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BIOLOGICAL CONTROL OF VEGETABLE AND FRUIT FUNGAL DISEASES BY BACTERIAL ANTAGONISTS

Principal Investigators: Profs. Ilan Chet, Leonid Chernin

Grantee Institution: The Hebrew University of Jerusalem (HUJ)

Collaborator: Prof. Zafar Ismailov
"FAN" Scientific Center of Development and Production, Samarkand State University, Samarkand, Republic of Uzbekistan (FAN)

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Executive Summary:

The main goals of research performed during the period covered by this report (fourth year of the project execution), were to continue the studies of the mechanisms responsible for the selected bacterial antagonist ability to serve as biocontrol agents of plant pathogenic fungi. In aim to go more insight to the role of cell-wall lytic enzymes and antibiotics as well of the regulatory systems responsible for the biocontrol-associated traits manifestation we developed a general strategy for gene-specific mutagenesis of the genes encoding the antifungal compounds mentioned above and regulatory proteins involved the compounds production. The elaborated approach includes cloning and sequencing of the corresponding genes; disrupting the cloned genes by insertion of an antibiotic cassette, recloning of the disrupted gene sequences into a suicidal type vector providing the specific selection of the bacteria in which the desired wild-type gene will be substitute via recombination with the mutated (disrupted one), selection of the corresponding knock-out mutants. Using this strategy we have obtained seven independent constructs for gene-specific mutagenesis of the desired genes. Five of them specifically suitable for directed mutagenesis of regulatory genes (*grrA, grrS, rpoS, rpoD*, and *phzI*) responsible for regulation of antifungal exoenzymes and antibiotics compounds in several biocontrol strains of various species of Gram-negative bacteria. Two other constructs providing directed mutagenesis of i) a 58-kDa endochitinase gene *chiA* described in several biocontrol strains of *Pantoae (Enterobacter) agglomerans*, *Serratia marcescens* and *Serratia plymutica* species and ii) the gene *prnC*, known as one of the four structure genes of an operon.encoding the biosynthesis of antifungal antibiotic pyrrolnitrin. The obtained constructs are currently in usage for gene-directed mutagenesis of biocontrol strains *P. agglomerans* IC1270 and *Pseudomonas aureofaciens* 449 showed previously wide-range spectrum of antagonism towards plant-pathogenic fungi. We also continued the work on enhancement of the strains biocontrol activity by genetic manipulations of the regulatory systems responsible for production of various antifungal compounds.

Section I

A) Research Objectives:

The specific research objectives for the first half of the fourth year of the project included: 1) partial cloning and sequencing of genes prnC and phzI, encoding respectively biosynthesis of antibiotic pyrrolnitrin in strain *P. agglomerans* IC1270 and N-acyl homoserine lactone (acyl-HSL) signal molecules involved in production of phenazine (Phz) antibiotics in strain *P. aureofaciens* 449; 2) disrupting the cloned genes by insertion of an antibiotic gentamycin (Gm) cassette; 3) recloning of the disrupted gene sequences into a suicidal type vector pEXT18 providing the specific selection of the bacteria in which the desired wild-type gene will be substitute via recombination with the mutated (disrupted) one; 4) selection of the corresponding knock-out mutants an study their antifungal activity in comparison to the wild type strain.

B) Research Accomplishments:

1. Partial cloning and sequencing of the regulatory gene *phzI* encoding the synthesis of N-acyl homoserin lactone (acyl-HSL) signal molecules responsibly for production of antifungal antibiotics of the phenazine group – HUJI. Numerous plant- and animal-associated bacteria regulate the expression of specific sets of genes in response to their own population densities, a phenomenon termed quorum sensing. Most quorum-sensing systems thus far identified in gram-negative bacteria employ *N*-acylhomoserine lactones (acyl-HSL) as signaling molecules. These signals accumulate with increasing cell density and upon reaching a threshold concentration bind a transcriptional regulator that in turn activates or represses target gene expression. Over 30 bacterial species have been shown to use quorum-sensing circuits to regulate diverse functions, including bioluminescence, virulence factor production, plasmid conjugal transfer, biofilm formation, motility, symbiosis, and antibiotic production

The root-associated biological control bacterium *Pseudomonas aureofaciens* 30-84 produces a range of exoproducts, including phenazines. Phenazine antibiotic biosynthesis is regulated in part by the PhzR-PhzI quorum-sensing system. Mutants defective in *phzR*, encoding a transcription regulator, or *phzI*, encoding acyl-HSL, produce very low levels of phenazines. Recently we have started to investigate this

phenomenon in several other strains of *P. aureofaciens* independently isolated in various regions of the former USSR. Several of these isolates (e.g. 64, 66, and 449) showed potency as biocontrol agents of several plant pathogenic fungi, including ability to control *Sclerotinia sclerotiorum* white mould disease in cucumber under greenhouse conditions.

Genomic DNA of strain *P. aureofaciens* 449 was used as template in a polymerase chain reaction (PCR) for the amplification of the DNA sequences analogues of gene *phzI*. The corresponding PCR product was ligated into pGEM-T Easy (Promega) vector, the ligation mixtures were electroporated into *E. coli* strain XL1-Blue and corresponding selected clones were subjected to sequencing using SP6 and T7 primers. The obtained 734-bp sequence revealed high level of homology with gene *phzI* from relatively similar strain 30-84.

2. Partial cloning and sequencing of the structure gene *prnC* encoding one of the enzymes of biosynthesis of antibiotic pyrrolnitrin in strain *P. agglomerans* IC1270 – HUJI and FAN. Genomic DNA of strain IC1270, a broad-spectrum antagonist of plant pathogens, produces and secretes a set of chitinolytic enzymes and antifungal antibiotic pyrrolnitrin (Prn), was used as template in a polymerase chain reaction (PCR) for the amplification of the DNA sequences analogues of gene *prnC*. The corresponding PCR product was ligated into pGEM-T Easy (Promega) vector, the ligation mixtures were electroporated into *E. coli* strain XL1-Blue and corresponding selected clones were subjected to sequencing using SP6 and T7 primers. The obtained 720-bp sequence revealed high level of homology with gene *prnC* from Prn-producing strain Pf5 of *Pseudomonas fluorescens*.

3. Consruction of specific cassettes for gene-directed mutagenesis of the structure and regulatory genes responsible for production of antifungal compounds –HUJI and FAN. Previously we have used *Enterobacter agglomerans* strain IC1270, a broad-spectrum antagonist of plant pathogens, produces and secretes a set of chitinolytic enzymes and antifungal antibiotic pyrrolnitrin, for cloning of gene chiA encoding synthesis of a 58-kDa endochitinase and four regulatory genes comprising regulatory networks required for production of many exoenxymes and secondary metabolites in many biocontrol strains of Gram-negative bacteria. Two of these genes, designated in strain IC1270 as *grrA* and *grrS*, comprised the GacA-type response

regulator and GacS-type sensor kinase, two other encoding the transcription sigma factors sigma^{70} (RpoD) and sigma^{38} (RpoS), specific for gene expression at the exponential or stationary growth phase. The complete CDS of the four regulatory genes were sequenced. Knock-out mutants of strain *P. agglomerans* IC1270 for genes *grrA, grrS, rpoS, rpoD, chiA* and *prnC*, and of strain *P. aureofaciens* 449 gene *phzI* were obtained used a gene replacement system that allowed for positive selection of all steps involved in the gene replacement process. This system included the *Bacillus subtilis sacB* gene as counter-selectable marker, allowing for positive selection of the segregation of true mutants from the more frequently occurring merodiploids on medium containing 5% sucrose. The replacement vector, pEX18T, which was genetically engineered to facilitate the cloning and genetic manipulation of virtually any DNA fragment, was used in all experiments. The second important component of the system is a Gm-r cassette, used for disrupting of the target gene.

The developed strategy included: 1) restriction analysis of each of the desired gene sequence, 2) determination of the restriction sites suitable for insertion of a selectable Gm-r cassette; 3) insertion of Gm-r cassette into the gene sequence cloned into pGEM-T Easy vector; 4) recloning of the resulted disrupted gene sequence including Gm-r cassette, into pEXT18T replacement vector. The resulted plasmid contains the Gm-r -expressing cassette , is mobilizable and is curable from its host by sucrose^{R} selection since it carries the counterselectable *sacB* marker; 5) introduction of the resulted derivative of pEXT18Tplasmid into a helper strain S17-1 of E.coli, providing mobilization of the derivative plasmid into a desired recipient strain (P. aureofaciens 449 in case of mutagenesis of phzI gene and P. agglomerans IC1270 in case of mutagenesis of the each of other six (*grrA, grrS, rpoD, rpoS, chiA*, and *prnC*) target genes; 6) selection of the strains IC1270 and 449 exconjugants with insertion of the pEXT18T plasmid derivatives into the bacterial chromosome; 7) monitoring of the successful gene replacement by Southern DNA-hybridization analysis of chromosomal DNAs; 8) study of the phenotype of the obtained knock-out mutants, including testion of their ability to produce antifungal compounds (chitinase, Prn and Phz) and to suppress the plant fungal pathogens *in vitro* and under greenhouse conditions.

At the present time we already performed experiments covering items 1-6 of this program and obtained desired knock-out mutants which have to be further tested as described in items 7 and 8.

C) Scientific Impact Collaboration: The co-Principal investigators of the project, Prof. Z. Ismailov worked in the Israeli partners laboratory in Rehovot for about 6 month starting from November 2001 until end of April 2002 to perform mainly molecular cloning and gene-directed nutagenesis experiments. Additionally the Senior Researcher Dr. F. Kabulova visited HUJI for 10 days in December 2001 to continue laboratory and greenhouse trials.

D) Description of Project Impact: The results of both groups obtained during this period started to be implemented in Uzbek partners laboratory in the Samarkand State Univ. with goal to study the available biocontrol agents mechanisms of antifungal activity.

E) Strengthening of Developing Country Institutions: During this period Dr. Ismailov and his team acquired all necessary technical facilities and help for performing described molecular biology and genetic engineering experiments. Profs. I. Chet and L. Chernin consulted the Uzbek partners about technical details of the performing trials and discussed protocols of the experiments.

F) Future Work: We are planning to continue the project mainly according to the originally proposed objectives and time chart. The developed scheme of the gene-directed mutagenesis will be used for further analyses of the selected biocontrol agents mechanisms of action and enhancement of the strains biocontrol activity by genetic manipulations of the regulatory systems responsible for production of various antifungal compounds.

Section II

A) Managerial Issues: No significant managerial issues have arisen during this year, however the budget was somehow revised at October 2000.

B) Budget: Additional changes in budget items was made in aim to give more facilities for the Uzbek' group for training and to acquire equipment and materials. For this additional FAN'part of the Budget was include the to the item of the HUJI part of the budget specially devoted to the training of Uzbek partners in Israel. Such

rearrangement of the budget will essentially help the Uzbek partner to use the money more specifically in aim to offer for members of Prof. Ismailov' team an opportunity for training and collaborative research with Israeli partners. At the same time this changes in the budget will help to the Uzabek partners use the money for buying more reagents and small equipment's necessary for continuation of the project using the facilities available in Samarkand.

C) Special Concerns: Any protocols of special concerns were changed.

D) Collaboration, Travel, Training and Publications:

The materials, equipment (laboratory pipette-aid, plastic tubes, software, etc.), and scientific books purchased by funds provided by the project and delivered to Samarkand, are available for use to all members of the Samarkand State University' research group headed by Dr. Ismailov.

Publications:

1. Zhou, L. M. Ovadis, Z. Ismailov, I. Chet, and L. Chernin. 2001. Involvement of two-component GacA-GacS global regulation system, RpoD and RpoS sigma factors and N-acyl homoserine lactone quorum-sensing signals in control of chitinolytic activity of *Enterobacter agglomerans* strain IC1270, p. 20. In: Abstracts of the 3rd International Symposium on Chitin Enzymology, Senigallia (Ancona), Italy, 6 - 10 May 2001.
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4. Chernin, L., and I. Chet. 2001. Microbial enzymes in biocontrol of plant pathogens and pests, pp. 171-225. In: Enzymes in the Environment:

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5. Chernin, L· Z. Ismailov, and I. Chet. 2001. Genetic manipulations of bacterial enzymes and antibiotics to enhance biocontrol of phytopathogens, p. 3-4. In: Extended Summaries of Int. Conf. "Achievements of Biotechnology for the future of mankind", 11-18 June 2001, Samarkand, R. Uzbekistan.
6. Ritte, E., Lurie, S., Ismailov, Z., Chet, I., Chernin, L. 2002. Postharvest biological control of *Monilinia* and *Rhizopus* on peach by *Pantoae agglomerans,* p. 70. In: Abstracts of IOBC/WPRS Working Group Biological Control of Fungal and Bacterial Plant Pathogens, 22-25 May 2002, Pine Bay, Kusadasi, Turkey.
7. Ritte, E., Lurie, S., Droby, S., Ismailov, Z., Chet, I., Chernin, L. Biocontrol of postharvest fungal pathogens of peach and apple by *Pantoae agglomerans* strain IC1270. In: Proceedings of the Seventh Working Group Meeting "Influence of A-Biotic and Biotic Factors on Biocontrol Agents", Eds. Y. Elad, J. Köhl and D. Shtienberg, IOBC/WPRS Bulletin, Vol.1, pp. 199-203.
8. Kamensky, M., Ovadis, M., Chet, I., Chernin, L. Biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* in the greenhouse by a *Serratia plymuthica* strain with multiple mechanisms of antifungal activity. Ibid., pp. 229-233.
9. Zohar-Perez, C., Ritte, E., Chernin, L., Chet, I., and Nussinovitch, A. Entrapment of chitinolytic *Pantoae agglomerans* in cellular dried alginate-based carriers. Biotechnology Progress (in press).
10. Zohar-Perez, C., Chernin, L., Chet, I., and Nussinovitch, A. Structure of dried cellular alginate matrix containing fillers provides extra protection for microorganisms against UVC. Appl. Environ. Microbiol. (submitted).
11. Kamensky, M., Ovadis, M., Chet, I., Chernin, L. Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. Soil Biol. & Biochem. (submitted).