Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4466–4470, July 1981
Genetics

Expression and processing of bacterial β-lactamase in the yeast *Saccharomyces cerevisiae*

(yeast transformation/2-μm DNA vector/hybrid plasmids/antibiotic resistance genes/eukaryotic gene expression)

RAINER ROGGENKAMP*, BIRGIT KUSTERMANN-KUHN†‡, AND CORNELIS P. HOLLENBERG*

*Institut für Mikrobiologie, Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, Federal Republic of Germany; and †Max-Planck-Institut für Biologie, Abteilung Beermann, Spemannstraße 34, D-7400 Tübingen, Federal Republic of Germany

Communicated by Wolfgang Beermann, March 31, 1981

ABSTRACT **The mode of expression in *Saccharomyces cerevisiae* of the bacterial antibiotic resistance gene coding for β-lactamase (EC 3.5.2.6) is described. Yeast transformants, containing hybrid plasmid pMP78-1 consisting of pBR325 in a 2-μm DNA vector, synthesize an active β-lactamase protein. The enzyme was purified about 100-fold over crude extracts. With regard to activity, molecular weight, and binding to specific antibodies the yeast β-lactamase was indistinguishable from the purified enzyme from *Escherichia coli*. Because the bacterial enzyme is synthesized as a preprotein with subsequent maturation, the results suggest that *S. cerevisiae* is able to convert the preprotein to the mature β-lactamase. This was confirmed by *in vitro* experiments showing that the bacterial preprotein can be processed by crude extracts of *S. cerevisiae*.**

The functional expression of a number of bacterial genes in yeast has recently been established (1–5). Using a 2-μm DNA vector, we were able to show that a number of antibiotic resistance genes are expressed in *Saccharomyces cerevisiae* transformants (1, 2). In the case of the chloramphenicol and kanamycin resistance genes, we observed an increased resistance to the corresponding antibiotics, whereas we demonstrated the active gene product of the ampicillin resistance gene, the β-lactamase (EC 3.5.2.6), by both biological and enzymatic assays. The synthesis of the bacterial β-lactamase in yeast was especially surprising because in bacteria this enzyme is translated as a preprotein (6, 7) carrying a signal peptide that is cleaved off during secretion into the periplasmic space (8, 9), in agreement with the signal hypothesis (10). The presence of active β-lactamase in yeast could be due either to enzymatic activity of the preprotein of the β-lactamase or to processing by the yeast cell of an inactive precursor to the active mature enzyme. To resolve this question we investigated the structure of the active β-lactamase in yeast. In this communication we show that the bacterial β-lactamase preprotein is inactive and present evidence that the active β-lactamase synthesized in yeast is identical to the mature enzyme in *Escherichia coli*.

MATERIALS AND METHODS

Materials, Strains, and Media. Staphylococcal protein A, labeled with ^{125}I, and [^{35}S]methionine were obtained from Amersham-Buchler (Braunschweig, Federal Republic of Germany); phenylmethylsulfonyl fluoride was purchased from Boehringer Mannheim; nitrocefin was a gift of Glaxo-Allenburys Research (Greenford, England), courtesy of C. H. O'Callaghan. Molecular weight standards and protein assay kits were obtained from Bio-Rad (Munich, Federal Republic of Germany). *E. coli* containing plasmid pBR322 was grown in minimal medium (11) supplemented with 25 μg of ampicillin per ml. Yeast transformants were grown in yeast nitrogen base (Difco), 2% glucose, and L-histidine (20 μg/ml) under selective conditions—i.e., without leucine.

Purification and Assay of β-Lactamase. Osmotic shock lysates of *E. coli* were used as starting material. The purification procedure was essentially as described by Melling and Scott (12) as modified in the laboratory of J. R. Knowles (personal communication). Yeast β-lactamase of YT6-2 was purified in the same way from crude extracts, obtained by using a Braun homogenizer (Braun, Melsungen, Federal Republic of Germany). After the second DEAE-cellulose chromatography the enzyme preparations were concentrated by dialysis against a saturated solution of ammonium sulfate (adjusted to pH 7.0 with NH_4OH) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Enzymatic assays were performed spectrophotometrically by measuring the decrease of absorbance of nitrocefin at 395 nm (13). The specific activity is defined as the decomposition of 1 μmol of nitrocefin per min per mg of protein. Protein was determined by using the Bio-Rad assay kit, with bovine gamma globulin as a standard.

Polyacrylamide Gel Electrophoresis. Nondenaturing gels were prepared and run as described by Maurer (14). Protein bands of β-lactamase were visualized directly by incubating the gels in 0.1 M potassium phosphate buffer containing 5 mg of nitrocefin per ml and 0.5% dimethyl sulfoxide. Gel electrophoresis in the presence of sodium dodecyl sulfate in 12.5% polyacrylamide was performed essentially as described by Laemmli (15). Samples of protein were preincubated with phenylmethylsulfonyl fluoride at a final concentration of 1 mg/ml for 30 min at 0°C before denaturation with sodium dodecyl sulfate. Gels were routinely stained with Coomassie blue. For the determination of enzyme activities in gel bands the proteins were renatured at 4°C by the following procedure: gels were soaked in 50% trichloracetic acid for 1 hr, then in 7% (wt/wt) acetic acid for 1 hr with two changes, followed by soaking for another hour in 7% acetic acid containing 0.5% Triton X-100. Acetic acid was then washed out for several hours with 0.02 M potassium phosphate buffer (pH 7.2) until the pH was equal to that of the buffer. The activity of β-lactamase was visualized as for the native gels described above.

Immunological Procedures. Specific β-lactamase antisera were prepared by immunization of rabbits, using conventional methods. For detection of β-lactamase with specific antibodies, sodium dodecyl sulfate/polyacrylamide gels were covered immediately after the run with a 1% agarose gel containing 2.5 mM sodium borate buffer at pH 8.4, 0.85% NaCl, and 50 μl of spe-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

‡ Present address: Institut für Hirnforschung, Sektion Neurochemie, Universität Tübingen, D-7400 Tübingen, Federal Republic of Germany.

cific antiserum per ml. Proteins were allowed to diffuse for 16 hr at 4°C. The agarose gel was carefully removed and washed for 48 hr at 4°C in borate-buffered NaCl (see above) and subsequently in the same solution containing 3 μCi (1 Ci = 3.7 $\times 10^{10}$ becquerels) of ^{125}I-labeled protein A per ml for 4 hr at room temperature. Unbound protein A was washed out as above for 48 hr at 4°C. Dried gels were exposed to Kodak X-Omat films at −70°C. For immunoprecipitation 5 μl of the minicell lysate was incubated with 20 μl of specific antiserum for 1 hr at room temperature and 12 hr at 4°C. The precipitate was collected by centrifugation at 6000 × *g* and washed twice with borate-buffered NaCl (see above) and once with the same buffer containing 1% Triton X-100.

Synthesis of β-Lactamase in Minicells and in a Cell-Free System. Polypeptides whose synthesis was directed by plasmid pBR322 in minicells were analyzed by sodium dodecyl sulfate electrophoresis and subsequent autoradiography as described (16). Cell-free transcription and translation using plasmid pBR322 as template were performed essentially as described by Zubay *et al.* (17). Assay kits were kindly provided by Juliane Alt, Institute of Botany, University of Düsseldorf. [^{35}S]Methionine-labeled polypeptides were analyzed as described above.

RESULTS

Yeast Transformants Producing β-Lactamase. The YT6 yeast transformants used in this study were obtained by transforming strain AH22, a double *leu2* mutant, with pMP78-1 (Fig. 1), using the method of Beggs (19). Plasmid pMP78 consists of pBR325 and *Hin*dIII fragment 3 of 2-μm DNA carrying the *Leu2* gene. Plasmid pMP78-1 contains the intact chloramphenicol resistance gene in addition to the β-lactamase gene. Studies on plasmid stability (20) have shown that even under selective conditions pMP78 is not stably maintained in the transformants. After 30 generations of selective growth we observed a loss of pBR325 DNA in 10% of the cells. Apparently pMP78 recombines with endogenous 2-μm DNA and pBR325 is lost, probably as a consequence of intramolecular recombination. The end product of this recombination is a 2-μm DNA that carries the *Leu2* gene. In many cultures this 2-μm DNA-*leu2* plasmid is present together with pMP78 and normal 2-μm DNA (20). The loss of pBR325 sequences can be considerably reduced by using a yeast host strain that has no 2-μm DNA and a recombinant plasmid that carries the whole 2-μm DNA as a vector (20).

To start a culture of YT6-2 we used colonies that showed a high production of β-lactamase when tested directly on plates (21). This selection assured cultures yielding the highest specific activities for β-lactamase in crude extracts. Experiments with transformants YT34 that carry pMP110, a recombinant plasmid similar to pMP78 but containing pBR322 instead of pBR325, showed in general more β-lactamase activity in plate assays. However, the specific activity in crude extracts was as high as for YT6-2.

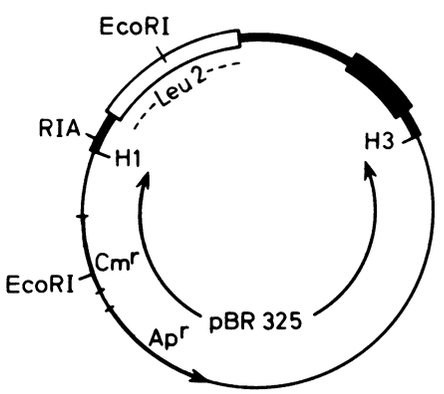

FIG. 1. Recombinant plasmid pMP78-1 (1) consists of pBR325 (18) and *Hin*dIII fragment 3 of 2-μm DNA carrying the *Leu2* gene from pJDB219 (19). The 2-μm DNA is drawn as a thick line. RIA represents the *Eco*RI restriction endonuclease site and H1 and H3 represent the *Hin*dIII restriction endonuclease sites on 2-μm DNA as described (16). Ap^r, Ampicillin resistance; Cm^r, chloramphenicol resistance.

Purification and Activities of β-Lactamase. The total activities of β-lactamases in yeast transformant YT6-2 and *E. coli* pBR322 were found to differ by a factor of 500 when the activities of crude extracts and osmotic shock lysates from the same quantity of cells (wet weight) were compared (Table 1). Likewise, the specific activity in *E. coli* was found to be about 500 times higher than that in yeast (Table 1), when we took into account that the specific activity of β-lactamase in osmotic shock lysates of *E. coli* was 10-fold higher than that in an *E. coli* crude extract. Elution patterns on DEAE-cellulose columns were the same for both bacterial and yeast enzymes; the β-lactamase activity was always found in a single peak. The yeast enzyme was stably maintained during the enrichment procedure, as judged from the yield of total activity, which was comparable to that of the *E. coli* enzyme. Apparently, the yeast β-lactamase is not degraded by protease action, although the yeast cell is known to contain a variety of proteases. The final enzyme preparations were analyzed by sodium dodecyl sulfate electrophoresis. The β-lactamase from *E. coli* was present as a single protein band, whereas the yeast enzyme, although enriched about 100-fold, appeared only as a minor band (Fig. 2, lanes A and B). When the same amount of yeast activity was loaded on the gels, the Coomassie blue staining intensities of the yeast enzyme band and the *E. coli* band were similar (data not shown). Thus, it can be concluded that the differences in total and specific activity in yeast and in *E. coli* reflect a lower content of β-lactamase in the yeast cell rather than a lower specific activity.

Relationship of Precursor and Mature β-Lactamase of *E. coli*. To compare the active yeast β-lactamase with the bacterial β-lactamase gene products, we analyzed the expression of

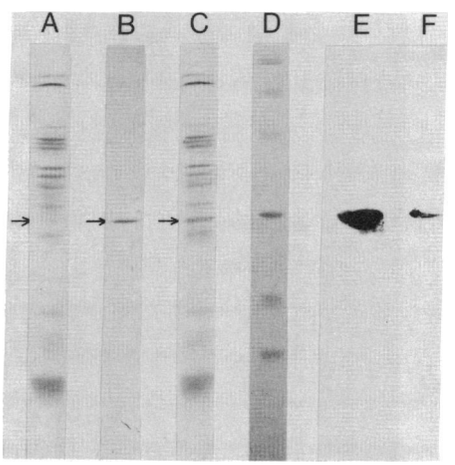

FIG. 2. Molecular weight determination of β-lactamase from yeast transformant YT6-2 after sodium dodecyl sulfate electrophoresis. Arrows indicate the position of the nitrocefin color reaction. Lane A, partly purified yeast β-lactamase (100 μg). Lane B, purified β-lactamase from *E. coli* (1 μg). Lane C, mixture of proteins in lanes A and B. Lane D, molecular weight markers: phosphorylase b, 95,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,300. Lane E, autoradiography of ^{125}I-labeled protein A immunoprecipitate from purified *E. coli* β-lactamase (5 μg). Lane F, like lane E, but with partly purified yeast β-lactamase (120 μg). Exposure time was 20 hr.

Table 1. Purification of β-lactamase from *E. coli* carrying plasmid pBR322 and from yeast transformant YT6-2

Strain	Purification stage	Total protein, mg	Specific activity, units/mg	Vol, ml	Total activity, units	Yield, %
E. coli(pBR322)	Osmotic shock lysate	207	22	230	4600	100
Yeast YT6-2	Crude extract	2800	0.004	125	11	100
E. coli(pBR322)	Exp. 1. DEAE-cellulose	66	48	80	3200	70
Yeast YT6-2		200	0.028	200	5.6	51
E. coli(pBR322)	Exp. 2. DEAE-cellulose	8.4	190	0.4	1600	35
Yeast YT6-2	and concentration	2.7	0.5	0.2	1.3	12

In both experiments, 60 g of cells (wet weight) was used.

pBR322 in minicells. After sodium dodecyl sulfate electrophoresis of the synthesized polypeptides a doublet and a prominent band of 2500 higher molecular weight were always observed (Fig. 3, lane A). All bands could be quantitatively precipitated by specific antiserum (Fig. 3, lane B), which proves that they are products of the β-lactamase gene. According to the data available from the nucleotide sequence of the β-lactamase gene and the amino acid sequence of the enzyme, the signal peptide consists of 23 amino acids (6, 7). Thus, we conclude, that the band with a molecular weight approximately 2500 higher than the doublet represents the preprotein. This was confirmed by an *in vitro* transcription and translation experiment using pBR322 as template. The labeled product shows only one major band, which occurs at the same position as that of the preprotein (Fig. 3, lane C). As expected, the coupled transcription–translation system produces only the preprotein because the secretion mechanism (9) required for processing is not present.

The mature β-lactamase could be identified directly by activity with the nitrocefin color reagent and the fact that it comigrates with purified β-lactamase (Fig. 3). Enzymatic activity was never observed at the position of the preprotein, although this protein was a major band among the labeled polypeptides. Consequently, the preprotein has no β-lactamase activity or an activity too low to be detected under our experimental conditions. The occurrence of a doublet representing the mature β-lactamase is due to incomplete denaturation, which was observed as well for the purified β-lactamase, denatured at 20°C (Fig. 3, lane D). The denaturation seems to depend also on salt and detergent concentration and can be complete even at 20°C, leading to a single band at the position of the faster-migrating band of the doublet (Fig. 2, lane B). Denaturation of purified enzyme at 90°C always results in a single protein band at this position.

Identification of Yeast β-Lactamase in Polyacrylamide Gels. The position of β-lactamase activity could be identified on polyacrylamide gels by using the nitrocefin color reagent. In nondenaturing gels the yeast enzyme appeared as a sharp single band and comigrated with the purified β-lactamase of *E. coli*, suggesting that the two enzymes are identical (Fig. 4). To substantiate this assumption, electrophoresis in the presence of dodecyl sulfate was performed. Although recovery of enzyme activity was low after renaturation, the β-lactamase bands could clearly be identified with nitrocefin and, in addition, with spe-

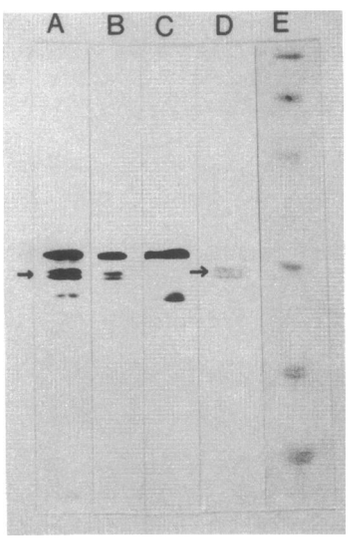

FIG. 3. Autoradiography of polypeptides whose synthesis is directed by plasmid pBR322 in minicells and in a cell-free system. Exposure time was 2 days. Lane A, minicell lysate (10 μl); lane B, immunoprecipitate of minicell lysate (5 μl); lane C, cell-free transcription and translation products (from 1 μg of pBR322); lane D, purified *E. coli* β-lactamase (5 μg); lane E, markers as in Fig. 2. Arrows indicate the position of the nitrocefin color reaction. One microliter of the minicell lysate corresponds to 10 μl of a minicell suspension with an optical density of 1 at 610 nm.

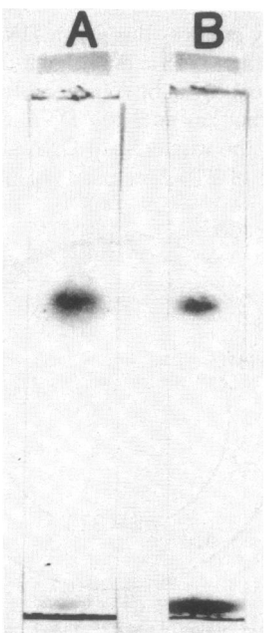

FIG. 4. Localization of β-lactamase from yeast transformant YT6-2 with nitrocefin on 15% polyacrylamide gels under nondenaturing conditions. Lane A, partly purified yeast β-lactamase (100 μg); lane B, purified β-lactamase from *E coli* (1 μg).

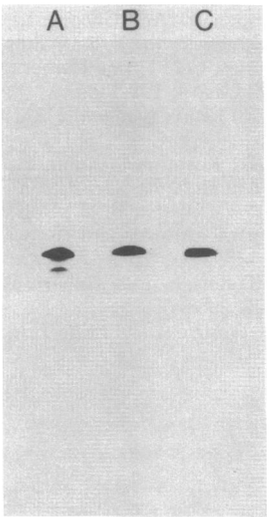

FIG. 5. Processing of bacterial β-lactamase preprotein with crude extracts of *S. cerevisiae*. [^{35}S]Methionine-labeled preprotein of β-lactamase synthesized *in vitro* with pBR322 as a template was incubated with a crude extract of yeast strain AH22 (200 μg of protein) in a total volume of 15 μl for 3 hr at 25°C. Autoradiography was performed for 2 days. Lane A, mixture as described above; lane B, yeast crude extract heated for 5 min at 95°C; lane C, 20 mM Tris·HCl, pH 7.0, instead of yeast crude extract.

cific antiserum (Fig. 2, lanes E and F). The yeast β-lactamase activity is found in a single protein band that migrates to exactly the same position as the purified enzyme from *E. coli*. This was most clearly demonstrated when mixtures of bacterial and yeast β-lactamases were loaded on a gel (Fig. 2, lane C). Only a single band showing activity of β-lactamase with the nitrocefin color reaction was observed. The results clearly demonstrate that the yeast β-lactamase has the same molecular weight as the bacterial enzyme. The fact that the yeast enzyme and that of *E. coli* have a similar specific activity and the same molecular weight proves that the yeast enzyme represents the processed form of β-lactamase. Furthermore, it can be concluded that *S. cerevisiae* is able to cleave a prokaryotic signal peptide.

Processing of Bacterial β-Lactamase Preprotein by Crude Extracts of *S. cerevisiae*. The identity of the yeast β-lactamase from transformant YT6-2 and the bacterial enzyme led to the suggestion that the corresponding processing mechanism must be present in yeast. In order to obtain additional confirmation, an *in vitro* experiment was performed. The [^{35}S]methionine-labeled preprotein, which was obtained by *in vitro* transcription and translation of pBR322, was incubated with crude extracts of yeast strain AH22. Subsequent analysis with sodium dodecyl sulfate electrophoresis and autoradiography of the dried gel showed that a new protein was generated at a position identical to that of the mature β-lactamase (Fig. 5). The appearance of the newly formed protein was dependent on time and could be detected as early as after 1 hr of incubation. After 3 hr substantial amounts of processed enzyme were produced. The specificity of the cleavage suggests that this reaction represents the actual processing mechanisms observed *in vivo*.

DISCUSSION

In the present study we have shown that the eukaryotic organism *S. cerevisiae* is able to functionally express a prokaryotic gene whose primary product in the bacterium is a preprotein that undergoes maturation in connection with its secretion into the periplasmic space.

In the transformed yeast cell, the β-lactamase preprotein most probably is the primary translation product. We have presented evidence suggesting that the bacterial preprotein of β-lactamase has no enzymatic activity. This rules out the possibility that the preprotein is responsible for enzyme activity in yeast and indicates that some form of processing must occur. Two additional lines of evidence support this conclusion. First, we have shown in denaturing sodium dodecyl sulfate gels that the yeast β-lactamase activity has the same molecular weight as the mature *E. coli* enzyme. Second, we have demonstrated that bacterial preprotein can be processed *in vitro* to mature β-lactamase by a yeast crude extract. The molecular weight determinations in sodium dodecyl sulfate gels with internal reference did not reveal any difference in molecular weight between the yeast and *E. coli* enzymes. The signal peptide cleavage sites thus cannot differ by more than a few amino acids. The exact position of cleavage can be established only by sequencing the NH_2 terminus of the enzyme.

In connection with published reports showing correct processing of eukaryotic precursor proteins in *E. coli* (22), our findings substantiate the general notion that the processing of precursor molecules, at least as far as these are involved in membrane transport, follows a mechanism universal in prokaryotic and eukaryotic organisms.

Processing of eukaryotic preproteins in *E. coli* cells has been shown for the insulin gene products. Insulin (22) has been shown in *E. coli* to be produced as a mature protein and to be secreted through the plasma membrane. Processing and secretion in eukaryotic cells is believed to be mediated by the rough endoplasmic reticulum and concluded by the Golgi apparatus. No details are known about transport mechanisms in yeast. Though presumably the rough endoplasmic reticulum has a function similar to that in higher eukaryotes, it is not clear to what extent its function is directed to the secretion of protein molecules. A processing of the pre-β-lactamase might occur at the endoplasmic reticulum and the mature enzyme might stay in the cell.

The presence in yeast of a protein processing mechanism that recognizes the signals from heterologous systems is of importance to the study of membrane function and to research directed towards the application of yeast as the host for the expression of foreign gene products.

Although the ampicillin resistance gene cannot be used as a selective marker in yeast, it can provide a very useful marker for the presence of recombinant plasmids in yeast transformants, because β-lactamase can be directly determined in colonies (23). The functional expression in yeast of the kanamycin resistance gene on Tn*601* in pCRI (1, 5) and the chloramphenicol resistance gene of pBR325 (1, 2), on the other hand, offer a direct possibility for selection in *S. cerevisiae*. Their use seems to be especially promising for wild strains without markers.

We gratefully acknowledge Birgit Nordhaus for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

1. Hollenberg, C. P. (1979) in *Extrachromosomal DNA*, ICN–UCLA Symposia on Cellular and Molecular Biology, eds. Cummings, D., Borst, P., Dawid, S., Weissman, S. & Fox, C. F. (Academic, New York), Vol. 15, pp. 325–338.
2. Hollenberg, C. P. (1979) in *Plasmids of Medical, Environmental and Commercial Importance*, eds. Timmis, K. N. & Pühler, A. (Elsevier/North-Holland, Amsterdam), pp. 481–494.
3. Cohen, J. D., Eccleshall, T. R., Needleman, R. B., Federoff, H., Buchferer, A. B. & Marmur, J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1078–1082.
4. Panthier, J. J., Fournier, P., Heslot, H. & Rambach, A. (1980) *Curr. Genet.* 2, 109–113.
5. Jiminez, A. & Davies, J. (1980) *Nature (London)* 287, 869–871.
6. Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3737–3741.

7. Ambler, R. P. & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3732–3736.
8. Talmadge, K., Stahl, S. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3369–3373.
9. Koshland, D. & Botstein, D. (1980) *Cell* **20**, 749–760.
10. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
11. Curtiss, R. (1965) *J. Bacteriol.* **89**, 28–40.
12. Melling, J. & Scott, G. K. (1972) *Biochem. J.* **130**, 55–62.
13. O'Callaghan, C. H. & Morris, A. (1972) *Antimicrob. Agents Chemother.* **2**, 442.
14. Maurer, H. R. (1971) *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis* (de Gruyter, Berlin), pp. 44–45.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Hollenberg, C. P. (1978) *Mol. Gen. Genet.* **162**, 23–34.
17. Zubay, G., Chambers, D. A. & Cheong, L. C. (1970) in *The Lactose Operon*, eds. Beckwith, J. R. & Zipser, D. (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY), pp. 375–391.
18. Bolivar, F. (1978) *Gene* **4**, 121–136.
19. Beggs, J. D. (1978) *Nature (London)* **275**, 104–109.
20. Erhart, E. & Hollenberg, C. P. (1981) *Curr. Genet.* **3**, 83–89.
21. Hollenberg, C. P., Kustermann-Kuhn, B., Mackedonski, V. & Erhart, E. (1980) in *Molecular Genetics in Yeast*, Alfred Benzon Symposium, eds. von Wettstein, D., Friis, J., Kielland-Brandt, M. & Stenderup, A. (Munksgaard, Copenhagen), Vol. 16, in press.
22. Talmadge, K., Kaufmann, J. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3988–3992.
23. Chevalier, M. R. & Aigle (1979) *FEBS Lett.* **108**, 179–180.