Ann. Inst. Pasteur/Microbiol.
1987, **138**, 537-547

EFFECTS OF TEMPERATURE INACTIVATION OF PENICILLIN-BINDING PROTEIN 2 ON ENVELOPE GROWTH IN *ESCHERICHIA COLI*

D. Buchnik (1), **C.L. Woldringh** (2) and **A. Zaritsky** (1) (*)

(1) *Department of Biology, Ben-Gurion University of the Negev, PO Box 653, Beer-Sheva 84105 (Israel), and*
(2) *Department of Electron Microscopy and Molecular Cytology, University of Amsterdam, Plantage Muidergracht 14, 1018 TV Amsterdam*

SUMMARY

The transition from rod-shaped to spheroidal cells was studied in a temperature-sensitive strain (SP45) of *Escherichia coli* K12, carrying a mutation (*pbp*A) in the gene coding for penicillin-binding protein 2 (PBP-2). This transition imposed by the restrictive temperature was associated with reduction of peptidoglycan/surface area and of cellular osmotic stability.

Addition of nalidixic acid (20 μg/ml) at the temperature shift from 30 to 42°C resulted in lysis of some cells and appearance of spheroidal bulges along the cylinders in other cells, consistent with the hypothesis of envelope weakening due to inactivation of PBP-2.

KEY-WORDS: *Escherichia coli,* Protein, Penicillin, Cell wall; PBP-2, Inactivation, Spheroidal form.

INTRODUCTION

Faster growing bacteria on richer media are larger [14] because the cell grows exponentially in mass [4], while its division is coupled to chromosome replication, which is initiated at a relatively constant cell size [12] and is ter-

Submitted May 2, 1987, accepted August 29, 1987.

(*) Corresponding author.

minated a constant time later [6]. Cell shape and relative dimensions, on the other hand, are not simply reflections of cell cycle events, but are also dependent upon the rate and mode of envelope growth [3, 22].

Rod-shaped bacteria extend by elongation and divide in a perpendicular plane. This is probably why selective blocking of division, by a great variety of chemical or physical agents (at doses sub-inhibitory for growth), results in long filaments with uniform diameters [17]. Such cells, however, do change their diameter with nutritional conditions [22, 24]. The mechanism responsible for this modulation of cell diameter and for shape determination has been the subject of numerous investigations, with ambiguous conclusions [3, 17, 22, 24]. Other factors which modify cell width should therefore be instructive in resolving the mechanism involved.

A series of proteins with high affinities for various β-lactam compounds, the so-called penicillin-binding proteins (PBP) have been separated from membranes of *Escherichia coli* [1]. Their physiological roles were assigned by exploiting highly-specific drugs and temperature-sensitive mutants [19]. The member of this series that is relevant to shape formation is PBP-2. This has been found to bind mecillinam exclusively at exceedingly low concentrations (0.01-0.1 μg/ml) and to be responsible for maintaining the bacillary cell shape in *E. coli* [9].

To study the involvement of PBP-2 in *E. coli* morphogenesis we chose strain SP45, which had been isolated [18] as a spontaneous mutant on mecillinam (and is thus resistant to the drug) at 30°C. It contains a mapped-point mutation [20] and cannot grow at 42°C. Like other temperature-sensitive *pbp*A mutants [8], SP45 passes through a shape change similar to that affected by low mecillinam concentrations upon transfer to the restrictive temperature. Its behaviour was compared with that of the isogenic wild-type strain KN126, to distinguish between specific effects on the cells of the defective PBP-2 and those of the higher temperature.

MATERIALS AND METHODS

Bacterial strains, growth conditions and measurements.

Two strains of *E. coli* K12 were exploited: KN126 (F^-, *ilv*, *mal*T, λ^R, *sup*D(126)(Ts), *tyr*A(am), *trp*E9829(am)) and SP45 (a *pbp*A(Ts) derivative of KN126).

Batch cultures were grown as previously described [23] in 50 ml Luria broth (LB), each contained in a 250 ml Erlenmeyer. Viability was determined by plating on Luria agar (1.5 %) plates, after 24 h incubation at 30°C.

A_{450} = absorbance at 450 nm.
DAP = *m*-diaminopimelic acid.
LB = Luria broth.
M = average cell mass.
NAL = nalidixic acid.
PBP = penicillin-binding protein.
PG = peptidoglycan.
S = average cell surface area.
V = average cell volume.
η = membrane microviscosity.
τ = doubling time.

Relative average cell mass (M) was expressed by the ratio between A_{450} (absorbance at 450 nm) to cell titre and represented as $A_{450}/10^9$ cells per ml.

Total protein was shown to be reliably represented by A_{450} in both strains growing in LB at both temperatures (data not displayed), with an average of 105 μg protein/A_{450} per ml of culture.

Microscopy and dimension determinations.

Dimensions and shape comparisons were executed by the aid of either a phase microscope (Zeiss, model AR) at a magnification × 320, or an electron microscope (Jeol, model 100B) at × 12,000, as described [21]. Cell dimensions were determined by moving a pen around the perimeter of the cell's image projected onto a screen, both attached to a computer which computes the two main axes of each cell from its image perimeter, and calculates both surface area and volume of cylinders with hemispherical polar caps (for bacilli), or prolates (for spheroids).

Peptidoglycan density.

Total peptidoglycan was inferred from the amount of *m*-diaminopimelic acid (DAP), determined by amino acid analysis (Beckman, model 121) of hydrolysed sacculi, as described before [23].

Peptidoglycan surface density was obtained by dividing total peptidoglycan by total surface area [(av. cell surface) × (nb of cells/ml) × 100 ml] in the same sample, and expressed as molecules DAP/nm^2.

RESULTS

Growth, size and shape at restrictive temperature.

In order to explore the participation of PBP-2 in cell-shape determination at a physiological level, growth of SP45 was compared with that of its wild-type isogenic parent KN126, at 30°C and after transfer to 42°C.

Both strains were cultivated in low cell concentrations with identical doubling times at the permissive temperature (τ = 48 – 50 min). M of the mutant was smaller by 25-30 % (table I, fig. 1).

Marked differences in the behaviour of the two strains were observed upon transfer to the restrictive temperature. The new steady-state growth rate of KN126 (τ = 25 min) was achieved immediately for both cell number and mass, thereby retaining the same M as in 30°C, concordant with the behaviour of *E. coli* ML30 [15]. In the mutant culture, the rate of cell division also accelerated with the temperature shift. However, this rate steadily declined for about 80 min (fig. 1). During the same period, both strains increased their total cell mass at constant and identical rates. The rate of mass growth started to decline only after division finally halted (at 80 min), and reached zero at about 220 min. At about 7 h, a drop in optical density indicated lysis of some cells.

TABLE I. — **Average cell mass, volume, surface area and peptidoglycan concentration in *E. coli* strains SP45 and KN126, at 30 and 42°C.**

Strain	Temp. (°C)	min at 42°C	M	V	M/V	S	nb cells measured	PG/S
KN126	30	—	6.8	1.94	3.51	10.41	195	0.59
KN126	42	40	6.9	2.35	2.94	11.28	188	0.47
KN126	42	105	6.9	1.82	3.79	8.89	231	0.85
SP45	30	—	4.8	1.28	3.75	7.58	276	0.69
SP45	42	60	7.4	2.82	2.62	10.60	246	0.41
SP45	42	108	12.2	4.83	2.53	12.64	130	0.61

M = relative value of average cell mass expressed in terms of $A_{450}/10^9$ cells per ml.
V = average cell volume in μm^3.
M/V = relative value of average cell buoyant density.
S = average cellular surface area in μm^2
PG/S = peptidoglycan surface density expressed as molecules peptidoglycan per nm^2 of cell surface area.

Change in cell shape of SP45 was evident after 40-50 min at 42°C, because the cell diameter gradually increased and the cylinders converted to ovoids. At about 100 min, the cell diameter became almost identical to the length (pictures not shown), while colony-forming ability started to decline (fig. 1).

Osmotic fragility.

The morphological changes of SP45 at the restrictive temperature, associated with growth anomalies (culminating in cell death), might have been due to weakening of its envelope, which would suggest that growth at 42°C sensitizes the mutant cells to environmental osmotic variations. The decline in viability was indeed faster by suspending SP45 cells in distilled water (fig. 2): after 160 min at 42°C, 90 % of the viable bacteria in the control (suspended in phosphate buffer) lost their ability to form colonies.

Cell dimensions and peptidoglycan concentration.

The osmotic fragility of SP45 at 42°C suggested that its envelope was weakened. To directly confirm the hypothesis that the envelope weakening was a reflection of reduced peptidoglycan surface density, cell dimensions and total peptidoglycan were determined at 30 and at 42°C.

The M of SP45 at 30°C was 25-30 % smaller than that of KN126, which was concordant with the respective M ratios (table I), and indicated a similar cell buoyant density in both strains at 30°C.

No significant differences in the dimensions of KN126 at the two temperatures were observed (table I). However, when the mutant cells had attained a spherical shape (at 108 min) the M was 3.77-fold its original value, while the surface area measured only 1.67-fold.

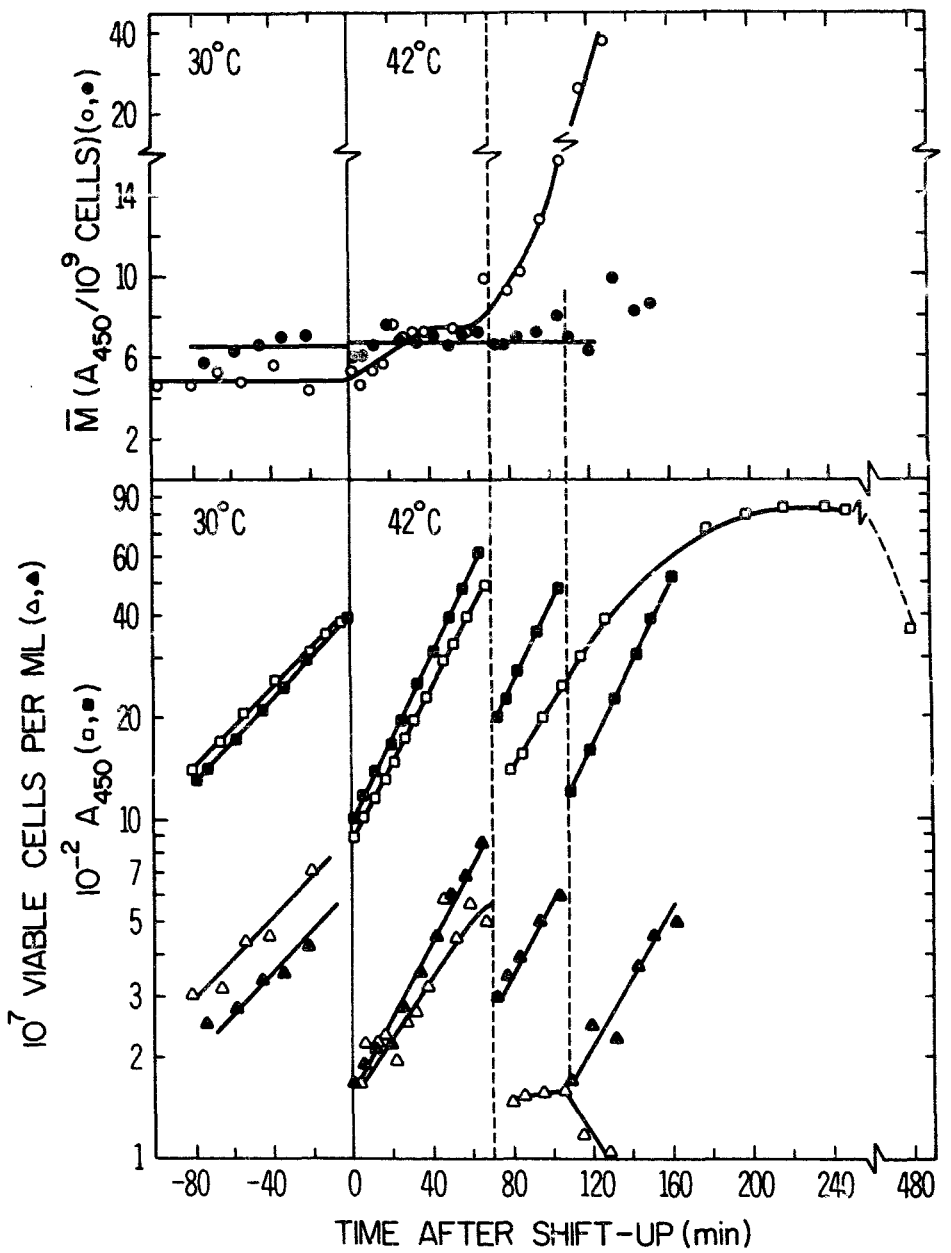

FIG. 1. — *Growth of* E. coli *KN126 (closed symbols) and of SP45 (open symbols) at 30 and at 42°C.*

Vertical dashed lines indicate 4-fold dilutions in fresh prewarmed medium.

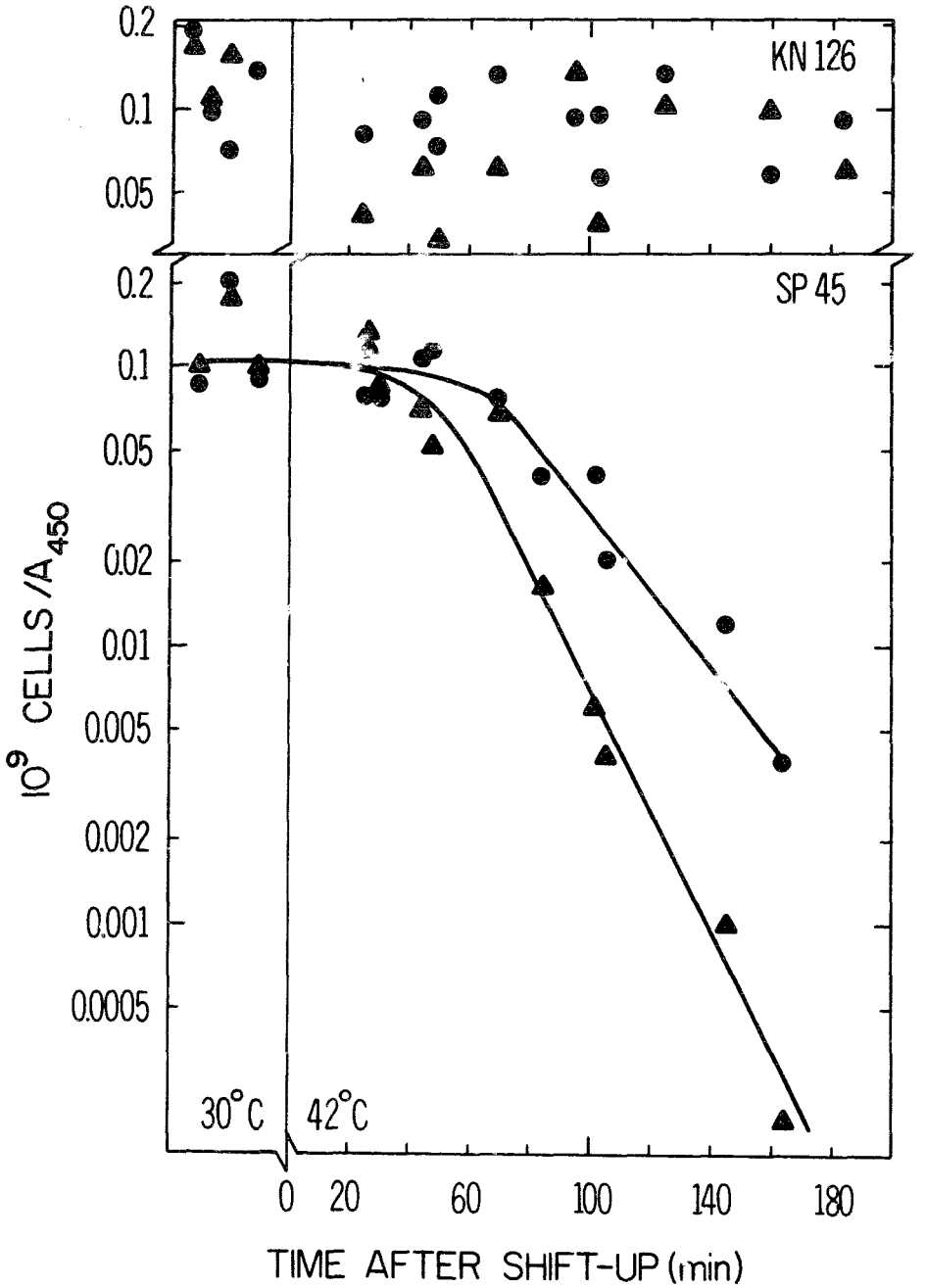

FIG. 2. — *Osmotic sensitivity of SP45 compared with that of KN126.*

Samples, withdrawn at the indicated times before or after the temperature shift, were diluted in cold (4°C) 65 mM phosphate buffer, pH 7.4 (circles), or in cold (4°C) distilled water (triangles), and plated on agar. Colonies were counted after 40-48 h incubation at 30°C.

The same samples were used for peptidoglycan determinations, which were related to unit surface area (table I). Both strains showed similar peptidoglycan surface densities at 30°C and similar reductions in this parameter 40-60 min after the shift to 42°C. At 108 min however, peptidoglycan surface concentration in the mutant, although similar to its original value, was found to be ca. 30-35 % lower than in KN126.

The effects of nalidixic acid.

The high temperature inflicted detrimental effects upon growth of the mutant, apparently through the weakening of the envelope. Inhibition of DNA synthesis also induces changes in bacterial envelopes: growth becomes unbalanced, divisions halt and cells convert into «snakes» [2]. The additive effects of both these factors were studied, to better understand the possible involvement of DNA synthesis in the mechanism of morphogenesis.

After addition of nalidixic acid (NAL, Calbiochem) only cells which have terminated chromosome replication progress to division [7]. Residual division and subsequent filamentation was also observed in the present study (graph not shown) after NAL was added.

Addition of NAL after transfer to 42°C resulted in immediate inhibition of divisions in both strains, followed by an exponential decline in colony-forming ability at 20 and 70 min respectively, (fig. 3). With the SP45 culture only, lysis of about 75 % cells occurred between 40-70 min (fig. 3).

Cell shape was affected by both factors (pictures not displayed): elongation occurred during the first 40 min, and only later did the temperature effect dominate when a central bulge appeared and swelled while distal cell ends remained cylindrical but stopped elongating.

DISCUSSION

Investigating the rod-sphere transition due to PBP-2 inactivation in *E. coli,* has previously been performed by addition of mecillinam to growth medium [5, 9, 11], although some work with *ts* mutants has also been reported [8, 13]. Combining data from these reports displays the following picture: low mecillinam concentrations inhibit growth at about 220 min [5, 9, 11], block division at about 80 min [5, 9] and cause shape changes which commence at about 50 min [9]. Similar effects were encountered in the present, complementary study, when PBP-2 was inactivated by the restrictive temperature in the *pbp*A mutant SP45 [18].

The osmotic sensitivity of SP45, built-up over the 60 min after its transfer to 42°C (fig. 2), indicates weakening of the cell wall. This is also evident from the ghosts observed during preparation for electron microscopy (pictures now shown). This weakening is likely to have evolved from reduced differential

rate of peptidoglycan synthesis relative to surface area increase during the same period, compared to that in KN126 (table I). This interpretation is supported by the higher sensitivity of strain KN126 during the first 50 min after shift-up (fig. 2), the same period in which peptidoglycan surface density is temporarily reduced (table I). It is also probable that this weakening is a major cause of the observed shape change, since identical growth in the presence of 5 % NaCl suppresses this phenomenon (unpublished observations), apparently due to a compensating effect of higher external osmotic pressure [5].

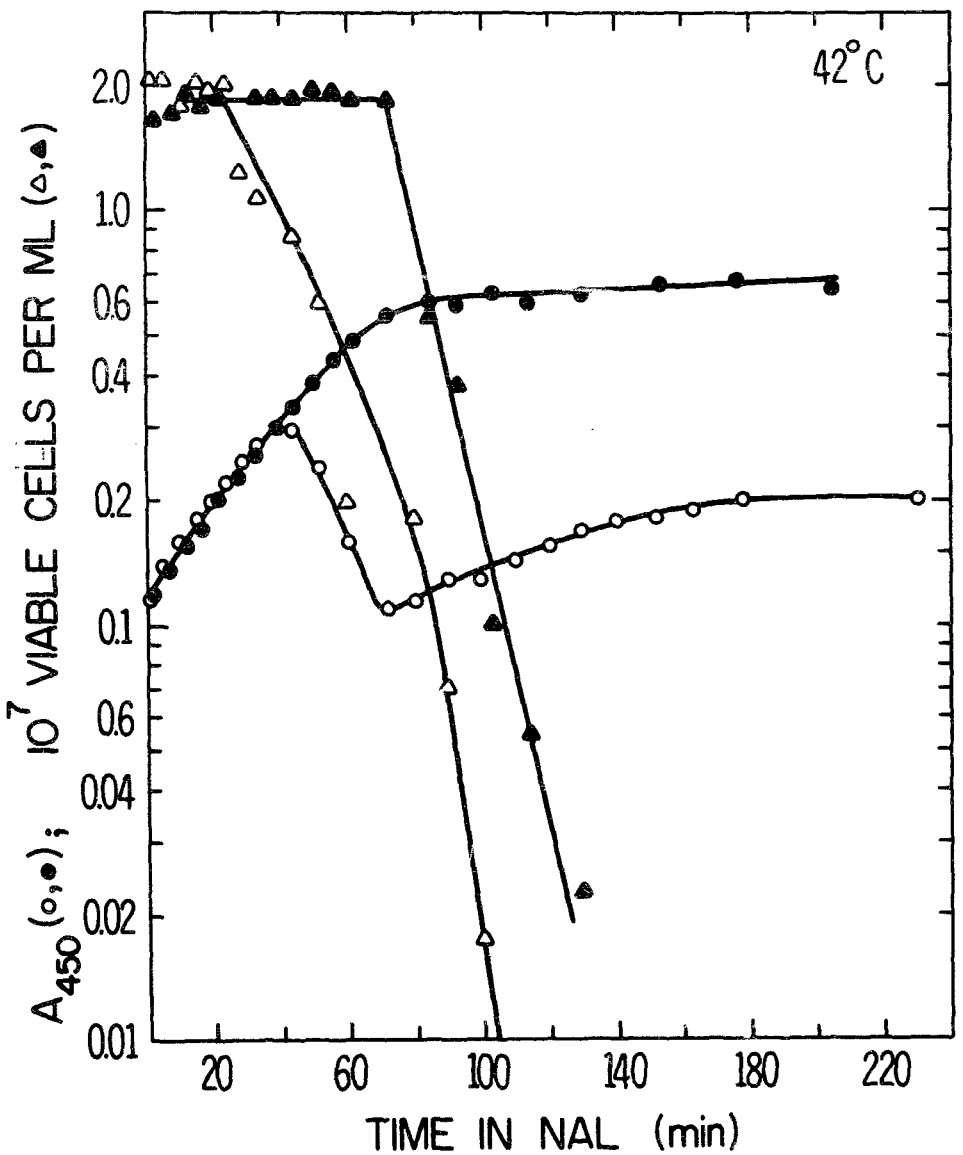

FIG. 3. — *Growth of KN126 (closed symbols) and of SP45 (open symbols) after a temperature shift from 30 to 42°C, in the presence of 20 μg/ml nalidixic acid.*

Peptidoglycan surface density has not been found to change in *E. coli* B/r growing under different nutritional conditions at 37°C [23]. This fact may mean that it is responsible for cell-shape determination or maintenance. It is shown here that higher density is required at a higher temperature in KN126 (40 % more at 42°C than at 30°C; table I). The SP45 envelope weakening observed may thus be due to its inability to restore the required higher density (table I). The fast lysis of mutant cells after simultaneous addition of NAL at temperature shift (fig. 3), confirms the envelope-weakening explanation for the shape change observed. Cell elongation continues for 50 min only, and bulbous swellings, characterizing damaged PBP-2 [3], presumably indicate the location of an envelope growth zone. The NAL treatment reveals such damage in a single cell by blocking its division. The size range of these bulges obtained in elongated cells is similar to that of the spheroids formed at 42°C without NAL. This is consistent with the hypothesis that inauguration of new growth zones is inhibited at the restrictive temperature, and that the observed shape changes result from growth at the zones existing before treatment.

Fatty acid compositions in *E. coli* membranes varies depending on the growth temperature [10], to preserve a constant membrane microviscosity (η) [16]. Soon after a temperature shift, activities of membranal proteins change, probably due to the change in η upon such a shift [24, 25]. It is conceivable that the reduction in peptidoglycan density in the wild-type after the shift reflects the decrease in η (see [26]). This explanation implies a lower-than-usual η in the mutant at the high temperature, which is a testable prediction.

ACKNOWLEDGEMENT

This work was partially supported by Ben-Gurion University Research Fund (A.Z.), and has been performed in partial fulfillment for an MSc thesis (D.B.).

B.G. Spratt has kindly provided the strains used. Computing cell dimensions and peptidoglycan determinations were performed in the laboratories of Prs N.B. Grover and D. Mirelman respectively; their help is gratefully acknowledged.

RÉSUMÉ

EFFET DE LA THERMOINACTIVATION DE LA PROTÉINE D'AFFINITÉ POUR LA PÉNICILLINE N° 2 SUR LA SYNTHÈSE DE L'ENVELOPPE DE *ESCHERICHIA COLI*

La transition de la forme cylindrique à celle sphéroïdale chez les bactéries a été étudiée à l'aide de la souche thermosensible (SP45) de *Escherichia coli* K12 portant une mutation (*pbp*A) dans le gène codant pour la protéine d'affinité pour la pénicilline (penicillin-binding protein 2 ou PBP-2).

Cette transition imposée par la température restrictive a été associée à la réduction de la surface du peptidoglycane et à celle de la stabilité osmotique cellulaire.

L'addition de l'acide nalidixique (20 µg/ml) pendant l'élévation de la température de 30 à 42°C, a eu pour résultat la lyse de certaines cellules et l'apparition de gonflements sphéroïdaux le long des cylindres chez les autres cellules, conformément à l'hypothèse de l'affaiblissement de l'enveloppe dû à l'inactivation de la PBP-2.

MOTS-CLÉS: Protéine, Pénicilline, Paroi cellulaire, *Escherichia coli;* PBP-2, Inactivation, Forme sphéroïdale.

REFERENCES

[1] BLUMBERG, P.M. & STROMINGER, J.L., Interaction of penicillin with the bacterial cell: penicillin binding proteins and penicillin sensitive enzymes. *Bact. Rev.*, 1974, **38**, 291-335.

[2] COHEN, S.S. & BARNER, H.O., Studies on unbalanced growth in *E. coli. Proc. nat. Acad. Sci.* (Wash.), 1953, **40**, 885-893.

[3] DONACHIE, W.D., BEGG, K.J. & SULLIVAN, N.F., *in* «Microbial development» (R. Losick & L. Shapiro) (pp. 27-62). Cold Spring Harbor Laboratory, New York, 1984.

[4] ECKER, R.E. & KOKAISL, G., Synthesis of protein, ribonucleic acid and ribosomes by individual bacterial cells in balanced growth. *J. Bact.*, 1969, **98**, 1219-1226.

[5] GREENWOOD, D., Effect of osmolarity on the response of *E. coli* to mecillinam. *Antimicrob. Agents a. Chemother.*, 1976, **10**, 824-826.

[6] HELMSTETTER, C.E., COOPER, S., PIERUCCI, O. & REVELAS, E., On the bacterial life sequence. *Cold Spr. Harb. Symp. quant. Biol.*, 1968, **33**, 809-822.

[7] HELMSTETTER, C.E. & PIERUCCI, O., Cell division during inhibition of DNA synthesis. *J. Bact.*, 1968, **95**, 1627-1633.

[8] IWAYA, M., GOLDMAN, R., TIPPER, D.J., FEINGOLD, B. & STROMINGER, J.L., Morphology of an *E. coli* mutant with a temperature-dependent round cell shape. *J. Bact.*, 1978, **136**, 1143-1158.

[9] JAMES, R., HAGA, J.Y. & PARDEE, A.B., Inhibition of an early event in the cell division cycle of *E. coli* by FL-1060, an imidinopenicillanic acid. *J. Bact.*, 1975, **122**, 1283-1292.

[10] MARR, A.G. & INGRAHAM, J.L., Effect of temperature on the composition of fatty acids in *E. coli. J. Bact.*, 1962, **84**, 1260-1267.

[11] MATSUHASHI, S., KAMIRYO, T., BLUMBERG, P.M., LINNETT, P., WILLOUGHBY, E. & STROMINGER, J.L., Mechanism of action and development of resistance to a new amidinopenicillin. *J. Bact.*, 1974, **117**, 578-587.

[12] PRITCHARD, R.H., BARTH, P.T. & COLLINS, J., Control of DNA synthesis in bacteria, *in* «Microbial Growth», XIX Symposium of the Society for General Microbiology (P.M. Meadow & S.J. Pirt) (pp. 263-297). Cambridge University Press, Cambridge, 1969.

[13] SATTA, G., BOTTA, G., CANEPARI, P. & FONTANA, R., Early initiatin of DNA replication and shortening of generation time associated with inhibition of lateral wall formation by mecillinam. *J. Bact.*, 1981, **148**, 10-19.

[14] SCHAECHTER, M., MAALØE, O. & KJELDGAARD, N.O., Dependency on medium and temperature of cell size and chemical composition during balanced growth of *S. typhimurium. J. gen. Microbiol.*, 1958, **19**, 592-606.

[15] SHEHATA, T.E. & MARR, A.G., Effect of temperature on the size of *E. coli* cells. *J. Bact.*, 1975, **124**, 859-862.

[16] SINENSKY, M., Homeoviscous adaptation — a homeostatic process that regulates the viscosity of membrane lipids in *E. coli. Proc. nat. Acad. Sci.* (Wash.), 1974, **71**, 522-525.

[17] SLATER, M. & SCHAECHTER, M., Control of cell division in bacteria. *Bact. Rev.*, 1975, **38**, 199-221.

[18] SPRATT, B.G., Resistance of *E. coli* to β-lactam antibiotics by a decrease in the affinity of a killing target. *Nature* (Lond.), 1978, **274**, 713-715.

[19] SPRATT, B.G., Penicillin-binding proteins and the future of β-lactam antibiotics. *J. gen. Microbiol.*, 1983, **129**, 1247-1260.

[20] SUZUKI, H., NISHIMURA, Y. & HIROTA, Y., On the process of cellular division in *E. coli:* a series of mutants of *E. coli* altered in the penicillin-binding-proteins. *Proc. nat. Acad. Sci.* (Wash.), 1978, **75**, 664-668.

[21] WOLDRINGH, C.L., DE JONG, M.A., VAN DEN BERG, W. & KOPPES, L., Morphological analysis of the division cycle of two *E. coli* substrains during slow growth. *J. Bact.*, 1977, **131**, 270-279.

[22] ZARITSKY, A. & PRITCHARD, R.H., Changes in cell size and shape associated with changes in the replication time of the chromosome of *E. coli*. *J. Bact.*, 1973, **114**, 824-837.

[23] ZARITSKY, A., WOLDRINGH, C.L. & MIRELMAN, D., Constant peptidoglycan density in the sacculus of *E. coli* B/r growing at different rates. *FEBS Letters*, 1979, **98**, 29-32.

[24] ZARITSKY, A., GROVER, N.B., NAAMAN, J., WOLDRINGH, C.L. & ROSENBERGER, R.F., Growth and form in bacteria. *Comments mol. Cell. Biophys.*, 1982, **1**, 237-260.

[25] ZARITSKY, A., Membrane microviscosity might be involved in bacterial morphogenesis. *Speculat. Sci. Technol.*, 1983, **6**, 465-470.

[26] ZARITSKY, A., PAROLA, A.H., ABDAH, M. & MASALHA, H., Homeoviscous adaptation, growth rate and morphogenesis in bacteria. *Biophys. J.*, 1985, **48**, 337-339.