Biochem. J. (1985) 231, 145–149 (Printed in Great Britain)

Propanediol oxidoreductases of *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*

Aspects of interspecies structural and regulatory differentiation

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The enzyme propanediol oxidoreductase, which converts the lactaldehyde formed in the metabolism of fucose and rhamnose into propane-1,2-diol under anaerobic conditions, was investigated in *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. Structural analysis indicated that the enzymes of *E. coli* and *K. pneumoniae* have the same M_r and pI, whereas that of *Salm. typhimurium* also has the same M_r but a slightly different pI. One-dimensional peptide mapping showed identity between the *E. coli* and *K. pneumoniae* enzymes when digested with α-chymotrypsin, *Staphylococcus aureus* V8 proteinase or subtilisin. In the case of *Salm. typhimurium*, this held only for the subtilisin-digested enzymes, indicating that the hydrophobic regions were preserved to a considerable extent. Anaerobically, the three species induced an active propanediol oxidoreductase when grown on fucose or rhamnose. An inactive propanediol oxidoreductase was induced in *Salm. typhimurium* by either fucose or rhamnose under aerobic conditions, and this was activated once anaerobiosis was established. An inactive propanediol oxidoreductase was also induced in *E. coli* under aerobic conditions, but only by growth on fucose. The inactive enzyme was not induced by either of the sugars in *K. pneumoniae*.

INTRODUCTION

Propanediol oxidoreductase is the key enzyme in the fermentation of fucose and rhamnose by cells of *Escherichia coli* (Cocks *et al.*, 1974; Boronat & Aguilar, 1979). The sugars are metabolized by the sequential action of an isomerase (Green & Cohen, 1956; Takagi & Sawada, 1964*a*), a kinase (Heath & Ghalambor, 1962; Takagi & Sawada, 1964*b*) and an aldolase (Ghalambor & Heath, 1962; Chiu & Feingold, 1969), which yield, in each case, 1 mol of dihydroxyacetone phosphate and 1 mol of L-lactaldehyde per mol of sugar phosphate cleaved.

Aerobically, both products of the aldolase-catalysed reaction are introduced in the general metabolism: dihydroxyacetone phosphate via the glycolytic pathway and L-lactaldehyde by its oxidation to lactate by the action of lactaldehyde dehydrogenase (Sridhara & Wu, 1969). Anaerobically, L-lactaldehyde has to be reduced to propane-1,2-diol by the action of propanediol oxidoreductase to balance the NAD reduction in the absence of O_2 (Cocks *et al.*, 1974).

Fucose and rhamnose metabolism thus converge, after the aldolase cleavage, into a common pathway. Both pathways have their sets of specific enzymes, although it is not clear whether those enzymes belonging to the common sections of the pathway, namely lactaldehyde dehydrogenase and propanediol oxidoreductase, are also duplicated (Ros & Aguilar, 1984; Chen & Lin, 1984*b*).

Propanediol oxidoreductase activity is induced during the anaerobic growth of cells by either of the two methylpentoses, although, surprisingly, fucose but not rhamnose is also able to induce an inactive propanediol oxidoreductase aerobically (Boronat & Aguilar, 1981). The aerobic enzyme is activated by means of a post-transcriptional mechanism so far not determined, once the anaerobic condition is established (Chen *et al.*, 1983; Chen & Lin, 1984*a*).

Salmonella typhimurium and *Klebsiella pneumoniae* are known to ferment fucose and rhamnose, and it has been shown that the fermentation mechanism operates in these two species as described in *E. coli:* through propane-1,2-diol production and excretion with the concomitant induction of propanediol oxidoreductase activity (Badía *et al.*, 1985).

MATERIALS AND METHODS

Bacteria

The *Escherichia coli* used in this study was a K12 strain also known as E15 (Bachmann, 1972). *Salmonella typhimurium* LT2 was obtained from A.T.C.C. (reference E 23564). *Klebsiella pneumoniae* C3, formerly *Citrobacter intermedius* C3 (Imperial *et al.*, 1982), was given to us by Dr. J. Guinea, Department of Microbiology, School of Pharmacy, University of Barcelona.

Chemicals

L-Lactaldehyde was prepared and purified in our laboratory as described previously (Boronat & Aguilar, 1979). L-Rhamnose, L-fucose, NADH, subtilisin and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *Staphylococcus aureus* V8 proteinase was from Miles Laboratories, Stoke Poges, Slough, Berks., U.K., and α-chymotrypsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride was obtained from Carlo Erba, Milan, Italy. Sodium deoxycholate was from BDH Chemicals, Poole, Dorset, U.K., and Nonidet P40 from Fluka, Buchs, Switzerland. Ampholines were from Pharmacia

Fine Chemicals, Uppsala, Sweden, and the Bradford protein assay and electrophoresis materials were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other chemicals were of the purest grade available from commercial sources.

Growth of cells and preparation of cell extracts

Carbon sources were added to a basal inorganic medium (Boronat & Aguilar, 1979) at 0.01 M concentration for aerobic growth and 0.02 M for anaerobic growth. Aerobic growth was carried out at 37 °C in 2-litre Erlenmeyer flasks, partially filled (200 ml each), and with strong shaking. Anaerobic growth was carried out at the same temperature in flasks completely filled and gently stirred magnetically. Growth was monitored at 420 nm.

Cells were harvested at the end of the exponential phase by centrifugation, washed in 10 mM-Tris/HCl buffer, pH 7.3, and suspended in 4 times their wet weight of the same buffer. The suspension was sonically disrupted in an MSE sonicator, set at an amplitude of 18–24 μm for periods of 30 s/ml of cell suspension in a tube chilled to 0 °C. After centrifugation at 100000 *g* for 60 min at 4 °C (to remove NADH oxidase) the supernatant fraction was used for enzyme assays.

Assay procedures

Spectrophotometric assay of propanediol oxidoreductase activity was performed at 25 °C by monitoring the conversion of NADH into NAD^+ (absorbance decrease at 340 nm) in the presence of lactaldehyde. The activity was measured in an assay mixture that consisted of 2.5 mM-L-lactaldehyde, 100 mM-sodium phosphate buffer, pH 7.0, and 0.125 mM-NADH. The C_3 substrate was omitted from the blank control mixture. All reactions were started by addition of the enzyme. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of substrate/min.

Concentration of the protein in cell extracts was determined by the method of Lowry *et al.* (1951) or by the method of Bradford (1976), as indicated; bovine serum albumin was used as standard.

Immunological techniques

Antisera against propanediol oxidoreductase were obtained as described previously (Boronat & Aguilar, 1981). For enzyme purification procedures (see below), the antibodies were partially purified. The immunoglobulin fraction of the serum was precipitated by 35% saturation with $(NH_4)_2SO_4$, centrifuged for 30 min at 10000 *g* and the pellet resuspended in one-half its original volume of 0.15 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.5. This immunoglobulin preparation was dialysed exhaustively against the same buffer and stored at −20 °C. Quantitative immunoelectrophoresis was performed as described by Laurell (1966). Crossed immunoelectrophoresis and crossed immunoelectrofocusing were performed as described by Chua & Blomberg (1979) and Smith *et al.* (1977) respectively.

Peptide mapping

For peptide mapping, propanediol oxidoreductase was purified by immunoprecipitation of the enzyme in the crude extract of each strain. The incubation mixture contained 100 mM-NaCl, 0.1% (v/v) Nonidet P40, 1% (w/v) sodium deoxycholate, 1 mM-phenylmethanesulphonyl fluoride and appropriate amounts of crude extract and globulins (specific antibodies). The immunoprecipitates were spun down in an Eppendorf centrifuge, washed five times with 0.15 M-NaCl and finally resuspended in the electrophoresis sample buffer (Laemmli, 1970). After electrophoresis, the bands were detected by immersing the gel in 10 vol. of 1 M-KCl in cold water. Bands of propanediol oxidoreductase were sliced out of the gel and eluted electrophoretically into a dialysis bag (Stephens, 1975). The contents of each bag were precipitated with 9 vol. of cold acetone (−20 °C) to remove the excess of sodium dodecyl sulphate, and the protein was dansylated by the procedure of Tijssen & Kurstak (1979).

Portions (10 μg) of dansylated propanediol oxidoreductase were subjected to limited proteolysis at 37 °C for 25 min (Cleveland *et al.*, 1977) with either 1 μg of *Staph. aureus* V8 proteinase, 0.25 μg of α-chymotrypsin or 0.003 μg of subtilisin. The peptides were resolved in a 15–20% (w/v) polyacrylamide-gel gradient and the bands were photographed under long-wave u.v. light.

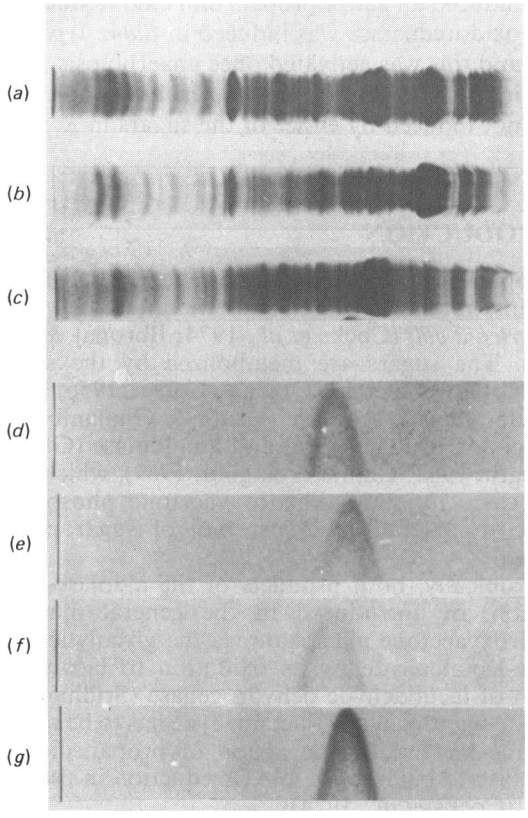

Fig. 1. Crossed immunoelectrophoresis of propanediol oxidoreductases from *E. coli*, *K. pneumoniae* and *Salm. typhimurium*

A sodium dodecyl sulphate/10%-(w/v)-polyacrylamide-gel electrophoresis of crude extracts of *E. coli* (*a*), *K. pneumoniae* (*b*) and *Salm. typhimurium* (*c*) containing 125 μg of total protein and stained with Coomassie Blue are shown in the upper part of the Figure. Parallel unstained strips containing 25 μg of total protein were immunoelectrophoresed with 1.5% (v/v) of the *E. coli* propanediol oxidoreductase antibodies, and are shown in (*d*), (*e*) and (*f*) respectively. In the bottom-most immunoelectrophoresis gel (*g*) a mixture of equal parts of each extract amounting to a total of 25 μg of protein was run.

RESULTS AND DISCUSSION

Comparative structural analysis

Cells of *E. coli*, *K. pneumoniae* or *Salm. typhimurium* were grown anaerobically on rhamnose. Extracts were prepared as indicated in the Materials and methods section and subjected to a crossed immunoelectrophoresis against anti-(*E. coli* propanediol oxidoreductase) antibodies. A single 'rocket' was formed with a protein that in each case had the same M_r (Fig. 1) and corresponded to that of a standard of purified *E. coli* propanediol oxidoreductase (Boronat & Aguilar, 1979) with M_r 39000 (results not shown). Moreover, if a mixture of the extracts, one-third of each, was applied to the same system, a single 'rocket' was again apparent, indicating the absence of molecular species with different M_r.

When these same extracts were subjected to a crossed immunoelectrofocusing, where the total amount of protein was separated by electrofocusing between pH 3.5 and 5.5 instead of by electrophoresis, a 'rocket' formed at a pI of 4.7 for the enzymes of *E. coli* and *K. pneumoniae*. *Salm. typhimurium* gave, in contrast, a 'rocket' at the significantly different pI of 4.3 (Fig. 2).

Further structural comparison of the three propanediol oxidoreductases was made by developing the one-dimensional peptide mapping for each enzyme. For this, propanediol oxidoreductase from each species was purified to homogeneity by immunological precipitation and electrophoresis (see the Materials and methods section). Purity was assessed by gel electrophoresis with either purified propanediol oxidoreductase (45 μg) of each strain or a mixture of them (15 μg each). A unique band was apparent under such conditions. The purified and dansylated enzymes were then digested with three different proteinases, *Staph. aureus* V8 proteinase, α-chymotrypsin and subtilisin, and the peptides obtained were resolved by electrophoresis in a polyacrylamide-gel gradient as indicated above (Fig. 3).

Band patterns obtained with the enzymes of *E. coli* or *K. pneumoniae* were indistinguishable, no matter which proteinase was used in the corresponding digestion; this indicates a high degree of similarity between these two proteins. The enzyme of *Salm. typhimurium* showed, in contrast, a clearly differentiated band pattern when digested with *Staph. aureus* V8 proteinase or α-chymotrypsin. However, when the enzyme was digested with subtilism, no difference whatsoever was apparent among the patterns obtained with any of the three species (Fig. 3). Structural comparison by these methods of the fucose-induced enzyme offered results indistinguishable from that obtained with the rhamnose-induced enzyme.

The experiment presented in Fig. 1 shows that the enzymes from these species cannot be distinguished from each other by their molecular mass. However, the possibility of differences in the sequences remained and

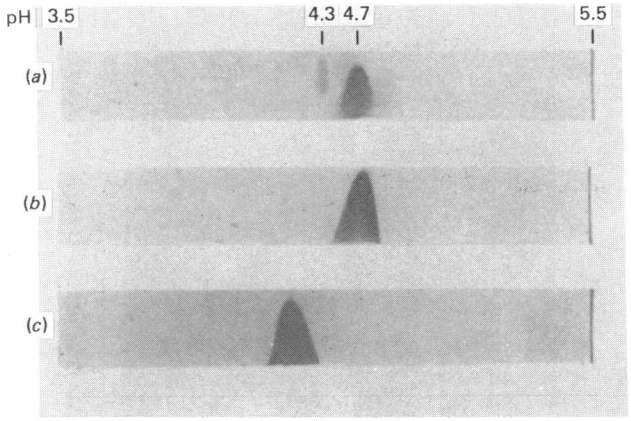

Fig. 2. Crossed immunoelectrofocusing of propanediol oxidoreductases from *E. coli*, *K. pneumoniae* and *Salm. typhimurium*.

Crude extract proteins were separated by isolectric focusing in a pH gradient from 3.5 to 5.5. Unstained strips containing 25 μg of total protein were subsequently immunoelectrophoresed with 1.5% (v/v) of anti-(*E. coli* propanediol oxidoreductase) antibodies. 'Rockets' of immunoprecipitation formed at the pI of the propanediol oxidoreductase from *E. coli* (*a*), *K. pneumoniae* (*b*) and *Salm. typhimurium* (*c*).

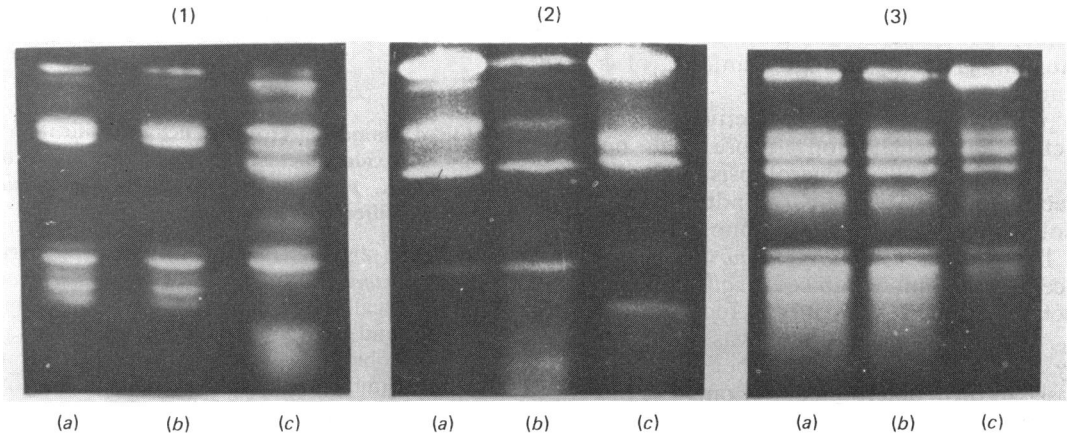

Fig. 3. Peptide mapping of propanediol oxidoreductases from *E. coli*, *K. pneumoniae* and *Salm. typhimurium*

A comparative study by one-dimensional peptide mapping of the purified propanediol oxidoreductases from *E. coli* (*a*), *K. pneumoniae* (*b*) and *Salm. typhimurium* (*c*) is presented for three different digestions of the enzyme. Panels show the band pattern corresponding to the peptides obtained by limited digestion of the enzymes with *Staph. aureus* V8 proteinase (1), α-chymotrypsin (2) or subtilisin (3).

was subsequently proved for the *Salm. typhimurium* enzyme by the electrofocusing experiment of Fig. 2. These differences in the amino acid composition and sequence of the enzyme of *Salm. typhimurium* were further confirmed by one-dimensional peptide mapping when digestion was performed with α-chymotrypsin or *Staph. aureus* V8 proteinase (Fig. 3).

The fact that no differences were found in the band pattern of the one-dimensional peptide mapping when the digestion was performed with subtilisin indicates that, in spite of the differences in the *Salm. typhimurium* enzyme, there is a strong conservation for the clusters of hydrophobic amino acids that act in the polypeptide chain as targets for this proteinase. According to McConkey's (1982) hypothesis on the quinary structure of proteins, these hydrophobic regions might be needed for the relationship between the enzyme and other cellular structures, such as membranes.

The decrease in antigenic determinants in *Salm. typhimurium* propanediol oxidoreductase as compared with the *E. coli* enzyme (Badía *et al.*, 1985) against which antibodies were prepared is in accordance with the changes in certain regions of the polypeptide chain and consequently in the structure of the protein. Immunological similarities between the *E. coli* and the *K. pneumoniae* enzymes are not surprising, in view of the structural similarities between these two proteins, although for unknown reasons the enzymic activities differ considerably, as discussed elsewhere (Badía *et al.*, 1985).

Control of propanediol oxidoreductase

To study the characteristics of the induction of propanediol oxidoreductase, the cells were grown aerobically and anaerobically on fucose and rhamnose. Extracts of each culture were prepared, and determination of the enzymic activity and immunological quantification of the enzyme were performed in all cultures. Of course, the immunological dissimilarities between *Salm. typhimurium* propanediol oxidoreductase and the enzymes of the other two species had to be taken in account for the immunological quantification.

A propanediol oxidoreductase protein calibration (not shown) permitted us to confirm the proportionality between the amount of protein and the area of the 'rocket', as well as to estimate a correction factor for the *Salm. typhimurium* protein; this gave a 'rocket' 30% longer than that given by the same amount of *E. coli* protein (Lamy *et al.*, 1983).

E. coli produced an induced inactive propanediol oxidoreductase aerobically on fucose but not on rhamnose. In these cells, anaerobiosis activated the inactive fucose-induced enzyme or induced an active propanediol oxidoreductase on rhamnose (Fig. 4 and Table 1). In contrast, *Salm. typhimurium* seemed to produce a certain amount of induced inactive propanediol oxidoreductase aerobically not only on fucose but also on rhamnose, whereas the enzyme was not induced aerobically by *K. pneumoniae* on either of the sugars. Anaerobiosis, in turn, determined, not only the presence of propanediol oxidoreductase 'rocket' in all cultures, but that of its enzymic activity, which increased at least 4–5-fold as compared with the aerobic basal activities (Fig. 4 and Table 1).

As shown, aerobic growth on fucose induces in *E. coli* an inactive propanediol oxidoreductase that becomes activated when anaerobic conditions are established. This indicates that the activity of the fucose-induced enzyme is under post-transcriptional control (Boronat & Aguilar, 1981; Chen & Lin, 1984*a*). In contrast, the activity of the rhamnose-induced enzyme is under transcriptional

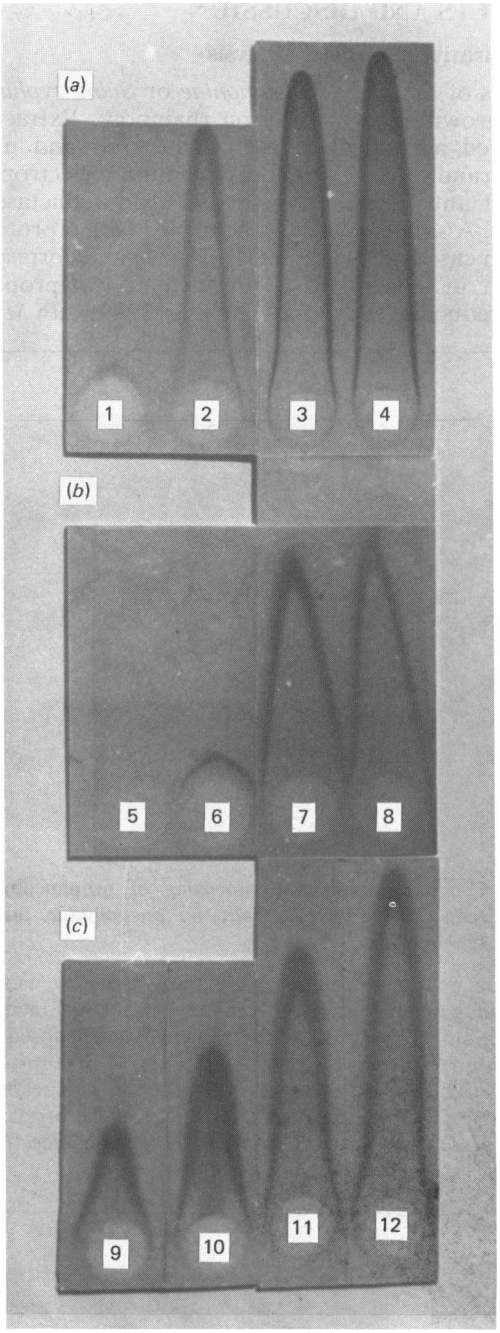

Fig. 4. Immunoelectrophoretic quantification of propanediol oxidoreductase protein in extracts of *E. coli*, *K. pneumoniae* and *Salm. typhimurium* grown under different conditions

Crude extracts (25 μg of total protein) of *E. coli* (*a*), *K. pneumoniae* (*b*) and *Salm. typhimurium* (*c*) cells grown aerobically on rhamnose (1, 5 and 9), aerobically on fucose (2, 6 and 10), anaerobically on rhamnose (3, 7 and 11) and anaerobically on fucose (4, 8 and 12) were applied to wells in an immunoelectrophoresis gel that contained 1.5% (v/v) of anti-(*E. coli* propanediol oxidoreductase) antibodies.

Table 1. Quantification of propanediol oxidoreductase activity in extracts of cells of the three species grown in different conditions

For experimental details see the text.

		Specific activity (units/mg of protein)			
	Growth condition ...	Aerobic		Anaerobic	
Strain	Carbon source	Rhamnose	Fucose	Rhamnose	Fucose
E. coli		0.05	0.12	0.60	0.65
K. pneumoniae		0.03	0.05	0.14	0.15
Salm. typhimurium		0.04	0.12	0.44	0.46

control (Boronat & Aguilar, 1981). Interestingly, *Salm. typhimurium*, which has high concentrations of propanediol oxidoreductase protein when grown aerobically, displays a post-transcriptional regulation for both fucose-induced and rhamnose-induced enzymes, whereas the *K. pneumoniae* enzyme displays transcriptional control when the cells are grown on either methylpentose.

It would seem that a post-transcriptional regulatory mechanism would be more advantageous and correspond to a more advanced evolutive step than transcriptional mechanism. The high efficiency in 'tuning' the enzymic activity of a permanently present protein by this mechanism, especially in cases where changes in nutrient availability may require a precise and rapid adaptation, would support this hypothesis. Nevertheless, in terms of cell economy, the permanent synthesis of an inactive protein is wasteful for cells subjected to progressive impoverishment of their environment. Under these circumstances the maintenance of a permanent enzymic endowment to be activated when required would be disadvantageous for the cell and would induce a selective pressure to evolve a transcriptional rather than a post-transcriptional control mechanism.

We thank Dr. G. Lorén and Dr. M. Viñas for helpful discussion and Laboratorios Ferrer Internacional for co-operation in the preparation of the enzyme antibody. This work was supported by Grant no. 1619 of the Comisión Asesora de Investigación Científica y Técnica, Spain.

REFERENCES

Bachmann, B. J. (1972) Bacteriol. Rev. **36**, 525–557
Badía, J., Ros, J. & Aguilar, J. (1985) J. Bacteriol. **161**, 435–437
Boronat, A. & Aguilar, J. (1979) J. Bacteriol. **140**, 320–326
Boronat, A. & Aguilar, J. (1981) J. Bacteriol. **147**, 181–185
Bradford, M. (1976) Anal. Biochem. **72**, 248–254
Chen, Y. M. & Lin, E. C. C. (1984*a*) J. Bacteriol. **157**, 341–344
Chen, Y. M. & Lin, E. C. C. (1984*b*) J. Bacteriol. **157**, 828–832
Chen, Y. M., Lin, E. C. C., Ros, J. & Aguilar, J. (1983) J. Bacteriol. **129**, 3355–3362
Chiu, T. H. & Feingold, D. S. (1969) Biochemistry **8**, 98–108
Chua, N. H. & Blomberg, F. (1979) J. Biol. Chem. **254**, 215–223
Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106
Cocks, G. T., Aguilar, J. & Lin, E. C. C. (1974) J. Bacteriol. **118**, 83–88
Ghalambor, M. A. & Heath, E. C. (1962) J. Biol. Chem. **237**, 2427–2433
Green, M. & Cohen, S. S. (1956) J. Biol. Chem. **219**, 557–568
Heath, E. C. & Ghalambor, M. A. (1962) J. Biol. Chem. **237**, 2423–2426
Imperial, J., Parés, R. & Cole, J. A. (1982) FEMS Microbiol. Lett. **13**, 247–252
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lamy, J., Compin, S. & Lamy, J. N. (1983) Arch. Biochem. Biophys. **223**, 584–603
Laurell, C. B. (1966) Anal. Biochem. **15**, 45–52
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
McConkey, E. H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3236–3240
Ros, J. & Aguilar, J. (1984) J. Gen. Microbiol. **130**, 687–692
Smith, C. J., Söderholm, J. & Wadstrom, T. (1977) Application Note 269, LKB Produkter, Stockholm
Sridhara, S. & Wu, T. T. (1969) J. Biol. Chem. **244**, 5233–5238
Stephens, R. E. (1975) Anal. Biochem. **65**, 369–379
Takagi, Y. & Sawada, H. (1964*a*) Biochim. Biophys. Acta **92**, 10–17
Takagi, Y. & Sawada, H. (1964*b*) Biochim. Biophys. Acta **92**, 18–25
Tijssen, P. & Kurstak, E. (1979) Anal. Biochem. **99**, 97–104

Received 29 November 1984/28 May 1985; accept 10 June 1985