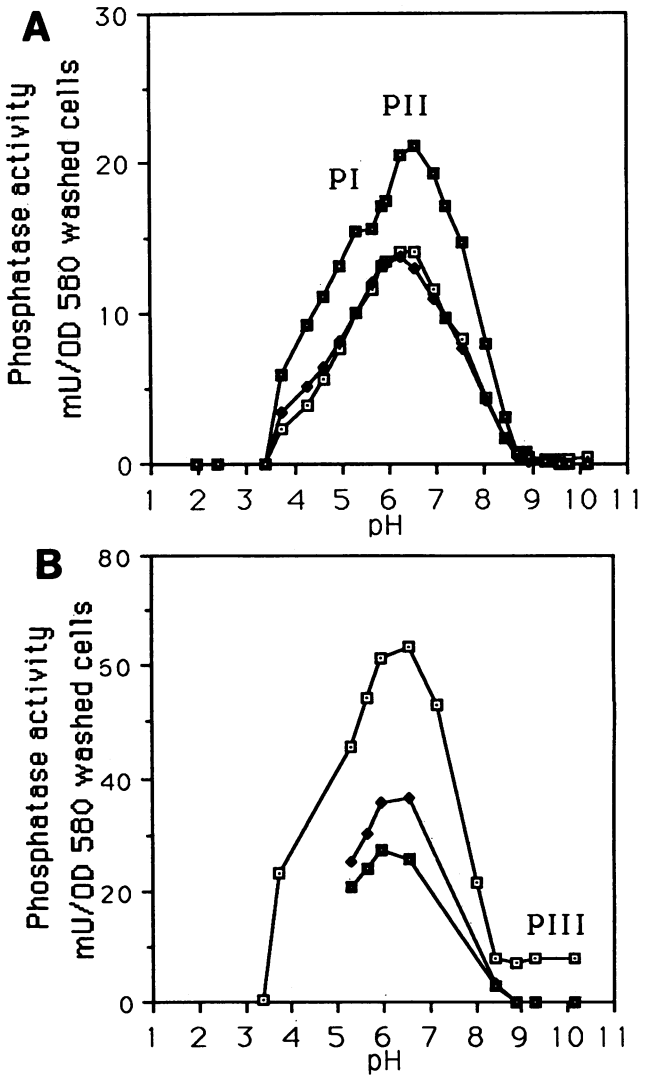


FIG. 3. Dependence of phosphatase activity upon pH in *P. fluorescens*. Phosphatase activity was measured by pNPP hydrolysis at different pHs with three buffer systems: 50 mM potassium hydrogenophthalate, pH 2.00 to 3.85; 50 mM Tris-maleate, pH 3.85 to 8.05; and 50 mM Tris-HCl, pH 8.00 to 10.40. MF3 cells were grown at 17.5°C in batch culture under slow rotatory agitation in three different media: citrate mineral salts (■), semisynthetic phosphate-limited proteose peptone (⊡), and semisynthetic 100 mM phosphate proteose peptone (◆). Phosphatase activity was determined on intact washed cells sampled during exponential phase (A) and during stationary phase (B). Results from washed cell suspensions are expressed in milliunits per $OD_{580}$ unit.

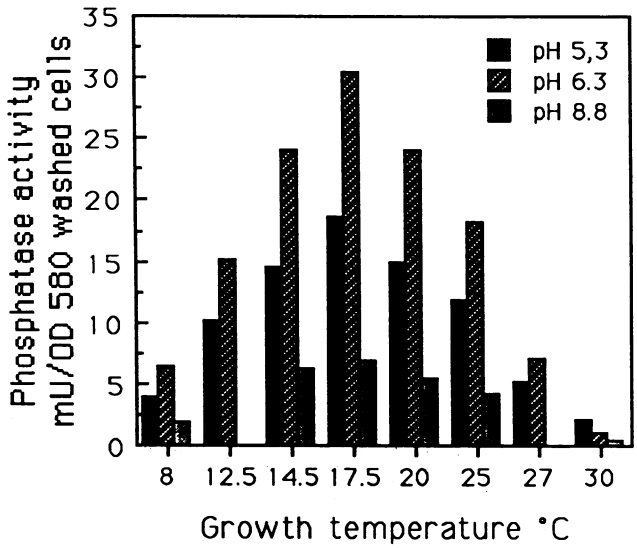


FIG. 4. Effects of growth temperature on periplasmic phosphatase activity. *P. fluorescens* MF3 was grown at different temperatures in batch culture, in a citrate mineral salts medium for pH 5.3 and 6.3 activities and in a semisynthetic phosphate-limited proteose peptone medium for pH 8.8 activity. Results from washed cell suspensions are expressed in milliunits per $OD_{580}$ unit (pH 8.8 phosphatase activities were not determined at 12.5 and 27°C).

pII, and pIII. A weak alkaline phosphatase activity, pIII (pH 9), was detectable only during the stationary phase and only in medium depleted of phosphate. Thus, this activity was repressed by phosphate and during exponential growth.

Two acidic phosphatase activities, pI (pH 5.3) and pII (pH 6.3), had common characteristics: these activities are the same in citrate medium in exponential and stationary phase, whereas they are increased in stationary phase in the other media. They were maximal in phosphate-limited proteose peptone medium during stationary phase. However, during exponential growth, in all three media the activities were well detectable, and the presence of phosphate in the medium (proteose peptone plus phosphate versus proteose peptone) did not decrease their production. Hence, phosphate deprivation seems to increase these activities only during the stationary phase. Although the purpose of this experiment was not to study the physiological conditions for an optimal production of acidic phosphatase activities, it shows that the regulation of this production is complex. Both acidic phosphatase activities (pI and pII) are sensitive to the growth phase, to the presence of phosphate in the medium, and to the composition of the medium itself. We do not yet know how many enzymes are responsible for such a pH-related activity pattern.

Since the complex regulation of acidic phosphatase activity was different from the regulation of alkaline phosphatase activity, these three activities were chosen to study the effects of growth temperature. As described in Materials and Methods, phosphatase activity was further assayed at pH 5.3 and 6.3 in exponentially growing cells in citrate medium and at pH 8.8 in stationary cells grown in proteose peptone medium. The effects of growth temperature on phosphatase activities are shown in Fig. 4.

Again, the optimal growth temperature was 17.5°C for both acidic and alkaline phosphatase activities. A regular increase in activity was observed when growth temperature increased from 8 to 17.5°C. Above 17.5°C, activity decreased to the minimum value observed at 30°C, which is, by contrast, the optimal growth temperature.

TABLE 2. Acid phosphatase activities produced in carbon-limited continuous cultures and in batch cultures by *P. fluorescens* cells from strain MF3 at 25 and 30°C

| Growth temp (°C) and culture[a] | $\mu$ ($h^{-1}$) | Phosphatase activity[b] (mU/$OD_{580}$ U) | |
|---|---|---|---|
| | | pH 5.3 | pH 6.3 |
| 30 | | | |
| Batch | 0.56[c] | 2.00 | 1.00 |
| Continuous | 0.35 | 1.90 | 1.43 |
| | 0.18 | 2.00 | 2.30 |
| 25 | | | |
| Batch | 0.52[c] | 12.0 | 18.0 |
| Continuous | 0.38 | 12.9 | 17.5 |
| | 0.26 | 12.8 | 20.0 |
| | 0.18 | 14.0 | 22.6 |

[a] Batch and continuous cultures were carried out in citrate minimum mineral salts medium.

[b] Determination of phosphatase activities at pH 5.3 and 6.3 was performed as described in Materials and Methods. Phosphatase activity in continuous cultures was measured at steady state for different dilution rates (growth rates). Activity assays were performed twice for each established steady state, at an interval of two residence times. Since measured activities did not change with time, values indicated in this table are the average of two determinations.

[c] Data from batch cultures are values for $\mu_{max}$.

It is interesting to note that pH 6.3 phosphatase activity was always higher than pH 5.3 activity for all the temperatures studied, except 30°C. This is the only distinction we could make between the temperature effects on the two acidic pH phosphatase activities. Although the alkaline phosphatase activity exhibited much lower values than acidic activities, the production of this enzyme appeared to be influenced in the same way by the growth temperature.

To ensure that pNPP diffuses freely into the periplasmic space at all temperatures, experiments were performed on toluenized and nontoluenized cells and showed no difference in the activity levels.

**Effects of growth rate on acidic phosphatase activities.** Carbon source-limited continuous cultures of *P. fluorescens* MF3 at 25 and 30°C were examined for acidic phosphatase activities at different dilution rates (growth rates). In order to compare these activities with those produced in batch cultures, dilution rates as close as possible to maximal growth rates determined for batch cultures at 12.5, 17.5, and 20°C (i.e., 0.18, 0.26, and 0.38 $h^{-1}$, respectively) were chosen.

At 25 and 30°C, growth rate variations had no significant effect on pH 5.3 phosphatase activity, and the activities in continuous cultures were almost identical to those measured in batch cultures at the same growth temperatures, (Table 2).

In contrast, a small but reproducible increase of pH 6.3 phosphatase activity was observed at lower growth rates in continuous cultures at 25 and 30°C. At these temperatures, activities in continuous cultures with different growth rates were higher than activities in batch cultures (i.e., when $\mu = \mu_{max}$) at the same temperatures. In addition, at the lowest growth rate (0.18 $h^{-1}$) assayed in continuous culture at 30°C, pH 6.3 activity was higher than pH 5.3 activity, although the former was always lower in batch cultures at this temperature (Table 2).

In order to emphasize the distinction between the effect of growth temperature and the effect of growth rate on pH 6.3

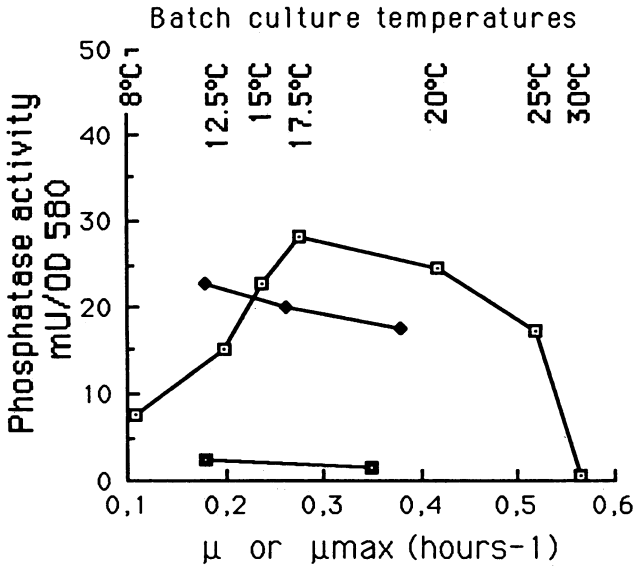


FIG. 5. pH 6.3 phosphatase activity as a function of maximal growth rate in batch cultures at different growth temperatures and as a function of growth rate in continuous cultures at 25 and 30°C. All conditions are described in footnote *a* to Table 2. ⊡, batch cultures; ◆, 25°C; ◘, 30°C.

phosphatase activity, we plotted results from continuous and batch cultures on the same graph (Fig. 5).

**Effects of temperature on a foreign phosphatase activity.** Plasmid pMF1 was transferred by conjugation to *P. fluorescens* MF3 as described in Materials and Methods (strain MF3 did not originally show any detectable hyperacidic phosphatase activity). AppA$^+$ Km$^r$ Rif$^r$ transconjugants were obtained, and one of them was chosen and controlled for the presence of a recombinant plasmid by plasmid extraction and by transformation into *E. coli* HB101. The recombinant strain MF301 was cultivated in the absence of selective pressure at 30°C. Clones obtained after 30 generations were all Km$^r$ AppA$^+$, demonstrating that this recombinant plasmid is stably maintained as the parental plasmid.

pH 2.5 acidic phosphatase activity expressed from *appA* was measured in MF301 cells at three different growth temperatures in citrate mineral salts medium. The activity was maximally expressed when the cultures entered the stationary phase, as is also the case in the original host (7). No activity was found in the extracellular medium. In contrast to the results obtained with the native periplasmic phosphatases, no difference was observed between the activities obtained at 17.5 and 30°C. However, a fourfold drop in activity occurred at 8°C (Table 3). This result could reflect a decrease in the copy number of the plasmid at this temperature or a real effect of temperature on the production of the enzyme.

Therefore, the same experiment was performed with strain MF302, in which *appA*, borne on pGM50 and expressed under the control of the constitutive promoter of gene *tet* from pBR322, was integrated into the chromosome of MF3. The pH 2.5 acid phosphatase activity was lower than that obtained with strain MF301, because of the single copy of the gene, but was expressed during exponential growth. Table 3 shows that in this case the activity increased slightly from 8 to 30°C (whereas the native phosphatase activities of this strain were maximal at 17.5°C).

TABLE 3. Effects of growth temperature on *E. coli* pH 2.5 acidic phosphatase activity produced by recombinant *P. fluorescens* cells

| Strain[a] | pH 2.5 acidic phosphatase activity[b] (mU/$OD_{580}$ U) at: | | |
|---|---|---|---|
| | 8°C | 17.5°C | 30°C |
| MF301, stationary phase | 40 | 180 | 180 |
| MF302 | | | |
| Exponential phase | 5.5 ± 2 | 6.5 ± 1.8 | 7.5 ± 1.7 |
| Stationary phase | 7 ± 2 | 7.5 ± 3 | 9.5 ± 1.8 |

[a] *P. fluorescens* MF301 and MF302 were grown at three different temperatures in batch culture in citrate mineral salts medium.

[b] pH 2.5 acidic phosphatase activity in washed cell suspensions was assayed as described in Materials and Methods.

## DISCUSSION

Like most *P. fluorescens* strains, strain MF0 (and MF3) produces an extracellular protease(s). Maximal levels of protease activity assayed in cell-free culture supernatants were reached in stationary phase, and 17.5°C was shown to be an optimal growth temperature for protease activity. This result agrees with previous results showing the optimal production temperature to be 20°C (13, 18), 21°C (19), or 18°C (8) in *P. fluorescens* strains, whereas the optimal growth temperature averaged 30°C.

Different phosphatase activities showed the same dependency upon growth temperature. In addition, not only do these enzymatic activities obviously correspond to at least three different enzymes, but they also are regulated in quite different manners. Both acidic (pH 5.3 and 6.3) phosphatase activities are constitutive, whereas protease activity is inducible and alkaline phosphatase is repressed by phosphate; furthermore, proteolytic activity is extracellular, whereas phosphatase activities are cell associated. Thus, their common maximal temperature of production might be significant with regard to a specific mechanism of regulation.

It could be argued that the observed effect of growth temperature on enzyme activity is attributable to an effect of growth rate, since growth rate varies with temperature. Two lines of evidence are against this point. First, although growth rates in batch culture at 25 and at 30°C were similar (0.52 and 0.56 $h^{-1}$), the phosphatase activities were much higher (pH 5.3, 6-fold; pH 6.3, 18-fold) at 25°C. Cytoplasmic endoproteases have been shown to be produced at 32°C in *Arthrobacter* strain $S_1$-55 (24), and *P. fluorescens* might produce similar cytoplasmic proteases above 27°C (23a). These proteases could account for the decrease of phosphatase activities at 30°C. Second, continuous culture experiments showed that the dilution rate has a weak effect on pH 6.3 activity production and no significant effect on pH 5.3 activity; even at 25°C, it was not possible to determine an optimal dilution rate. Furthermore, when growth rate was reduced to below 0.28 $h^{-1}$, either by lowering batch culture temperature or by decreasing the dilution rate, opposite effects were observed. In the first case the pH 6.3 phosphatase activity sharply decreased, whereas in the second case it slightly increased. These two arguments corroborate the hypothesis that growth temperature and growth rate act independently on phosphatase activities.

The activities of the enzymes assayed in this study are a reflection of synthesis, export, and activation/inactivation. If there is an optimal temperature for the export of enzymes in *P. fluorescens*, the production of heterologous exported

protein should display the same optimal temperature. In MF301 the multicopy plasmid contains the *appA* gene coding for the pH 2.5 hyperacidic *E. coli* phosphatase activity under the control of its own promoter. The activity of the enzyme depended on the growth temperature, but 17.5°C was no longer an optimal temperature since there was no difference between 17.5 and 30°C. However, although plasmid pTB107 is stably maintained at 8°C, the decrease of activity displayed at 8°C might reflect a decrease in the copy number of the recombinant plasmid at this temperature. When the gene *appA* was integrated in the host's chromosome, the activity of the heterologous phosphatase regularly and slightly increased from 8 to 30°C.

Thus, a foreign gene of mesophilic origin has been expressed and the protein has been exported in a psychrotrophic strain at a temperature below the minimal growth temperature of the original host. Similar results have been obtained by Leriche (16a) with promiscuous plasmids and by Kolenc et al. (14), who successfully transferred a TOL plasmid from the mesophilic *Pseudomonas putida* PaW1 to the psychrotrophic *Pseudomonas* strain QS1. In addition our results eliminate the hypothesis that the temperature effect acts at the level of the export machinery.

Thus, several differently regulated exported enzymatic activities display an optimum at 17.5°C which is neither related to the growth rate modifications nor due to the functioning of the export machinery. Further studies are now in progress to determine at what level that temperature effect does take place.

Some environmental stimuli have been shown to lead to a bacterial response that allows bacterial growth under modified conditions via a complex regulation pattern involving a specific sensor, transducing signals, regulator(s), and newly synthesized or activated proteins responsible for initiating such an adaptative response (21, 28). Among these stimuli, temperature (22) is known to be a determinant of growth rate and provides a good example for such a regulation, as illustrated by the heat shock response (22) and by the more recently described cold shock response (9, 12). It has been shown with mesophilic bacteria that the synthesis of most proteins does not vary within a normal temperature range, i.e., within a range in which the log of growth rate varies proportionally to the inverse of the temperature. But above the highest limit or below the lowest limit (10) of this range, newly synthesized proteins appear; hence, their designation as heat shock proteins or cold shock proteins, respectively (9, 23).

However, the effects of temperature on the production of the enzymes studied in this work may be different from that mentioned above. Indeed, *P. fluorescens* is psychrotrophic, and the effect of temperature is observed within an intermediate temperature range instead of at extreme temperatures. Our results suggest the existence of a mechanism of temperature regulation which might be specific to the psychrotrophy.

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