Brazilian Journal of Genetics, 19, 4, 545-549 (1996)
(Revista Brasileira de Genética)

Effect of sigma factor S (σS) on the stability of penicillin-binding protein 3 (PBP3) of *Escherichia coli* K12*

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ABSTRACT

The stability of penicillin-binding protein 3 (PBP3), a cell septum synthesizing protein, was analyzed at different incubation temperatures in three *Escherichia coli* K12 strains carrying a PBP3-overproducing plasmid. The stability of PBP3 was significantly reduced in stationary phase cells shifted to 42°C for 4 h, compared to samples incubated at 28 or 37°C. The half-life of PBP3 in the C600 strain was 60 min at 42°C, while samples incubated at 28 or 37°C had PBP3 half-lives greater than 4 h. Analysis of the PBP3 content in mutants deficient in *rpoS* (coding for the stationary phase sigma factor, σS) and *rpoH* (coding for the heat shock sigma factor, σ32) genes after shift to 42°C showed that stability of the protein was controlled by σS but not by σ32. These results suggest that control of the PBP3 levels in *E. coli* K12 is through a post-transcriptional mechanism regulated by the stationary phase regulon. We demonstrated that stability of PBP3 in *E. coli* K12 involves degradation of the protein. Moreover, we observed that incubation of cells at 42°C significantly reduces the stability of PBP3 in early stationary phase cells in a process controlled by σS.

INTRODUCTION

There are seven different proteins which covalently bind β-lactam antibiotics in the inner membrane of *Escherichia coli* K12, the penicillin-binding proteins (PBPs) (Spratt, 1977). PBPs are involved in the final stages of peptidoglycan synthesis and perform essential functions such as septum formation, maintenance of the rod cell shape and the rigid nature of the cell envelope representing, therefore, the lethal targets of β-lactam antibiotics (Spratt, 1977; Park, 1987).

Penicillin-binding protein 3 (PBP3) is a low copy number cell division-associated protein, with an Mr of 60 kDa, specifically required for synthesis and assembly of septal peptidoglycan (Botta and Park, 1981; Spratt and Cromie, 1988). Based on its role in the cell division process, PBP3 was expected to be required for only a brief period during the cell cycle and therefore its production should be precisely regulated. However, previous attempts to understand molecular mechanisms modulating enzymatic activity, intracellular level, or specific location of PBP3 have failed to explain how this protein is regulated in *E. coli* K12 (Buchanan, 1981; Wientjes *et al.*, 1983).

PBPs in exponential phase cells are rather stable and have half-lives greater than 4 h at 37°C (Buchanan, 1980). However, overnight grown cells contain only a fraction of the PBP3 levels detected during the exponential phase (Buchanan and Sowell, 1982; De La Rosa *et al.*, 1982). Reduction of PBP3 levels in the stationary phase requires an alternative sigma factor,

* This paper is dedicated to Prof. Carlos Chagas Filho, founder of the Instituto de Biofísica, on the occasion of its 50th anniversary.

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σS, coded by the *rpoS* gene (Dougherty and Pucci, 1994). However, these measurements were based on the binding of PBP3 to radioactive penicillin, thus the decrease in PBP3 levels may be due either to a blockage of enzymatic activity or to a specific degradation process of the protein by cellular proteases.

In this work we tried to elucidate the fate of PBP3 in cells overexpressing the protein, by means of a cloned gene copy carried on a plasmid, using specific polyclonal antibodies and labeling reactions with radioactive penicillin during transition to stationary phase at different incubation temperatures and with the presence, or not, of *de novo* protein synthesis.

MATERIAL AND METHODS

Bacterial strains and growth conditions

E. coli C600 and MC4100 strains were from our laboratory strain collection. *E. coli* ZK126 (W3110 Δ*lacU*169 *tna*-2) and its isogenic *rpoS* derivative, ZK1000 (ZK126 *rpoS::kan*) were obtained from Dr. R. Kolter (Bohannon *et al.*, 1991) whilst strain KY1429 (MC4100 *rpoH*6(Am) *zhf::Tn*10) was received from Dr. K. Young (Young *et al.*, 1989). The PBP3-overproducing plasmid, pMS316, was kindly provided by Dr. Hirochi Hara (Houba-Hérin *et al.*, 1985). Strains were grown in Luria-Bertani broth (LB) supplemented with chloramphenicol (25 μg ml^{-1}) for plasmid maintenance. Experimental cultures were prepared in 100 ml LB with 2% inocula, using overnight grown cells incubated at 28°C in an orbital-water-bath shaker. Growth was monitored at 600 nm in a model 100-40 Hitachi spectrophotometer until a density of 1.5 was reached. Cultures were then divided into aliquots. Kanamycin and spectinomycin were added to some of them to a final concentration of 50 μg ml^{-1} each, and others were kept without antibiotics. Cultures were immediately shifted to 37°C, 42°C or kept at 28°C, and further incubated for 4 h. No significant lysis were detected under these conditions.

Detection of PBP3

The PBP3 content was determined by immunoblot analysis and by labeling assays with [^{3}H]-benzylpenicillin. Aliquots of the cultures were centrifuged, washed once with phosphate buffer and sonically disrupted under cooling. Immunoblot analyses were carried out with cell extracts, after protein estimation (Bradford, 1976), and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide resolving gels. Proteins were transferred to nitrocellulose membranes following standard procedures (Sambrook *et al.*, 1989). PBP3 on blots was visualized by probing with a specific mouse polyclonal antibody (dilution 1:5,000) kindly supplied by Dr. J.T. Park. Blot development was achieved with a conjugated goat anti-mouse IgG peroxidase (dilution 1:10,000; Sigma, St. Louis, USA) and a chemiluminescence detection system (Amersham, UK). Detection of PBP3 with [^{3}H]-benzylpenicillin (21 Ci/mmol, Amersham) was carried out using isolated membrane fractions, as previously described (Spratt, 1977; Ferreira *et al.*, 1987).

RESULTS

In wild-type cells carrying a PBP3-overproducing plasmid there was no significant difference between the PBP3 levels of cells harvested at mid and late exponential phase at a growth temperature of 28°C. A growth phase-dependent reduction of PBP3 was detected only in samples maintained for longer periods after the onset of the stationary phase. Determination of the PBP3 levels by immunoblot analysis showed that incubation of *E. coli* cells at 42°C, but not 28°C or 37°C, resulted in a significant reduction in the amount of PBP3 levels (ca. 40% of the levels detected in samples incubated at 28°C) (Table I). In the presence of protein synthesis inhibitors at 42°C, the levels of PBP3 in the three strains fell even further to 0 to 22% of the values found in samples at 28°C or 37°C (Table I). The half-lives of PBP3 in the C600 strain in samples kept at 28°C

Table I - Relative amounts of overproduced PBP3 in *Escherichia coli* K12 strains grown under different conditions. Cells were harvested and the PBP3 content determined by densitometric analysis of the immunoblots after a 4 h incubation period.

Strain	Inhibition of protein synthesis	Amount of total PBP3 (%)[a]		
		Incubation temperature		
		28°C	37°C	42°C
C600	No	100	116	39
	Yes	100	95	9
MC4100	No	100	120	27
	Yes	100	90	0
ZK126	No	100	100	46
	Yes	100	112	22

[a] Relative concentration of PBP3. The values detected in samples kept at 28°C were used as reference (100%) for the samples kept at 37 and 42°C. Results are average values of two independent experiments.

or at 37°C were greater than 4 h while incubation at 42°C reduced the half-life of PBP3 to approximately 60 min as evaluated by immunoblots and labeling assays (Figure 1).

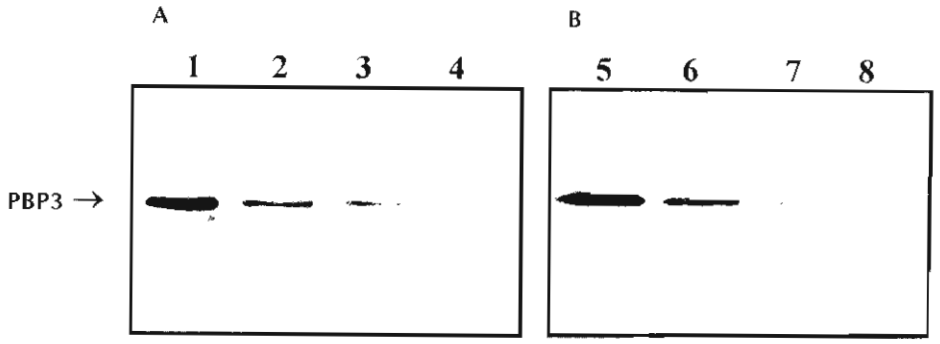

Figure 1 - Detection of PBP3 by immunoblot (A) and labeling with radioactive penicillin (B) in stationary phase cells incubated at 42°C in the presence of protein synthesis inhibitors. Cells were incubated at 28°C to stationary phase at time zero (lanes 1 and 5) and further incubated at 42°C for: 1 h (lanes 2 and 6), 2 h (lanes 3 and 7), and 4 h (lanes 4 and 8). Equivalent amounts of total protein were loaded on each lane.

The amount of PBP3 was immunologically followed in strains deficient in the alternative sigma factors σS (*rpoS*) or σ32 (*rpoH*) in early stationary phase cells at 42°C to demonstrate the possible involvement of the heat shock and stationary phase regulons. When the two mutant strains over-expressing PBP3 were shifted to 42°C, only one of them, KY1429 (*rpoH*), showed the temperature-dependent reduction of PBP3 observed in the isogenic parental strain MC4100 (Figure 2). Degradation of PBP3 was prevented by the *rpoS* mutation in the ZK1000 strain either in the presence or absence of *de novo* protein synthesis inhibitors (Figure 2). This result indicates that the observed temperature-dependent degradation of PBP3 is under the control of the σS regulon.

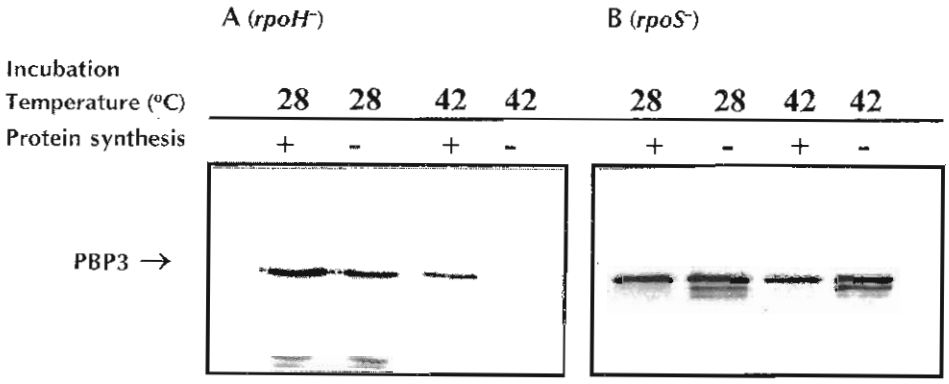

Figure 2 - Detection of PBP3 by immunoblot analysis of KY1429 (A) and ZK1000 (B) strains. Cultures were incubated at 28°C to stationary phase, then subjected to temperature shift in the presence and absence or protein synthesis as indicated. Each lane contained 1 μg of total protein.

DISCUSSION

The *rpoS* gene product, σS, is specifically required for transcription of genes under conditions of nutrient starvation as experienced by cells at the stationary phase (Loewen and Hengge-Aronis, 1994). The σS defines a regulon comprising at least 30 genes acting on different aspects of the bacterial metabolism, such as DNA repair, cell morphology, virulence, osmoprotection and thermotolerance (Loewen and Hengge-Aronis, 1994). In our experiments we demonstrated that σS participates in the control of PBP3 stability in early stationary phase cells incubated at 42°C. Curiously, no extragenic sequences usually found in promoters recognized by this sigma factor could be found in the upstream region of the PBP3 coding gene (Dougherty and Pucci, 1994). Therefore, any effect of σS on the control of PBPs stability might be indirect, probably by activation or inactivation of gene(s) regulating the action of proteases, or liability of the protein to protease action at different temperatures.

Incubation of *E. coli* cells at 42°C, but not at 28°C or 37°C, resulted in reduced stability of PBP3 in early stationary phase cells. However, cells kept longer at the stationary phase, as overnight grown cells, show the same reduction in the levels of PBP3, irrespective of the growth temperature (De La Rosa *et al.*, 1982; Dougherty and Pucci, 1994). Such a reduction of the PBP3 content in stationary phase cells may represent an energy saving measure, since non-dividing cells could use amino acids and energy generated by degradation of cell division-associated proteins. Since the growth rate of cells at 42°C is higher than at lower temperatures, the observed reduced stability of PBP3 at elevated temperatures could represent an anticipation of the phenomenon suffered by late stationary phase cells at lower temperatures. In this case, the observed reduced stability of PBP3 would reflect the same behavior of the protein in stationary phase cells, and not a temperature-dependent process. The lack of involvement of the heat shock regulon in the reduced

stability of PBP3 in early stationary phase cells further supports this possibility.

Reduction of PBP3 levels could also reflect altered transcription of the coding gene upon shift to different temperatures. However, previous reports based on the use of a gene report system showed that incubation of exponential phase cells at elevated temperatures resulted in increased transcription of the gene (Ogura *et al.*, 1991). Therefore, the levels of PBP3 in cultures shifted to 42°C in the presence of protein synthesis inhibitors indicate that reduction of intracellular PBP3 operates at the post-transcriptional level and may be due to the action of proteases. Cell envelope proteases act on different PBPs of *E. coli* K12. PBP1b and PBP7 can be cleaved by the outer membrane protease OmpT upon damage to the cell envelope (Henderson *et al.*, 1994). In addition, the Prc protease removes the last 11 C-terminal amino acid residues of PBP3 in a non-essential maturation process at the cytoplasmic membrane (Hara *et al.*, 1991). Although no evidence is yet available, Prc and OmpT proteases are good candidates for the temperature-dependent degradation of PBP3 in stationary phase cells.

Cell lysis induced by two β-lactam antibiotics could be prevented by activation of the heat shock response (Powell and Young, 1991). Since no alteration in the PBP labeling pattern was detected in heat-shock-induced cells, the lysis-suppressive event does not seem to operate through mechanisms affecting activity and/or stability of PBPs. We further demonstrated that inactivation of the heat shock response did not affect the stability of PBP3, since mutants defective in the *rpoH* gene did not show any alteration in the down-regulation process of the protein in stationary phase cells incubated at 42°C.

During the septation process, PBP3 acts in concert with other cell division genes such as *ftsA*, *ftsZ*, *ftsQ* (Naninga, 1991) and *ftsH* (Santos and Almeida, 1975; Ferreira *et al.*, 1987). In an experiment designed to evaluate the interaction of cell division gene products and PBP3, several cell division mutants of different *fts* genes were transformed with a PBP3-overproducing plasmid. When these strains were shifted to the restrictive temperature, only the *ftsH* mutant could restore septation but not viability (Ferreira *et al.*, 1987). Moreover, analysis of PBP3 content in the *ftsH* mutant strain showed that the amount of the protein accumulated in these cells was considerably lower than that detected in other cell division mutants (Ferreira *et al.*, 1987). It is thus possible that an intact FtsH protein plays a role in the stability of PBP3 in *E. coli* K12. Similarly, previous reports showed that specific mutations in the *ftsA* gene could reduce the amount of penicillin bound to PBP3 in the cell envelope of *E. coli* K12 cells (Tormo *et al.*, 1986). Determination of PBP3 half-life in *ftsH* and *ftsA* mutants as well as in other cell division mutants should reveal the possible involvement of cell division-associated genes in the σS-dependent stability of PBP3.

Elucidation of the regulatory mechanisms and the identification of additional genes involved in the control of PBP3 in *E. coli* K12 may contribute to the understanding of the cell division process in this organism.

ACKNOWLEDGMENTS

We acknowledge the helpful technical assistance of Celso Pereira and Eduardo Camacho. This work was supported by CNPq and FINEP grants.

RESUMO

A estabilidade da proteína ligadora de penicilina 3 (PBP3), responsável pela formação do septo, foi analisada em três cepas de *Escherichia coli* K12 superprodutoras de PBP3, submetidas a diferentes temperaturas. Culturas em fase estacionária, quando transferidas a 42°C por 4 h, tiveram seus níveis de PBP3 drasticamente reduzidos, quando comparadas a culturas mantidas a 28 ou 37°C. Na ausência de síntese protéica, a meia-vida da PBP3 incubada a 28 ou 37°C é maior que 4 h, enquanto a incubação a 42°C reduz este valor para cerca de 60 min na cepa C600. A análise dos níveis de PBP3 em cepas mutantes para o gene *rpoS* (que codifica o fator sigma de fase estacionária, σS) ou para o gene *rpoH* (que codifica o fator sigma da resposta ao choque térmico, σ32), após transferência para 42°C, mostrou que a estabilidade da proteína é dependente de σS mas não de σ32. Os resultados sugerem que os níveis de PBP3 são regulados pós-transcripcionalmente por mecanismos mediados pelo regulon de fase estacionária. Estas observações podem contribuir para melhor compreensão dos processos envolvidos no controle da divisão celular e metabolismo do peptideoglicano de *E.coli*.

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(Received September 22, 1995)