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Isolation and some properties of the nucleoprotein complex of *Propionibacterium freudenreichii* subsp. *shermanii*

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Abstract — A nucleoprotein complex (NPC) from *P. shermanii* VKM-103 was purified to homogeneity by chromatography on DEAE-Sepharose FF and analysed by SDS-PAGE and HPLC. The NPC had a Mr of 44 kDa and exerted a protective and reactivating action on *E. coli* AB1157, after treatment with different stresses including: heating, UV-irradiation, exposure to salts of heavy metals and ethanol. The cell-free extract, that served as the source of the NPC also showed a reactivating and protective effect in different pro- and eukaryotic organisms inactivated by different stress factors. Boiling the NPC for 2 min and treatment by pronase increased its UV-protective effect, but led to the loss of its reactivating effect. In contrast, treatment with DNAse caused a marked decrease in the UV-protection property; RNAse had only a small effect. © Inra/Elsevier, Paris.

***Propionibacterium freudenreichii* subsp. *shermanii* / antistress factor / protective and reactivative effect**

Résumé — Isolation et caractérisation du complexe d'ADN–protéine (CAP) de *Propionibacterium freudenreichii* subsp. *shermanii*. Un complexe d'ADN–protéine (CAP ; 44 kDa) a été isolé de *P. shermanii* VKM-103, purifié en utilisant une méthode chromatographique (DEAE-Sepharose FF) et analysé par SDS-PAGE et HPLC. Le CAP a produit un effet protecteur et a réactivé la bactérie *E. coli* AB1157 après un traitement avec des agents de stress (chauffage, rayons UV, sels des métaux lourds, éthanol). L'extrait cellulaire ayant servi comme source du CAP a également produit un effet protecteur et réactivant chez divers organismes pro et eukaryotes inactivés par les agents de stress. Le chauffage du CAP (2 min) et le traitement avec la pronase ont augmenté l'effet protecteur contre les rayons UV, mais l'effet réactivant a disparu. Par contre, le traitement avec la DNAse a causé un abaissement marqué de l'effet protecteur. La RNAse avait un effet négligeable. © Inra/Elsevier, Paris.

***Propionibacterium freudenreichii* subsp. *shermanii* / facteur anti-stress / effet protecteur et réactivant**

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1. INTRODUCTION

Bacteria periodically experience life-threating stresses in a variety of natural situations. How bacteria survive these environmental stresses is a fundamental question in biology. In order to increase their chance of surviving these encounters, the molecular response of the bacteria is often linked to the synthesis of a set of evolutionary conserved proteins, stress-induced proteins or heat-shock proteins.

As shown by Gottesma [3], many of the genes of *E. coli* are organized in regulons in which unlinked genes are coordinately controlled by a common regulatory gene. Different stress systems, such as heat shock, oxidation stress, the SOS (Save Our Souls) regulon and stringent regulon, respond to the same environmental stimuli [11].

Earlier, we [12,13] showed that a dialysed cell-free extract obtained from *P. freudenreichii* subsp. *shermanii* VKM-103 exerted a protective action on UV-inactivated *E. coli* AB1157 and mutant strains with various deficiencies in their repair systems (PolA$^-$, RecA$^-$, UvrA$^-$), just as in eukaryotic organisms inactivated by UV-B and UV-C radiation [2].

Cellular responses to external stresses are often mediated by heat-shock proteins some of which possess chaperone functions. Therefore, we suggested that a protein may exert an universal action, showing protective and reactivative effects in organisms inactivated by unrelated stresses and our findings support this suggestion.

The objectives of the present study were: (1) to study the reactivative and protective actions of antistress factors of *P. freudenreichii* subsp. *shermanii* in microorganisms under different stresses; (2) isolation, purification and partial characterization of the antistress factors and (3) to study the reactivative and protective actions of the purified antistress factors.

2. MATERIALS AND METHODS

2.1. Strains and growth media

P. freudenreichii subsp. *shermanii* VKM-103, from the Russian Academy of Science, *Candida guilliermondii* VSB-656, from the collection of 'VNII Synthes Belok', Moscow and *Saccharomyces cerevisiae* KM-231, from the collection of the Department of Microbiology, Moscow State University, were used. The propionic acid bacteria were cultivated for 72 h under anaerobic conditions in a chemically defined medium of the following composition (g·100 mL): glucose 1.5, $(NH_4)_2SO_4$ 0.3, tryptone (Difco) 0.1, yeast extract (Difco) 0.05, KH_2PO_4 0.1, Na_2HPO_4 0.2, $CaCl_2$ 0.002, $MgSO_4$ 0.002, NaCl 0.002, $CoCl_2$ $6H_2O$ 0.001, pH 6.8–7.0. *E. coli* was grown aerobically in nutrient broth (Oxoid N2) at 200 rpm and on the surface of nutrient agar (Oxoid) at 37 °C. *C. guilliermondii* and *S. cerevisiae* were grown for 7 h at 230 rpm at 32 °C in nutrient wort. Nutrient wort was prepared by the standard method: germinated grains of barley were milled, flooded with water and kept 10–12 h at 55–58 °C with permanent mixing. During this time, sugars (mainly maltose), amino acids and polypeptides accumulate as a result of the hydrolysis of starch and proteins by the corresponding amylolytic and proteolytic enzymes. The nutrient wort also contains nucleic acids, vitamins, organic acids, mineral salts, carbohydrates in large amounts (about 80 % of maltose, glucose, dextrines, etc.). The liquid is filtered and the sugar content in the filtrate is determined with Balling's areometer; 1° Balling corresponds approximately to a sugar content of 1 %. We used 6°B nutrient wort.

2.2. Preparation of cell extracts of *P. freudenreichii* subsp. *shermanii*

The bacteria were harvested by centrifugation at 10 000 *g* for 20 min, washed twice with 0.05 mol·L^{-1} sodium phosphate buffer, pH 7.4 and resuspended in the same buffer to the initial optical density. This suspension was diluted 3-fold in the same buffer, sonicated (22 kHz, 40 s × 5 at 4 °C) and centrifuged at 144 000 *g* for 1 h at 4 °C. The extracts were then dialysed (1:500) against the same buffer at 10 °C overnight to remove the low molecular mass constituents. The solutions to be tested were sterilized by filtration through a Millipore filter (0.22 µm) (Sigma, USA).

The dialysed cell-free extract (CFE) was salted out with $(NH_4)_2SO_4$. Active fractions were obtained in the range of 20–40 % (AF1) and 60–80 % (AF2) and were subjected to the further separation and purification described below. Protein concentration was determined according to Lowry et al. [6] and spectrophotometrically [14], comparing the absorbance at 235 and 280 nm. Nucleic acids were determined spectrophotometrically at 260 nm [14].

2.3. UV-irradiation

Overnight cultures (50 mL) of *E. coli* were washed, resuspended in 5 mL of 0.05 mol·L^{-1} sodium phosphate buffer, pH 7.4 and incubated at 37 °C for 1h to deplete endogenous substrates. A suspension of bacteria (1 to 3×10^8 cells·mL^{-1}) was UV-irradiated in a Petri dish, 10 cm in diameter. The source of UV-radiation (254 nm) was a BUV-30 bactericidal lamp (Moscow, Moscow State University, Russia). The intensity of the radiation was 10 W·m^2. The flux was measured by a UV radiometer 'UFD-4' with magnetic photoelement (Moscow University, Moscow, Russia). All dilutions and radiation procedures were performed in the dark.

2.4. Exposure conditions and cell survival assay

The cell suspensions of *E. coli* used in studying heat shock, solvent shock, oxidative shock and effect of Cd^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} were prepared as for the UV-irradiation assays.

Logarithmic-phase cells of *C. guilliermondii* and *S. cerevisiae* were washed twice with distilled water (1 500 *g* for 5 min) and resuspended in a mineral medium [10] supplemented with 1 % (w/v) sucrose to a concentration of 10^5 cells·mL.

The reactivative effect of the CFEs on the bacteria inactivated by different stress factors was estimated as follows: cell suspensions of test cultures were incubated for 15 min in the presence of the stress factor, then washed, resuspended in 0.05 mol·L^{-1} phosphate buffer, pH 7.0 containing 20 µg CFE (as protein)·mL^{-1} for bacteria and 400 µg CFE·mL^{-1} for yeasts. In the case of the purified nucleoprotein complex (NPC), 40–80 µg·mL^{-1} of this protein were added to the bacterial suspension. After 10 min incubation, the viable cells were enumerated and compared with an untreated control. The protective effect was determined in the same way except that CFE or NPC was added before the stress treatment. The % viability was measured with reference to an untreated control. Yeast cell viability was estimated by the microcolony method [1]. The experiments were performed in triplicate.

2.5. Analytical methods

2.5.1. SDS-PAGE

One-dimensial SDS-PAGE was performed according to Laemmli [4] using a resolving gel containing 15 % (w/v) acrylamide. Gels were fixed in 30 % ethanol – 10 % glacial acetic acid solution for 1 h and silver stained.

2.5.2. High performance liquid chromatography (HPLC)

The extent of protein purification was also determined by distributive HPLC using an Ultropac TSK G-4000SW (7.5 × 300 mm column) (LKB, Uppsala, Sweden). The eluent was 0.04 mol·L^{-1} sodium phosphate buffer, pH 7.0 containing 0.2 mol·L^{-1} NaCl and 10 % (v/v) ethanol at a flow rate 0.5 mL·min^{-1}. The eluate was monitored at 226 nm. Two-mL fractions were collected and assayed for protective (reactivative) activity.

2.5.3. Determination of molecular mass of proteins

Molecular mass of proteins was measured by SDS-electrophoresis, using a calibrative set of proteins (14.4–94 kDa) (Pharmacia, Sweden), and by HPLC (see above) using a calibrative curve. Bovine serum albumin 67 kDa, ovalbumin 43 kDa, carboxypeptidase B 34.3 kDa, trypsin 23.8 kDa and soybean inhibitor of trypsin 20.1 kDa were used as standards.

2.5.4. Purification

The $(NH_4)_2SO_4$ precipitates, AF1 and AF2, were dissolved in 2 mL of 0.04 mol·L^{-1} sodium phosphate buffer, pH 7.0 containing 0.2 mol·L^{-1} NaCl (buffer A) to which 1 mol·L^{-1} PHMSF in methanol was added. After incubation for 1 h at 4 °C, the solution was dialysed three-fold against

the initial buffer. This treatment was undertaken to prevent the hydrolysis of proteins by serine proteinases, which were present in large quantities. Carboxypeptidase activity was not detected in these fractions. After this treatment and dialysis, serine proteinase activity was not found in the protein solutions when determined according to Worthington [15]. After dialysis, the protein solution was separated on Sephacryl S-200 SF (1.6 × 90 cm) in buffer A at a rate of 10 cm·h^{-1}. The process was monitored spectrophotometrically at 279 nm. Active fractions were pooled and the solution was dialysed against 0.03 mol·L^{-1} Tris-HCl buffer, pH 7.0 (buffer B). Dialysed solutions of active protein were loaded on DEAE-Sepharose FF (1.6 × 30 cm), after equilibration with buffer B. Elution was performed by a linear gradient of 1 mol·L^{-1} NaCl in buffer B at a flow rate of 25 cm·h^{-1}. Fractions corresponding to the main peaks on the chromatogram were pooled, dialysed against water and lyophilized. The activity of the lyophilized preparations were tested as described above.

3. RESULTS AND DISCUSSION

In *table I*, reactivation of heated suspensions of *E. coli, S. cerevisiae* and *C. guilliermondii* by a CFE from *P. shermanii* VKM-103 is shown. Sublethal heat shock and ethanol stress induce practically identical changes in protein synthesis in yeasts [8, 9] An antistress effect of *P. shermanii* CFE in ethanol-inactivated cells of *E. coli* was also obtained (*table II*). The CFE exerted protective and reactivative effects in the case of oxidation stress induced by H_2O_2 (*table III*) The responses to oxidation stress are of particular interest because active oxygen species are generated not only by environmental agents, but also by normal aerobic metabolism. H_2O_2 relates to active oxygen species and as can be seen in *table III*, CFE exerts a remarkable protective action on *E. coli* inactivated by H_2O_2. H_2O_2 induces primarily an oxidation stress but also a secondary SOS response [11]. The protective action of the CFE was inversely proportional to the rate of cell survival. The CFE helped in those cases where the cell's repair systems were in difficulty and could not cope with the task. When survival of H_2O_2 inactivated cells was 5.3 %, preincubation with CFE increased it to 16.7 % (data not shown) and, when survival was 0.6 %, preincubation with CFE increased cell survival ten-fold, to 6.7 % (*table III*).

Salts of heavy metals, i.e. $CdCl_2.2H_2O$, $ZnSO_4$, $CoCl_2.6H_2O$ and $CuSO_4$ were also used as stress factors. It was shown [11] that $CdCl_2$ induces 17 proteins and provokes SOS-, heat shock- and oxidation stress

Table I. Reactivation of *E. coli, S. cerevisiae* and *C. guilliermondii*, inactivated by heating, after 15 min of postincubation with cell-free extract (CFE) of *P. shermanii* VKM-103.

Tableau I. Activation de *E. coli, S. cerevisiae* et *C. guillermondii* inactivé par le chauffage après la postincubation avec l'extrait cellulaire de *P. shermanii.*

Microorganism	Conditions of heating		Viability %	
	T°	Time, min	Without CFE	With CFE
E. coli	45	30	74	91
	45	90	23	85
S. cerevisiae	42	30	66	93
	42	90	4.5	9.5
C. guilliermondii	42	60	30	33
	42	90	20	29

In the case of *E. coli*, 20 µg of protein per mL and in the case of yeasts 400 µg of protein per mL of suspension were added.

Pour *E. coli*, 20 µg·mL^{-1} de protéine sont ajoutées. Pour les levures, 400 µg·mL^{-1} de protéine sont ajoutées.

Table II. Protective and reactivative effects of the cell free extract in ethanol stressed *E. coli* AB1157.
Tableau II. Effet protecteur et réactivant de l'extrait cellulaire chez *E. coli* AB1157 inactivé par l'éthanol.

Experimental conditions	Average cell number / mL × 10^6, (X ± SE)	Survival %
Control (untreated cells)	468 ± 25.8	100
Incubation with ethanol (a)	30.0 ± 1.7	6
Preincubation with CFE then incubation with ethanol	182 ± 11.4	38
Incubation with ethanol (b)	17.2 ± 1.0	3
Incubation with ethanol, washing, postincubation with CFE	21 ± 1.3	14

Final concentration of ethanol / concentration finale d'éthanol : (a) 4 % ; (b) 5 %.

Table III. Effect of the cell free extract of *P. shermanii* on the survival of hydrogen-peroxide (10 mmol·L^{-1}) inactivated *E. coli* AB1157.
Tableau III. Effet de l'extrait cellulaire de *P. shermanii* sur la survie des cellules d'*E. coli* inactivées par H_2O_2 (10 mmol·L^{-1}).

Experimental conditions	Average cell number × 10^6 × mL^{-1} (X ± SE)	Survival %
Control (untreated cells)	46.0 ± 1.0	100
Incubation with H_2O_2, 15 min	0.3 ± 0.03	0.6
Preincubation with CFE 10 min, washing, then incubation with H_2O_2 15 min	3.09 ± 0.95	6.7
Incubation with H_2O_2, washing, then incubation with CFE 10 min	0.75 ± 0.95	1.47

responses in *E. coli*; the latter two responses predominate. $CdCl_2$ was found [5] to be the most potent agent in inducing the synthesis of adenylated nucleotides, accumulation of which is consistent with a role of alarmones in the oxy-R-mediated response.The other heavy metals tested exerted a similar action on cells.

The CFE of *P. shermanii* exerted a protective and reactivative action on *E. coli* AB1157 inactivated by exposure to the heavy metals mentioned above (*figure 1*). The data raise a question about the mechanism involved. Purification of the substance was attempted to determine its mode of action.

The primary purification of active factor was performed by salt fractionation of CFE of *P. shermanii*. Active substances were found in the protein sediments obtained at

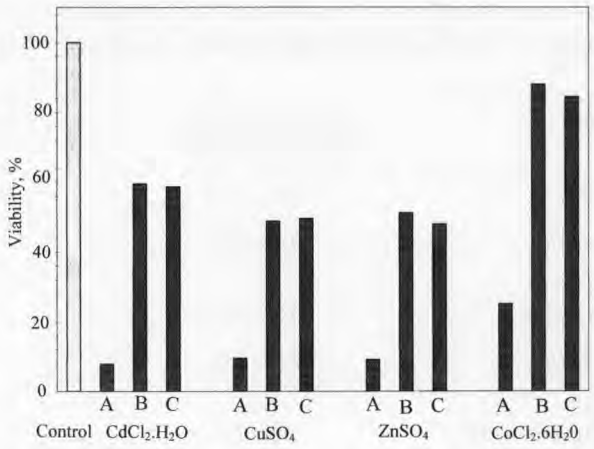

Figure 1. Protective and reactivative action of the cell-free extract (CFE) of *P. shermanii* VKM-103 on *E. coli* AB1157 inactivated by exposure to the salts of heavy metals. **A**. Without CFE. **B**. With preincubation. **C**. With postincubation.

Figure 1. L'influence protectrice et réactivante de l'extrait cellulaire de *P. shermanii* VKM-103 sur les cellules d'*E. coli* AB1157 inactivées par les sels des métaux lourds. **A**. Sans extrait cellulaire. **B**. Avec préincubation. **C**. Avec postincubation.

20–40 % (AF1) and 60–80 % (AF2) saturation with $(NH_4)_2SO_4$. Fractions AF1 and AF2 were further subjected to gel-filtration on Sephacryl. Fractionation on Sephacryls S-200 and S-300 did not give a good separation, but distribution of active proteins on the column allowed us to get rid of 30–40 % of inactive proteins without considerable loss of activity. This is the reason gel-filtration was involved in protein purification and as a result fractions AF1-2 and AF2-2 were obtained.

Both fractions (AF1-2 and AF2-2) were chromatographed on DEAE-Sepharose FF (Pharmacia, Sweden) at pH 7.0. Elution of bound material was performed with a linear gradient of NaCl (1 mol·L^{-1} in buffer B). Purification profiles of the individual proteins (sub-fractions) of *P. shermanii* is shown in *figures 2a* and *b*. Subsequent testing of the sub-fractions obtained showed that 7 sub-fractions possessed reactivating activity and the highest activity was revealed in sub-fraction AF2-2.5. HPLC and SDS-PAGE analyses of these sub-fractions were performed under standard conditions [4]. These analyses showed that preparation AF2-2.5 is a single protein, while the other active sub-fractions were mixtures of proteins and peptides of different molecular mass. The molecular mass of the compound in AF2–2.5 was 44 ± 2 kDa as shown with both HPLC and SDS-PAGE. Using spectrophotometric measurements, it was found that fraction 2–2.5 was a nucleoprotein complex (NPC) composed of 25 % of protein and 75 % nucleic acids.

This preparation showed a protective and reactivative action in UV-irradiated (254 nm) and Cd- and ethanol-inactivated *E. coli* (*table IV*) and also a reactivative effect of *E. coli* cells, inactivated by heating (50 °C, 60 min) (*table V*). Postincubation of the cells subjected to heat shock with the purified factor increased cell survival by approximately two-fold.

The preparation lost activity after treatment with DNAse, but not with RNAse. In

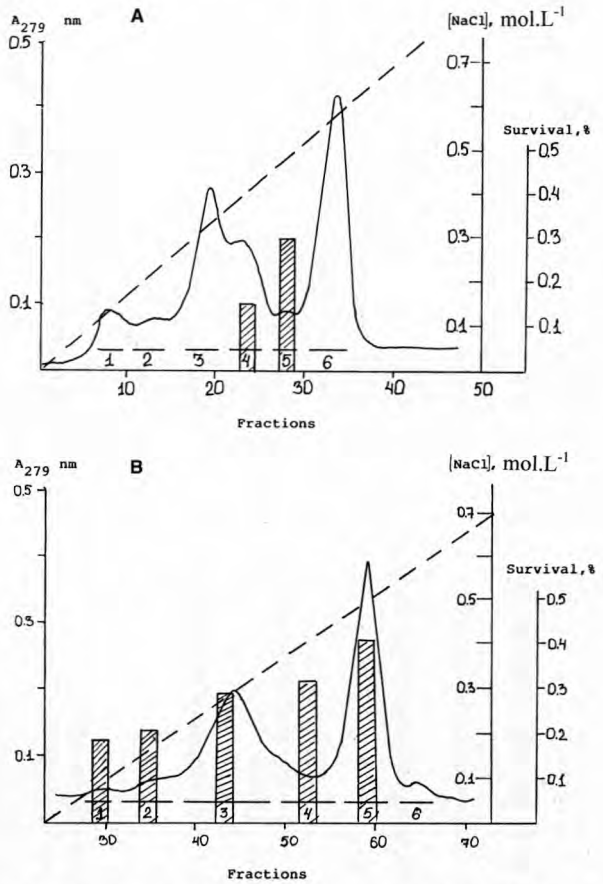

Figure 2. Separation of AF1-2 and AF2-2 fractions by anion-exchange chromatography on DEAE-sepharose. **A**. Separation of active proteins of AF1-2. **B**. Separation of active proteins of AF2-2. Continuous line-boundaries of subfractions; subfractions are numbered; shade columns show cell viability in % in the presence of respective subfractions.

Figure 2. Séparation des fractions qui contiennent AF1-2 et AF2-2 par chromatographie d'échange d'ions sur la DEAE sepharose. **A**. Séparation des protéines actives d'AF1-2. **B**. Séparation de protéines actives d'AF2-2. La ligne continue délimite les subfractions ; elles sont numérotées ; les colonnes grises montrent la survie des celulles (%) en présence des subfractions respectives.

contrast, treatment with pronase increased the protective effect of the preparation almost two-fold (*figure 3*). The increase of UV-protection after treating the preparation with pronase may be explained by a change in the conformation of the complex leading to the release of nucleic part that evidently served as a UV-screen [7], since nucleic products have maximal adsorbtion at 250–260 nm.

Boiling the preparation separated it into a supernatant, containing mainly nucleic acid and a precipitate, containing mainly denaturated protein. The supernatant, when added to irradiated cells, exerted a remarkable protective, but not a reactivative effect, suggesting that the nucleic acid portion of the preparation served, at least after separation from the protein part of the complex, as a UV-screen (*table VI*). From *table VI*,

Table IV. Protective and reactivative effect of the purified preparation in UV-, Cd- and ethanol-inactivated *E. coli* AB1157.
Table IV. Effet protecteur et réactivant de la préparation purifiée sur les cellules d'*E. coli* AB1157 inactivées par rayons UV, Cd et éthanol.

Stress	Antistress protection	Cell survival %	Division index (a)
UV-irradiation, 60 $J \cdot m^{-2}$	Without protection	0.09	
	Preincubaion 5 min with preparation (20 $\mu g \cdot mL^{-1}$)	0.27	3.1
	Preincubation 5 min with preparation (60 $\mu g \cdot mL^{-1}$)	0.49	5.7
	Postincubation 10 min with preparation(60 $\mu g \cdot mL^{-1}$)	0.14	1.65
Exposure to 4 % ethanol for 15 min	Without protection	25.1	
	Preincubation 10 min with preparation (60 $\mu g \cdot mL^{-1}$), then exposure to ethanol, 15 min	59.7	2.4
	Postincubation 10 min with preparation (60 $\mu g \cdot mL^{-1}$)	60.0	2.5
Exposure to 2 $mmol \cdot L^{-1}$ $CdCl_2$ for 15 min	Without protection	23.9	
	Preincubation 10 min with preparation (60 $\mu g \cdot mL^{-1}$), then exposure to $CdCl_2$, 15 min	89.9	3.4
	Postincubation 10 min with preparation (60 $\mu g \cdot mL^{-1}$)	41.5	1.8

(a) The division index is defined as the ratio of cell number forming colonies in the presence and absence of the protective agent.
(a) L'index de division est défini comme le nombre relatif de cellules qui forment des colonies en présence et en l'absence de l'agent protecteur.

Table V. Reactivation of *E. coli* AB1157 inactivated by heating (50 °C, 60 min) after postincubation with the purified preparation.

Tableau IV. Réactivation des cellules d'*E. coli* inactivées par le chauffage (50 °C, 60 min) après postincubation avec la préparation purifiée.

Antistress protection	Cell survival %	Division index (a)
Control (without heating)	100	
Without protection	25.1	
Postincubation 10 min (80 μg·mL^{-1} of protein)	41.5	1.8

(a) See *table IV* / voir *tableau IV*.

Table VI. Effect of the preparation after boiling on survival of UV-inactivated cells of *E. coli*.

Tableau VI. Effet de la préparation (après le chauffage) sur la survie de cellules d'*E. coli* inactivées par les rayons UV.

Antistress protection	Cells survival %	Division index (a)
Preincubation		
Control (without protection)	0.16	
Incubation with supernatant after boiling of the preparation (containing mainly nucleic acid product), 100 μg·mL^{-1},10 min	6.3	39
Control	0.12	
Incubation with the solution of sediment obtained after boiling and separation of sediment from the preparation 100 μg of protein/mL, 10 min	0.56	4.7
Postincubation		
Control (without postincubation)	0.037	
Incubation with the preparation without any treatment	0.06	1.65
Control (without postincubation)	0.07	
Incubation with supernatant, obtained after boiling of the preparation (containing mainly nucleic acid product)	0.08	0

(a) See *table IV* / voir *tableau IV*.

it is seen that the nucleic acid part of the NPC served only as a UV-screen and did not exert an effect on postincubation (reactivation). NPC without any treatment and especially its protein part display reactivative and protective effects.

The isolation of the compound responsible for protective and reactivative action of the CFE from *P. shermanii* opens prospectives for understanding the mechanism of antistress action.

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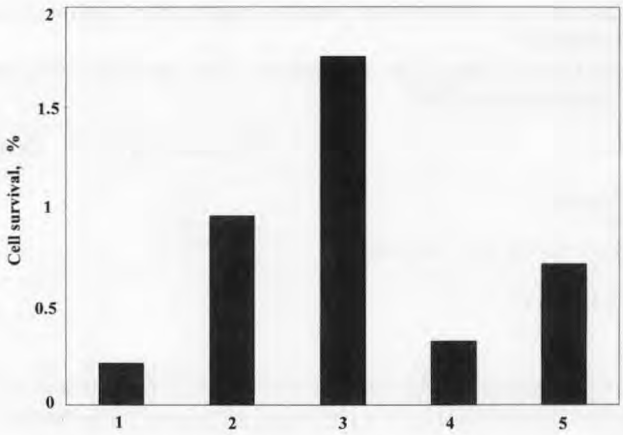

Figure 3. Protective effect of the nucleoprotein complex (NPC) in UV-inactivated *E. coli* AB1157 after enzymatic treatment. **1**. Control (without protective factor). **2**. Preincubation with nucleoprotein complex (NPC). **3**. NPC treated with pronase. **4**. NPC treated with DNAse. **5**. NPC treated with RNAse.

Figure 3. Effet protecteur du complexe après traitement enzymatique sur les cellules d'*E. coli* AB1157 inactivées par les rayons UV. **1**. Contrôle (sans le facteur protectif). **2**. Préincubation avec (CAP). **3**. CAP traité avec la pronase. **4**. CAP traité avec la DNAse **5**. CAP traité avec la RNAse.

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