Stringer, C. D., Norton, I. L., & Hartman, F. C. (1981) *Arch. Biochem. Biophys. 208*, 495.
Takabe, T., & Akazawa, T. (1975a) *Plant Cell Physiol. 16*, 1049.
Takabe, T., & Akazawa, T. (1975b) *Arch. Biochem. Biophys. 169*, 686.
Zurawski, G., Perrot, B., Bottomley, W., & Whitfeld, P. R. (1981) *Nucleic Acids Res. 9*, 3251.

Asparaginase II of *Saccharomyces cerevisiae*: Comparison of Enzyme Stability in Vivo and in Vitro†

Kyu Won Kim and Robert J. Roon*

ABSTRACT: Asparaginase II of *Saccharomyces cerevisiae* is a cell wall mannan containing glycoprotein. Recent studies have demonstrated that asparaginase II activity increases in exponentially growing cell cultures and then decreases as the cells enter the stationary phase. Enzyme inactivation has been attributed to a Zn^{2+}-dependent protease which is synthesized de novo during the late exponential phase [Pauling, K. D., & Jones, G. E. (1980) *J. Gen. Microbiol. 117*, 423–430; Pauling, K. D., & Jones, G. E. (1980) *Biochim. Biophys. Acta 616*, 271–282]. We have investigated the mechanism of asparaginase II inactivation using both whole cell suspensions and highly purified enzyme. Our data indicate that the rate of asparaginase II inactivation in cell suspensions is primarily influenced by pH changes that occur as a consequence of cell growth and glucose fermentation and that enzyme inactivation is not dependent on Zn^{2+} or on de novo protein synthesis. Also, in vitro studies with purified enzyme show kinetics of inactivation that are similar to those observed in vivo. Consequently, involvement of a yeast protease in the inactivation process is relatively unlikely.

Asparaginase II is a phosphomannan protein which is secreted into the cell wall of *Saccharomyces cerevisiae* (Dunlop & Roon, 1975; Jones, 1977a,b; Dunlop et al., 1976, 1978, 1980a). Synthesis of the enzyme is regulated by the nitrogen supply (Dunlop & Roon, 1975; Dunlop et al., 1980a,b; Kang et al., 1982). Asparaginase II activity can vary over 100-fold, with very low levels being observed in cell growing on a readily metabolized nitrogen source such as glutamine and high levels being found in cells starved for nitrogen or grown on a poor nitrogen source such as proline. Recently, it was reported that during exponential growth of *Saccharomyces* of minimal ammonia medium, asparaginase II is synthesized in high levels and then is inactivated during the time of transition to the stationary phase (Pauling & Jones, 1980a,b). This biphasic pattern of enzyme synthesis and inactivation has been confirmed by studies in our laboratory (Roon et al., 1982).

In studying the inactivation of asparaginase II, Pauling & Jones (1980a,b) found that enzyme inactivation was prevented by the addition of a protein synthesis inhibitor (cycloheximide), metal chelator (EDTA),[1] or sulfhydryl reagents (pOHMB, iodoacetate). Also, the rate of asparaginase II inactivation was decreased in cells treated with a protease inhibitor, TPCK. Furthermore, the addition of Zn^{2+} to cells treated with EDTA stimulated the rate of enzyme inactivation. On the basis of these data, Pauling and Jones suggested that asparaginase II is degraded by a Zn^{2+}–dependent protease which is synthesized de novo during the late exponential phase of growth.

The possibility that asparaginase II is degraded by a proteolytic system is an intriguing one for at least two reasons. First, the elucidation of such an inactivation system could shed further light on the mechanisms whereby enzymes of nitrogen catabolism are regulated. Proteolysis could provide a novel physiological mechanism for the (down) regulation of asparaginase II when its catalytic function (ammonia production) is no longer necessary. Second, proteolysis of asparaginase II might serve as a model for the turnover of other yeast exoenzymes such as invertase and acid phosphatase (Schekman, 1982). It would be of considerable interest to understand the mechanism whereby a proteolytic enzyme was able to degrade asparaginase II, an exoenzyme which is presumably entrapped within the mannan/glucan matrix of the yeast cell wall. Exoenzymes such as asparaginase II are secreted into the yeast cell wall in the bud portion of growing cells perhaps within a periplasmic-like space (Tkacz & Lampen, 1973; Field & Schekman, 1980). The exoenzymes are accessible to exogenous substrates of low molecular weight. However, it is presently unclear to what extent these exoenzymes would be accessible to freely diffusible extracellular proteases or to proteases secreted into the yeast cell wall subsequent to exoenzyme deposition. Thus, a convincing demonstration of the protease-dependent turnover of asparaginase II within the yeast cell wall would raise many questions concerning the physical characteristics of this cellular structure and the turnover of the exoenzymes entrapped therein.

The present report documents our studies on the inactivation of asparaginase II with whole cell suspensions and highly purified enzyme preparations. Although in many respects we have confirmed the experimental results of Pauling & Jones (1980a,b), a detailed comparison of the kinetics of asparaginase II inactivation in vivo and in vitro does not support their proteolysis model. Rather, our data suggest that asparaginase II is denatured as the result of the decrease in pH which occurs

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received November 29, 1982.* These studies were supported in part by a grant from the University of Minnesota, Graduate School.

[1] Abbreviations: TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; YNB, yeast nitrogen base (without amino acids and ammonium sulfate); pOHMB, *p*-(hydroxymercuri)benzoate.

0006-2960/83/0422-2704$01.50/0

as yeast enter the stationary phase. We found that the stability of asparaginase II at low pH is decreased by sulfate ion and somewhat improved by the addition of asparagine, but we can detect no requirement for metal ions or for de novo protein synthesis in this inactivation process.

Materials and Methods

Organisms. The haploid yeast strains X2180-1A (*MATa, SUC2 mal gal2 CUP1*) was obtained from the Yeast Genetics Stock Center, Donner Laboratory, University of California, Berkeley, CA. Strain X2180–1A is isogenic with strain S288C which was used by Pauling and Jones to study asparaginase II inactivation (Pauling & Jones, 1980a,b).

Growth Conditions. The standard chemically defined medium contained, per liter, 30 g of D-glucose, 2 g of yeast nitrogen base (Difco no. 033-15-9, without amino acids and ammonium sulfate), 1.32 g of ammonium sulfate, and 1.69 g of L-glutamic acid, monosodium salt. Yeast cultures were grown at 23 °C with slow rotary shaking at 150 rpm from a 0.5% inoculum. The cells were harvested by centrifugation after 12–16 h when the absorbance at 660 nm was 0.1–0.2. The harvested cells were used immediately.

Derepression and Inactivation of Asparaginase II in Cell Suspensions. The medium contained 3% D-glucose and 0.2% Difco yeast nitrogen base (without amino acids and ammonium sulfate). The pH of the medium was adjusted to 7.0 with 10 N KOH. Cells were suspended to an absorbance of 0.4 at 660 nm (ca. 0.12 mg/mL, dry weight) and allowed to incubate at 30 °C. At the times indicated in individual experiments, 10-mL samples were removed and assayed for asparaginase II activity.

In experiments where the cultures were maintained at a constant pH, 20 mM potassium phosphate and 50 mg/L cycloheximide were included in the media.

Inactivation of Purified Asparaginase II. In vitro inactivation studies were conducted in 10 mM potassium phosphate, pH 3.2. At the times specified samples were removed, mixed with 4 volumes of ice-cold 50 mM potassium phosphate, pH 7.0, and placed in an ice bath until assays could be performed.

Purification of Asparaginase II. The asparaginase II preparations used in this study were extracted from *Saccharomyces cerevisiae* and purified by a previously published method (Dunlop et al., 1978). The fractions used in this study correspond to the highest purity material characterized in previous investigations.

Analytical Methods. Asparaginase II Activity. Whole cell suspensions and purified enzyme preparations were assayed at 23 °C for the ability to convert L-asparagine and hydroxylamine into L-aspartic acid β-hydroxamate as described previously (Dunlop et al., 1980a,b). Asparaginase II activity is reported as nanomoles of the β-hydroxamate synthesized per minute per milligram of cells (dry weight).

Results

Synthesis and Inactivation of Asparaginase II in Growing Cell Cultures. Pauling and Jones reported a biphasic pattern of asparaginase II synthesis and degradation during growth of *Saccharomyces* (Pauling & Jones, 1980a,b). In our initial studies, shown in Figure 1, the control cultures gave a similar biphasic pattern of enzyme expression. Because the inactivation of asparaginase II was reportedly catalyzed by a Zn^{2+}-dependent enzyme system, we tested the effect of EDTA. The metal chelator was added to cell cultures after 4 h of incubation, at a time when the rate of asparaginase II synthesis was maximal. When EDTA was added as the tetrasodium salt, asparaginase II synthesis was stimulated, and the subsequent inactivation phase did not occur. However, when EDTA was added as the free acid, the pattern of enzyme synthesis was similar to that of the control culture, and the rate of inactivation was slightly stimulated.

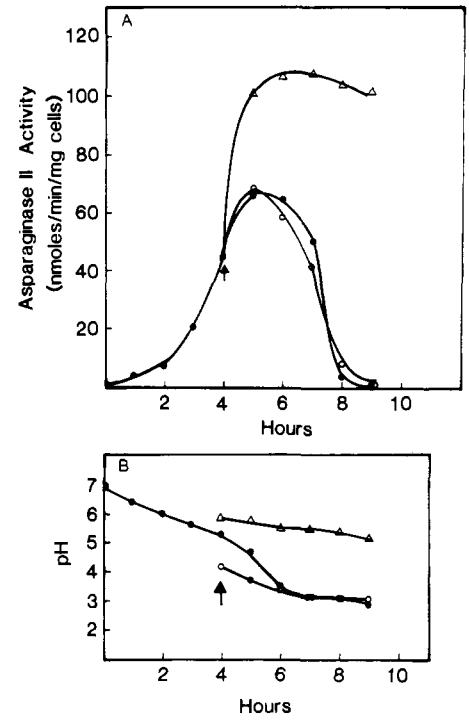

FIGURE 1: Synthesis and inactivation of asparaginase II in vivo: influence of EDTA. Yeast cells were grown in 3% glucose, 0.2% YNB, and 10 mM $(NH_4)_2SO_4$, initial pH 7.0. After 4 h of growth at 30 °C the following additions were made: control, no addition (●), 5 mM EDTA, tetrasodium salt (△), and 5 mM EDTA, free acid (○). At the times indicated 10-mL samples were removed, the yeast cells were assayed for asparaginase II activity (A), and the pH of the culture media was determined (B).

When the pH was monitored in these cultures, it was determined that the addition of the tetrasodium salt of EDTA resulted in an initial increase in pH, and subsequent pH values were sustained above pH 4.5 for the duration of the experiment. In contrast, the addition of EDTA in the free acid form resulted in an initial drop in pH. As the incubation was continued the pH of this latter culture converged with that of the control culture. After 2 h, both cultures were in the pH range 3.3–3.5. During the next 2 h the pH of both cultures fell below 3.1 and most of the asparaginase II activity was lost.

These data suggested two things: (1) Since the addition of EDTA in the free acid form did not prevent asparaginase II inactivation, the requirement of Zn^{2+} in this process is somewhat questionable. (2) The fact that EDTA in the tetrasodium form could prevent asparaginase II inactivation raised the possibility that it was the effect of the compound in maintaining a high pH rather than its chelating capacity that was of importance in preventing inactivation.

To test this possibility, we repeated the growth experiment and supplemented the cultures with a variety of compounds which have been reported to influence the inactivation of asparaginase II (Pauling & Jones, 1980a,b). The compounds were added after 4 h of growth and the cultures incubated for an additional 5 h. Table I gives the asparaginase II activity and pH values exhibited by these cultures at the completion of the incubation period. The data suggest a possible correlation between effect on pH and asparaginase II activity. Those cultures in which the pH was maintained at 3.4 or above exhibited significant retention of asparaginase II activity,

Table I: Effect of Various Reagents on Asparaginase II Activity and pH of Growing Yeast Cells[a]

reagent added	asparaginase II specific activity[b]	pH
none	2	2.95
10 mM $(NH_4)_2SO_4$	3	3.00
10 mM $ZnSO_4$	2	2.90
5 mM EDTA, free acid	1	2.90
5 mM EDTA, disodium salt	35	3.30
5 mM EDTA, tetrasodium salt	112	4.60
50 μg/mL cycloheximide	68	4.20
10 mM L-glutamine	72	3.60
10 mM L-asparagine	69	4.60
10 mM L-glutamate (Na^+ salt)	62	3.40
10 mM L-aspartate (K^+ salt)	68	3.40
1 mM TPCK	5	3.10
5 mM pOHMB	70	4.60
20 mM potassium phosphate, pH 7.0	115	5.20

[a] Yeast cells were suspended in 3% glucose, 0.2% YNB, and 10 mM $(NH_4)_2SO_4$, initial pH 7.0. Reagents added at 4 h when the pH was 5.0 and asparaginase II activity was 62 nmol min^{-1} (mg of cells)$^{-1}$. Asparaginase II activity and the pH were measured after 5 h of additional growth. [b] Specific activities are given as nmol min^{-1} (mg of cells)$^{-1}$.

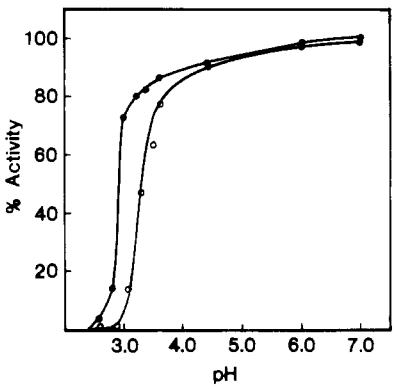

FIGURE 2: Effect of pH on asparaginase II activity remaining in cell cultures. Cell cultures which had been nitrogen starved for ca. 4.5 h, with an initial asparaginase II activity of ~90 nmol min^{-1} (mg of cells)$^{-1}$, were suspended to 1 mg/mL (dry weight) in 3% glucose, 0.2% YNB, 20 mM potassium phosphate, and 50 μg/mL cycloheximide ± 10 mM $(NH_4)_2SO_4$, pH as indicated. After a 1-h incubation at 30 °C the cells were collected by centrifugation and washed with an equal volume of 20 mM potassium phosphate, pH 7.0. Asparaginase II activity was determined in cultures which had been incubated with (○) and without (●) 10 mM $(NH_4)_2SO_4$.

whereas in the cultures in which the pH fell below 3.2 the enzyme became inactivated. Interestingly, the mode by which these compounds affected pH varies significantly. For example, potassium phosphate probably acts strictly as a buffer, whereas cycloheximide inhibits protein synthesis which indirectly inhibits glucose fermentation and prevents the accompanying drop in pH.

Pauling and Jones have suggested that asparaginase II inactivation requires de novo protein synthesis (Pauling & Jones, 1980a,b). To examine this possibility, yeast cultures exhibiting high asparaginase II activity (4.5-h cultures on the time scale shown in Figure 1A) were collected and suspended in growth medium of various pHs containing 50 μg/mL cycloheximide. These cultures were assayed for asparaginase II activity after a 1-h incubation period. The results of this study are shown in Figure 2. Two properties of the system are apparent: (1) Asparaginase II activity is rapidly lost in cell suspension held in complete media below pH 3.2 despite the presence of cycloheximide. This suggests that de novo protein synthesis is not a prerequisite for asparaginase II inactivation. (2) At a given pH value the rate of inactivation is more rapid in complete medium containing 10 mM $(NH_4)_2SO_4$ than in media lacking this compound.

Table II: Asparaginase II Activity in Nongrowing Yeast Suspensions at Low pH[a]

reagent added	asparaginase II activity (% control)
none	89
5 mM $(NH_4)_2SO_4$	23
5 mM Na_2SO_4	29
5 mM $ZnSO_4$	27
10 mM NH_4Cl	95
10 mM NaCl	93
5 mM $ZnCl_2$	95
10 mM L-asparagine	92
10 mM L-asparagine + 5 mM $(NH_4)_2SO_4$	59
5 mM EDTA	89
5 mM EDTA + 5 mM $ZnSO_4$	26

[a] Yeast cells having a derepressed asparaginase II activity of ca. 95 nmol min^{-1} (mg of cells)$^{-1}$ were suspended in medium containing 3% glucose, 0.2% YNB, 20 mM potassium phosphate, and 50 μg/mL cycloheximide, pH 3.2. Asparaginase II activity was determined after a 1-h incubation in the presence of the reagents listed.

To further elucidate how $(NH_4)_2SO_4$ increases the rate of asparaginase II inactivation, the experiment was repeated at pH 3.2 with a variety of other salts replacing $(NH_4)_2SO_4$. The data given in Table II indicate that it is the sulfate ion rather than the ammonium ion which destabilizes asparaginase II, since ammonium chloride does not increase the rate of inactivation. Similarly, zinc sulfate and sodium sulfate promote inactivation, but their corresponding chloride salts do not. The addition of EDTA does not prevent the inactivation caused by $ZnSO_4$ [or by $(NH_4)_2SO_4$ or Na_2SO_4; data not shown]. The addition of enzyme substrate, L-asparagine, partially prevented the enzyme inactivation which occurs in the presence of sulfate ion.

These experiments were repeated at pH 4.5 and 2.7. At pH 4.5 asparaginase II is not significantly inactivated even in the presence of 10 mM sulfate ion. At pH 2.7 most activity is lost even in the absence of sulfate and/or in the presence of enzyme substrate, L-asparagine (data not shown).

Our results are not compatible with a requirement for an easily removable zinc component or for de novo protein synthesis in the inactivation of asparaginase II. However, our in vivo data do not eliminate the possible involvement of a constitutive, cell wall acidic protease. To investigate this possibility we conducted detailed kinetic studies using highly purified preparations of asparaginase II (Dunlop et al., 1978, 1980a,b). The results of this study are given in Figure 3. In panel A it can be seen that asparaginase II inactivation is stimulated by ammonium sulfate but not by ammonium chloride. Similarly panel B shows that inactivation is stimulated by zinc sulfate but not by zinc chloride. The inactivation is not prevented by the inclusion of EDTA in the reaction mixture. Finally, it can be seen that the sulfate-dependent inactivation is partially prevented by the inclusion of enzyme substrate, L-asparagine (or D-asparagine). Thus, the pattern of asparaginase II inactivation observed with purified enzyme preparations is very similar to that observed in vivo.

Discussion

The in vivo studies we have conducted indicate that the inactivation of asparaginase II does not require de novo protein synthesis and is not directly influenced by metal chelators. The in vitro studies further suggest that the inactivation process

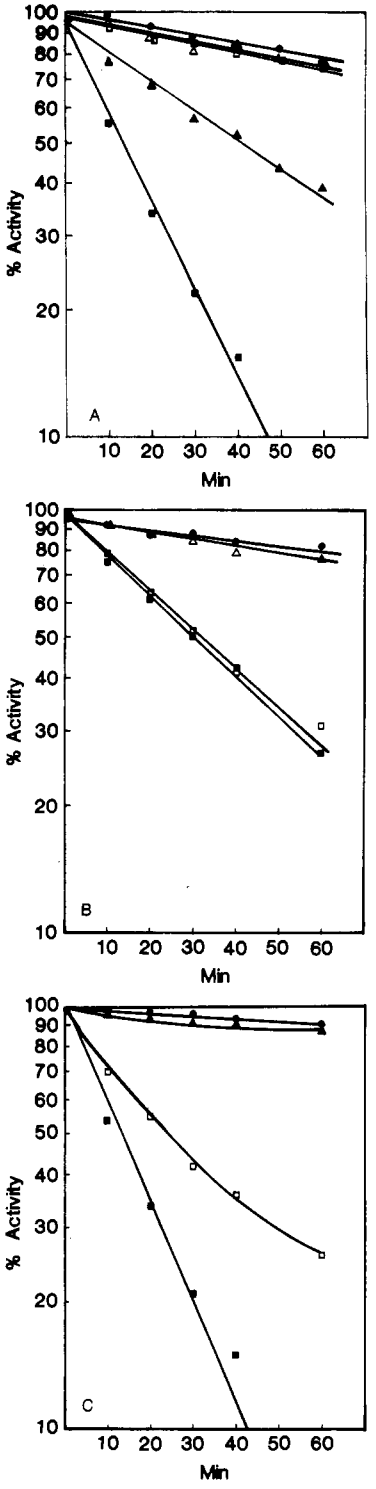

FIGURE 3: Inactivation of asparaginase II in vitro. Kinetics of asparaginase II inactivation were determined with purified enzyme suspended in 10 mM potassium phosphate, pH 3.2. (A) The following additions were made: control, no addition (●), 10 mM NH_4Cl (△), 20 mM NH_4Cl (□), 5 mM $(NH_4)_2SO_4$ (▲), and 10 mM $(NH_4)_2SO_4$ (■). (B) The following additions were made: control (●), 5 mM $ZnCl_2$ (△), 5 mM $ZnSO_4$ (□), and 5 mM $ZnSO_4$ plus 5 mM EDTA (■). (C) The following additions were made: control (●), 10 mM L-asparagine (▲), 10 mM $(NH_4)_2SO_4$ (■), and 10 mM $(NH_4)_2SO_4$ plus 10 mM L-asparagine (□).

results from simple denaturation rather than proteolytic attack. The enzyme preparations we used were equal in purity to the fractions of highest specific activity previously studied (Dunlop et al., 1978, 1980a,b) and gave single broad bands on polyacrylamide gel electrophoresis. Because these preparations contain a high/heterogeneous carbohydrate component, it is difficult to obtain sharp bands on gel electrophoresis or give correspondingly rigorous assessments of enzyme purity. It is possible that a proteolytic component may have copurified with asparaginase II. However, since the purified material gave essentially the same kinetics of inactivation as the cell-bound enzyme and in light of the insensitivity (in our study) of the inactivation process to TPCK (Pauling & Jones, 1980a,b), we conclude that there is at present no firm evidence supporting proteolytic involvement in asparaginase II inactivation.

There is a striking parallel between our data with respect to asparaginase II inactivation and data recently reported on the inactivation of a repressible acid phosphatase in *Saccharomyces* (Bostian et al., 1982). Both of these enzymes are cell wall glycoproteins, but they serve different metabolic roles, nitrogen catabolism vs. phosphate catabolism, and their biosynthesis is regulated by their respective catabolic products. Both enzymes show a similar pH profile for inactivation. What is more intriguing is that the rate of inactivation of both enzymes in vivo or in vitro is stimulated by ammonium sulfate and decreased by L-asparagine. Whereas the relationship between asparaginase II and L-asparagine is clear, the physiological significance of L-asparagine interaction with acid phosphatase is somewhat obscure. Stifling the urge to speculate on potential evolutionary relationships between the two enzymes, we await further structural and regulatory data before offering any rationale for these behavioral similarities.

Acknowledgments

We thank Moira Murdoch for her skilled technical assistance. Our deepest heartfelt appreciation goes to Dr. Dennis Livingston for critically reviewing the manuscript.

Registry No. Asparaginase, 9015-68-3.

References

Bostian, K. A., Lemire, J. M., & Halvorson, H. O. (1982) *Mol. Cell. Biol. 2*, 1–10.

Dunlop, P. C., & Roon, R. J. (1975) *J. Bacteriol. 122*, 1017–1024.

Dunlop, P. C., Roon, R. J., & Even, H. L. (1976) *J. Bacteriol. 125*, 999–1004.

Dunlop, P. C., Meyer, G. M., Ban, D., & Roon, R. J. (1978) *J. Biol. Chem. 253*, 1297–1304.

Dunlop, P. C., Meyer, G. M., & Roon, R. J. (1980a) *J. Biol. Chem. 255*, 1542–1546.

Dunlop, P. C., Meyer, G. M., & Roon, R. J. (1980b) *J. Bacteriol. 143*, 422–426.

Field, C., & Schekman, R. (1980) *J. Cell Biol. 86*, 123–128.

Jones, G. E. (1977a) *J. Bacteriol. 129*, 1165–1167.

Jones, G. E. (1977b) *J. Bacteriol. 130*, 128–130.

Kang, L., Keeler, M. L., Dunlop, P. C., & Roon, R. J. (1982) *J. Bacteriol. 151*, 29–35.

Pauling, K. D., & Jones, G. E. (1980a) *J. Gen. Microbiol. 117*, 423–430.

Pauling, K. D., & Jones, G. E. (1980b) *Biochim. Biophys. Acta 616*, 271–282.

Roon, R. J., Murdoch, M., Kunze, B., & Dunlop, P. C. (1982) *Arch. Biochem. Biophys. 219*, 101–109.

Schekman, R. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 243–246.

Tkacz, J. S., & Lampen, J. O. (1973) *J. Bacteriol. 113*, 1073–1075.