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SUMAR – CONTENTS – SOMMAIRE – INHALT

REGULAR ARTICLES

SHORT COMMUNICATION

ORAL PRESENTATION ABSTRACTS

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Evaluation of genotoxic and antimicrobial effect induced by fungicide Dithane M-45

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SUMMARY. The cyto-genotoxic potential of fungicide Dithane M-45 on the root meristem cells of *Tulipa praestans* Hoog. cv. 'Unicum' and antimicrobial effects on some bacterial strains were investigated for concentrations lower than those currently used in agricultural practice.

The results indicate a stimulating effect of the treatments with this fungicide on mitotic division, associated with a higher frequency of chromosomal aberrations for all concentrations tested, and for all root harvesting periods. The high sensitivity of root meristem cells of *Tulipa praestans* cv. 'Unicum' to the fungicide action suggest the potential of this species to be used as a plant-system for detecting the mutagenicity and genotoxicity, mainly the clastogenic and aneugenic effects, of various chemicals. However, only a slight antibacterial effect against the tested bacteria was observed in the paper disc and agar well diffusion assays.

Keywords: Antimicrobial effect, cytotoxicity, dithane, genotoxicity, tulipa

Introduction

For a long time, chromosomal aberrations were a criteria for assessing the reproductive process in plants and have been correlated with the morphological and taxonomic changes, fertility - sterility relationships, mutations and other characteristics.

Genotoxicity and cytotoxicity testing of chemical compounds on various plant species is a simple, fast and sensitive enough technique (Grant, 1982), validated by the United Nations Environment Program (UNEP), World Health Organzation (WHO) and US Environmental Protection Agency (US EPA) (Türkoğlu, 2009) to be efficient for monitoring of environmental pollutants (Ma, 1999; Yi and Meng, 2003; Bolle *et al*., 2004).

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The pesticides, widely used throughout the world, contain a wide variety of different substances both in terms of composition and in terms of their properties, being used to kill or remove the harmful organisms. The extensive use of pesticides to control pests, and their long persistence in the environment, led to the initiation of research on developing systems for testing and evaluation of their cyto-genotoxic potential. While the use of pesticides has become a necessity, their ingredients induce acute toxic effects in different non-target organisms, as well as in experimental systems (Sudhakar *et al*., 2001). In the case of plants, pesticides can act as a mutagen to induce the occurrence of chromosomal aberrations and cytotoxicity (Chauhan *et al*., 1999; Yuzbasioglu, 2003; Popescu *et al*., 2013; Şuţan *et al*., 2014). The active substances of pesticides can have adverse or killing effects not only on target organisms (plant pathogens and pests), but can be also harmful to non-target organisms such as the soil bacteria. Among all bacteria from soil, some species that are involved in biodegradation process and useful for soil fertility can be sensitive to chemical substances. Some soil bacterial strains are plant pathogens, or humans and animals opportunistic pathogens. When is exposed to pesticides and fungicides, the soil microbiota might be adversely affected (Hussain *et al*., 2009).

Knowing that many pesticides, respectively insecticides, fungicides or herbicides are highly effective in inducing chromosomal aberrations, we initiated a study on the cyto-genotoxic effects of pesticide Dithane, in various concentrations, on the genetic material in somatic cells that are in mitotic division, in the species *Tulipa praestans* Hoog cv. 'Unicum'.

In this paper, we present relevant data to support the reliable use of the above mentioned plant system for detecting genotoxic effects of pesticides, evaluating changes in the mitotic index and identifying different types of chromosomal aberrations occurred as result of the exposure of meristematic root cells to pesticide action. Also, we estimated the effects of pesticide Dithane M-45 on several bacterial strains isolated from cultivated soil.

Materials and methods

Dithane M-45 is a very well-known multisite fungicide, which belong to the ethylene bisdithiocarbamates group. The active ingredient of fungicide tested is 80% mancozeb, as a coordination product of Zinc ion and Manganese ethylene bisdithiocarbamate.

Mancozeb is a broad spectrum contact fungicide with protectant activity against a wide range of plant diseases.

Evaluation of cytogenotoxic activity

The plant used as test material was *Tulipa praestans* cv. 'Unicum' (2n = 16). Three clean and healthy bulbs of *Tulipa praestans* cv. 'Unicum' were chosen for each treatment group. After dry scales of bulbs were removed, onion bulbs were grown in freshly tap water, at room temperature. When the roots reached 1.5-2 cm in length,

they were treated with different concentrations of aqueous solution of Dithane M-45 containing 300, 600 and 900 ppm of active ingredients for 6 hours period. The concentrations were chosen to be lower than those doses used in agricultural field to control different diseases. The control was prepared by exposing the seeds to water only. After 6 hours of treatment went by, roots were transferred into fresh tap water for 24 h, and 48 h respectively.

The roots were collected and fixed in Carnoy 1:3 acetic acid-ethyl alcohol mixture for overnight and then preserved in 70% alcohol at 4ºC for cytological studies. The root tips were hydrolyzed in 1 N HCl at 60^0C for 12 minutes, followed by staining with 2% aceto-orcein at 60^0C for 14 minutes. After proper fixation and staining, appropriate squash preparations were made for each of the treatment and control. Effect of chemical treatment and control on different chromosome plates were observed under light microscope. All observations were made from temporarily prepared slides. To determine the effects of this fungicide on mitotic index, 3000 cells were scored in control group and in each treated group.

Mitotic index (MI) was computed by determining the mitotic cell frequency (prophase, metaphase, anaphase and telophase) by the total number (3000 cells) of cells observed and multiplying the result by 100.

Cytological abnormalities were also observed and scored. Photomicrographs of cells showing chromosomal aberrations as well as showing normal mitosis were taken using an CX31 Olympus microscope.

Percentage of cells showing chromosomal abnormalities such as sticky chromosomes, laggard chromosomes, multipolar anaphases, as well as aberrant interphases (binucleate cells) were recorded at the appropriate mitotic stages.

Statistical analysis

Results are presented as the mean ± standard error of more independent experiments. Statistical significance analysis of the obtained data was performed by the use of analysis of variance (one way ANOVA). Additionally, Duncan's multiple range test was used to compare the means of the treatments. Significant differences were set at $P \leq 0.05$.

Evaluation of antibacterial effect

The disc diffusion method and agar wells method were used for testing the bacterial sensitivity to Dithane M-45 (Valgas *et al*., 2007).

Five bacterial strains isolated from cultivated field soil samples and identified with API test kit were used in our experiment: *Pseudomonas (Chryseomonas) luteola* (95.4%), *Elizabethkingia meningoseptica (Chryseobacterium meningosepticum)* (85%)*, Ewingella americana* (80.4%), *Weeksella virosa* (87.6%), and *Pasteurella pneumotropica* (93.1%).

Pseudomonas (Chryseomonas) luteola is a non-fermenting, Gram negative, strict aerobic rod, motile with polar multitrichous flagella. Colonies are yellow on solid media, either smooth or wrinkled (Hansen and Nielsen, 2011). It is frequently found especially in damp environment (water, soil) and it can cause septicaemia, endocarditis, peritonitis and other infections mainly in patients with immunosuppressive therapy or health disorders. The susceptibility to tetracycline was registered for some clinical strains that were resistant to cephalosporins, but other studies indicate resistant strains to tetracycline (Chihab *et al*., 2004; Doublet *et al*., 2010).

Elizabethkingia meningoseptica (formerly known as *Chryseobacterium* or *Flavobacterium meningosepticum)* is an opportunistic pathogen, that can cause meningitis, sepsis, pneumonia, infection of skin or other organs (Ceyhan and Celik, 2011), mainly in newborns or immunocompromised patients. It is a Gram negative, aerobic, non-fermenting and non-motile, oxidase - positive rod, widely distributed in nature (soil, freshwater and saltwater) and in hospital environments. Because in clinical cases the bacteria are inherently resistant to many antibiotics, *E. meningoseptica* can be considered a potential nosocomial pathogen (Dias *et al.*, 2010).

Ewingella americana is a Gram negative rod of Enterobacteriaceae Family, common in some ecological niches (like some type of food: vegetables, meat, mushroom), but also in human blood, respiratory tract secretions, faeces, urine or wounds (Rozhon *et al*., 2012). It is lactose fermenting, oxidase negative, indole negative and catalase positive, facultative anaerobic rod (Hassan *et al*., 2012). In some clinical cases this bacterium can produce severe infections because the antibiotic resistance.

Weeksella virosa is a Gram negative aerobic rod, oxidase positive, indole positive and catalase positive bacterium. It is unable to grow on MacConkey agar. It can be isolated from urine, blood, cerebrospinal fluid, from the genital tract or ears, eyes, rectal area and can be associated with pneumonia, peritonitis, urinary tract infections and bacteremia (Slenker *et al*., 2012). Some clinical isolates can resist to tetracycline, other strains can be susceptible to this antibiotic (Reina *et al.,* 1990).

Pasteurella pneumotropica is an opportunistic Gram negative pathogen, non-motile, cocobacillary to rod-shaped organism, which can cause rather latent infections in rodents such as upper respiratory infections and pyogenic syndrome (Harlev *et al*., 2009). It can be found in soil, and some studies point out the *Pasteurella pneumotropica* capacity of polychlorinated biphenyls (PCBs) degradation.

After obtaining an overnight culture by incubation at 37°C on nutrient agar, and then a bacterial suspension into 2 mL sterile saline buffer for each bacterial strain, we used all five bacterial suspensions both in filter paper disc diffusion method (PD) and agar wells diffusion method (AW).

In filter paper disc diffusion method dry and sterilised filter paper discs (6 mm diameter) were used (Deliu, 2010; Parihar *et al*., 2010). Each bacterial strain was homogenously mixed and uniformly inoculated with the help of a sterile cotton swab on

dried surface of nutrient agar in Petri dishes (4 mm level depth). We allowed the medium surface to dry (only a few minutes) and the antibiotics to reach room temperature before applying the discs. Then, we discharged the antibiotic disc and the filter paper discs with flamed and cooled forceps and we gently pressed the disc into the solid medium with the help of the same forceps. The distance between discs was about 25 mm. The disc with Tetracycline (30 µg per disc from Bioanalyse) was placed on the inoculated surface as a positive control and sterile distilled water was used as a negative control.

Four filter paper discs were placed on the medium. We impregnated each discs with 5µL of a certain concentration of Dithane M-45 (respectively 300 ppm, 600 ppm and 900 ppm), and the negative control with sterile distilled water by using micropipettes. Then, the inoculated plates were incubated inverted, for 24 h at 37°C, under aerobic conditions.

In the agar wells diffusion method, the bacterial suspensions were homogenously distributed on the nutrient agar in plates by using a sterile cotton swab, after which we cut four 6 mm diameter wells in the solid medium (using a sterile glass capillary tube), spaced at about 25 mm apart from one another (Valgas *et al*., 2007).

In every agar well we put 5 µL of Dithane M-45 (300 ppm, 600 ppm and respectively 900 ppm) and of negative control, by using a micropipette. As in the filter paper disc diffusion method, Tetracycline (Bioanalyse) was used as positive control (30 µg per disc). We allowed the solution to diffuse in the medium and then the plates were incubated inverted for 24 h at 37°C.

The antibacterial effect of Dithane M-45 was evaluated by reading the experimental results. The confluent bacterial growth was observed for each tested bacterial strain. The diameters of inhibition zones were measured in mm from the underside of the plates as a clear zone surrounding the discs, and agar wells, respectively.

Results and discussion

The results obtained in experiments performed in order to highlight the cytogenetic effects of the fungicide Dithane in *Tulipa praestans* cv. 'Unicum' showed a statistically significant increase in the general mitotic index following treatments with the fungicide Dithane in concentrations of 300 ppm, 600 ppm, and 900 ppm, respectively, as compared to the control (Fig. 1).

Transfer of the rooted tulip bulbs to tap water after 6 hours of treatment with Dithane had a stimulating effect, the number of cells undergoing mitotic division being higher and significantly higher after 24 hours and respectively 48 hours of maintenance in tap water, depending on the concentration of the fungicide.

A significantly increase in the general mitotic index values was found in the experimental variant with Dithane in concentration of 900 ppm, both immediately after treatment (5.65 %), and after the transfer of rooted bulbs to tap water for 24 hours (5.86 %) or 48 hours (7.3 %) .

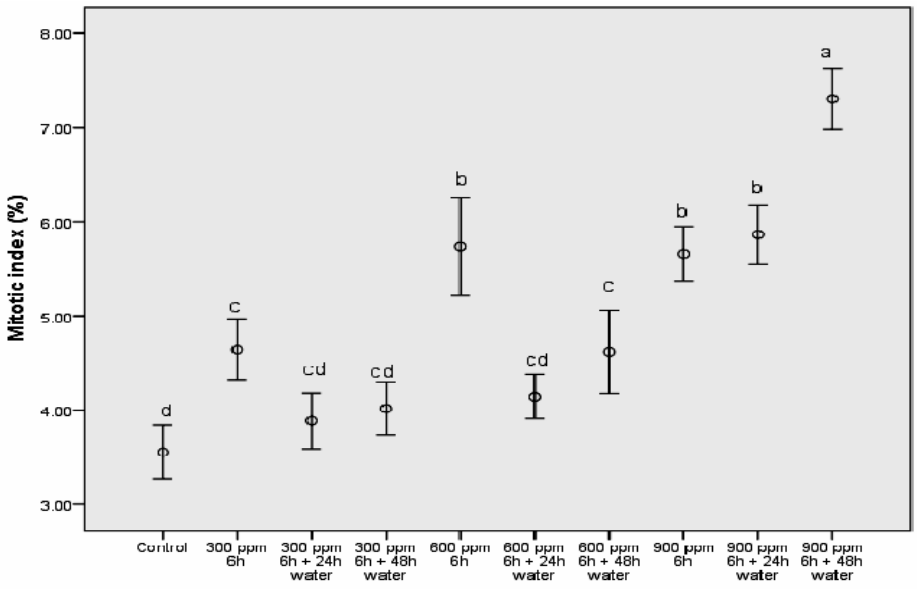

Figure 1. The variation of mitotic index in apical root meristems of *Tulipa praestans* cv. 'Unicum' treated with the fungicide Dithane. The standard errors are ilustrated in the graph as error bars; a, b, c, d: assessment of the significance of differences, by Duncan's Multiple Range Test, $p<0.05$.

Similar results were obtained when assessing the cyto-genotoxic potential of the fungicide Dithane M-45 in Narcissus pseudonarcissus (Şuţan *et al.,* 2014). Using a different formula of the pesticide Dithane (750 g/kg), Asita and Makhalemele (2009) found an inhibitory effect on mitotic division, which could be the consequence of different concentrations tested.

In the case of treatment of the roots with solutions of Dithane in various concentrations (300 ppm, 600 ppm, 900 ppm), the change in the mitotic index was associated with the induction of mitotic and chromosomal aberrations such as vagrants and laggards, metaphases with sticky or depolymerized chromosomes, anaphases with chromosome fragments, C-metaphases, asters in anaphase, bridges in anaphase, multipolar anaphases (Fig. 2). Also, there were observed variations in the number of chromosomes (Fig. 3).

The frequency of chromosome aberrations varied widely depending on the concentration of the fungicide and the time of collection of roots, the highest percentage (0.90 %) being determined for the treatment with 600 ppm Dithane for 6 hours + 48 hours of recovering in tap water. Regardless of the fungicide concentration, the transfer of roots in tap water after the treatment with Dithane was correlated with an increased frequency of chromosome aberrations (Table 1).

Table 1.

The frequency of chromosome aberrations induced in *Tulipa praestans* cv. 'Unicum' following treatment with different concentrations of Dithane.

Treatment duration	**Concentration of Dithane solution**	**Chromosome aberrations (%)**				**Total number of chromosome aberrations** (% ± ES)
		Prophase	**Metaphase**	**Anaphase**	**Telophase**	
Control	0	0	0	0	0	0 ± 0^{c}
6 h	300 ppm	0	0,01	0,02	0,01	$0,01 \pm 0,03^{c}$
6 h + 24 h in water		0	0,02	0,02	0,01	$0,01 \pm 0,05^{c}$
6 h + 48 h in water		0	0,07	0,06	0	$0,03 \pm 0,019^{c}$
6 h	600 ppm	0	0,16	0,26	0,23	$0,16 \pm 0,058^{bc}$
6 h + 24 h in water		0	0,28	0,34	0,21	$0,21 \pm 0,074^{bc}$
6 h + 48 h in water		0	0,36	0,33	0,20	$0,22 \pm 0,081^{bc}$
6 h	900 ppm	0	0,27	0,34	0,32	$0,23 \pm 0,078^{bc}$
6 h + 24 h in water		0	0,66	0,82	0,42	$0,48 \pm 0,178^{b}$
6 h + 48 h in water		0,26	1,60	1,43	0,33	$0,90 \pm 0,354^{a}$
Total number of chromosome aberrations (% ± ES)		$0,03 \pm 0,006^{b}$	$0,34 \pm 0,153^{a}$	$0,36 \pm 0,142^{a}$	$0,17 \pm 0,09^{a}$	$0,226 \pm 0,056$

It is considered that the formation of sticky chromosomes involves chromatin protein matrix rather than nucleotide sequences. Generally, a consequence of the induction of chromosome stickiness is the formation of ana-telophase chromosome bridges. The aggregate state of the genetic material is irreversible, often resulting in significant decrease of the mitotic index, as well in cell death (Fernandes *et al*., 2009). In our study, the number of cells with sticky chromosomes was extremely low, which can be correlated with the higher values of the mitotic index in treatments with Dithane.

Chromosome breakage can be associated with either an increased affinity to mutagens of some regions of DNA, the presence of highly fragile sites, or the composition of some specific DNA sequences, probably related to the nuclear matrix (Kihlman, 1966). The results of numerous studies showed that chromosome breakage induced by the alkylating agents is produced typically in the heterochromatic regions. In our study with Tulipa praestans, the chromosome breakage occurred both in the terminal and middle regions. The largest chromosomal fragments were observed

following treatment with Dithane in concentration of 900 ppm. The presence of the C-metaphases may be a consequence of partial or complete inhibition of the spindle formation (Fiskesjö, 1993; Fernandes *et al*., 2009), which also explain the formation of cells with a variable number of chromosomes (from 2n = 24 to 2n = 37). In a single cell, the observed chromosome number was lower (2n = 7) than the basic chromosome number in the genus Tulipa (x = 12) (Fig. 3). In this context, it should be noted that, in the case of some C-metaphases, chromosomes with achromatic lesions were observed (Fig. 2).

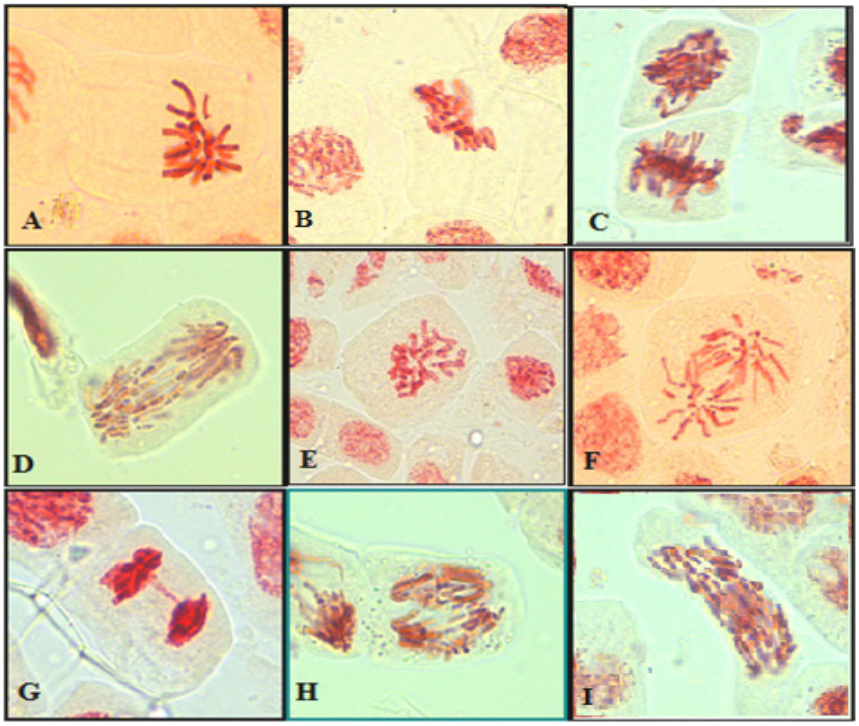

Figure 2. Chromosome aberrations induced by the fungicide Dithane M-45 in root meristem cells of *Tulipa praestans* cv. 'Unicum'. (A) vagrant; (B) sticky chromosomes; (C) chromosome fragments in metaphase; (D) chromosome fragments in anaphase; (E) C-metaphase; (F) aster-like arrays of chromosomes in anaphase; (G) chromosome bridges; (H) multipolar anaphase; (I) abnormal anaphase.

Anaphase chromosome bridges, seen in both anaphase and telophase, might be the consequence of the existence of cohesive chromosome ends, chromosome rearrangements, or chromosome fusions, in which case the chromosome bridges can be multiple and may persist until telophase (Giacomelli, 1999; Marcano *et al*., 2004).

Kuriyama (1982) noted that the polyploidization agents induce the increase of microtubule organizing centers (MTOCs). The MTOCs replicates throughout the cell cycle, simultaneously with the DNA replication (Ghadimi *et al*., 2000). These structures are responsible for cell polarity and chromosome segregation in anaphase, which maintain the numerical chromosome stability and genomic integrity organisms (Sumara *et al*., 2004). The presence of supernumerary organizing centers of microtubules, accompanied by the inhibition of cytokinesis and deregulation of the nuclear spindle formation (Fig. 2), causes unequal chromosome segregation and formation of multipolar anaphases (Ochi *et al*., 2003). In the studies carried out with *Tulipa praestans* cv. 'Unicum', we have seen the occurrence of multipolar anaphases (Fig. 2).

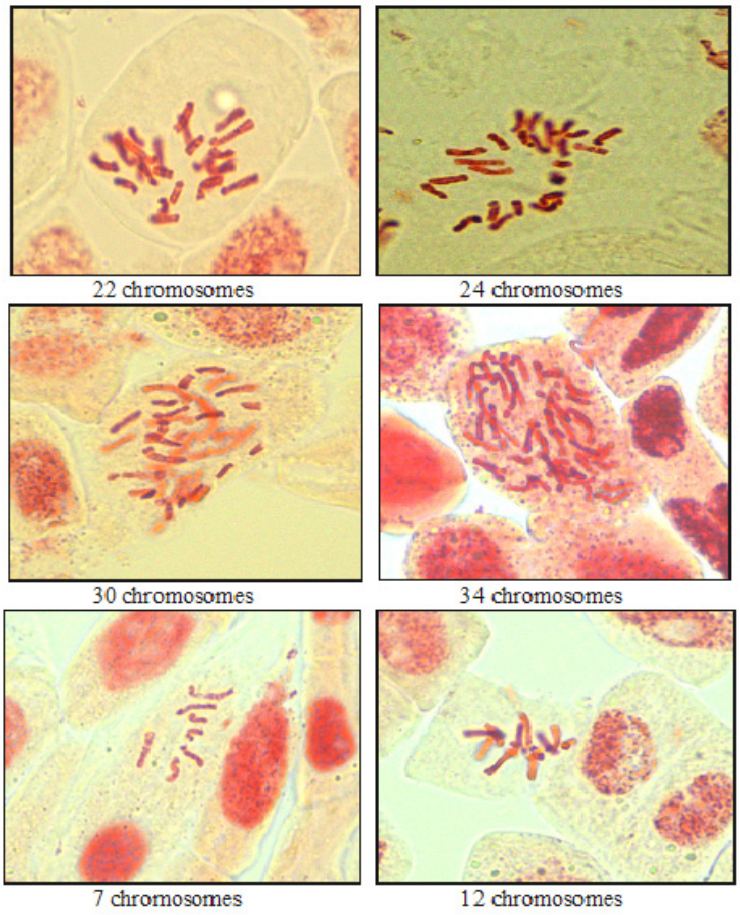

Figure 3. Variation of the chromosome number in root meristem cells of *Tulipa praestans* cv. 'Unicum'.

The antimicrobial activity of different concentrations of Dithane M-45 is illustrated as the diameter of inhibition zone measured in mm, in Table 2.

All three concentrations of Dithane M-45 had only a poor effect against the tested bacterial strains. The largest inhibition zone was found for Dithane M-45 900 ppm on *Weeksella virosa* and *Elizabethkingia meningoseptica*. The diameters of inhibition zones were higher with increasing pesticide concentration (Fig. 4, 5).

All five bacterial strains tested in this study, which are inhabitant in soil, can be potential pathogens for humans or animals. Because of their origin, they exhibit sensitivity to tetracycline (according CLSI standards a diameter larger than 15 mm shows a sensitive strain), while the clinical isolates could be resistant to this antibiotic.

Table 2.

Antimicrobial activity of Dithane M-45 in paper disc diffusion method (PD) and agar wells diffusion method (AW).

Variants/ Control	**Tested microorganisms (mm, diameter of inhibition zone)**									
	Pseudomonas luteola		*Elizabethkingia meningoseptica*		*Ewingella americana*		*Weeksella virosa*		*Pasteurella pneumotropica*	
	PD	AW	PD	AW	PD	AW	PD	AW	PD	AW
Dithane M-45 300 ppm	7	7	7	8	-	-	7	9	-	-
Dithane M-45 600 ppm	7	7	7	8.5	7	-	7	9	-	-
Dithane M-45 900 ppm	7.5	8	7.5	9	7.5	-	8	9.5	7	7
Tetracycline 30 µg per disc (positive control)	28		28		24		26		26	

In other studies on the effects of Dithane M-45 on soil bacterial strains (Deliu, 2010), in which higher concentrations of the fungicide were used, the largest inhibition zone was found for Dithane M-45 0.16% and 0.32% (up to 30 mm), while Dithane M-45 0.08% led to inhibition zones which mostly ranged between 7 - 10 mm.

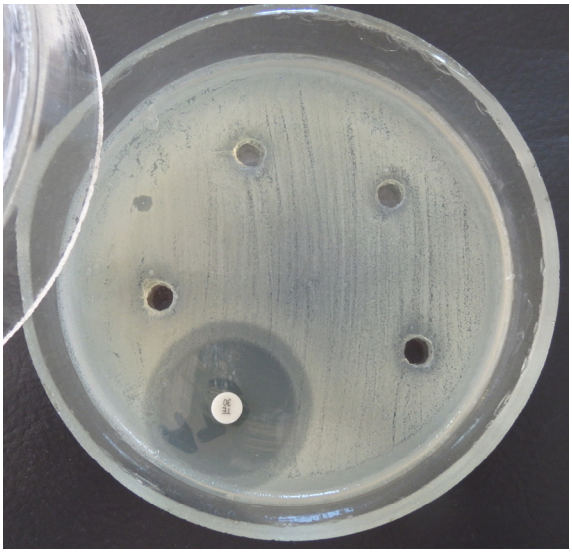

Figure 4. Antimicrobial activity of Dithane M-45 on *Weeksella virosa* - agar wells method.

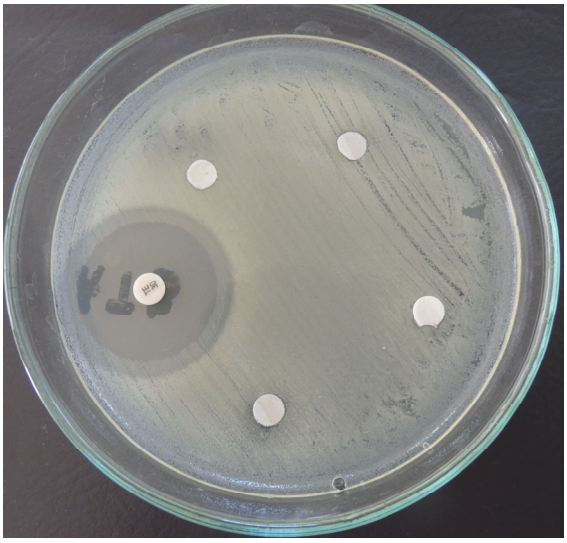

Figure 5. Antimicrobial activity of Dithane M-45 on *Elizabethkingia meningoseptica* - filter paper discs method.

Conclusions

The meristematic root cells of *Tulipa praestans* cv. 'Unicum' proved to be sensitive to the mitogenic effect of the fungicide Dithane, which resulted in a significant increase of the percentage of cells undergoing the cell division. Completion of at least two cell cycles subsequently to the fungicide treatment had also a stimulatory effect, the frequency of cells in one of the phases of mitotic division being in these treatments approximately double as compared to the control.

REFERENCES

Bolle, P., Mastrangelo, S., Tucci, P., Evandri, M. G. (2004) Clastogenicity of atrazine assessed with the *Allium cepa* test. *Environ. Mol. Mutagen.,* **43**(2), 137-141

Ceyhan, M., Celik, M. (2011) *Elizabethkingia meningosepticum* (*Chryseobacterium meningosepticum*) infections in children, *Int. J. of Pediatrics*, volume 2011, Article ID 215237

Chauhan, L. K. S., Saxena, P. N., Gupta, S. K. (1999) Effects of deltamethrin on the ultrastructure of root meristem cells of *Allium cepa*, *Pesticide Biochemistry and Physiology*, **64**, 81-189

Chihab, W., Alaoui, A.S., Amar, M. (2004) *Chryseomonas luteola* identified as the source of serious infections in a Moroccan University Hospital. *J. Clin. Microbiol.,* **42**(4), 1837-1839

Deliu, I. (2010) Studies regarding the resistance of some bacterial strains from soil to chemical factors, *Lucrări Ştiinţifice Seria Horticultură,* **53**(2), 619-624

Dias, M., Prashant, K., Pai, R., Scaria, B. (2010) *Chryseobacterium meningosepticum* bacteremia in diabetic nephropathy patient on hemodialysis, *Indian J. Nephrol.,* **20**(4), 203-204

Doublet, B., Robin, F., Casin, I., Fabre, L, Le Fleche, A., Bonnet, R., Weill, F. X. (2010) Molecular and biochemical characterization of the natural chromosome-encoded class A β-Lactamase from *Pseudomonas lutea*, *Antimicrobial Agents and Chemother.,* **54**(1), 45-51

Fernandes, T. C. C., Mazzeo, D. E. C., Marin-Morales, M. A. (2009) Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent - trifluralin herbicide, *Ecotoxicol. and Environ. Safety,* **72**, 1680-1686

Fiskesjö, G. (1993) Technical methods section. *Allium* test I: A 2-3 day plant test for toxicity assessment by measuring the mean root growth of onions (*Allium cepa* L.), E*nviron.l Toxicolo. and Water Quality,* **8**, 461-470

Giacomelli, F. R. B. (1999) *Evaluation of the meiotic behavior in varieties of oat (Avena sativa) recommended for the south area,* Dissertation (Master's degree in Genetics) - State University of Maringá

Hansen, A. K., Nielsen, D. S. (2011) *Handbook of Laboratory Animal Bacteriology*, 2nd Edition, CRC Press, Boca Raton, pp 300

Harlev, M., Mujahed, M., Waner, T., Kalman R. (2009) A novel protocol for treatment and elimination of *Pasteurella pneumotropica* infection from rodent colonies with subclinical latent infection, *Israel J. Veterinary Medi.,* **64**(4), 108-113

Hassan, S., Amer, S., Mittal, C., Sharma, R. (2012) *Ewingella americana*: an emerging true pathogen, *Case Report in Infectious Diseases*, vol. 2, Article ID 730720

Hussain, S., Siddique, T., Saleem, M., Arshad, M., Khalid, A. (2009) Impact of pesticides on soil microbial diversity, enzymes, and biochemical reactions, *Advances in Agronomy,* **102**, 159-200

Kihlman, B. A. (1966) *Actions of chemical on dividing cells*, 1st Edition, Prentice-Hall, New Jersey, Englewood Cliffs, pp 88

Kuriyama, R. (1982) Effect of colcemid on the centrioloe cycle in Chinese hamster ovary cells, *J. Cell Sci.,* **53**, 155-171

Marcano, L., Carruyo, I., Del Campo, A., Montiel, X. (2004) Cytotoxicity and mode of action of maleic hydrazide in root tips of *Allium cepa* L, *Environ. Res.,* **94**, 221-226

Ghadimi, B. M., Sackett, D. L., Difilippatonio, M. J., Schröck, E., Neumann-Jauho, T. A. (2000) Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations, *Genes Chromosomes Cancer*, **27**, 183-190

Ma, T. H. (1999) The international program on plant bioassays and the report of the follow-up study after the hands-on workshop in China, *Mutation Res.,* **426**(2), 103-106

Ochi, T., Suzuki, T., Isono, H., Schlagenhaufen, C., Goessler, W., Tsutsuí, T. (2003) Induction of structural and numerical changes of chromosome, centrosome abnormality, multipolar spindles and multipolar division in cultured Chinese hamters V79 cells by exposure to a trivalent dimethylarsenic compound, *Mutation Res.*, **530**, 59-71

Parihar, P., Parihar, L., Bohra, A. (2010) *In vitro* antibacterial activity of fronds (leaves) of some important pteridophytes, *J. Microbiol Antimicrobials*, **2**(2), 19-22

Popescu, A., Şuţan, A. N., Deliu, I. (2013) Genotoxicity and mutagenicity testing of chemical substances by the plant-based bioassays, *Proceedings of the 19th International Conference "The Knowledge-Based Organization",* Sibiu, Romania, pp 361-366

Reina, J., Gil, J., Salva, F., Gomez, J., Alomar, P. (1990) Microbiological characteristics of *Weeksella virosa* (Formerly CDC group IIf) isolated from the human genitourinary tract, *J. Clinic. Microbiol.,* **28**(10), 2357-2359

Rozhon, W., Khan, M., Poppenberger, B. (2012) The natural antibiotic resistances of the Enterobacteriaceae *Rahnella* and *Ewingella*, In: *Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium*, Pana, M. (ed), InTech

Slenker, A. K., Hess, B. D., Jungkind, D. L., DeSimone, J. A. (2012) Fatal case of *Weeksella virosa* sepsis, *J. Clinic. Microbiol.,* **50**(12), 4166-4167

Sudhakar, R., Ninge Gowda, K. N., Venu, G. (2001) Mitotic abnormalities induced by silk dyeing industry effluents in the cells of *Allium cepa*, *Cytologia*, **66**, 235-239

Sumara, I., Giménez-Abián, J. F., Gerlich, D., Kraft ,C., La Torres, T. (2004) Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. *Curr. Biolo.,* **14**, 1712-1722

Şuţan, N. A., Popescu, A., Mihăescu, C., Soare, L. C., Marinescu, M. V. (2014) Evaluation of cytotoxic and genotoxic potential of the fungicide Ridomil in *Allium cepa* L, *Analele Ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi, Seria a II-a. Biologie vegetală*, **60**(1), 5-12

Şuţan, N. A., Yanik, T., Popa, E. (2015) Comparative genotoxicity of two fungicide formulations in meristemtic root cells of *Narcissus pseudonarcissus* cv. 'Samanta', *Full Paper Proceeding GIMAR*, **1**, 216-225

Türkoğlu, S. (2009) Genotoxic effects of mono-, di-, and trisodium phosphate on mitotic activity, DNA content, and nuclear volume in *Allium cepa* L, *Caryologia,* **62**(3), 171-179

Valgas, C., de Souza, S. M., Smânia, E. F. A., Smânia-Jr., A. (2007) Screening methods to determine antibacterial activity of natural product, *Braz, J. Microbiol.,* **38**, 369-380

Yi, H., Meng, Z. (2003) Genotoxicity of hydrated sulfur dioxide on root tips of *Allium sativum* and *Vicia faba*, *Mutation Res.,* **537**(1), 109-114

Yuzbasioglu, D. (2003) Cytogenetic effects of fungicide afugan on the meristematic cells of *Allium cepa* L, *Cytologia*, **68**(3), 237-243

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 19-27)

Western Blot analysis of detoxifying enzymes, cytochrome c and sirtuin expression in patients suffering from mitochondrial complex I deficiency

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SUMMARY. In recent years, a great deal of interest has been given to sirtuins and their role in mitochondrial metabolism, especially their implication in the process of aging. By activating detoxifying enzymes such as manganese-dependent superoxide dismutase (MnSOD) or copper-zinc superoxide dismutase (CuZnSOD), sirtuins are able to trigger enzymatic cascades leading to the reduction of cellular reactive oxygen species (ROS). This is of high importance as ROS is one of the main factors implicated in cellular aging.

In addition, it has been recently shown that mitochondrial complex I activity influences the expression and function of sirtuins. Complex I or the NADH dehydrogenase is the largest complex of the mitochondrial respiratory chain and a major regulator of energy production. Also, it is the main site for ROS production. That being said, it is important to determine not only the exact connection between sirtuins and ROS production, but also the way complex I affects sirtuin expression and activation.

In our study we analyzed selected cell lines carrying mitochondrial DNA mutations in genes encoding for complex I subunits. Our main goal was to identify potential correlations between sirtuin expression, ROS production and specific mitochondrial DNA mutations. This has been done by measuring antioxidant enzymes and sirtuin expression using Western Blot technique. The results demonstrate that complex I deficiencies do have an impact on ROS production and sirtuin expression. Moreover, we identified a particular correlation between SIRT3 and MnSOD.

Keywords: Mitochondria, oxidative stress, sirtuins

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Introduction

To maintain its physiological parameters, as well as for various actions performed in everyday life, the human body needs energy. Since the very first eukaryotic cell, energy has been provided by sub-cellular structures known as mitochondria.

The main function of mitochondria is mostly energy (ATP) production. Apart from this, it is also involved in reactive oxygen species (ROS) production, Ca^{2+} homeostasis and cellular apoptosis. These processes depend on the structural and functional integrity of the mitochondrial respiratory chain, also known as the oxidative phosphorylation system (OXPHOS). OXPHOS is made up of five multi-subunit enzymatic complexes, expressed from both nuclear and mitochondrial genes. The five complexes use reduced coenzymes and molecular oxygen to produce cellular energy. Mitochondrial OXPHOS activity is dependent on the NAD^+/NADH ratio that is crucial for the efficiency of the mitochondrial metabolism (Alberts *et al.*, 2014).

ROS production is a side effect of energy production, an increase in its levels leading to important cellular damage. Consequently, cells possess detoxification enzymes, such as manganese-dependent superoxide dismutase (MnSOD or SOD2) or Cu-Zn superoxide dismutase (CuZnSOD or SOD1) to protect against toxic levels of ROS. Also, c-type cytochromes, components of the electron transport chain (ETC), act as ROS scavengers. Cytochrome c levels may vary in a ROS-dependent manner (Atlante *et al.*, 2000).

The main producer of ROS in the mitochondria is complex I of the ETC, the largest and the most evolved enzymatic complex of the respiratory chain. Among its 45 subunits, only 14 are crucial for the catalytic function. Half of those crucial subunits are nuclear-encoded (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8) whereas the other half results from the expression of the mitochondrial genome (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) (Fassone and Rahman, 2012). Due to its important role, complex I has to be well regulated. Apart from transcriptional and translational regulation, there is also post-translational regulation, which involves phosphorylation and especially acetylation. Indeed 20% of mitochondrial proteins own an acetylation site, NDUFA9 one of the complex I subunits being one of them (Ahn *et al.*, 2008). Acetylation is dependent on sirtuin activity.

Sirtuins belong to the class III histone deacetylase family with NAD^+-dependent protein lysine deacetylase activity, which has been shown to play an important role in many physiological processes. These enzymes are characterized by highly conserved NAD^+ binding and catalytic domain (Haigis and Sinclair, 2010; Li *et al,* 2015). Therefore, it is clear that sirtuins also require a balanced NAD^+/NADH ratio, for their activation, thus being dependent on the metabolic state of the cell. Their function as deacetylases allows to activate or inactivate target proteins. One of the most intriguing roles of sirtuins is related to the regulation of cellular ROS level (Pahirar *et al.*, 2014). By activating detoxifying enzymes such as MnSOD or CuZnSOD, sirtuins are able to trigger enzymatic cascades, which lead to the reduction of cellular reactive oxygen species (ROS).

Nowadays seven mammalian sirtuins are known (SIRT1-7). Their localization is dependent on tissue or cell specificity as well as physiological conditions: SIRT1, 6, 7 are located mainly in the nucleus, SIRT1 and SIRT2 in the cytosol, and SIRT3, 4 and 5 in the mitochondria (Verdin *et al.*, 2010). This study focuses more on the characterization of SIRT3 expression in skin fibroblasts from patients carrying mitochondrial DNA mutations. SIRT3 has been chosen owing to its localization in the mitochondria.

We perfomed Western Blotting in order to determine whether there is a connection between the expression of SIRT3, MnSOD, CuZnSOD and cytochrome c, as well as the implications that mitochondrial mutations have on their levels.

Materials and methods

Sample preparation. Fibroblasts were obtained from skin biopsies taken after acquiring informed consent from seven patients with mitochondrial complex I deficiencies. Four samples with four diferent mitochondrial mutations encoding complex I subunits (Table 1) were selected for further Western Blotting analysis. As control, healthy fibroblasts obtained also from skin biopsies were used.

Table 1.

Biological material used for Western Blotting: samples from four patients with diferent mitochondrial mutations and two healthy controls

Complex I subunits	Mutation	Codes used in this study	Clinical phenotype
ND4	11778	ND4 11778 DCF	Only visual impairment
ND6	14487	ND6 14487 BM	Severe
ND1	3460	ND1 3460 BM	Mild
	3709	ND1 3697 BL	Severe
WT1, WT2	Healthy fibroblasts / controls (wild type)		

Fibroblasts were cultivated at 37 °C (5% CO_2) on DMEM-12 AmnioMAX media (Institut de Biotechnologies Jacques Boy) supplemented with 10% Fetal Bovine Serum Standard Quality (Institut de Biotechnologies Jacques Boy), 25 mg of uridine and 50 mg of pyruvate. Cells were grown in 75 cm^2 tissue culture flasks and were used after they reached 75 % confluence. To prepare the cells, two confluent flasks (about 7 x 10^6 cells/ flask) were trypsinized for 7 minutes at 37 °C (2 ml/ flask). Trypsin was inactivated with 4 ml of cell media. Cells were counted with the Z™ Series COULTER COUNTER (Beckman Coulter Inc.) and then, harvested by centrifugation (800xg, 5 minutes). The cell pellets were stored at -80 °C until further use.

Western Blot assay. Western Blotting has been used to quantify the expression of MnSOD, CuZnSOD, cytochrome c and SIRT3, based on their molecular weight.

We used Tris Sodium-dodecylsulfate (SDS) with a pH of 6.8 (1 M Tris-Base and 30 mM SDS) and one with a pH of 8.8 (300 mM Tris-Base and 30 mM SDS). Next, we made the following buffers: (i) Sample buffer: 1 M Tris-SDS with pH 6.8, 30 mM SDS, 10% Glycerol, 1% Bromophenol Blue and 5% 2β-Mercapto Ethanol; (ii) Radioimmunoprecipitation assay (RIPA) buffer (pH 8): 150 mM NaCl, 1% Triton X-100, 0.5% Sodiumdeoxycholate, 0.1% SDS and 50 mM Tris; (iii) Electrophoresis buffer (pH 8.3): 272 mM Tris-Base, 1.92 M Glycine, 35 mM SDS; (iiii) Blotting buffer (pH 8.3): 20 mM Tris-Base, 150 mM Glycine, 20% Ethanol.

Two types of running gels were prepared: (i) 8% Acrylamide gel: 12.5 ml Tris-SDS (pH 8.8), 6.65 ml Acrylamide (30%), 5.85 ml distilled water; (ii) 15% Acrylamide gel: 12.5 ml Tris-SDS (pH 8.8), 12.5 ml Acrylamide (30%). 250 µL of APS (ammonium persulfate) 10% and 12.5 µL of TEMED (Tetramethyl-ethylenediamine, Eurobio, Les Ulis, France) were added. Quickly, the gels were poured between the two plaques. Isopropanol was applied on top of them. The stacking gel (Acrylamide 3%) was prepared and 100 µL of APS and 10 µL of TEMED were then added and the mixture poured on top of the polymerized running gel (after waiting at least 30 minutes).

In order to prepare the samples, we mixed them with a 20 µl of RIPA buffer per millions of cells with antiprotease(1x CompleteTM Protease inhibitor cocktail tablets, Roche, Mannheim, Germany). They were then centrifuged (8000xg, 20 min, 4 °C) and the supernatant was recovered. Next, 30 µg of sample were diluted into an antiprotease solution (1x) in order to reach 10 µL. Eventually, samples were ½ diluted into sample buffer. This mix was then heated at 100 °C for 5 minutes.

Then, we started the electrophoretic migration on the polyacrylamide gel: the first well was filled up with a molecular weight marker (PageBlueTM Protein Staining Solution, Euromedex, Souffelweyersheim, France), while in the rest of the wells we deposited the samples. The pre-migration was started under an 100 V electric field, during 10 minutes. Next, a 150 V electric field enabled the separation of proteins over 90 min. The electrophoresis tank contained electrophoresis buffer diluted in distilled water.

Nitrocellulose membranes were cut, washed in blotting buffer and then placed on a sponge on Western Blot transfer membrane (Bio-Rad, Hercules, USA*)*. The migration gel was carefully transferred onto the membrane after cutting off and throwing the stacking gel away. An additional sponge was used to cover the gel. The transfer was then started at 260 mA for 90 min.

For antibody incubation, the membranes were transferred in a box containing a 1:1 mixture of blocking buffer (LI-COR™) and PBS 1x and placed on a stir plate for 2 h. After that, each membrane was washed twice with PBS 1X and Tween (1%) (Sigma Aldrich, Lyon, France), for 5 minutes. The antibodies, specific for each

protein of interest, came from mouse, rabbit or goat (Table 2). We used 5 ml in each box and left it for at least 2 hours. Next the membrane was again washed twice with PBS 1x + Tween 1%. Next, 5 ml of secondary antibody were added in each box and removed 45 minutes later. The washing process was repeated. Revelation was performed according to the settings below with the Odyssey Fc imaging device by LI-COR. The bands were quantified by integration of pixel intensity and normalized to reference proteins (α-Tubulin, and VDAC) which served as an internal control. The Western Blotting analysis was repeated three times for each set of samples.

Table 2.

Antibodies and immunostaining conditions

Target	Supplier	Primary antibody		Wavelength / Duration of image aquisition
		Dilution	Species	
α-Tubulin	Calbiochem	1/2000	Mouse	700 nm / 2 min
MnSOD	Abcam			
Cytochrome c				
CuZnSOD		1/2000	Rabbit	800 nm / 7 min
SIRT 3	Cell signaling	1/1000		

Results and discussion

Using skin fibroblasts from patients suffering from complex I deficiency due to mitochondrial DNA mutations, we performed cell culture and Western Blot in order to assess the expression of selected proteins according to their specific roles: two detoxification enzymes (MnSOD,CuZnSOD), cytochrome c and one metabolic enzymes regulator (SIRT 3). We used α Tubulin, a cytoskeletal protein, as a reference protein to control the loading on the gel, and VDAC as a reference protein for cytochrome c, due to its role as a mitochondrial porin. A comparison between levels of expression of the same proteins from healthy fibroblasts and the ones found in patient cells harboring mitochondrial DNA mutations has been made.

The oxidative stress was assessed by quantifying the expression of the antioxidant enzymes (MnSOD and CuZnSOD) and cytochrome c which has an antioxidant role (Atlante *et al.*, 2000).

MnSOD expression compared to α-tubulin in patient cell lines harboring mitochondrial DNA mutations, showed some variations for each cell sample (Fig. 1). For example, the MnSOD band corresponding to ND1 BM presents an increased signal. After quantification, we produced a graphic that would help in interpreting the results. A slight decrease of 10% compared to the control group (WT), can be noticed for the first patient cell line which is ND1 BM. Also, both ND1 BL and ND4 DCF present a reduction of 30% and over 80% respectively. However, a significant increase of 30% can be seen for ND6 BM cell lines.

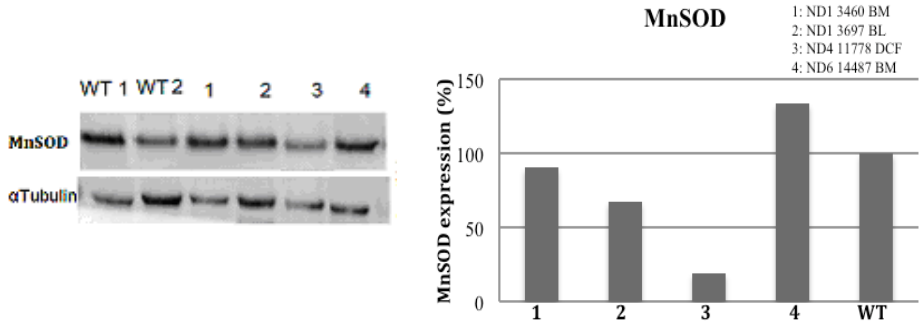

Figure 1. MnSOD levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations.

For CuZnSOD, the control bands seemed to have approximately the same signal strength (Fig. 2). We noticed some differences among patient cell lines. ND1 BM presents with the lowest level, all the others being increased, or at least equal to the control. ND1 BM and ND6 BL present an increase of over 140%.

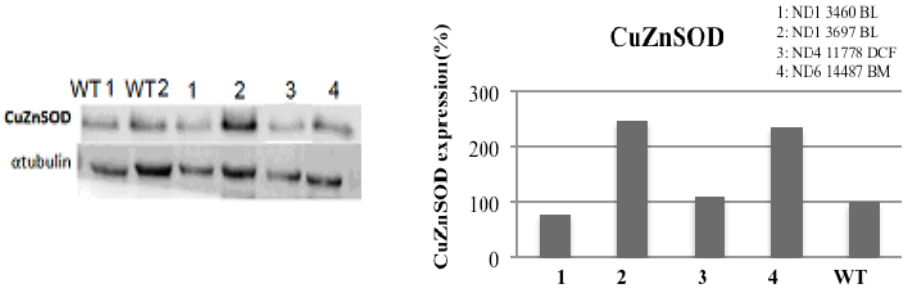

Figure 2. CuZnSOD levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations.

Cytochrome c expression presented similar bands on the Western blot picture when compared to VDAC expression (Fig. 3). Cytochrome c / VDAC ratio was slightly increased in both patients carrying a mutation in the ND1 subunit (ND1 BM and ND1 BL) compared to WT. An important increase of 420% was observed for ND6 BM. ND4 DCF was almost identical to the control.

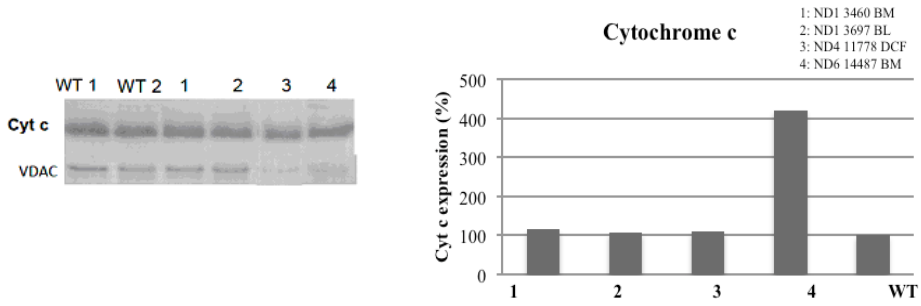

Figure 3. Cytochrome c levels normalized to VDAC in patient cell lines harboring mitochondrial DNA mutations.

We went on by quantifying SIRT3 expression in our patient cell lines. ND1 BL and ND4 DCF showed a decreased expression of SIRT3 with 20% and 30% respectively, compared to the control group. However, ND1 BM and ND6 BM had an increased level of SIRT3 expression with about 25% (Fig. 4).

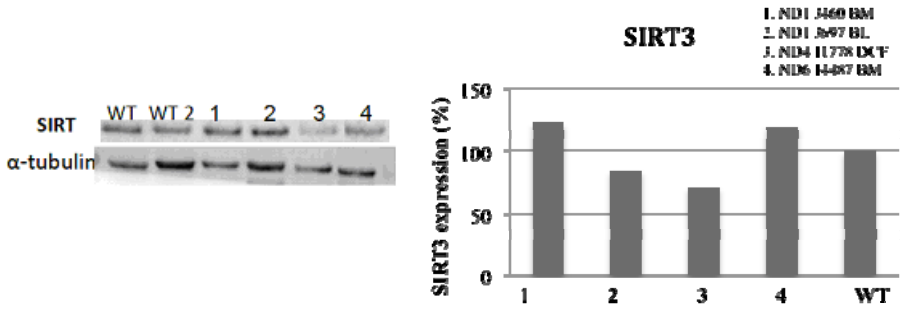

Figure 4. SIRT3 levels normalized to α-tubulin in patient cell lines harboring mitochondrial DNA mutations

Analyzing our results, SIRT3 seemed to vary on a mutation-dependent basis. Indeed, as showed on the diagram above, its expression was decreased compared to WT for ND1 3697 BL and ND4 11778 DCF whereas SIRT3 expression was induced in ND1 3460 BM and ND6 14487 BM.

In addition, a strong resemblance in the expression pattern of SIRT3 and MnSOD was observed. For each type of mutation, the variation in SIRT3 expression was similar to the change in MnSOD expression, not CuZnSOD. This observation is even more intriguing considering that both MnSOD and SIRT3 are exclusively

mitochondrial proteins, CuZnSOD being a cytoplasmic enzyme. Moreover, it is known that SIRT3 activates MnSOD by deacetylation (Bause and Haigis, 2012; McDonnell *et al.*, 2015). Lately, it has been proposed that SIRT3 would enable the induction of antioxidant genes, among which those encrypting MnSOD, through PGC-1α (Kong *et al.*, 2010). Thus, SIRT3 would not only regulate the acetylation of the protein MnSOD, but would also be able to impact its expression at a transcriptional level.

Furthermore, we observed that patients affected by a different mutation in the same subunit, displayed different biochemical profiles related to oxidative stress. For example, ND1 3460 BM and ND1 3697 BL had very different level of expression of proteins of interest such as MnSOD, CuZnSOD and Cytochrome c. The biochemical profile therefore seemed to be dependent on the location and nature of the mutation. Moreover, the biochemical profiles of patients displaying a severe phenotype are very heterogeneous, making it difficult to correlate the clinical phenotype to oxidative stress.

On the whole, the study of sirtuin regulation is an area with plenty of future possibilities, the better understanding of sirtuin function and regulation giving new insights into the process of cellular aging. Also, this knowledge would aid the discovery of new molecules acting on sirtuin expression for many illnesses, especially mitochondrial diseases, cancer and neurodegenerative diseases. Moreover, sirtuins could be the key to reducing cellular stress, thus ensuring a longer cellular life.

Conclusions

Although some major progresses have lately been made regarding the diagnosis of mitochondrial impairments, they is still no treatment available. Better understanding of the biochemical consequences of complex I deficiencies can provide a crucial support in developing new therapeutic approaches.

In our study, the oxidative stress in complex I-deficient patients was indirectly assessed through the Western blott estimation of MnSOD, CuZnSOD and cytochrome c expression. We observed that patients affected by a different mutation in the same subunit, displayed different immunoblotting profiles related to oxidative stress. Also, we noticed a possible correlation between SIRT3 and MnSOD expression in our patient cell lines carrying mitochondrial DNA mutations encoding complex I subunits.

As already mentioned, more research into this area is needed in order to be able to discover the true metabolic implications of sirtuin function.

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REFERENCES

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., Walter, P. (2014) *Molecular Biology of the Cell, 6th Edition,* Garland Science, New York, pp 1464

Ahn, B. H., Kim, H. S., Song, S., Lee, I. H., Liu, J., Vassilopoulos, A., Deng, C. X., Finkel, T. (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis, *Proc. Natl. Acad. Sci. USA,* **105**, 14447-14452

Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E., Passarella, S. (2000) Cytochrome c is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and can operate as a ROS Scavenger and as a respiratory substrate in cerebellar neurons undergoing excitotoxic death, *J. Biol. Chem.,* **275**, 37159-37166

Bause, A. S., Haigis, M. C. (2012) SIRT3 regulation of mitochondrial oxidative stress, *Exp. Gerontol.,* **48**, 634-639

Fassone, E., Rahman, S. (2012) Complex I deficiency: clinical features, biochemistry and molecular genetics, *J. Med. Genet.*, **49**, 578-590

Haigis, M., Sinclair, D. (2010) Mammalian Sirtuins: Biological Insights and Disease Relevance, *Annu. Rev. Pathol.,* **5**, 253-295

Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., Fang, F., Chang, Y. (2010) Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis, *PloS ONE,* **5**, e11707

Li, Y., Liu, T., Liao, S., Li, Y., Lan, Y., Wang, A., Wang, Y., He, B. (2015) A mini-review on Sirtuin activity assays, *Biochem. Biophys. Res. Commun.,* **467**, 459-466

McDonnell, E., Peterson, B. S., Bomze, H. M., Hirschey, M. D. (2015) SIRT3 regulates progression and development of disseases of aging, *Trends Endocrinol. Metab.,* **26**, 486-492

Parihar, P., Solanki, I., Mansuri, M. L., Parihar, M. S. (2014) Mitochondrial sirtuins: emerging roles in metabolic regulations, energy homeostasis and diseases, *Exp. Gerontol.,* **61**, 130-141

Verdin, E., Hirschey, M. D., Finley, L. W., Haigis, M. C. (2010) Sirtuin regulation of mitochondria: energy production, apoptosis and signaling, *Trends. Biochem. Sci.,* **35**, 669–675

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 29-32)

Investigation of Methicillin-Resistant *Staphylococcus aureus* strains from Satu Mare using Molecular Biology Techniques

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SUMMARY. *Staphylococcus aureus* is a clinically important bacteria. It causes mild to severe, often life-threatening infections. A total of 51 *Staphylococcus* strains were analyzed in this study using polymerase chain reaction. 33% of the strains were confirmed as *S. aureus*, and none of these were resistant to methicillin.

Keywords: *mecA*, MRSA, *nucA*, PCR, *Staphylococcus aureus*

Introduction

The *Staphylococcus aureus* is a sphere shaped, non-motile, Gram-positive bacterium, which forms grape-like clusters. Due to their antibiotic resistance, the methicillin-resistant *S. aureus* (MRSA) strains are widespread both in hospitals and ambulatory units. These strains were documented first in 1961 (Ito *et al.,* 1999).

The *mecA* gene encodes a modified penicillin-binding protein, the PBP2a, which has low affinity to penicillin antibiotics (Song *et al*., 1987). The *mecA* gene together with the *mecI* and *mecR1* genes form the *mec* operon, which is located on the SCC*mec* mobile genetic element (Ito *et al.,* 2004). This has several classes and sub classes (Ito *et al.*, 2001).

The aim of this study is to investigate the presence of MRSA is Satu Mare County. It is necessary to know what kind of antibiotics can be considered to be taken by the patients.

Materials and methods

The analysed *Staphylococcus* strains are isolated and determined using classical microbiological methods in the CityMed laboratory, from the Satu Mare county Public Health Department.

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Using the multiplex polymerase chain reaction (PCR) technique we amplified a 270 bp long region of the thermonuclease gene (*nucA*), which is specific to *S. aureus* strains, and a 533 bp long region of the *mecA* gene. In case of the *nucA* gene the presence of amplification products confirm that the analysed strains are *S. aureus*, and the *mecA* amplicon demonstrates that the studied strains are resistant to methicillin and other penicillin group antibiotics (cross-resistance) (Matthews and Tomasz, 1990). First we used a colony PCR method, which lacks DNA extraction and a suspension of bacteria were directly added to the PCR mix, and second the PCR was repeated with isolated DNA. Later the experiments were repeated using purified DNA from selected isolates. The PCR mix contained the following components:

10 µl 5x buffer
3 µl $MgCl_2$ with 1.5 mM final concentration
1 µl dNTP 0.2 final concentration
0.5 µl mecA I primer 1 µM final concentration
0.5 µl mecA R primer 1 µM final concentration
0.5 µl nucA R primer 1 µM final concentration
0.5 µl nucA I primer 1 µM final concentration
GoTaq polymerase 1.25 U

40 µl in a reaction tube + 10 µl sample DNA

We used the following PCR programme:

Initiative denaturation: 5 minutes 94º C
Denaturation: 30 seconds 92º C
Annealing: 30 seconds 52º C
Extension: 60 seconds 72º C
} 30 cycle
Final elongation: 5 minutes 72º C

Results and discussion

Using colony PCR we found seven MRSA suspect strains. Repeating the amplification with DNA from these suspect strains we could not confirm the presence of methicillin-reisitant *S. aureus*. From the total of 51 isolates only 33% were confirmed as *S. aureus* (Fig. 1). The investigated strains in majority were isolated from nasal discharge (Fig. 2).

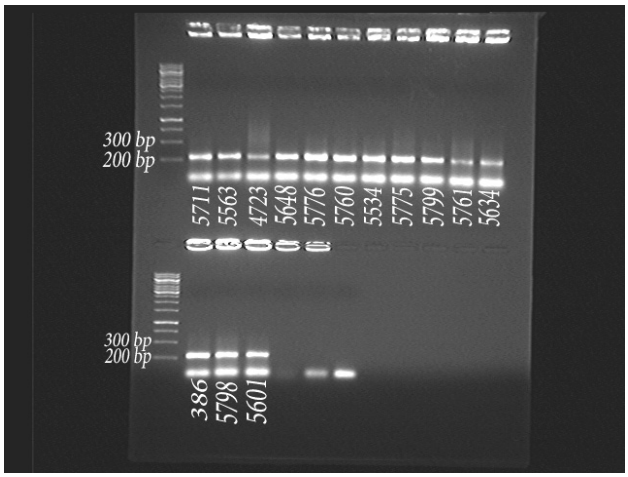

Figure 1. Separation by agarose gel electroforesis (1%) of multiplex PCR products. The first lane represents molecular size ladder (GeneRuler 100 bp DNA ladder, Fermentas). In case of *S. aureus*strains a clear band can be observed with an expected size of 270 bp. The last three lanes are negative controls without sample DNA.

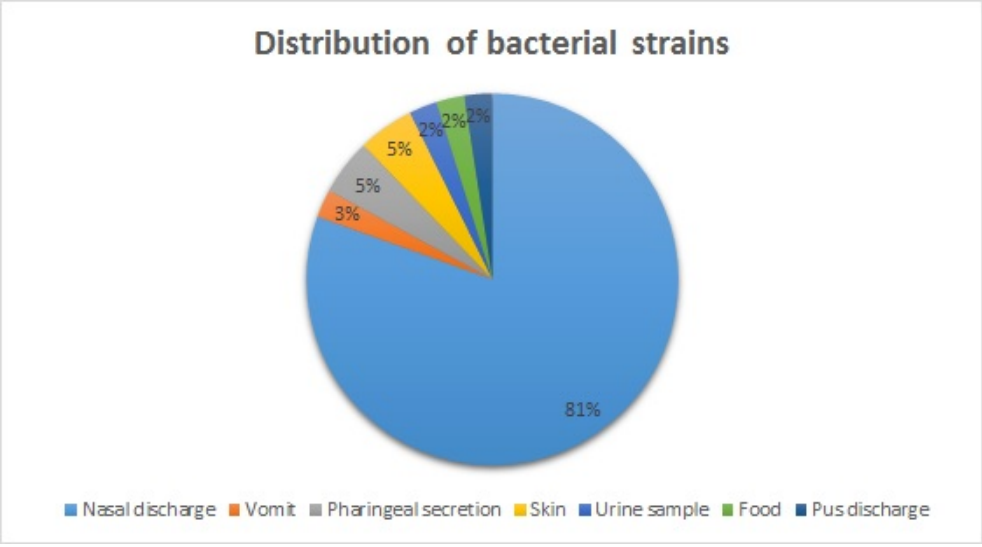

Figure 2. Distribution of bacterial strains between source of isolation. The generality of the strains stem from nasal discharge 81%, presenting that this bacteria appears mostly in the respiratory tracts. A few isolates stem from pharingeal secretion, 5%, skin, 5%, food, 2%, pus discharge, 2% and urine sample, 2%.

We could not identify methicillin-resistant *S. aureus* strains.

Conclusions

Using molecular biology methods only 33% of the strains were confirmed as *S. aureus*. The other are probably coagulase-negative staphylococcus strains. It is difficult to make distinction between the coagulase-positive and coagulase-negative strains, because using classical methods we can not see the slight difference which can be seen with molecular methods. For example: using PCR.

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REFERENCES

Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H., Hiramatsu, K. (2004) Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, ccrC, *Antimicrobial Agents and Chemotherapy*, **48**(7), 2637-2651

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C., Hiramatsu, K. (2001) Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*, *Antimicrobial Agents and Chemotherapy*, **45**(5), 1323-1336

Ito, T., Katayama, Y., Hiramatsu, K. (1999) Cloning and nucleotide sequence determination of the entire mec DNA of pre-methicillin-resistant *Staphylococcus aureus* N315, *Antimicrobial Agents and Chemotherapy*, **43**(6), 1449-1458

Matthews, P., Tomasz, A. (1990) Insertional inactivation of the mec gene in a transposon mutant of a methicillin-resistant clinical isolate of *Staphylococcus aureus*, *Antimicrobial Agents and Chemotherapy*, **34**(9), 1777-1779

Song, M. D., Wachi, M., Doi, M., Ishino, F., Matsuhashi, M. (1987) Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion, *FEBS Letters,* **221**(1), 167-171

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 33-38)

Detection of beta-lactamase resistance genes in a hospital chlorinated wastewater treatment system

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SUMMARY. In this study, by amplification of several specific resistance genes we aimed at evaluating the presence of beta-lactamase resistance genes in the influent and effluent of a hospital chlorinated wastewater treatment system. Four types of beta-lactamase resistance genes (blaPER, blaVIM, blaNDM-1 and SHV) were detected in the influent water sample, two of which (blaVIM and SHV) being detected in the effluent as well. Our results indicate the reduced effectiveness of wastewater treatment, as several resistance genes can be found in the water discharged after the chlorination treatment.

Keywords: Antibiotic resistance genes, beta-lactam antibiotics, chlorination, hospital wastewater

Introduction

Beta-lactam antibiotics are active agents against many Gram-positive as well as Gram-negative microorganisms (Thomson and Bonomo, 2005). These antimicrobials are widely used to treat bacterial infections in humans, thus, in most countries they are the largest group of antibiotics used by hospitals (ECDC 2013) potentiating the emergence of resistance strains able to withstand high concentrations of antibiotics. Clinical settings can be a potential source for spread and development of antibiotic resistance (Pauwels and Verstraete, 2006) due to the dispersal of antibiotic resistance genes by vertical or horizontal gene transfer to bacteria that are related or unrelated evolutionary and ecologically (Gomes *et al*., 2013). Chlorination is a widely used disinfection method, nonetheless recent studies indicated that chlorination can enhance changes in ARG (antibiotic resistance genes) abundance and diversity (Jia *et al.*, 2015). The aims of this study were to evaluate and compare the presence of beta-lactamase resistance genes in a hospital wastewater collected from a public hospital in Romania, before (influent water) and after the treatment by chlorination steps (effluent wastewater).

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Material and methods

The water samples were collected from the influent and effluent of a chlorination wastewater treatment system from a public hospital in Cluj-Napoca, Romania, in sterile 1 L containers and transported on ice to the laboratory, for further analyses. To concentrate the microbial biomass, the water was filtered onto MCE filtration membranes (47 mm in diameter and 0.2 μm pore size). Genomic DNA was extracted from the membranes by using ZR Soil Microbe DNA Miniprep (ZymoResearch, USA) according to the manufacturer's protocol. Beta lactamase specific genes were amplified by PCR using several primer pairs (Table 1). PCR mixture contained 2 μl genomic DNA, 0.25 μM of each primer, 5 μl of 5x MyTaq Reaction Buffer, 1.5 U of MyTaq Red DNA Polymerase, and water to a final volume of 25 μl. The amplification protocol included a initial denaturation step of 5 min at 95 ^{o}C, followed by 35 cycles of denaturation 30 sec at 95 ^{o}C, primer annealing at 56-58 ^{o}C (specific for each primer pair), and extension for 30 sec at 72 ^{o}C, and a final extension step of 10 min at 72 ^{o}C. PCR products of the expected size were verified by 2 % agarose gel electrophoresis and were visualized under UV after ethidium bromide staining.

Table 1.

Primers used for amplification of Beta-lactamase resistance genes.
Forward and reverse primer sequences are given.

Gene	Gene classification (Beta-lactamase Classes)	Sequence of forward primer (5'-3')	Sequence of reverse primer (5'-3')	References
blaPER	A	TGCTGGTTGCTGTTTTTGTGA	CCTGCGCAATGATAGCTTCAT	Jiang *et al*., 2013
blaPSE	A	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC	Zhu *et al*., 2013
blaCTX-M	A	GGAGGCGTGACGGCTTTT	TTCAGTGCGATCCAGACGAA	Zhu *et al*., 2013
blaIMP	B	AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	Zhu *et al*., 2013
blaOXA-10	D	CGCAATTATCGGCCTAGAAACT	TTGGCTTTCCGTCCCATTT	Zhu *et al*., 2013
blaVIM	B	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	Zhu *et al*., 2013
blaOXA-48	D	GTAGCAAAGGAATGGCAA	CCTTGCTGCTTATTGTCA	Naas *et al*., 2012
blaKPC	A	GATACCACGTTCCGTCTGG	GCAGGTTCCGGTTTTGTCTC	Hindiyeh *et al*., 2008
blaNDM-1	B	ATTAGCCGCTGCATTGAT	CATGTCGAGATAGGAAGTG	Naas *et al*., 2011
SHV	A	GCGAAAGCCAGCTGTCGGGC	ATTGGCGGCGCTGTTATCGC	Jiang *et al*., 2013
ampC	C	CAGCCGCTGATGAAAAAATATG	CAGCGAGCCCACTTCGA	Zhu *et al*., 2013

Results and discussion

By PCR amplification using primer pairs specific for beta-lactamase resistance genes we detected four beta-lactamase resistance genes blaVIM, blaNDM-1, SHV and blaPER, in the influent sample, whereas SHV and blaVIM genes were found in the effluent sample (Table 2). Over the past few years metallo-beta-lactamase (MBL) producing isolates have emerged worldwide and are associated with outbreaks in healthcare settings. They cause serious infections such as bacteremia and ventilator associated pneumonia, particularly in patients admitted to the ICU (De *et al*., 2010).

Table 2.

Detection of beta-lactamase resistance genes in the influent and effluent of the hospital chlorinated wastewater treatment system.

Resistance gene	Influent	Effluent
blaPER	+	-
blaPSE	-	-
blaCTX-M	-	-
blaIMP	-	-
blaOXA-10	-	-
blaVIM	+	+
blaOXA-48	-	-
blaKPC	-	-
blaNDM-1	+	-
SHV	+	+
ampC	-	-

blaVIM and blaNDM are the most common MBL genes, encoded by integron borne mobile gene cassettes. The VIM-1 enzyme has very broad substrate specificity, presence of blaVIM conferring resistance to broad array of beta-lactams (ampicillin, carbenicillin, piperacillin, mezlocillin, cefotaxime, cefoxitin, ceftazidime, cefoperazone, cefepime, and carbapenems). Microorganisms expressing NDM-1 (New Delhi metallo-β-lactamase) are mostly multi-drug resistant. The NDM-1 gene confers resistance to beta-lactam antibiotics including last-resort carbapenem antibiotics. Lateral transfer of the plasmid-associated gene, blaNDM, has allowed it to be passed between *Enterobacteriaceae* genera commonly found in the human microbiome, including *Escherichia coli*,

Enterobacter cloacae, and *Klebsiella pneumoniae*. blaPER encodes an extended-spectrum β-lactamase (ESBL), conferring resistance to penicillins, cefotaxime, ceftibuten, ceftazidime, and the monobactam aztreonam but sparing resistance to carbapenems and cephamycins and has been found in *Aeromonas* spp., *Acinetobacter baumannii*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and the *Enterobacteriaceae* in Asia and Europe (Poirel *et al*., 2005). Another extended-spectrum β-lactamase encoding gene identified both in the influent and effluent samples from the hospital wastewater treatment was SHV gene. The SHV enzymes are named after the sulfhydryl variable active site and are commonly associated with *K. pneumoniae*. Initially, these bacteria contained a single ESBL gene, but now multiple ESBL genes are commonly present in a single strain, further complicating the process of detection (Samaha-Kfoury and Araj, 2003).

Our results indicated the presence of beta-lactam resistance genes blaVIM and SHV in the effluent of the wastewater treatment system suggesting that microorganisms possessing beta-lactamase resistance genes might be present in spite of the chlorination step. It is hypothesized that although chlorination is used on a large scale for disinfection, it can significantly alter the antibiotic resistome. Although the total relative abundance of antibiotic resistant bacteria is reduced, antibiotic resistance genes increase in abundance (Jia *et al*., 2015).

This study is a first step in a more complex experiment regarding screening of influent and effluent hospital wastewater for different groups of antibiotic resistance genes and the effect of wastewater treatment and chlorination of the fate of antibiotic resistance bacteria and ARG.

Conclusion

Several resistance genes have been detected by molecular methods in the effluent of a hospital wastewater treatment system, suggesting microorganisms possessing beta-lactamase resistance genes could withstand treatment and chlorine disinfection. The persistence of antibiotic resistant microbes in the hospital wastewater could negatively impact the environment as it discharges in the municipal sewage system.

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REFERENCES

De, A. S., Kumar, S. H., Baveja, S. M. (2010) Prevalence of metallo- β-lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in intensive care areas in a tertiary care hospital, *Indian J. Crit. Care Med.,* **14**, 217-19

Gomes, E. S., Schuch, V., de Macedo Lemos, E. G. (2013) Biotechnology of polyketides: New breath of life for the novel antibiotic genetic pathways discovery through metagenomics*, Braz. J. Microbiol.,* **44**(4), 1007-1034

Hindiyeh, M., Smollen, G., Grossman, Z., Ram, D., Davidson, Y., Mileguir, F., Vax, M., David, B. D., Tal, I., Rahav, G., Shamiss, A., Mendelson, E., Keller, N. (2008) Rapid detection of blaKPCcarbapenemase genes by real-time PCR, *J. Clinical Microbiol.,* **46**(9), 2879-2883

Jia, S., Shi, P., Hu, Q., Li, B., Zhang, T., Zhang, X. X. (2015) Bacterial community shift drives antibiotic resistance promotion during drinking water chlorination, *Environ. Sci. Technol. Article, ASAP DOI*: 10.1021/acs.est.5b03521.

Jiang, L., Hu, X. L., Xu, T., Zhang, H. C., Sheng, D., Yin, D. Q. (2013) Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China, *Sci. of the Total Environ.*, **458**, 267-272

Naas, T., Ergani, A., Carredr, A., Nordmann, P. (2011) Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples, *Antimicrobial Agents and Chemotherapy*, **55**(9), 4038-4043

Naas, T., Cotellon, G., Ergani, A., Nordmann, P. (2013) Real-time PCR for detection of blaOXA-48 genes from stools, *J. of Antimicrob. Chemother.*, **68**(1), 101-104

Poirel, L., Cabanne, L., Vahaboglu, H., Nordmann, P. (2005) Genetic environment and expression of the extended-spectrum β-lactamase blaPER-1 gene in gram-negative bacteria, *Antimicrob. Agents Chemother.,* **49**: 1708–1713

Pauwels, B., Verstraete, W. (2006) The treatment of hospital wastewater: an appraisal, *J. Water and Health,* **4**(4), 405–416

Samaha-Kfoury, J. N., Araj, G. F. (2003) Recent developments in b-lactamases and extended spectrum b-lactamases, *BMJ*, **327**, 1209–1213

Thomson, J. M., Bonomo, R. A. (2005) The threat of antibiotic resistance in Gram-negative pathogenic bacteria: beta-lactams in peril, *Curr. Opin. Microbiol.*, **8**(5), 518-524

Wan, M., Chou, C. (2015) Class 1 integrons and the antiseptic resistance gene (qacеδ1) in municipal and swine slaughterhouse wastewater treatment plants and wastewater—associated methicillin-resistant *Staphylococcus aureus*, *Int. J. of Environ. Research and Public Health*, **12**(6), 6249-6260

Zhu, Y.-G., Johnson, T. A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R. D., Hashsham, S. A., Tiedje, J. M. (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms, *Proc. Natl. Acad. Sci. USA*, **110**(9), 3435-40

Web sources

Surveillance report. Point prevalence survey of the healthcare-associated infections and antimicrobial use in European acute care hospitals 2011-2012. ECDC 2013: http://www.ecdc.europa.eu/en/publications/Publication/healthcare-associated-infections antimicrobial-use-PPS.pdf

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 39-43)

MIO-enzyme toolbox: cloning, expression and purification of recombinant *Rt*PAL

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SUMMARY. The recent advances in the molecular engineering of aromatic ammonia lyases (ALs) and aminomutases (AMs) attracted increased interest towards their applications in the treatment of phenylketonuria and/or the synthesis of non-natural amino acids. Herein we describe the cloning, isolation and purification of recombinant L-phenylalanine ammonia-lyase from *Rhodosporidium toruloides* for future biocatalytic and therapeutic applications.

Keywords: MIO–enzyme, non-natural amino acid, phenylalanine ammonia-lyase, phenylketonuria

Introduction

The recent advances in the molecular engineering of aromatic ammonia lyases (ALs) and aminomutases (AMs) attracted increased interest towards their applications in the treatment of phenylketonuria (Gamez *et al*., 2005; Babich *et al*., 2013) and/or the synthesis of non-natural amino acids (Turner, 2011). All these enzymes have in common an auto-catalically formed 5-methylene-3,5-dihydro-imidazole-4-one (MIO) electrophilic prosthetic group (Rétey, 2003) and show high structural and sequence similarities (Poppe, 2013; Bánóczi *et al*., 2015). Our research activities focus on the design and isolation of more stable ammonia lyases and their substrate scope extension towards novel valuable substrates, one of the interested MIO-enyzmes being phenylalanine ammonia-lyase from *Rhodosporidium toruloides* (Prosekov *et al*., 2014).

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Materials and methods

LB medium, tryptone, yeast extract, agarose, antibiotics (tetracycline, chloramphenicol, carbenicillin), imidazole, IPTG, HEPES, Tris, PMSF, NaCl, KCl, NaH_2PO_4, Na_2HPO_4, SDS, EDTA, TEMED, glycerol, β-mercaptoethanol, Coomassie Brilliant Blue are reagents used in experiments and were purchased from companies Sigma, Aldrich, Carl Roth GmbH, Poch, Liofilchem, Serva.

Lysozyme, DNAse, RNAse were purchased from the company Carl Roth GmbH and cOmplete Protease Inhibitor Cocktail Tablets from Roche.

Molecular cloning and expression optimization

The synthetic gene encoding for *Rt*PAL was synthesized by Life Technologies according to the sequence of the desired protein and optimized for expression *in E. coli.* (Figure 1). The synthetic gene encoding *Rt*PAL, containing an enterokinase cleavage site at the N-terminus, was cloned in the pET-19b_J906 expression vector using XhoI and Bpu1102I cloning sites. The obtained plasmid was transformed in *E.coli* XL-1 Blue heat competent cells as well as in different *E.coli* host strains (Rosetta (DE3) pLysS, BL21(DE3) pLysS, Origami 2) in order to optimize the expression yields. Different concentrations of IPTG (0.1mM, 0.5mM and 1mM) and different fermentation temperatures (20°C, 30°C and 37°C) were tested during the expression optimization.

Protein production, isolation and purification

The recombinant plasmids, containing the *N*-terminal $(His)_{10}$-tag were produced in *E. coli* Rosetta (DE3) pLysS cells using LB media supplemented with the carbenicilin and chloramphenicol.

First a preculture was prepared by the inoculation of 100 ml of sterile LB medium, containing carbenicillin (50 μg/ml) and chloramphenicol (30 μg/ml) with the bacterial cells from the agarplate, followed by overnight incubation at 37°C and shaking at 200 rpm. 8 × 0.5 L of LB medium (in 2L flasks) was inoculated with 2% (*v/v*) of the preculture and grown at 37°C, 200 rpm until OD_{600} reached 0.7-0.8. Protein expression was induced *via* the addition of 0.1 mM IPTG, and the cell growth was maintained at 25°C for another 8 h, reaching an OD_{600} value of 3.6. Cells were harvested by centrifugation (30 min, 4500 rpm), followed by their resuspension (with vortex and pipetting) in 100 ml lysis buffer (50 mM Tris, 300 mM NaCl, 0.5 mM EDTA; pH 8) supplemented with RNAse (3 mg), Lysozyme (10 mg), PMSF (20 mg/ 1 ml EtOH) and 1 tablet of complete protease inhibitor from Roche. The cells were lysed by sonication (2 sec pulse, 40% intensity, 30 min, $T < 20\ ^0C$) and cell debris, respectively membrane fractions were removed by centrifugation (15000 rpm, 35 min). The supernatant was loaded on Ni-NTA affinity chromatography, using appoximately 2 ml of Ni-NTA superflow resin from Qiagen and the protocol described by the

manufacturer. The *Rt*PAL protein with the *N*-terminal His-tag eluted with the 400 mM imidazole fraction. The protein was 4 × fold concentrated through amicons with 10kDa cut-off, followed by their further purification with size-exclusion chromatography, using SEC200 10/300 GL column and 20 mM Tris and 150 mM NaCl, pH 7.5 as eluent. The homotetrameric protein eluted at 10.5-12 ml retention volumes. The protein was stored until further use at -20^{0}C, with 10% glycerol. The purity of the isolated protein was determined through SDS-PAGE, using 12% Tris-glycine Laemmli gels.

Enzyme activity measurements

Activity of *Rt*PAL was determied spectrophotometrically, by monitoring the production of trans-cinnamic acid at 290 nm, using Quartz cuvettes of 1,4 ml and an UV-VIS Cary 50, Varian spectrophotometer.

Results and discussion

Based on the sequence of *Rt*PAL (PDB code:1Y2M) (Figure 1a) we designed a gene sequence with an enterokinase cleavage site for directional cloning into the pET19b(+) vector, using XhoI and BpuI102 cloning sites. The designed gene sequence was obtained through gene synthesis services, and was cloned succesfully into the expression vector, obtaining the novel recombinant plasmid encoding *Rt*PAL (Figure 1b) with an *N*-terminal His(10)-tag and an enterokinase cleavage site, serving for the His-tag removal ulterior to protein purification. The recombinant plasmid was tranformed through heat-shock into different *E.coli* competent cells in order to optimize the expression yields. During expression optimization, besides the influence of the host cells (*E.coli* Rossetta, BL21, C41 all with (DE3), pLysS modifications) we investigated the influence of inducer (IPTG) concetration and of the temperature upon the expression yields. The optimal conditions were found to be *E.coli* Rosetta (DE3) pLysS as host strain, 0.1 mM IPTG and 25 ^{o}C fermentation temperature.

Using the optimal conditions we performed the expression and purification of the *Rt*PAL enzyme. The enzyme containing the *N*-terminal His_{10}-tag was purified with Ni-affinity chromatography followed by size-exclusion chromatography. The purity of the protein, verified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis proved to be > 90%, while its tetrametric, native fold was determined through its molecular weight estimated from its elution volume from the SEC200 10/300 GL column and the calibration curve (molecular weight as function of retention volume) of the column. (Figure 2).

1a.

MGHHHHHHHHHHSSGHIDDDDKHMLEMAPSLDSISHSFANGV
ASAKQAVNGASTNLAVAGSHLPTTQVTQVDIVEKMLAAPTDS
TLELDGYSLNLGDVVSAARKGRPVRVKDSDEIRSKIDKSVEFLR
SQLSMSVYGVTTGFGGSADTRTEDAISLQKALLEHQLCGVLPSS
FDSFRLGRGLENSLPLEVVRGAMTIRVNSLTRGHSAVRLVVLEA
LTNFLNHGITPIVPLRGTISASGDLSPLSYIAAAISGHPDSKVHVV
HEGKEKILYAREAMALFNLEPVVLGPKEGLGLVNGTAVSASMA
TLALHDAHMLSLLSQSLTAMTVEAMVGHAGSFHPFLHDVTRPH
PTQIEVAGNIRKLLEGSRFAVHHEEEVKVKDDEGILRQDRYPLR
TSPQWLGPLVSDLIHAHAVLTIEAGQSTTDNPLIDVENKTSHHG
GNFQAAAVANTMEKTRLGLAQIGKLNFTQLTEMLNAGMNRG
LPSCLAAEDPSLSYHCKGLDIAAAAYTSELGHLANPVTTHVQPA
EMANQAVNSLALISARRTTESNDVLSLLLATHLYCVLQAIDLRA
IEFEFKKQFGPAIVSLIDQHFGSAMTGSNLRDELVEKVNKTLAKR
LEQTNSYDLVPRWHDAFSFAAGTVVEVLSSTSLSLAAVNAWKVA
AAESAISLTRQVRETFWSAASTSSPALSYLSPRTQILYAFVREELG
VKARRGDVFLGKQEVTIGSNVSKIYEAIKSGRINNVLLKMLA

1b.

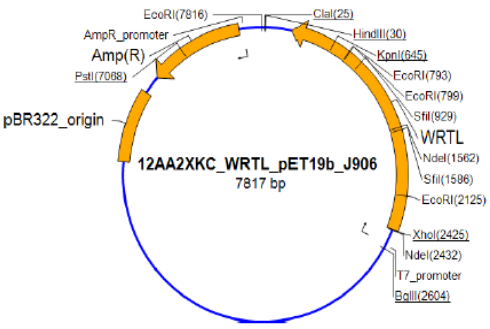

Figure 1. 1a. The amino acid sequence of the designed recombinant *Rt*PAL and **1b.** The genetic map of the created recombinant plasmid containing the gene encoding *Rt*PAL

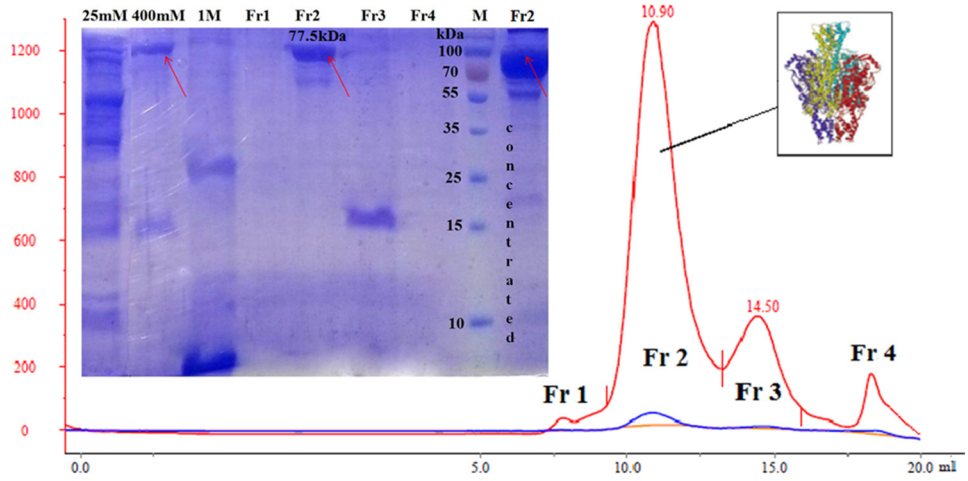

Figure 2. Purification of *Rt*PAL on SEC200 size-exclusion column and the SDS-PAGE gel containing samples from the purification steps of *Rt*PAL

The use of protease inhibitors during all of the isolation and purification steps proved to be crucial, in order maintain the integrity of the enzyme.

The enzyme activity and the kinetic parameters of the purified enzymes was determined towards the natural substrate *L*-phenylalanine. The values of the maximal velocity (v_{max}) and of the Michaelis-Menten constant *(Km)*: v_{max} = 0.47 $\mu M \times s^{-1}$

K_m=648 *mM,* k_{cat}=3.8 s^{-1} are in accordance with the values from literature (Babich *et al*., 2013), proving the success of the cloning, production, isolation and purification processes.

Conclusions

The succesfull cloning, expression, isolation and purification of phenylalanine ammonia lyase from *Rhodosporidium toruloides* was successfully achieved, obtaining enzyme with >90% purity and enzymatic activity similar with values reported in the literature.

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REFERENCES

Babich, O. O., Pokrovsky, V. S., Anisimova, N. Y., Sokolov, N. N., Prosekov, A. Y. (2013) Recombinant l-phenylalanine ammonia lyase from *Rhodosporidium toruloides* as a potential anticancer agent: l -Phenylalanine Ammonia Lyase Anticancer Activity, *Biotechnol. Appl. Bioc.*, **60**, 316–322

Bánóczi, G., Szabó, C., Bata, Z., Filip, A., Hornyánszky, G., Poppe, L. (2015) Structural modeling of phenylalanine ammonia-lyases and related MIO-containing enzymes – An insight into thermostability and ionic interactions, *Studia UBB Chemia*, vol. 4, 213–228

Gamez, A., Sarkissian, C., Wang, L., Kim, W., Straub, M., Patch, M., Chen, L., Striepeke, S., Fitzpatrick, P., Lemontt, J. (2005) Development of pegylated forms of recombinant phenylalanine ammonia-lyase for the treatment of classical phenylketonuria, *Mol. Ther.,* **11**, 986–989

Poppe, L. (2013) MIO-containing ammonia-lyases and 2,3-aminomutases. *BioTrans. conference presentation,* Manchester, UK

Prosekov, A. Y., Babich, O. O., Pokrovsky, V. S., Ivanova, A. S., Novoselova, V. M. (2014) Efficient expression of recombinant l-phenylalanine ammonia-lyase from *Rhodosporidium toruloides* using *Escherichia coli*, *J. Appl. Biotechnol.,* **2**, 2327-0640

Rétey, J. (2003) Discovery and role of methylidene imidazolone, a highly electrophilic prosthetic group, *Biochim. Biophys. Acta*, **1647**, 179-184

Turner, N. (2011) Ammonia lyases and aminomutases as biocatalyst for the synthesis of α- amino and β-amino acids, *Curr. Opin. Chem. Biol.*, **15**, 234-240

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Evaluation of bio-resources: monitoring *Arthrospira* growth in supplemented brackish water

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SUMMARY. *Arthrospira* is a cyanobacterium with high nutrient values that is mono cultured on a commercial scale. A new empirical growth medium was formulated by incorporating selected nutrients of the standard Zarrouk's medium in natural brackish water. Growth of *Arthrospira* in this medium was observed on the basis of protein and pigment contents and microscopic examination. Results sustain the use of this nutrient medium as cost effective method for economically important cyanobacterium cultivation, furthermore possible contamination risks were identified.

Keywords: *Arthrospira*, brackish water, chlorophyll, growth

Introduction

Arthrospira fusiformis Woronichin is a species of planktonic photosynthetic filamentous cyanobacterium (Vonshak *et al*., 1988, Lefort *et al*., 2014). *Arthrospira* cells present a high nutritive value (contain essential nutrients: proteins, minerals, provitamins, polyunsaturated fatty acids), therefore are widely used as food supplements (Belay, 1993; Ogato and Kifle, 2014). Due to its ability to tolerate a wide range of salinity levels, a good quality monoculture can be achieved and competitor growth can be limited by regulating salt concentrations (Zeng and Vonshak, 1998; Ahmed *et al*., 2014). A major drawback of commercial scale production is the high cost of growth medium (Raoof *et al*., 2006; Mahrouqi *et al*., 2015). Thus, our future aim is to modify and accommodate a natural brackish water basin, for cost effective *Arthrospira* production in outdoor pond system. This paper focuses on growth monitoring of two *Arthrospira* strains in supplemented brackish water, on the biodiversity shift induced by *Arthropsira* in this natural habitat, and which species can compete with *Arthrospira*, contaminating the mono culture.

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Materials and methods

Water from an alkaline basin in Dezmir (approx. surface area of 400 m^2, pH of 8.57-9.11, salinity 21.2-26.6 g/L) was filtered, chemically analyzed and supplemented with C, N and P to develop an empirical, nutrient enriched growth medium (EGM) that resembles Zarrouk (Z) medium, typically used for *Arthrospira*. Strains were provided as *Arthrospira platensis* AICB 49 isolated from Egypt (growth rate comparable to those used in industrial production) (Fig. 1 a) and *Arthrospira fusiformis* AICB 606 (isolated from an alkaline lake, Cluj County) (Fig. 1 b-c) by AICB (Culture Collection of Algae of the Institute of Biological Research - Dragoş et al., 1997). For the experiments parallel series of different combinations of Z medium, EGM, one of the *Arthrospira* strains, and homogenized mat were made. Series were cultured in 3 replicates, in volumes of 200 ml, at room temperature (22-24°*C*), under constant illumination (315 $\mu mol \cdot m^2 \cdot s^{-1}$), for 14 days. Samples were collected daily for specific growth rate parameters: pH, optical density (678 nm) and chlorophyll *a*. Initial and final analysis included dry weight measurements, chlorophyll *a*, *b*, *c* and total chlorophyll determinations (Ritchie, 2006; Ritchie, 2008; Keresztes *et al*., 2010). Taxa were identified from enrichment cultures by optical and electron microscopy examinations (Ettl and Gärtner, 2014).

Results and discussion

The EGM, developed by complementing brackish water with anions (HCO_3^-, NO_3^-, PO_4^{3-}), can sustain the growth of *Arthrospira* at a rate comparable or even higher than standard growth medium. This was supported by the daily chlorophyll *a* concentrations (μg/ml) and biomass dry weight (mg/L) measured after 14 days. Growth curves, based on optical density measurements, showed a slower growth start than in case of Z medium, and also a delayed stationary phase. AICB 606 strain had a higher growth rate both in EGM and Z compared to AICB 49 strain, also higher dry weight at the end of experiments, 563.88 mg/L in EGM and 450.81 mg/L in Z compared to values of AICB 49, 439.78 mg/L in EGM and 206.24 mg/L in Z respectively, suggesting that AICB 606 is pre-adapted to the specific chemistry of this area.

Growth of *Arthrospira* as dominant photosynthetic microorganism, led to appreciable increase in pH (from the initial 8.6-9.1 up to 11) and implicitly to reduction of the native flora that is a potential growth competitor, therefore a biomass contaminant. Initially, a total of 33 taxons were identified in the alkaline water basin. Cyanobacteria dominated the habitat (15 taxa), followed by diatoms (8 taxa) and green algae (3 taxa) (Fig. 2). The risk of monoculture contamination was assessed by measuring chlorophyll *a, b* and *c* levels in the biomass. This allows to distinguish between cyanobacteria (containing only chlorophyll *a*), green algae (containing chlorophylls *a* and *b*) and other groups (that also contain chlorophyll *c1* and *c2*).

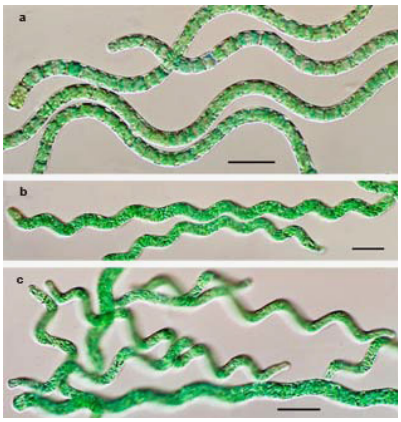

Figure 1. a- *Arthrospira platensis* AICB 49;
b-c – *Arthrospira fusiformis* AICB 606. Bar dimension: 20 µm.

Low chlorophyll *c* values indicate that there is no notable risk of contamination of the biomass, although *Chlorella homosphaera* (containing chlorophyll *a* and *b*) seems to be capable to grow both in EGM and Z. Eight cyanobacterial taxa were detected in the final experimental culture, revealing that they are an evident risk factor even if their presence cannot be distinguished by chlorophyll measurements. Five species were identified as possible competitors of AICB 606, in the EGM: *Synechocystis crassa*, *Synechocystis salina*, *Synechococcus* sp., *Oscillatoria limnetica* and *Pseudanabaena minima.*

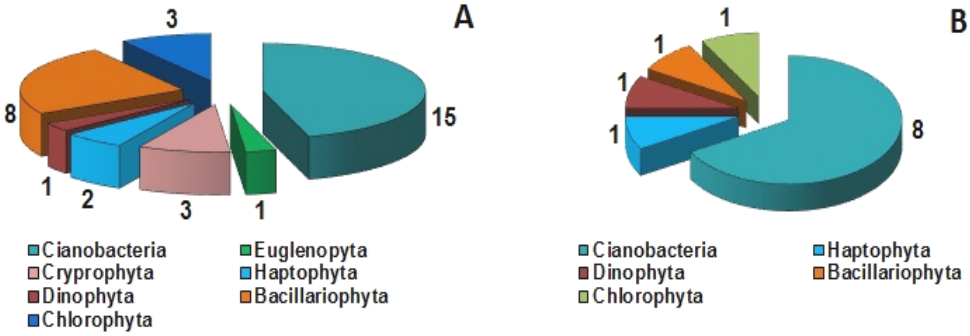

Figure 2. Total number of phyla and taxa identified in the brackish Dezmir basin (**A**) and in the experimental cultures (**B**)

Conclusions

A new empirical growth medium was developed that can support a rapid and constant growth of *Arthrospira* at levels comparable or even higher than standard nutrient medium, AICB 606 yielding higher growth rates in every experimental condition than AICB 49. Among potentially contaminating taxa, *Synechocystis crassa*, *Synechocystis salina* (cyanobacteria) and *Chlorella homosphaera* (green algae) have high probability of impeding the establishment of a stable monoculture.

Acknowledgements. This work was supported by POSDRU/187/1.5/S/155383 research scholarship and by a grant from the Romanian Ministry of National Education, project PN 09-360201.

REFERENCES

Ahmed, M., Moerdijk-Poortvliet, T., Wijnholds, A., Stal, L. J., Hasnain, S. (2014) Isolation, characterization and localization of extracellular polymeric substances from the cyanobacterium *Arthrospira platensis* strain MMG-9, *Eur. J. Phycol.,* **49**(2), 143–150

Belay, A., Yishimichi, O., Kazuyuki, M., Hidenori S. (1993) Current knowledge on potential health benefits of *Spirulina*, *J. of Appl. Phy.,* **5**, 235-241

Dragoș, N., Péterfi, L. Ș., Momeu, L., Popescu, C. (1997) *An introduction to the algae and the culture collection of algae at the Institute of Biological Research*, Cluj University Press, Cluj-Napoca

Ettl, H., Gärtner, G. (2014) *Syllabus der Boden-, Luft- und Flechtenalgen*, 2nd Edition, Springer Spektrum, Berlin, pp 773

Lefort, F., Calmin, G., Crovadore, J., Falquet, J., Hurni, J. P., Osteras, M., Haldemann, F., Farinelli, L. (2014) Whole-genome shotgun sequence of *Arthrospira Platensis* strain paraca, a cultivated and edible cyanobacterium, *Genome Announc.,* **2**(4), e00751-14

Mahrouqi, H. A., Naqqiuddin, M. A., Achankunju, J., Omar, H., Ismail A. (2015) Different salinity effects on the mass cultivation of *Spirulina* (*Arthrospira platensis*) under sheltered outdoor conditions in Oman and Malaysia, *J. Algal Biomass Utln*., **6**, 1-14

Ogato, T., Kifle, D. (2014) Morphological variability of *Arthrospira (Spirulina) fusiformis* (Cyanophyta) in relation to environmental variables in the tropical soda lake Chitu, Ethiopia, *Hydrobiologia,* **738**, 21–33

Raoof, B., Kaushik, B. D., Prasanna, R. (2006) Formulation of a low-cost medium for mass production of *Spirulina*, *Biomass Bioenerg.,* **30**, 537-542

Ritchie, R. J. (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents, *Photosynth. Res*., **89**, 27-41

Ritchie, R. J. (2008) Universal chlorophyll equations for estimating chlorophylls *a, b, c,* and *d* and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents, *Photosynthetica,* **46**, 115-126

Vonshak, A., Guy, R., Guy, M. (1988) The response of the filamentous cyanobacterium *Spirulina platensis* to salt stress, *Arch. Microbiol.,* **150**, 417–420

Zeng, M. T., Vonshak, A. (1998) Adaptation of *Spirulina platensis* to salinity-stress, *Comp. Biochem. Physiol.*, **120**, 113–118

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 49-51)

HPPR gene expression in *Salvia sclarea* L. from Republic of Moldova

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SUMMARY. *Salvia sclarea* L. has received attention for its broad range of pharmacological activities and usage in cosmetics industry and food as for fragrances. However, little is known about the genetics of the secondary metabolites synthesis in that plant. In this research, the real-time PCR was used to investigate the expression of *HPPR* gene involved into acid rosmarinic metabolic pathway. An increased transcriptional activity of gene in hybrids compared to parental forms was identified.

Keywords: Hydroxyphenylpyruvate reductase (HPPR) gene, rosmarinic acid, *Salvia sclarea L*

Introduction

Rosmarinic acid (RA) is a natural phenolic compound widely distributed in the plant kingdom contained in many *Lamiaceae* herbs. This substance has attracted interest due to its biological activities especially concerning its antioxidant, anti-inflammatory, antibacterial properties (Petersen, 2003).

According to EUROPAM, the annual cultivated area of *Salvia sclarea* L. (*Lamiaceae*) in Moldova is arund 2000 ha. The production and secondary metabolites yield is very variable (Dweck, 2000). The success of major compounds content increasing by sage breeding programs could be ensured through ability to regulate the RA synthesis metabolic pathway.

The ability to assess accurately genetic differences between parents and subsequently to predict progeny performance could enhance the efficiency of breeding programs. The investigation of the molecular basis provides opportunities for further research in a wide range of areas. In this study it has been investigated the transcriptional activity of *HPPR (hydroxyphenylpyruvate reductase)* gene governing the RA biosynthesis which includes to involve both the *phenylpropanoid* and a *tyrosine-derived* pathway.

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Materials and methods

Twenty-eight genotypes of clary sage from *Aromatic and Medicinal Plants Collection* of the Institute of Genetics, Physiology and Plant Protection, ASM, including 13 hybrids and 15 parental forms were evaluated (Martea, 2014). The design of specific primers was performed using ***PRIMER3*** tool.

Total RNA was extracted from a bulk of five plants of each genotype using TriReagent according to the manufacturer's instruction. Real Time PCR analysis was performed with gene-specific primers and Maxima SYBR Green/ROX qPCR Master Mix on a DTprime Real-time cycler. All samples were analyzed in three replicates performed in three different runs. The relative expression was calculated via the $2^{-\Delta Ct}$ method.

Results and discussion

The analysis of *HPPR* transcriptional activity showed that relative expression ranged from 0,015 to 0,3 conventional units. The highest transcript accumulation level of *HPPR* gene was detected in *[S. s. Turkmen/N S_7 x (K-36 x 0-41) F_2 x 0-19)F_1 x 0-22) B_4 x L-15)F_8]**F_1*** (H1) while the lowest quantities were observed in case of *(K-36 x 0-41) F_2 x 0-19)F_1 x 0-22) B_4 x L-15) F_8* (P14) (Fig. 1).

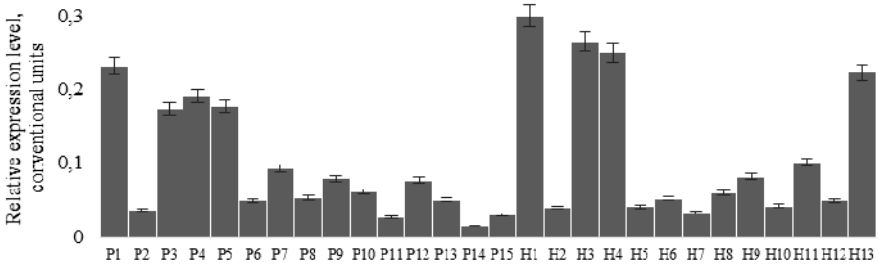

Figure 1. Relative expression of *HPPR* gene for clary sage genotypes

The quantity of transcripts in parental forms was relatively lower compared to clary sage hybrids. Results obtained for genetic groups correlated with hybrid vigor in clary sage. Hybrid vigor is substantial and important for most commercial traits in plants. Thus, 7 of the 13 investigated hybrids have shown quantitative values of the transcriptional activity higher than such in parental forms. The characterization of genetic variability and estimation of the genetic relationships among varieties are essential to clary sage breeding programs. Thus, these findings could represent a substantial advantage to predict the heterosis expected from crosses at all levels.

Conclusions

The level of relative expression of the *HPPR* gene was determined and its involvement in the biosynthesis of acid rosmarinic in *Salvia sclarea* L. was demonstrated.

Our results showed an increased transcriptional activity of HPPR gene in hybrids compared to parental forms, selection of parental forms is an important first step in any breeding programs. At the same time, the generation of genetic information for medicinal plant and application of molecular breeding approaches are necessary for species cultivated in Republic of Moldova.

REFERENCES

Dweck, A. C. (2000) The folklore and cosmetics use of vatious *Salvia* Species, In: *Sage. The genus Salvia,* Kintzios, S. E. (ed), Harwood Academic Publishers, Amsterdam, The Netherlands, pp 1-25

Martea, R. (2014) Polimorfismul genetic în cadrul unor genotipuri de *S. sclarea* L., *Buletinul AŞM. Ştiinţe ale Vieţii*, **3**, 109-116

Petersen, M., Simmonds, M. S. (2003) Rozmarinic acid, *Phytochemistry,* **62**(2), 121-125

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 53-56)

The role of different positively and negatively charged ions on the stability of the histone nucleosome core particle

Attila Bende[1,✉]

SUMMARY. The role of different positively (Na^+, K^+, Mg^{2+}) and negatively (Cl^-) charged ions on the interaction between the negatively charged PO_4^- group of DNA and the positively charged histone protein side chains of lysine and arginine amino acids have been investigated using *ab initio* quantum chemical methods. The analysis of the intermolecular interaction have shown that the positively charged ions polarize the negative charges on the PO_4^- group which weaken the electrostatic interaction between the negative side of the DNA and the positive part of the histone protein. Similarly, the negatively charged Cl^- ion can drastically change the charge distribution on the positively charged lysine or arginine amino acids and again weaken the electrostatic interaction between the negative side of the DNA and the positive part of the histone protein.

Keywords: *Ab initio* methods, DNA, histone, ions, nucleosome

Introduction

Nucleosomes are the basic building blocks of the chromatins and the fundamental repeating units in the cell nucleus. Its crystal structure has been identified by the Richmond Group initially at 2.8 Å atomic resolution (Luger *et al.*, 1997) using X-ray diffraction experiments, which they subsequently refine at 1.9 Å resolution (Richmond *et al.*, 2003). According to this crystal structure, the double-stranded B-DNA superhelix (147 base pair long sequence) is wrapped around the nucleosome core built by eight histone proteins. A detailed structural investigation (Davey *et al.*, 2002) has shown that there are over 120 direct protein–DNA interactions as salt bridges between the main chain amides of the histone and the DNA backbone phosphates. These protein-DNA interactions are further enhanced by several hundred water mediated bridges where the water molecule is intercalated between the charged ends of the salt bridges.

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Materials and methods

The supramolecular system was built up considering two neighboring guanine molecules of a single-stranded DNA chain, together with two sugar and one PO_4^- groups which binds to the lysine or arginine amino acid side chains of the proteins. Positively charged (Na^+, K^+, Mg^{2+}) ions were set close to the PO_4^- fragment, while the negatively charged Cl^- ion was placed close to the amino groups groups of the amino acid side chains. Furthermore, three water molecules were also introduced in our supramolecular system placed close to the different ions. For the geometry optimization the two-layer ONIOM method (Maseras *et al.*, 1995; Svensson *et al.*, 1996; Dapprich *et al.*, 1999) was applied, implemented in the GAUSSIAN 09 program package (Frisch *et al.*, 2009). In the *model* system the PO_4^- group of the sugar-phosphate chain, the lysine or arginine side chains occurring in the protein chain, the three water molecules, as well as the positively and negatively charged ions are included, while the sugars and the guanine fragments were included only in the *real* system. The *model* system was described using the MP2/TZVP levels of theory, while for the *real* (supramolecular) system the HF/6-31G method was considered.

Results and discussion

The intermolecular interaction energies between the negatively charged PO_4^- group of DNA and the positively charged histone protein side chains of lysine and arginine amino acids including the Na^+, K^+, and the Cl^- ions were analized in our previous works (Bende *et al.*, 2007, 2008, 2012) and the values of these energies are presented Table 1.

Table 1.

Interaction energies (in eV-s) between the PO_4^- group of DNA and the lysine and arginine residues of the histone in the presence of different ions

Geometries	Interactions energies (in eV)	
	HF	MP2
$PO_4^-\cdots Lys^+/Arg^+$	-5.24/-4.79[a]	-5.64/-4.98[a]
$PO_4^-\cdots Lys^+/Arg^+$ with H_2O, K^+	-4.84/-4.38[b]	-5.04/-4.57[b]
$PO_4^-\cdots Lys^+/Arg^+$ with H_2O, K^+, Cl^-	-1.12/-0.95[c]	-1.15/-0.97[c]
$PO_4^-\cdots Lys^+/Arg^+$ with H_2O, Mg^{2+}	+0.33/+0.13	-0.02/-0.12

[a] See (Bende et al., 2007); [b] See (Bende et al., 2008); [c] See (Bende et al., 2012)

Conclusions

Using *ab initio* quantum chemistry methods, the intermolecular interaction energies between the the negatively charged PO_4^- group of DNA and the positively charged histone protein side chains of lysine and arginine amino acids. The results have shown that the presence of differently charged ions with different valence numbers could uniquely influence the strength of the DNA protein interaction inside the nucleosome. Accordingly, the positively charged ions with single valency (Na^+, K^+) could somewhat weaken the interaction but it remains strong enough in order to not come apart the wrapped DNA to the histone. The presence of an extra Cl^- ion close to the positively charged amino acids have a significant influence on the interaction strength, by reducing the magnitude of the interaction energy with almost 80%. The positively charged bivalent ions (Mg^{2+}) can even break the DNA-protein complex and hinder their re-formation and safe packing.

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REFERENCES

Bende, A., Bogár, F., Ladik, J. (2007) Charge transfer between the PO_4^- groups of DNA and the arginine$^+$ and lysine$^+$ side chains of proteins, *Chem. Phys. Lett.,* **437**, 117-119

Bende, A., Bogár, F., Ladik, J. (2008) The role of water and K^+ ion in the charge transfer between PO_4^- groups of DNA and the lysine$^+$ and arginine$^+$ side chains of histone proteins, *Chem. Phys. Lett.,* **463**, 211-213

Bende, A., Bogár, F., Ladik, J. (2012) Possible role of Cl^- ions in DNA–protein interactions in the nucleosomes, *Chem. Phys. Lett.,* **525–526**, 115–119

Dapprich, S., Komáromi, I., Byun, K. S., Morokuma, K., Frisch, M. J. (1999) A new ONIOM implementation in Gaussian98. Part I. The calculation of energies, gradients, vibrational frequencies and electric field derivatives, *J. Mol. Struct., (THEOCHEM)*, **461–462**, 1-21

Davey, C. A., Sargent, D. F., Luger, K., Maeder, A. W., Richmond, T. J. (2002) Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 Å resolution, *J. Mol. Biol.,* **319**, 1097-1113

Frisch, M. J. *et al.*, GAUSSIAN, Inc., Wallingford, CT, 2009

Luger, K., Mäder, W., Richmond, R. K., Sargent, D. F., Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution, *Nature*, **389**, 251-260

Maseras, F., Morokuma, K. (1995) IMOMM: A new integrated *ab initio* + molecular mechanics geometry optimization scheme of equilibrium structures and transition states, *J. Comput. Chem.,* **16**, 1170-1179

Richmond, T. J., Davey, C. A. (2003) The structure of DNA in the nucleosome core, *Nature,* **423**, 145-150

Svensson, M., Humbel, S., Froese, R. D. J., Matsubara, T., Sieber, S., Morokuma, K. (1996) ONIOM: A multilayered integrated MO + MM method for geometry optimizations and single point energy predictions. a test for diels−alder reactions and $Pt(P(t\text{-}bu)_3)_2 + H_2$ oxidative addition, *J. Phys. Chem.,* **100**, 19357-19363

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 57-60)

New method for the molecular recognition of thyroid hormones

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SUMMARY. The development of new methods that can detect a broad range of biomarkers became essential in modern medicine. The most frequent endocrine disorders include thyroid pathology. Stochastic sensors represent a unique class of single-molecule detectors and a promising candidate in biomedical analysis due to their ability to determine in one run more than one analyte. In clinical practice the main analytes used for the diagnosis and evolution of thyroid disease are free L-T_3, L-T_4 and TSH. A fast screening method based on stochastic sensors was proposed for the enantiorecognition of free L-T_3, L-T_4, D-T_4 and TSH. Stochastic microsensors based on a mixture between two inulins (IN, TEX) and two ionic liquids (IN-L-Ala-C_4-L-lac, IN-L-Phe-L-lac) immobilized on diamond paste (DP) were used for the assessment of thyroid hormones in whole blood samples. IN-L-Phe-C_4-L-lac based microsensors showed the highest sensitivity for the assay of D-T_4, L-T_4 and TSH, while the highest sensitivity for L-T_3 was obtained by using the stochastic microsensors based on IN-L-Ala-L-lac. The quantification limits obtained for thyroid hormones were: 10^{-12} mol/L for L-T_4, $4x10^{-13}$ mol/L for L-T_3, $6x10^{-12}$ for D-T_4 mol/L and $5x10^{-15}$ g/mL for TSH.

The microsensors determined the thyroid hormones in whole blood samples with high reliability: recoveries higher than 95.00%, and RSD (%) lower than 1.00%. The microsensors had great features in biomedical analysis for pattern recognition of thyroid hormones. This will help early detection of related diseases.

Keywords: Enantiorecognition, inulins, stochastic microsensors, thyroid hormones

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Introduction

Stochastic sensors represent a promising candidate in early diagnosis and prevention due to their capacity to perform qualitative and quantitative analysis (Movileanu, 2014). The principle of the stochastic sensors is based on the channel conductivity and consists of modulation of the ionic current induced by reversibly binding analytes of interest to the wall of the channel (Stefan-van-Staden *et al.*, 2013). From the obtained diagram (Fig. 1) it could be recognized the signature of the analyte expressed by t_{off} and its concentration revealed by t_{on} values. Using diamond paste as a matrix in the construction of stochastic sensors represents a relatively new method, having the advantage of a better fixation of the analyte passing the channel.

ELISA and CLEIA/ECLEIA are the most frequently used methods in clinical practice for the determination of F-T4, F-T3 and TSH. In order to detect the three hormones, three different detection kits are needed, while using our method one can determine in one run all the analytes with a lower cost. Several methods that have been used for the assay of free L-T3, free L-T4 and TSH include high performance liquid chromatography (Gondova *et al.*, 2011; Jin *et al.*, 2007), radioimmunoassay (Giorgiou *et al.*, 1994), liquid chromatography (Wang *et al.*, 2003), equilibrium dialysis (Sapin *et al.*, 2003) and electrochemical methods (Wang *et al.*, 2014).

In this paper we demonstrate the ability of the proposed microsensors to fast detect free triiodothyronine (L-T_3), levothyroxine (L-T_4), dextrothyroxine (D-T_4) and thyroid stimulating hormone (TSH) in whole blood samples.

Materials and methods

Two diamond paste sensors based on a mixture of inulins and ionic liquids (IN-L-Phe-C_4-L-lac and IN-L-Ala-L-lac) were designed by modifying diamond paste. The concentration ranges of standard solutions were obtained by serial dilution. All the chronoamperometric measurements were recorded using a PGSTAT 302, software Ecochemie version 4.9. The unknown concentrations of L-T_3, L-T_4, D-T_4 and TSH were determined by inserting the value $1/t_{on}$ in the related equation of calibration.

Results and discussions

The response characteristics of the enantioselective electrochemical sensors based on inulins and ionic liquids are shown in Table 1. The linear concentration range of both microsensors based on IN-L-Phe-C4-L-lac and IN-L-Ala-L-lac covers the normal range of f-L-T_3 in serum given by ECLIA method. Also these two microsensors can detect the presence of L-T_4 with a very low limit of determination, but with a

linear concentration range under the normal used range by ECLIA method. Compared with the most frequently used techniques for the assessment of TSH, such as ELISA, the limit of determination obtained using the microsensors based on IN-L-Phe-C4-L-lac and IN-L-Ala-L-lac ($4x10^{-15}$ g/mL) is lower than the one reported using ELISA kit ($2,74x10^{-12}$ g/ml).

Conclusions

IN-L-Phe-C4-L-lac and IN-L-Ala-L-lac microsensors had the best sensitivity and limit of quantification, having the linear concentration range suitable for direct enantiorecognition of L-T3 in blood samples; therefore they can be used reliably as tools in the diagnostic of thyroid diseases. Compared with ELISA and chemiluminescence methods used in clinical laboratories for their determination, the main advantages are: (1) there is no need for sample pretreatment before assay, samples being used as taken from the patient; (2) low cost; (3) decreased time of determination.

Table 1.

Response characteristics of the microsensors

Microsensor based on	Signature of the enantiomer t_{off} (s)	Sensitivity (mol/L s^{-1})	Linear concentration range (mol/L)	Limit of quantification (mol/L)	Limit of detection (mol/L)	Equation of calibration; correlation coefficient*
L-T4						
IN-L-Phe-C4-L-lac/DP	0.7	$5.93x10^{9}$	10^{-12}-$4x10^{-12}$	10^{-12}	$7.81x10^{-14}$	1/ton=0.05+$5.93x10^{9}$; R=0.9831
IN-L-Ala-L-lac/DP	0.7	$5.44x10^{8}$	$8x10^{-12}$-10^{-10}	$8x10^{-12}$	$1.3x10^{-12}$	1/ton=0.03+$5.44x10^{8}$; R=0.9833
D-T4						
IN-L-Phe-C4-L-lac/DP	1.1	$2.2x10^{9}$	$6x10^{-12}$-10^{-11}	$6x10^{-12}$	$1.35x10^{-14}$	1/ton= $2.2x10^{9}$-0.01; R=0.999
IN-L-Ala-L-lac/DP	2.1	$2.45x10^{4}$	10^{-8}-10^{-6}	10^{-8}	$3.41x10^{-8}$	1/ton=0.03+$2.45x10^{4}$; R=0.941891
L-T3						
IN-L-Phe-C4-L-lac/DP	1	$4.02x10^{9}$	$4x10^{-12}$-$x10^{-12}$	$4x10^{-12}$	$8.16x10^{-13}$	1/ton=0.05+$4.02x10^{9}$; R=0.92953
IN-L-Ala-L-lac/DP	1	$3.08x10^{10}$	$4x10^{-13}$-10^{-12}	$4x10^{-13}$	$4.85x10^{-15}$	1/ton=0.03+$3.08x10^{10}$ R=0.9967
TSH						
		g/mL	**g/mL**	**g/mL**	**g/mL**	
IN-L-Phe-C4-L-lac/DP	0.5	$3.58x10^{10}$	$5x10^{-15}$-$5x10^{-13}$	$5x10^{-15}$	$1.01x10^{-16}$	1/ton=0.02+$3.58x10^{10}$; R=0.9972
IN-L-Ala-L-lac/DP	0.3	$3.36x10^{10}$	$5x10^{-15}$-$5x10^{-13}$	$5x10^{-15}$	$2.6x10^{-14}$	1/ton=0.03+$3.36x10^{10}$; R=0.9393

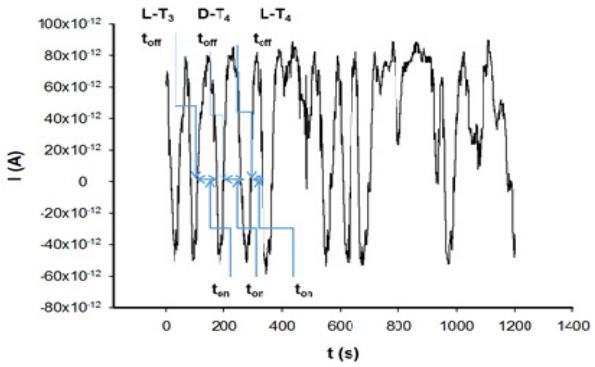

Figure 1. Example of a diagram recorded for the assay of f-L-T_3, f-L-T_4, and f-D-T_4

REFERENCES

Georgiou, S., Christofidis, I. (1996) Radioimmunoassay of free thyroxine (T4) using ^{125}I-labeled T4-IgG complex with very large molecular weight, *Clin. Chim. Acta*., **244**, 209-220

Gondová, T., Petrovaj, J., Sucha, M., Armstrong, D. W. (2011) Stereoselective HPLC determination of thyroxine enantiomers in pharmaceuticals, *J.of Liquid Cromatogr. Rel. TechnoL.,* **34**, 2304-2314

Jin, D., Zhang, M., Jin, S., Lee, M. K., Song, G. C., Back, G, Lee, Y. I. (2007) Enantioselective resolution of thyroxine hormone by high-performance liquid chromatography utilizing a highly fluorescent chiral tagging reagent, *Chirality*, **19**, 625-31

Movileanu, L. (1990) Watching single proteins using engineered nanopores, *Protein Pept. Lett.,* **21**, 235-246

Sapin, R. M., d'Herbomez, M. (2003) Free thyroxine measurement by equilibrium dialysis and nine immunoassays in sera with various serum thyroxine-binding capacities, *Clin. Chem*., **49**, 1531-1535

Stefan-van Staden, R. I., Balasoiu, S. C., Bazylak, G., van Staden, J. F., Aboul-Enein, H. Y., Radu, G. L. (2013) Inulins as electroactive materials for enantioanalysis of chiral drugs, *J. Electrochem. Soc*., **160**, B192-B195

Wang, R., Jia, Z. P., Hu, X. L., Li, Y. M., Chen, L. R., (2003) Determination of serum thyroxine enantiomers in patients by liquid chromatography with a chiral mobile phase, *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci*., **785**, 353-359

Wang, H. X., Wu, P., Dong, C., Wang, J., Wang, Y., Liu, J. C. (2014) Electrochemical biosensor based on interdigited electrodes for the determination of thyroid stimalting hormone, *Int. J, Electrochem. Sci*., **9**, 12-21

Web sources

http://www.synevo.ro/ft4-tiroxina-libera/
http://www.synevo.ro/ft3-triiodotironina-libera/
http://www.abcam.com/thyroid-stimulating-hormone-tsh-human-elisa-kit-ab100660.html

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 61-65)

The risk for colorectal cancer and polymorphisms of APC gene

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SUMMARY. Colorectal cancer (CRC) is a complex genetic disease which appears as a result of interactions between multiple genetic and non-genetic factors. In this study we have analyzed the association between four polymorphisms of APC gene (*rs41116, rs465899, rs2229992* and *rs2019720*) and colorectal cancer in Romanian population. After receiving the informed consent, blood samples were obtained from individuals with CRC (M:F=95:85) and from healthy persons (M:F=27:33). Genomic DNA was extracted from peripheral blood samples and the APC gene polymorphisms were assessed by PCR-RFLP. The promoter polymorphism rs2019720 was associated with CRC. Thus, the CC genotype (OR 2.307) and allele C (OR 1.843) increased the disease risk, while the AA genotype (OR 0.453) and allele A (OR 0.543) decreased the CRC risk. We found that the APC polymorphism *rs2019720* was associated with CRC in Romanian population.

Keywords: Adenomatous polyposis coli, colorectal cancer, polymorphisms

Introduction

The mutations in adenomatous polyposis coli (*APC*) gene are involved in the development of colorectal cancer (CRC) (Fearnhead *et al.,* 2001; Fodde, 2002). Germline mutations were linked to familial adenomatous polyposis (FAP), while somatic mutations occurs in approximately 80% of sporadic cases (Laurent-Puig *et al.,* 1998; Goss and Groden, 2002). Most frequently patients are carriers of frameshift mutations (68%) which are associated with synthesys of proteins with abnormal structure (e.g. truncated proteins) and functions. For example, the missense germline mutations, p.I1307K and p.E1317Q, were frequently identitifed in sampels from CRC patients (Frayling *et al*., 1998). Also, codons 1286 and 1450 represents hotspots for somatic mutations which underlying the occurrence of premature stop codons (Fodde, 2002; Laurent-Puig *et al.,* 1998).

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In addition to these mutations the role of single nucleotide polymorphisms (SNPs) of APC in genetic predisposition to CRC is a subjects of discussions (Almeida *et al.,* 1996; Tranah *et al.*, 2005). The variants of these SNP have a low penetrance and present significant interpopulational differences. As a consequence, the disease risk may not be identical in different ethnic groups (Chen *et al*., 2006). However, for several SNPs the association with CRC was reconfirmed in several independent studies (e.g. g.1458T>C, g.4479G>A and g.5268T>G, p.D1822V) (Slattery *et al*., 2001; Tranah *et al.*, 2005; Chen *et al*., 2006).

Until now no large-scale screening for APC gene variants in Romanian population has been conducted. In this study we have analyzed the relationship between four polymorphisms of APC gene (*rs41116, rs465899, rs2229992* and *rs2019720*) and CRC in Romanian population.

Materials and methods

Blood samples were obtained from 180 individuals with CRC and from 60 healthy subjects after receiving the informed consent (Table 1). Genomic DNA was extracted from peripheral blood samples and the APC gene polymorphisms were assessed by PCR-RFLP using already published protocols (Sieber *et al*., 2002; Cui *et al*., 2005). Briefly, each PCR reaction (10 μL) contains about 50 ng genomic DNA, 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 1 unit Taq DNA polymerase, 100 μmol/L dNTP and 0.5 μmol/L of each primer. The annealing temperature for each polymorphism was 55°C. The amplicons were digested with SspI (*rs41116*), MspI (*rs465899*) and RsaI (*rs2229992* and *rs2019720*) restriction enzymes.

Statistical analysis was performed using *StatsDirect* (version 2.8.0) software. Chi-square test (χ 2) was used to compare the distribution of genotypes and alleles in patients and control groups. A p value < 0.05 was considered statistically significant.

Table 1.

Characteristics of analyed subjects

	CRC patients	**CRC controls**
Median age	62.5	58.5
Interval	51-79	46-67
Men / Women	95 / 85	27 / 33

Results and discussion

The distribution of APC genotype are presented in Table 2. All investigated polymorphisms were distributed in accordance with the Hardy-Weinberg equilibrium.

Table 2.

The distribution of APC gene polymorphism in patients and control groups

	Genotypes	**CRC patients**	**CRC controls**	**OR (95%CI)**	**χ^2 (p)**
rs2019720	CC	66	14	**2.30** (1.17-4.53)	6.05 (***0.01***)
	CA	73	31	0.78 (0.43-1.42)	0.63 (0.42)
	AA	21	15	**0.45** (0.21-0.95)	4.49 (***0.03***)
	HWE (p)	0.9	0.79		
rs2229992	CC	38	11	1.38 (0.65-2.93)	0.74 (0.38)
	CT	85	37	0.70 (0.38-1.29)	1.28 (0.25)
	TT	37	12	1.2 (0.57-2.5)	0.24 (0.61)
	HWE (p)	0.42	0.07		
rs465899	GG	29	12	0.77 (0.36-1.65)	0.42 (0.51)
	GA	85	35	0.80 (0.44-1.47)	0.47 (0.48)
	AA	49	13	1.59 (0.79-3.21)	1.73 (0.18)
	HWE (p)	0.28	0.19		
rs41116	TT	49	14	1.45 (0.73-2.88)	1.13 (0.28)
	TC	83	32	0.94 (0.52-1.7)	0.03 (0.84)
	CC	28	14	0.69 (0.33-1.43)	0.96 (0.32)
	HWE (p)	0.48	0.6		

We observed that rs2019720 polymorphism is associated with CRC. Thus, CC genotype (OR 2.3) and C allele (OR 1.84) increased the disease risk, while the AA genotype (OR 0.45) and the A allele (OR 0.54) seems to be protective factors for CRC.

These results are in concordance with the data obtained by Huang and contributors that reported a significant difference between cases and controls only for rs2019720 polymorphism (Huang *et al*., 2012).

The rs2019720 polymorphism is located in the promoter region of the APC gene and is expected to be functional and to be a real marker for CRC predisposition. However, the haplotype analysis may describe much better the disease risk. Huang and collaborators found a strong LD between rs2019720 and rs6594646 but demonstrated that no haplotype is significant associated with CRC (Huang *et al*., 2012).

We previously tested by PCR multiplex the presence of mutations in APC gene in samples collected from 16 patients (10 men and 6 women) and 21 first and second degree relatives and found no mutation in the codon 1061 of APC gene (Toma *et al*., 2008). The data obtained during the present study complete the data regarding predisposition for CRC in Romanian population.

Conclusions

In this study the rs2019720 is associated with susceptibility to CRC in Romanian population.

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REFERENCES

Almeida, R., Morton, N., Fidalgo, P., Leitão, N., Mira, C., Rueff, J., Monteiro, C. (1996) APC intragenic haplotypes in familial adenomatous polyposis, *Clin. Genet.,* **50**(6), 483-485

Chen, S. P., Tsai, S. T., Jao, S. W., Huang, Y. L., Chao, Y. C., Chen, Y. L., Wu, C. C., Lin, S. Z., Harn, H. J. (2006) Single nucleotide polymorphisms of the APC gene and colorectal cancer risk: a case-control study in Taiwan, *BMC Cancer,* **6**, 83

Cui, D. H., Jiang, K. D., Jiang, S. D., Xu, Y. F., Yao, H. (2005) The tumor suppressor adenomatous polyposis coli gene is associated with susceptibility to schizophrenia, *Mol. Psychiatry,* **10**(7), 669-677

Fearnhead, N. S., Britton, M. P., Bodmer, W. F. (2001) The ABC of APC, *Hum. Mol. Genet*., **10**(7), 721-733

Fodde, R. (2002) The APC gene in colorectal cancer, *Eur. J. Cancer,* **38**(7), 867-871

Frayling, I. M., Beck, N. E., Ilyas, M., Dove-Edwin, I., Goodman, P., Pack, K., Bell, J. A., Williams, C. B., Hodgson, S. V., Thomas, H. J., Talbot, I. C., Bodmer, W. F., Tomlinson, I. P. (1998) The APC variants I1307K and E1317Q are associated with colorectal tumors, but not always with a family history, *Proc. Natl. Acad. Sci. USA,* **95**, 10722-10727

Goss, K. H., Groden, J. (2000) Biology of the adenomatous polyposis coli tumor suppressor, *J. Clin. Oncol.,* **18**(9), 1967-1979

Huang, X., Wang, Y., Yu, T., Liu, B., Li, X., Li, W., Chen, S., Zhao, Q., Li, X., Yang, F., Wang, Q., Wang, J., Xiao, Y., Xu, Y., Feng, G., Peng, Z., He, L., He, G. (2012) Association study of APC polymorphisms with colorectal cancer in Han Chinese, *Clin. Biochem.,* **45**(18), 1669-1672

Laurent-Puig, P., Beroud, C., Soussi, T. (1998) APC gene: database of germline and somatic mutations in human tumors and cell lines, *Nucleic Acids Res*., **26**, 269-270

Sieber, O. M., Lamlum, H., Crabtree, M. D., Rowan, A. J., Barclay, E., Lipton, L., Hodgson, S., Thomas, H. J., Neale, K., Phillips, R. K., Farrington, S. M., Dunlop, M. G., Mueller, H. J., Bisgaard, M. L., Bulow, S., Fidalgo, P., Albuquerque, C., Scarano, M. I., Bodmer, W., Tomlinson, I. P., Heinimann, K. (2002) Whole-gene APC deletions cause classical familial adenomatous polyposis, but not attenuated polyposis or "multiple" colorectal adenomas, *Proc. Natl. Acad. Sci. USA,* **99**(5), 2954-2958

Slattery, M. L., Samowitz, W., Ballard, L., Schaffer, D., Leppert, M., Potter, J. D. (2001) A molecular variant of the APC gene at codon 1822: its association with diet, lifestyle, and risk of colon cancer, *Cancer Res.,* **61**, 1000-1004

Tarafa, G., Prat, E., Risques, R. A., Gonzalez, S., Camps, J., Grau, M., Guino, E., Moreno, V., Esteller, M., Herman, J. G., Germa, J. R., Miro, R., Peinado, M. A., Capella, G. (2003) Common genetic evolutionary pathways in familial adenomatous polyposis tumors, *Cancer Res*., **63**(18), 5731-5737

Toma, M., Cimponeriu, D., Pompilia, A., Stavarachi, M., Beluşică, L., Radu, I., Gavrilă, L. (2008) Molecular analysis of mutations for the adenomatous polyposis coli (APC) gene in Romanian patients with colorectal cancer, *J. of Medicine and Life,* **1**(4), 423-428

Tranah, G. J., Giovannucci, E., Ma, J., Fuchs, C., Hunter, D. J. (2005) APC Asp1822Val and Gly2502Ser polymorphisms and risk of colorectal cancer and adenoma, *Cancer Epidemiol. Biomarkers Prev.,* **14**(4), 863-870

Wallis, Y. L., Morton, D. G., McKeown, C. M., Macdonald, F. (1999) Molecular analysis of the APC gene in 205 families: extended genotype-phenotype correlations in FAP and evidence for the role of APC amino acid changes in colorectal cancer predisposition, *J. Med. Genet.,* **36**, 14-20

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 67-70)

Microanatomical findings in intestinal atresia

Patricia Meşter[1,✉] and Constantin Ionescu[2]

SUMMARY. The birth of a baby with a congenital malfunction of the intestinal tract is a very difficult situation on one hand because of the therapy and on the other hand because of the small age of the patients. Intestinal atresia is a birth defect that affects both anatomical and functional integrity of the digestive tract. This kind of defect is the most common situation of congenital intestinal obstruction, seen in almost 1/3 of the new-borns, being associated in most of the cases with other anomalies. Our study was performed on 18 new-born bowel tissue samples and is based on the embriological theories of production of these defects. Our study found a pronounced microscopic necrosis of the mucous membrane with venous ectasies and hemoragic infiltrates of the submucosis. Also there was a decrease of ganglion cells in the myenteric plexus associated with hypertrophy of nerve fibers.

Keywords: Intestinal atresia, neurocristopathies, neuronal anomalies

Introduction

Intestinal atresia is a birth defect that affects functional and anatomical integrity of digestive tract. Its features are represented by a common radiological and clinical syndrome of neonatal obstruction of the bowel.

There has been a variety of theories regarding the etiology of this abnormality: developmental defects (digestive tube recanalization disorders (Tandler, 1903), inflammatory changes, fetal injuries (intussusception, volvulus, mesenteric defects), late maesenteric intrauterine vascular accidents as the cause of most jejunoileal atresias (Louw and Barnard, 1955).

Recent studies made by (Ure, 1997; Berger, 1998; Masumoto, 1999) support the hypothesis that congenital atresia and stenosis of the digestive tract are the result of the intestinal innervation disorders.

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Materials and methods

Our study presents macroscopic and microscopic anatomical data, resulted from the new-born bowel tissue samples harvested intraoperatively (18) with ages between 3 hours and 3 days, which had type I or type II of ileal or colic atresia after Bland Sutton classification. The pathological specimens were clinically evaluated for identifying the macroscopic lesions and for the microscopic study the samples were histopatologically prepared and examined with a MC 5 optic microscope (ocular 10X, objective 40X).

Results and discussion

The results were compared with samples of normal bowel taken from deceased new-born babies and the general aspect of bowel wall, blood vessels and nervous fibers was presented.

Our study found a pronounced microscopic necrosis of the mucous membrane with venous ectasies and hemoragic infiltrates of the submucosis. Also there was a decrease of ganglion cells in the myenteric plexus associated with hypertrophy of nerve fibers (Figs. 1,2).

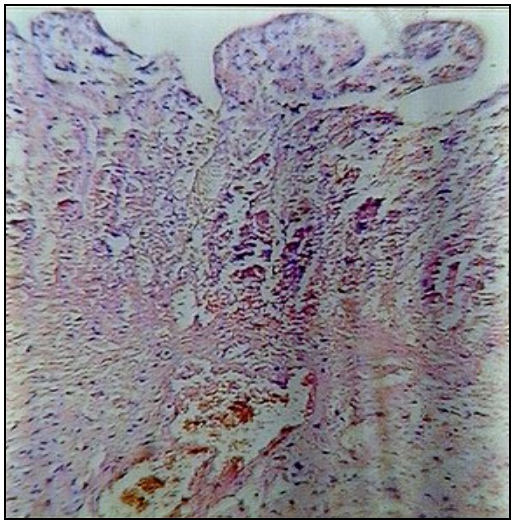

Figure 1. Aspect of small intestine. H&E staining, objective 10X.
Extended areas of hemoragic necrosis inside tunica mucosa
and areas of non-specific infiltrations of the submucosa.

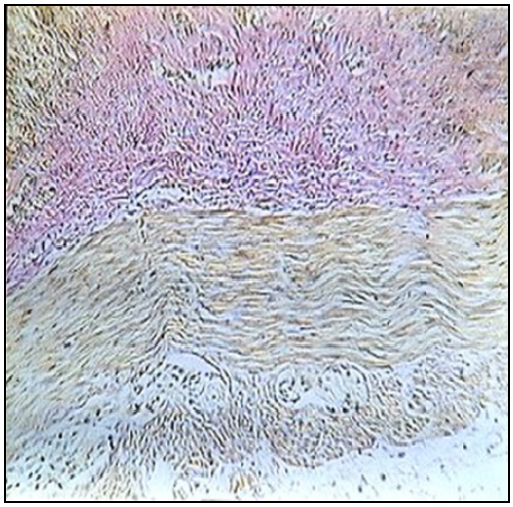

Figure 2. Aspect of small intestine. Van Gieson staining, objective 10X.
Submucosa showing discrete edema and diffuse lymphocytic infiltration.
The myenteric plexus shows few ganglion cells and many hypertrophic nerve fibers.

Conclusions

Intestinal atresia is the result of the nervous anomalies (aganglionosis and nervous fiber hypertrophy), which induce vascular anomalies, fact that was also shown in our macroscopic and microscopic study. We think that this kind of anomalies can be included in the category of nurocristopathies.

REFERENCES

Berger, S., Ziebell, P., Kessler, M., Hofmann-Von Kap-Herr, S. (1998) Congenital malformations and perinatal morbidity associated with intestinal neuronal dysplasia, *Pediatric surgery international*, **13**(7), 474-479

Louw, J. H., Barnard, C. N. (1955) Congenital intestinal atresia observations on its origin, *The Lancet*, **266**(6899), 1065-1067

Masumoto, K., Suita, S., Nada, O., Taguchi, T., Guo, R., Yamanouchi, T. (1999) Alterations of the intramural nervous distributions in a chick intestinal atresia model, *Pediatric research*, **45**(1), 30-37

Tandler, J. (1903) Zur entwicklungsgeschichte des menschlichen darmarterien, *Anat Anz* **23,** 132-134

Ure, B. M., Holschneider, A. M., Schulten, D., Meier-Ruge, W. (1997) Clinical impact of intestinal neuronal malformations: a prospective study in 141 patients, *Pediatric surgery international*, **12**(5-6), 377-382

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 71-72)

=== ORAL PRESENTATION ABSTRACTS ===

How to win a Nobel Prize: stumbling on the secret of cell division

Tim Hunt[1,✉]

I grew up in Oxford wanting to be a scientist, loving gadgets and processes like melting lead pipes or electrolyzing salt solutions to make poisonous and explosive gases. Luckily, I had excellent teachers who channeled these enthusiasms into a deeper and more formal understanding of chemistry and biology (physics, alas, was beyond my grasp) so that it was possible to study at Cambridge University and carry on there with a Ph.D. in biochemistry, on the business of the control of haemoglobin synthesis. I'll explain how I arrived at this—it was an accident—and also where I pursued the subject. It took ten years, many interesting side roads, a lot of travel and a devastating fire to solve the problem of how the synthesis of haem was coordinated with the synthesis of globin.

After that, it took another 7 years or so to find a really good new problem to work on, but on July 22nd 1982 I was teaching and researching at the Marine Biological Laboratory, Woods Hole, and saw to my amazement that a prominent protein, later called cyclin, disappeared just before fertilized sea urchin eggs divided for the first (and every subsequent) time they divided. Finding out what this protein was, and what it did, took another six or seven years of very exciting work, leading away from the control of protein synthesis to the control of cell division. Yet amazingly, the underlying mechanisms were identical, involving a class of enzymes known as protein kinases, which attach phosphate residues onto other proteins, thereby modifying their behavior. I've always liked biological switches and finding how they work.

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Most recently, however, I've been drawn to the study of the enzymes that remove phosphates from proteins and their control, which turn out to be very important in the switches that initiate and terminate cell division. The path was marked by unexpected discoveries all along the way, almost always stemming from sensible experiments designed to test something different!

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 73-74)

=== ORAL PRESENTATION ABSTRACTS ===

Switches and latches: controlling cell division

Tim Hunt[1,✉]

The process of mitosis involves a comprehensive reorganization of the cell: chromosomes condense, the nuclear envelope breaks down, the mitotic spindle is assembled, cells round up and release their ties to the substrate and so on and so forth. This reorganization is triggered by the activation of a protein kinase called Cyclin-Dependent Kinase 1 (CDK1). The end of mitosis is marked by the proteolysis of the cyclin subunit of CDK1, which terminates kinase activity. At this point, the phosphate moieties that altered the properties of hundreds of proteins to bring about the cellular reorganization are removed by protein phosphatases.

We recently began to pay attention to the control of these protein phosphatases, conscious that it was likely that they were shut off as cells enter mitosis, and reactivated when mitosis is complete, allowing return to interphase. It is difficult to see how proteins could be fully phosphorylated if both kinases and phosphatases were simultaneously active (much as filling a wash basin requires not only turning on the water taps, but also putting in the plug).

It emerged that at least one protein phosphatase, PP2A-B55, was shut off in mitosis. Depletion of this particular form of PP2A accelerated entry into mitosis, and blocked exit from mitosis. We have discovered how this phosphatase is regulated. It entails binding a small inhibitor protein (α-endosulfine or ARPP-19) that is phosphorylated by a protein kinase called Greatwall that is itself a substrate of CDK1. Failure to inhibit PP2A-B55 causes arrest of the cell cycle in G2 phase. I will explain how we found this out (proteomics played a key role) and discuss the

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role of this particular control mechanism in the control of mitosis. The “switches and latches” of my title refers to our still rather poor understanding of exactly how the timing of entry into mitosis is controlled, together with the realization that the Greatwall-α-Endosulfine circuit is not only required for entering mitosis, but also for staying there.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 75-76)

=== ORAL PRESENTATION ABSTRACTS ===

Current aspects and perspectives of Aquaporinology: 30 years after the discovery in Cluj-Napoca by Benga group of the first water channel protein (later called aquaporin1)

Gheorghe Benga[1, 2, 3, 4, 5, 6, ✉]

In 1985, together with my coworkers, I discovered in Cluj-Napoca, Romania, the first water channel protein (WCP) in the human red blood cell (hRBC) membrane, after a decade of systematic studies of water diffusion in the hRBC. The discovery was reported in 1986 in two landmark publications (Benga *et al.*, 1986a,b) and reviewed in subsequent years (Benga, 1988; 1989).

In 1988 the same protein was serendipitously isolated from hRBC by Peter Agre and coworkers working in Baltimore, USA, and called CHIP28 (*CH*annel forming *I*ntegral membrane *P*rotein of *28* kDa) (Denker *et al.*, 1988). This group recognized the role of the protein as a water channel only in 1992 (Preston *et al.*, 1992). In the same year other WCPs were discovered and cloned and the name "aquaporins" was proposed for this class of membrane proteins. The WCP first discovered by my group in 1985 and re-discovered by Agre group in 1992 was called aquaporin 1 (AQP1). Soon it became obvious that a large family of WCPs exists, as hundreds of such proteins have been discovered in organisms from all kingdoms of life, including unicellular organisms (archaea, bacteria, yeasts, and protozoa) and multicellular ones (plants, animals, and humans). WCPs belong to a superfamily of membrane proteins called MIPs (intrinsic membrane proteins) (Benga, 2012)."

In 2003, Peter Agre was awarded the Nobel Prize in Chemistry "for the discovery of water channels". An invited review of the history of the discovery of water channels proteins was published by Benga in September 2003, one month before the Nobel Prize for Chemistry was announced (Benga, 2003). The seminal contributions from 1985 of the Benga group were completely overlooked by the Nobel Prize Committee.

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The discovery of WCPs was a crucial event in science. As formulated by Wolburg *et al.* (2011): "The detection of water-specific membrane channels in red blood cells belongs to the fundamental discoveries in biology of the twentieth century (Benga *et al.*, 1986a,b; Denker *et al.*, 1988; Preston *et al.,* 1992)." As I have been working in the field for more than 25 years I could see that this domain of science became a very hot area of research embracing many branches of natural sciences, These proteins are now studied from the molecular and cellular level (structure-function relationships, expression in various cells, regulation) to the level of whole organisms and of populations. New very important aspects are uncovered every day, the diversity of hundreds of WCPs is revealed, with increasing practical implications, including the physiological and medical implications. Thousands of publications appeared on these topics. The study of water channel proteins became not only a very hot field of research with a lot of theoretical and practical issues, but also a new domain of natural sciences, domain for which I suggested the term of "aquaporinology" (Benga, 2013, 2014).

REFERENCES

Benga, G. (1988) Water transport in human red blood cells, *Prog. in Biophys. Mol. Biol.,* **51**, 193-245

Benga, G. (1989) Membrane proteins involved in the water permeability of human erythrocytes: binding of *p*-chloromercuribenzene sulfonate to membrane proteins correlated with nuclear magnetic resonance measurements, In: *Water transport in biological membranes*, Benga, Gh. (ed). CRC Press, Boca Raton, Vol. 2, pp 41-61

Benga, G. (2003) Birth of water channel proteins - the aquaporins, *Cell Biol. Int.,* **27**, 701-709

Benga, G. (2013) On the definition, nomenclature and classification of water channel proteins (aquaporins and relatives), *Mol. Asp. Med.,* **33**, 514-517

Benga, G. (2013) Aquaporinology (the study of water channel proteins - aquaporins and relatives) as a new domain of natural sciences, *Oltenia for Studies in natural Sciences*, **29**, 316-319

Benga, G. (2014) Aquaporinology, Acta Endocrinologica (Buc), **10**, 1-8

Benga, G., Popescu, O., Pop, V. I., Holmes, R. P. (1986a) *p*-(Chloromercuri) benzenesulfonate binding by membranes proteins and the inhibition of water transport in human erythrocytes. *Biochemistry,* **25**, 1535-1538

Benga, G., Popescu, O., Borza V., Pop, V. I., Mureşan, A., Mocsy, I., Brain, A., Wrigglesworth, J. M. (1986b) Water permeability of human erythrocytes. Identification of membrane proteins involved in water transport, *Eur. J. Cell Biol.* **41**, 252-262

Denker, B. M., Smith, B. L., Kuhaida, F. P., Agre, P. (1988) Identification, purification and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules, *J. Biol. Chem.,* **263**, 15634-15642

Preston, G. M., Carroll, T. P., Guggino, W. B., Agre, P. (1992) Appearance of water channels in *Xenopus* oocytes expressing red blood cell CHIP28 protein, *Science*, **256**, 385-387

Wolburg, H., Wolburg-Bucholz, K., Fallier-Becker, P., Noell, S., Mack, A. (2011) *Structure and functions of aquaporin-4-based orthogonal arrays of particles.* International Review of Cell and Molecular Biology, Elsevier, **287**, 1-41

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, LX, Sp. Iss., 2015 (p. 77-78)

=== ORAL PRESENTATION ABSTRACTS ===

Biotechnological improvement of drought and salt tolerance of crops: a new paradigm for increasing food production

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World population, at present about 7.4 x 10^9 inhabitants, is expected to grow by almost 30% over the next 35 years, to reach 9.2 x 10^9 people in 2050. According to FAO estimates, it will be necessary to increase agricultural production by at least 60% over 2005-2007 levels to meet the expected demand for food. This is probably one of the biggest challenges that mankind has ever faced; yet, if we look at the recent past, this goal – *a priori* – does not seem so difficult to achieve. Indeed, in 50 years, between 1960 and 2009, world population more than doubled – from 3 x 10^9 to 6.8 x 10^9 people – while it was still possible to increase the amount of food *per capita*, from 2200 Kcal/person/day to an average of more than 2800 Kcal/person/day. Obviously, this food is not distributed evenly: while the average food supply in Europe reached in 2009 almost 3400 Kcal/person/day, it was below 2000 Kcal/person/day in some developing countries in sub-Saharan Africa and South East Asia. A fairer distribution of food worldwide is largely a matter of political will and international solidarity – although it will imply significant economic and logistical questions that should also be addressed – and many people believe that there should be no technical problems to feed the world's population if we were able to share the available food resources. Unfortunately this reasoning is flawed: the methods used in the past during the so-called 'Green Revolution' (GR) of the 1960s and 1970s allowed a huge increase in the amount of food available to mankind, but cannot be applied anymore under the present, quite different circumstances. As a consequence of the GR, current agricultural production is mostly based on an excessive use of intensive production

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practices – that will not be sustainable in the long term – and is very dependent on large amounts of inputs, including the massive use of agrochemicals (pesticides, herbicides, chemical fertilizers), and of water for irrigation. In a world where the area of arable land is actually decreasing and irrigation water is becoming an increasingly scarce resource, the most obvious strategies to rapidly increase agricultural production (a significant increase of the global cropland area, or the relative area of land cultivated under irrigation) should be ruled out; the use of our actual high-input agriculture in marginal, low-fertility soils will be also unsustainable. In addition, the loss of genetic diversity of our crops and the forseeable effects of climate change contribute to make necessary a change of paradigm in plant breeding and agricultural practices. Innovative approaches will be required to boost crop yields and food production in the years ahead.

For all major crops, average yields are only a fraction – between 20 and 50% – of record yields; environmental abiotic stress conditions, especially drought and soil salinity, are the main factors responsible for these yield reductions. Therefore, generation of crops tolerant to water and salt stress appears to be the most effective strategy to increase crop productivity, and hence food supply, in the next few decades, by reducing yield losses and extending the area available for agriculture. To develop these tolerant varieties in the shortest possible time, all available means should be used: genetic engineering to generate GM plants with these drought and/or salt-tolerance phenotypes, as well as conventional breeding methods (i.e., sexual crosses and selection)... but with the help of the biotechnological tools developed in recent years that are now available to the plant breeder.

Up to now, apart from a few specific examples, neither traditional plant breeding nor genetic engineering has delivered widely used commercial stress-tolerant varieties. Nevertheless, research lines in progress are providing promising results and we should be confident that in the coming years the combination of both approaches will allow the improvement of abiotic stress tolerance for our major crops.

In this communication, positive and negative aspects of the 'Green Revolution' and the present situation regarding food supply will be reviewed. We will comment on the challenges facing crop production in the immediate future, in the context of global climate change and in the frame of a sustainable agriculture, without further degradation of the environment or depletion of the natural resources our next generations should inherit. Finally, the aforementioned strategies to increase crop productivity based on the development of drought and salt stress resistant varieties will be discussed, including reference to some successful examples and promising lines of reseach.