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In Vivo Regulation of Chromosomal β-Lactamase in *Escherichia coli*

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Chromosomal β-lactamase, a periplasmic enzyme of *Escherichia coli*, was studied with respect to its regulation in vivo. Both the activity and the amount of β-lactamase increased with growth rate. During a nutritional shift-down, chromosomal β-lactamase activity followed stable ribonucleic acid accumulation. After a nutritional shift-up the differential rate of β-lactamase synthesis did not increase immediately (like stable ribonucleic acid), but it did increase after a lag period of 30 min. To determine whether β-lactamase was under stringent control, strains carrying a temperature-sensitive valyl-transfer ribonucleic acid synthetase and differing only in the allelic state of the *relA* gene were shifted from a permissive to a semipermissive temperature. No influence by the *relA* gene product was found on β-lactamase synthesis. The regulation of this periplasmic enzyme is discussed in relation to that of some components of the translational apparatus.

It has been clearly demonstrated that a large number of proteins in *Escherichia coli* are coordinately regulated in that their differential rate of synthesis increases with growth rate in much the same manner as ribosomal RNA (26). This group includes such components of the translational machinery as ribosomal proteins, elongation factors Tu and G, and certain aminoacyl-tRNA synthetases (25, 26). Maaløe has termed the genes specifying these proteins as the "core" (O. Maaløe, *in* R. F. Goldberger, ed., *Protein Synthesis and Development*, in press). From the work of Pedersen et al. (26) it appears, however, that many genes outside the core are expressed in a manner similar to the expression of core operons. The function of none of these proteins is known. Two general types of mechanisms have been described for the regulation of the core genes, namely active and passive mechanisms. The former are those models in which some regulatory element, e.g., guanosine tetraphosphate, influences the transcription of the genes in a direct manner. A passive mechanism would be, for example, one in which the overall pattern of transcription of the genome indirectly influences the availability of the transcription for the core genes (17).

Gram-negative bacteria export two kinds of proteins, the proteins of the outer membrane and the soluble proteins located between the outer and inner membranes in a region called the periplasmic space. It has been demonstrated that at least some of these proteins are synthesized from membrane-bound polysomes (10, 11, 27). As has been shown in eucaryotic systems (3), the immediate translation product for export proteins has a molecular weight greater than that of the final mature protein (10). The chromosomal β-lactamase of *E. coli* is a periplasmic enzyme, the activity of which has been found to be positively correlated with growth rate (4). The structural gene for this enzyme, *ampC*, is apparently expressed constitutively; i.e., no inducers or effector molecules have been demonstrated to affect the synthesis of β-lactamase. Moreover, β-lactamase synthesis is strictly dependent on gene copy number (23, 24).

In this study we examined the in vivo regulation of chromosomal β-lactamase. We show that the synthesis of this enzyme is not affected by the stringent control system. Nevertheless, chromosomal β-lactamase exhibits a regulation pattern similar in some respects to that of the core proteins.

MATERIALS AND METHODS

Organisms. Strain LA51 (*pyrB thr leu his rpsL ampA1 ampC*$^+$) is an *ampA1* transductant of the *E. coli* K-12 strain PA2004 (R. Lavallé). The *ampA1* mutation increases the β-lactamase production by a factor of 30 compared with wild type. Strains BJ1, BJ2, and BJ3 were constructed by transducing the *ampA1* allele with P1*clr*100*cml* (20) into NF314 (*leu valS*$^+$ *relA*$^+$), NF536 [*leu valS*(Ts) *relA*$^+$], and NF537 [*leu valS*(Ts) *relA*], respectively. These strains are isogenic derivatives of *E. coli* B strain AS19 and were kindly provided by N. Fiil, Department of Microbiology, University of Copenhagen, Copenhagen, Denmark.

Media and growth conditions. Bacteria were in

most experiments grown in potassium morpholinopropane sulfonate (MOPS) medium (22) supplemented with 100 μg of the L isomer of each required amino acid per ml, 25 μg of uracil per ml, and 1.32 mM K_2HPO_4. The carbon sources were as follows: 0.4% glycerol, 0.4% glucose, or 0.4% glucose–0.2% Casamino Acids. Minimal medium E (30) was supplemented with 1 μg of thiamine per ml, 100 μg of the L isomer of each required amino acid per ml, 25 μg of uracil per ml, and 0.2% glucose or 0.2% glucose–0.2% Casamino Acids. LB medium of Bertani (1) was supplemented with medium E, 1 μg of thiamine per ml, and 0.2% glucose. All cultures were grown aerobically on a rotary shaker in Erlenmeyer flasks with a minimum ratio of culture volume to flask volume of 1:5. To obtain balanced growth, the cells were pregrown at the respective temperature in the same medium for at least 10 generations before each experiment. Bacterial mass was measured by optical density at 450 nm (OD_{450}) in a Zeiss PMQII spectrophotometer, using a 1-cm light path. The OD_{450} was always kept below 1.0. Samples of growing cultures were removed and diluted with medium to obtain readings in the range of 0.05 to 0.50 absorbance unit. Growth rates are expressed as k, the first-order constant, in units of $hour^{-1}$, as calculated from the expression $k = \ln 2/\text{mass doubling time in hours}$. Unless otherwise stated, the experiments were performed at 37°C.

In the shift-down experiment, 1 volume of cells was grown in MOPS–glucose–Casamino Acids to an OD_{450} of 0.5 and filtered through a sterile Nalgene membrane filter. The cells were rinsed with prewarmed MOPS–glycerol, and the filter was cut out with a scalpel and resuspended in 2 volumes of prewarmed MOPS–glycerol. The flask was vigorously shaken for about 10 s, and the filter was taken away.

Measurement of protein and RNA. Samples of 5 ml from the culture were made 5% in ice-cold trichloroacetic acid. After standing overnight at 4°C, the samples were centrifuged. The precipitates were washed once in 3 ml of ice-cold 5% trichloroacetic acid and then resuspended in 1 M NaOH and incubated at 37°C for 60 min. The protein content was determined by the method of Lowry et al. (16), using bovine serum albumin as a standard. The amount of RNA was measured by the orcinol procedure of Schneider (28), using yeast RNA as a standard.

Preparation of enzyme extracts. At different times, 20 ml of the culture was rapidly chilled, and the cells were collected by centrifugation at 12,000 × g for 10 min at 4°C. The cells were washed once in 5 ml of 50 mM Tris-hydrochloride buffer (pH 7.4) and resuspended in 1 ml of the same buffer. The cells were broken by sonic disruption with a Branson B12 Sonifier (six times, 15 s each) at a power setting of 5. The extracts were either assayed immediately for enzyme activities and protein content or frozen.

Determination of β-lactamase activity. β-Lactamase activity was determined as previously described (15). One unit of β-lactamase activity was defined as the enzyme activity that hydrolyzed 1 μmol of benzylpenicillin per h in 0.05 M phosphate buffer (pH 7.4) at 37°C. Specific β-lactamase activity was expressed as units per milligram of protein.

Immunoelectrophoresis of β-lactamase. The immunoelectrophoresis method of Laurell (12) was used with some minor modifications. Agarose was dissolved in a barbital-glycine-Tris buffer (pH 8.2) (29) at a concentration of 1% (wt/vol). This pH was used because of the high isoelectric point for β-lactamase (pI = 9.9 [unpublished data]). Ammonium sulfate-precipitated rabbit antiserum prepared against purified β-lactamase (14) was added at 45°C. Gels were prepared on glass plates (8 by 8 cm), and the electrophoresis was run overnight at 40 V/plate. The gels were washed, dried, stained, and destained. Cell extracts were prepared in the following way. Bacteria were grown in steady-state conditions to an OD_{450} of 1.0, and enzyme extracts were prepared from a 40-ml culture. The procedure was as described above for the preparation of enzyme extracts, with the exception that the cells were treated with a lysozyme–EDTA solution (100 μg/ml and 1 mM, respectively) and were sonically disrupted 12 times (15 s each). The same amount of protein was applied to each well in the agarose gel. The area under the precipitation line was taken as a relative value of the amount of β-lactamase.

Materials. All chemicals were of the highest grade commercially available. Benzylpenicillin (penicillin G) was kindly provided by AB Astra, Södertälje, Sweden. Zulkowsky starch (used in β-lactamase activity determinations) was from E. Merck AG, Darmstadt, Germany. MOPS and yeast RNA type II were purchased from Sigma Chemical Co., St. Louis, Mo. The Nalgene filter used in the shift-down experiment had a pore size of 0.20 μm and was from Nalge Sybron Corp., Rochester, N.Y.

RESULTS

Chromosomal β-lactamase levels in different media. Cultures of strain LA51 (*ampA1 ampC*$^+$) were harvested during steady-state growth in various media, and the activity of chromosomal β-lactamase was examined. Specific activity of chromosomal β-lactamase increased as a function of growth rate (Fig. 1). Thus, within a k range of 0.4 to 1.6 there was an increase in specific activity of approximately threefold. The amount of β-lactamase produced as measured by immunoelectrophoresis showed a good correlation with specific activity. It can therefore be concluded that the observed increase in specific activity was due to an increased amount of β-lactamase produced.

Changes in β-lactamase activity after a nutritional shift-up. Cells of strain LA51 were grown at steady-state conditions in MOPS–glycerol and shifted to MOPS–glucose–Casamino Acids. β-Lactamase activity and stable RNA and protein accumulation were followed. As expected, an immediate increase of stable RNA synthesis was found after the shift (Fig. 2a). Protein continued to accumulate after the shift at the preshift rate for about 30 min and then gradually increased to the postshift rate of synthesis. β-Lactamase exhibited a pattern similar

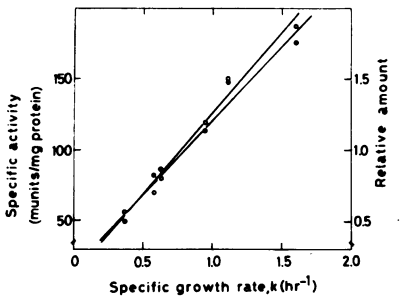

FIG. 1. *Levels of β-lactamase at different growth rates in E. coli K-12. The specific activity and the relative amount of β-lactamase is plotted as a function of the first-order constant for growth (k). The values clustered at k = 0.37 are for cells growing in MOPS-glycerol; k = 0.58, medium E plus glucose; k = 0.63, MOPS-glucose; k = 0.95, medium E plus glucose plus Casamino Acids; k = 1.11, MOPS-glucose-Casamino acids; k = 1.60, LB medium. Symbols: ○, specific activity of β-lactamase in LA51 (ampA1 ampC⁺); ●, relative amount of β-lactamase (measured by immunoelectrophoresis) in the same strain.*

to the accumulation of protein (Fig. 2b). The final postshift rate correlated well with the steady-state value for the same medium (Fig. 1 and data not shown). The differential rates of synthesis for stable RNA as well as for β-lactamase are given in Fig. 3. After the shift, stable RNA, unlike chromosomal β-lactamase, showed an immediate increase in the differential rate of synthesis.

Changes of β-lactamase activity after a nutritional shift-down. Cells of strain LA51 were grown at steady-state conditions in MOPS-glucose-Casamino Acids and shifted by filtration to MOPS-glycerol. Figure 4 demonstrates the accumulation of the same three parameters as given in Fig. 2. The onset of accumulation of protein and stable RNA appeared 90 and 200 min after the shift, respectively (Fig. 4a). This is the classical response after a nutritional shift-down, as described by Maaløe and Kjeldgaard (18). Interestingly, β-lactamase showed a response similar to that of stable RNA. Thus, the onset of accumulation of β-lactamase appeared much later than that of the bulk of proteins.

Is the *rel* system involved in the regulation of β-lactamase? It was of interest to determine whether chromosomal β-lactamase was influenced by the *rel* system. The AmpA phenotype introduced into three isogenic strains of *E. coli* B, NF314 (*relA*⁺ *valS*⁺), NF536 [*relA*⁺ *valS*(Ts)], and NF537 [*relA valS*(Ts)], gave the *ampA1* derivatives BJ1, BJ2, and BJ3, respectively. In the *valS*(Ts) strains, the level of charged $tRNA^{val}$ (i.e., valyl $tRNA^{val}$) decreases at a growth temperature above 32°C. It is known

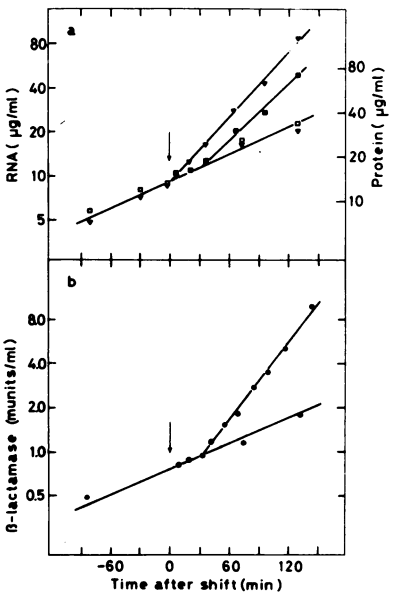

FIG. 2. *Nutritional shift-up in strain LA51. Cells were grown in MOPS-0.4% glycerol to an OD_{450} of 0.25, and part of the culture was added to an equal volume of prewarmed medium consisting of MOPS, 0.4% glucose, and 0.2% Casamino Acids. (a) Accumulation of stable RNA (▽, ▼) and protein (□, ■) as a function of time. (b) Accumulation of β-lactamase (○, ●) as a function of time. The arrows indicate the time when the culture was shifted. Open symbols indicate samples taken from the preshift medium, and closed symbols indicate samples taken from the postshift medium.*

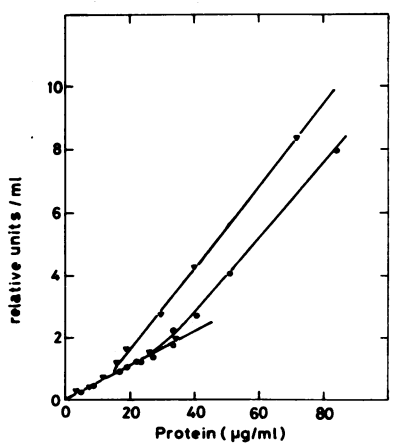

FIG. 3. *Differential rate of accumulation of stable RNA (▽, ▼) and β-lactamase (○, ●) in a nutritional shift-up in LA51. The experimental procedure was the same as that described in the legend to Fig. 2. Open and closed symbols indicate samples taken from preshift and postshift media, respectively.*

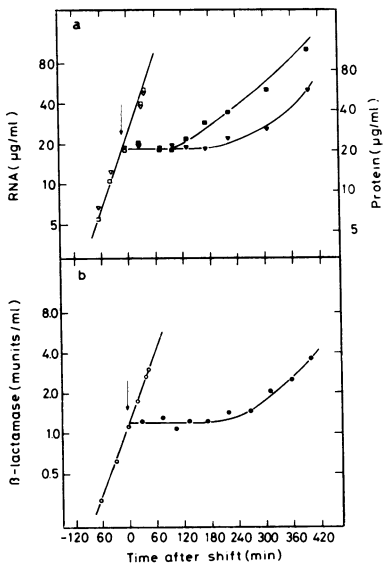

FIG. 4. *Nutritional shift-down in strain LA51. Cells were grown in MOPS–0.4% glucose–0.2% Casamino Acids to an OD_{450} of 0.5 and shifted to MOPS–0.4% glycerol as described in the text. (a) Accumulation of stable RNA (▽, ▼) and protein (□, ■) as a function of time. (b) Accumulation of β-lactamase (○, ●) as a function of time. Open and closed symbols indicate samples taken from preshift and postshift media, respectively.*

that deprivation of aminoacylated tRNA, i.e. accumulation of uncharged tRNA, allows the accumulation of stable RNA in the strain carrying the *relA* allele but not in the strain carrying the *relA*$^+$ allele. Steady-state cultures were grown in MOPS–glucose at the permissive temperature (29.5°C) and shifted to the semipermissive temperature (35.5°C), which allows a reduced level of protein synthesis. Accumulation of protein and stable RNA and activity of β-lactamase were followed. In the parental strain (BJ1), there was an immediate increase in the rate of accumulation of both stable RNA and protein after the shift (Fig. 5 and Table 1). Neither BJ2 [*relA*$^+$ *valS*(Ts)] nor BJ3 [*relA valS*(Ts)] showed any increase in the rate of accumulation of protein after the shift to 35.5°C. Instead, there was a slight decrease in protein synthesis. The rate of accumulation of stable RNA in BJ2 was reduced by 20% but increased in the *relA* strain by 30% relative to the rates before the temperature shift.

Figure 6 shows the activity of β-lactamase plotted against total protein concentration when cells were shifted from 29.5°C to the semipermissive temperature of 35.5°C. There was a rapid decrease in enzyme activity, and the new steady-state rate of production was not achieved until after 10 to 15 min. We have at present no explanation to this decrease in activity. During the entire experiment no enzyme activity was released into the medium. The differential rate of β-lactamase synthesis that was reached after the shift was slightly lower than the preshift value in the parental strain BJ1 (0.8 unit), but even more so in the *valS*(Ts) strains BJ2 and BJ3 (0.5 and 0.4 unit, respectively) (Fig. 6 and Table 1). Thus, no clear difference in the differential rate of synthesis of β-lactamase was found as a consequence of the *relA* allele.

DISCUSSION

In this study the in vivo regulation of an *ampA1* mutant derepressed for β-lactamase was examined. The wild-type level of β-lactamase is too low to be accurately monitored. It appears, however, that the *ampA1* mutation does not affect the dependency on growth rate, but derepresses the operon by the same factor irrespective of growth rate (data not shown). The *ampA1* mutation leads to an increased transcription of β-lactamase mRNA and does not affect the half-life of β-lactamase mRNA (unpublished data). The mutation *ampA1* is in close proximity to the structural gene *ampC* and is located on the same *Eco*RI fragment (unpublished data). We therefore suggest that *ampA1* is an up-promoter mutation or a mutation within a possible attenuator region.

It has been suggested by Maaløe that the chromosomal β-lactamase shows a pattern of regulation which is similar to that of stable RNA and proteins involved in the protein synthesis machinery, the so-called core components (Maaløe, in press). Thus, the level of chromosomal β-lactamase shows a dependence on growth rate similar to that seen for ribosomal proteins, rRNA, elongation factors G and Tu, certain aminoacyl-tRNA synthetases, and a number of unidentified proteins separated on two-dimensional gel electrophoresis (25, 26).

The relative copy number of the *ampC* gene is approximately 1.3 times higher in rich media than in poor media when the type of calculation employed by McKeever and Neidhardt is used (19). Thus, gene dosage effects cannot explain the observed threefold increase in β-lactamase production between a k of 0.4 and a k of 1.6. Furthermore, when the DNA synthesis was stopped by the addition of nalidixic acid in a shift-up experiment, the synthesis of β-lactamase was not inhibited. Instead, there was an even more rapid accumulation of the enzyme compared with a conventional shift-up, possibly due to inhibition of synthesis of other proteins (data not shown). This means that the differential rate

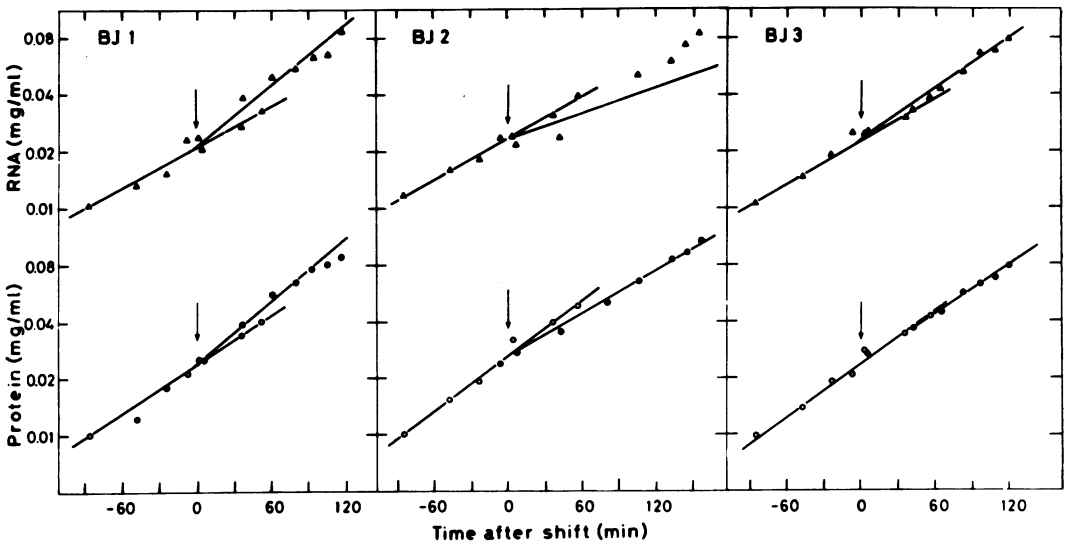

FIG. 5. *Accumulation of stable RNA and protein as a function of time in strains BJ1 (relA⁺ valS⁺ ampA1), BJ2 [relA⁺ valS(Ts) ampA1], and BJ3 [relA valS(Ts) ampA1]. Cells were grown in MOPS–0.4% glucose at the permissive temperature (29.5°C)* (△, ○) *to an* OD_{450} *of 0.8, when a portion of the culture was diluted four times in prewarmed media at the semipermissive temperature (35.5°C)* (▲, ●). *Symbols:* △ *and* ▲, *stable RNA;* ○ *and* ●, *protein.*

TABLE 1. *Synthesis of RNA, protein, and β-lactamase in strains BJ1, BJ2, and BJ3 after a shift in growth temperature from 29.5 to 35.5°C*

Bacterial strain	Temp (°C)	Differential rate of β-lactamase synthesis[a]	Relative rate of synthesis[b]		
			Protein	RNA	β-lactamase
BJ1 (*relA⁺ valS⁺ ampA1*)	29.5	1.38	1.00	1.00	1.0
	35.5	0.73	1.47	1.46	0.8
BJ2 [*relA⁺ valS*(Ts) *ampA1*]	29.5	1.79	1.00	1.00	1.0
	35.5	1.06	0.80	0.80	0.5
BJ3 [*relA valS*(Ts) *ampA1*]	29.5	1.40	1.00	1.00	1.0
	35.5	0.71	0.87	1.28	0.4

[a] The differential rate of synthesis was calculated as the slope obtained from plots such as those shown in Fig. 6. The postshift values were taken from the time when the new rate of synthesis was achieved (approximately 20 min after the temperature shift).

[b] The relative rate of synthesis was calculated as the product of the differential rate of synthesis and the initial rate of protein synthesis (*k* for protein) and normalized to the value obtained at 29.5°C.

of β-lactamase synthesis from each gene copy, like that of the core components, is much higher in rich media than it is in poor media.

It is well established that some components of the translational machinery that show growth rate dependence are under stringent control (2, 6–8, 13). We find during partial deprivation of charged $tRNA^{val}$ no differences in the differential rates of synthesis of β-lactamase in *relA⁺* and *relA* strains. This suggests that the *rel* system is not involved in the regulation of this enzyme. Upon low charging of aminoacyl tRNA, wild-type *relA⁺* strains immediately accumulate guanosine tetraphosphate, in contrast to a *relA*-carrying strain in which the level of guanosine tetraphosphate diminishes or stays the same (5). The level of the nucleotide guanosine tetraphosphate is known to rise during a nutritional or an energy source shift-down, and it has been suggested that it acts as an inhibitor of rRNA synthesis during such shifts (9, 21). It was therefore of interest that chromosomal β-lactamase followed stable RNA rather than the bulk of proteins during a nutritional shift-down. However, the experimental data given in this paper show that the regulation of chromosomal β-lactamase clearly differs from that of the core components. This was most notable when the response to a shift-up was examined. Core components in the translational apparatus show a virtually instantaneous increase in differential rate of synthesis during a transition to a medium permitting a higher growth rate (25), whereas for chromosomal β-lactamase a lag of about 30 min was found before the postshift rate of synthesis was achieved. Thus, only during balanced growth in different media and in the response to a shift-down does the chromosomal β-lactamase

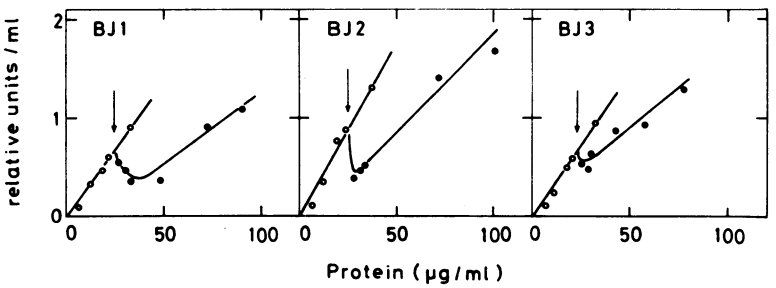

FIG. 6. *Differential rate of accumulation of β-lactamase (○, ●) in the experiment described in the legend to Fig. 5. Open and closed symbols indicate samples taken from preshift and postshift media, respectively.*

exhibit a pattern of regulation that appears coordinated with the regulation of the protein-synthesizing machinery.

It appears that the mode of in vivo regulation observed for chromosomal β-lactamase is not general for periplasmic enzymes. Thus, we have examined alkaline phosphatase under conditions identical to those described in this paper for β-lactamase. Under high-phosphate conditions, which generate repression of the enzyme, specific activity was invariant with growth rate. Likewise, enzyme activity followed the bulk of protein synthesis during a nutritional shift-down. Like chromosomal β-lactamase, no influence of the *rel* system was observed (data not shown).

The structural gene for chromosomal β-lactamase (*ampC*) as well as the regulatory *ampA1* mutation have recently been cloned on a derivative of plasmid pBR322. It is hoped that in vitro synthesis of β-lactamase and sequence analysis of control regions for *ampC* will yield further information as to the regulation of this periplasmic enzyme.

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