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ENZYMES FOR THE DEGRADATION OF RHAMNOGALACTURONAN I AS VIRULENCE FACTORS OF PHYTOPATHOGENIC BACTERIUM *Pectobacterium atrosepticum*

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Abstract

Plant pathogenic pectobacteria (*Pectobacterium* genus) are well-known all over the world as the causal agents of the cultural plant diseases called soft rots. Rot symptoms are related to the extensive plant tissue maceration due to the production by microorganisms of the plant cell wall degrading enzymes. Most of the pectobacteria-secreted enzymes catalyze the cleavage of homogalacturonan. This polysaccharide, that is a linear homopolymer, consists of galacturonic acid residues and is the most abundant (by mass) pectic polysaccharide of plant cell walls. The knockout of genes of homogalacturonan-degrading enzymes is known to lead to reduced virulence of pectobacteria. In addition, the modification of another pectic compound — rhamnogalacturonan I also occurs in the course of infection process caused by pectobacteria. This compound is a ramified heteropolymer, the backbone of which consists of alternate rhamnose and galacturonic acid residues, and side chains are represented by galactose or arabinose residues. However, the role of pectobacterial enzymes for rhamnogalacturonan I degradation in the development of soft rots has not been previously ascertained. The present study is dedicated to the investigation of the necessity of *P. atrosepticum* SCRI1043 enzymes degrading rhamnogalacturonan I for a full development of soft rots in the plants infected by pectobacteria. By directed mutagenesis, we have obtained mutant forms of *P. atrosepticum* SCRI1043 deficient in genes encoding rhamnogalacturonyl hydrolase (genome locus *eca3749*) that cleaves the backbone of rhamnogalacturonan I, and galactanase (genome locus *eca0852*) that breaks side chains of this polymer. For the target gene knockout, mutant loci were constructed by overlap-extension PCR. Most of the original gene was replaced by kanamicin-resistance cassette. The obtained construction was ligated into a mobilized suicide vector and the resulting plasmid was transferred into donor *E. coli* CC118 strain cells. The recombinant plasmid with the mutant locus was introduced into *P. atrosepticum* SCRI1043 cells by three-parental mating. The *P. atrosepticum* SCRI1043 clones, in which the original locus was replaced by the mutant one, and the donor plasmid was eliminated, were selected on the selective media. The mutant strains *P. atrosepticum* SCRI1043 3749 and *P. atrosepticum* SCRI1043 0852 caused significantly less damage to the plant tissues of *Brassica rapa* spp. *pekinensis* Cha Cha cv. compared to parental wild-type strain. Herewith, the strain mutant in *eca0852* locus encoding galactanase, the enzyme that cleaves side chains of rhamnogalacturonan I, was least virulent. The reduction in virulence, in this case, was not related to the suppression of homogalacturonan-degrading enzyme activity or less motility of bacteria. Thus, we have demonstrated that, first, rhamnogalacturonan I-degrading enzymes may be attributed to virulence factors of phytopathogenic pectobacteria, and second, the hydrolysis of the sides chains of rhamnogalacturonan I contributes more to the process of tissue maceration than the decay of the polymer backbone.

Keywords: *Pectobacterium atrosepticum*, pectic polysaccharides, rhamnogalacturonan I, glycosyl hydrolases

Members of *Pectobacterium* genus are one of the most harmful plant pathogens in the world [1]. These microorganisms cause diseases in plants called soft or wet rot [2, 3]. Key determinants of pectobacteria pathogenity are extracellular enzymes that degrade cell wall polysaccharides, of which enzymes destructing polygalacturonic acid (homogalacturonan) that are more diverse. Such polymer, mainly contained in medial plates, is the most spread pectin polysaccharide of plant cell walls [4]. Its destruction in the course of infection results in tissue maceration [2, 3, 5]. Numerous researches had shown that mutant forms of pectobacteria, in which secretion of polygalacturonan-destructing enzymes is absent or reduced, cannot cause symptoms of soft rots [6-8].

Along with genes encoding the homogalacturonan-destructing enzymes, pectobacteria genome has genes of degrading enzymes of other pectin polysaccharide, the rhamnogalacturonan I (RGU I). Unlike homogalacturonan (linear homopolymer consisting of residues of galacturonic acid), RGU I is a branched heteropolymer. Its backbone consists of the alternate rhamnose and galacturonic acid residues, and side chains attached to rhamnose are galactose or arabinose residues [4].

In our previous studies we had shown that RGU I plays an important role in pectobacteria colonization in vessels of primary xylem, where microorganisms form special biofilm-like multiple cell structures which we call bacterial emboli [9]. As apart from biofilms, in which extracellular matrix is mainly represented by bacterial exopolysaccharides [10-12], primary matrix of bacterial emboli is formed from RGU I [13]. Such polymer is released from plant cell walls due to receptive plant response, and forms certain microcosm for construction of bacterial emboli. With maturing of bacterial emboli, RGU I, as part of extracellular matrix, is replaced by extracellular pectobacteria polysaccharides [14]. It denotes dynamic transformation of RGU I at development of infection. However, for pectobacteria the role of RGU I degradation enzymes in pathogenesis was not demonstrated yet.

We had established for the first time that destruction of RGU I during the infection largely contributes to development of soft rot symptoms caused by *Pectobacterium atrosepticum*.

Purpose of present research is to verify the need for RGU-I destructing enzymes for development of soft rots in plants infected by pectobacteria.

Techniques. *Pectobacterium atrosepticum* strain SCRI1043 (earlier called *Erwinia carotovora* ssp. *atroseptica* SCRI1043) [15] (by courtesy of E.A. Nikolaychik) (Collection of Belarus State University), *Escherichia coli* and mutants at *eca0852* (Δeca0852) and *eca3749* (Δeca3749) loci were cultured at 28 °C in Luria-Bertani (LB) medium [16] containing 10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl (pH 7.5). When necessary, kanamycin (30 μg/ml), streptomycin (100 μg/ml), tetracycline (12.5 μg/ml) were added.

The target enzyme sequences were searched for by BLASTp algorithm in PDB (Protein Data Bank Europe, https://www.ebi.ac.uk/pdbe/) and UniProt (https://sparql.uniprot.org/). Phylogenetic tree was plotted by nearest neighbor method; bootstrap support was indicated on tree branches. Molecule phylogenetic analysis was conducted with MEGA 6.0 software (https://www.megasoftware.net/). Biochemical description of enzymes was taken from CAZy database (Carbohydrate-Active enZyme, http://www.cazy.org/).

Mutant locus for knockout of *eca0852* and *eca3749* genes was established using overlap extension polymerase chain reaction (PCR) method. Most part of gene coding area was removed and insertion in lieu of it kanamycin resistance cas-

sette was inserted as a marker for selection of mutant cells. The obtained structure was ligated into suicidal mobilizable vector pKNG101 (generously provided by Professor L.N. Moleleki, Pretoria University, RSA); the resultant plasmid was electroporated into cells of donor strain *E. coli* CC118. Recombinant mutant loci as part of obtained plasmid were introduced into *P. atrosepticum* SCRI1043 by triparental crossing. Cells with recombinant plasmid were selected on streptomycin- and kanamycin-containing media. Afterwards, cells, in which the second recombination took place with replacement of the target gene and elimination of donor plasmid, were selected on the sucrose-containing medium. It was followed by sampling clones resistant to kanamycin and sensitive to streptomycin. Mutation was verified by PCR method, and nucleotide sequence of mutant locus was determined as described above [8].

Virulence of pectobacteria mutants was assessed as the weight of affected (macerated) leaf tissues of cabbage (*Brassica rapa* spp. *Pekinensis*) Cha Cha cv. inoculated with microorganisms. Pectobacteria were grown in LB medium until the late logarithmic growth phase. Afterwards it was collected by centrifuging and resuspended in 10 mM magnesium sulphate solution less stressful for plants than sodium chloride [17]. Inoculum concentration was adjusted to 1×10^7-3×10^7 CFU/ml by serial dilution. Leaf surface was sterilized by Belizna bleaching solution (0.8% active chlorine) and by 70% ethanol. Next, leaves were rinsed by sterile water with small cuts made, in which 10 μl of bacterial suspension (1×10^5-3×10^5 CFU/ml) or sterile magnesium sulphate solution was injected. Infected leaves were placed in Petri dishes and incubated at 28 °C for 48 hours. Macerated plant tissues were extracted by scalpel and weighted. Results were obtained in minimum 10 biological replications and visualized in form of boxplots plotted by graphic package ggplot2 (https://ggplot2.tidyverse.org/). To assay pectate lyase activity, bacterial cells were cultured in synthetic D5 midium of the following composition with pectin as a sole source of carbon: 13.6 g/l KH_2PO_4, 1.0 g/l NH_4Cl, 0.3 g/l $MgSO_4$ (added as 100× stock solution after sterilization), 1.4 g/l NaOH, 2 g/l pectin, pH 7.5.

Pectate lyase activity was assayed as per described methodology [18]. Cell-free supernatant of cultures (50 μl) grown in D5 midium within 24 hours at 28 °C in a thermostat shaker-incubator (Orbi Safe, Sanyo, Japan) at 160 rpm/min was mixed with 450 μl of reaction mixture (pH 8.5) containing 50 mM Tris-HCl, 0.1 mM $CaCl_2$ and 0.05% polygalacturonic acid, incubated at 37 °C during 5 min. Absorption was measured at λ = 235 μm (spectrophotometer Solar PB2201B, ZAO SOLAR, Belarus). Quantity of enzyme catalyzing conversion of 1 μM substrate per minute was taken as a unit of activity. Specific activity was expressed in u/mg protein.

Swarming motility was assessed upon culturing pectobacteria in semi-liquid D5 midium containing 0.4% Pronadisa agar (Laboratorios CONDA, S.A., Spain) and 2 g/l of sucrose or pectin. At early stationary growth stage, 3 μl of bacterial culture was inoculated in semi-liquid agar and incubated at 28 °C with measuring of macro colony diameter in 24 hours.

Statistical analysis was conducted by standard mathematic methods (calculation of mean *M* and standard deviation $\pm\sigma$, comparison of means by Student's *t*-test) in Microsoft Excel 2000. Package ggplot2 was used for visualization of values of macerated tissue mass; confidence level of differences (p-value) was calculated by nonparametric Wilcoxon's test. Differences were statistically significanet at $p < 0.05$.

Results. Used strains of bacteria, plasmids, and primers are characterized in Table 1.

1. Strains, plasmids, and primers used for creation of mutant strains of *Pectobacterium atrosepticum* (*Pba*) deficient in genes encoding rhamnogalacturonyl hydrolase and galactanase

Name	Description
	Strains
Pectobacterium atrosepticum SCRI1043	Wild type [15]
SCRI1043Δ*3749*	Mutant of SCRI1043 with Km resistance cassette insertion in chromosome
SCRI1043Δ*0852*	Mutant of SCRI1043 with Km resistance cassette insertion in chromosome
Escherichia coli CC118	Host of suicidal vector pKNG101 (*ara*, *leu*) *araD lacX* 74 *galE galK PhoA20 thi*-1 *rpsE rpoB argE (am) recA1*, Sm^R (19)
HH26/pNJ5000	Mobilizing strain for conjugative delivery of suicidal vector pKNG101 into cells *Pba*, Tet^R [20]
	Plasmids
pKD4	Matrix for PCR-amplification of kanamycin resistance cassete, Km^R [21]
pKNG101	Suicidal mobilizable vector for inactivation of target genes, *pir*-ori R6K mobRK2 *sacB* Sm^R [22]
pKNG101Δ*3749*	Km^R, Sm^R, *sacB*, contains region of chromosome DNA *Pba* with deleted gene *eca3749*
pKNG101Δ*0852*	Km^R, Sm^R, *sacB*, contains region of chromosome DNA *Pba* with deleted gene *eca0852*
	Primers
upECA3749_F	5′-GCATGTTGACCGAGCTGTCC-3′
upECA3749_KmR	5′-GCCTACACAATCCGACTTCCCAATCCCACTCTTC-3′
dnECA3749_KmF	5′-CCCATGTCAGCCGTTAAGCGATATTCCCAATGTTGCCG-3′
dnECA3749_R	5′-CATGTCCCATCATTTCGCAAC-3′
Km3749_F	5′-GATTGGGAAGTCGGATTGTGTAGGCTGGAGCTGCTTC-3′
Km3749_R	5′-GGAATATCGCTTAACGGCTGACATGGGAATTAGC-3′
chek 3749_F	5′-GTTGCGGTTGGCACGATGG-3′
chek 3749_R	5′-CGAACAGATGGCAATACGTCGG-3′
upECA0852_F	5′-CTAAAGTGTTCTTATTCGATGAGCCC-3′
upECA0852_KmR	5′-CATGTCAGCCGTTAAGTGCTTTACCCAACCAATATCCG-3′
dnECA0852_KmF	5′-CCTACACAATCGCAAATTCTCCAAATGTATAACACCG-3′
dnECA0852_R	5′-CGTCCACTTTCTTACGCCCTC-3′
Km0852_F	5′-GGGTAAAGCACTTAACGGCTGACATGGGAATTAGC-3′
Km0852_R	5′-CATTTGGAGAATTTGCGATTGTGTAGGCTGGAGCTGCTTC-3′
chek 0852_F	5′-GTGTTGCGATTGGGCGGG-3′
chek 0852_R	5′-GTCTGTCGGTAACCAAAGAAAAGCG-3′

Note. Symbols ECA3749 and ECA0852 in primer indices denote gene loci of rhamnogalacturonyl-hydrolase and galactanase, accordingly; F and R are forward and reverse primers, respectively. Symbol up corresponds to amplification of area above the assumed deletion, dn — area next to the deleted fragment. Symbol Km denotes addition of first (F) or resulting (R) fragment of kanamycin resistance cassette of pKD4 plasmid located from the 5′-end of primer (at beginning of index) or at 3′-end of primer (at the end of index). Chek primers initiate amplification from proximal and distal areas of concerned locus to determine its size and sequencing. Detailed scheme of test was earlier described by Datsenko et al. [21].

As per annotations provided for in CAZy and UniProt databases, *P. atrosepticum* SCRI1043 genome has 8 genes encoding enzymes that destruct RGU I. Expression of such genes is increased upon colonization of the host plants by pectobacteria [23]. Genome loci *eca3749* and *eca0852* of *P. atrosepticum*, one of which encodes enzyme that destruct the backbone of RGU I, and the second one that destruct side chains, were selected for knockout of genes encoding RGU I-degrading enzymes. Sequence of the first locus was annotated as encoding rhamnagalacturonyl-hydrolase enzyme that destruct RGU I backbone. Subject to annotation, *eca0852* encodes galactanase that cleave side chains of RGU I. Phylogenetic analysis had proven that target ECA3749 and ECA0852 enzymes were close to protein groups with rhamnagalacturonyl-hydrolase and galactanase activity, respectively (Fig. 1).

The *eca0852* and *eca3749* deficient chromosome mutants of *P. atrosepticum* were constructed by allele exchange with the use of suicidal vector pKNG101 according to earlier described protocol [8]. To assess the influence of target mutations on ability of *P. atrosepticum* to cause maceration in plant tissues, leaves of cabbage were inoculated with wild and mutant pectobacteria. Weight of soft rot produced by such strains within 48 hours varied (Fig. 2). Both mutants caused maceration of plant tissue far less intensively than wild type bacteria. Herewith,

mutation in gene encoding galactanase ECA0852, had considerably stronger effect (see Fig. 2). Most likely, the reason is that hydrolysis of side RGU I chains largely contributes to the tissue maceration process than destruction of polymer backbone. Tissue maceration symptoms were not found in leaves inoculated with sterile 10 mM magnesium chloride solution.

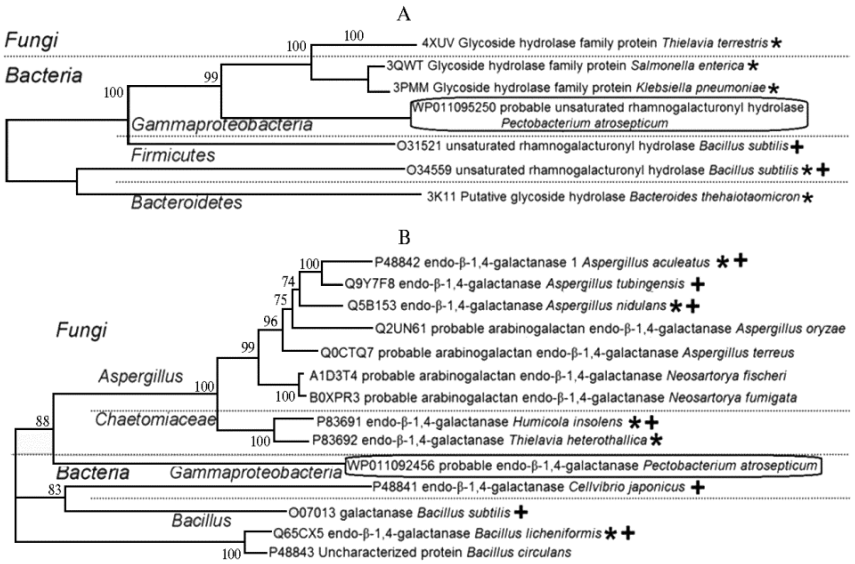

Fig. 1. Cladograms of amine acid sequences foremost similar to rhamnagalacturonyl-hydrolase of *Pectobacterium atrosepticum* (ECA3749, WP011095250) (A) **and β-1,4-endogalactanases of *P. atrosepticum* (ECA0852, WP011092456)** (B: "+" — proteins with known biochemical characteristics, "*" — proteins for which there are X-ray structural analysis in the literature sources.

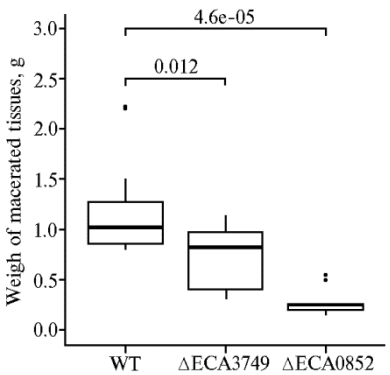

Fig. 2. Box-plots reflecting distribution of weight values in macerated tissues in leaves of cabbage (*Brassica rapa* spp. *Pekinensis*) Cha Cha cv. inoculated by wild-type strain *Pectobacterium atrosepticum* SCRI1043 (WT) **and its mutants for *eca3749* and *eca0852* loci encoding rhamnagalacturonyl-hydrolase and galactanase, respectively.** The dark horizontal line inside the box-plot denotes the median weight value of macerated tissue, upper and lower edges reflect the 1st and 3rd distribution quartiles of the analyzed values of variant, vertical lines indicte extreme value laying within the limits of one and a half interquartile ranges; black points are values exceeding the limits of one and a half interquartile ranges. Difference validities calculated by Wilcoxon's nonparametric test are provided above the parenthesis unifying box-plots.

2. Motility of wild-type strain *Pectobacterium atrosepticum* SCRI1043 and mutants for *eca3749* and *eca0852* loci (0.4 % agar)

Strain	Diameter of colonies, mm ($M\pm\sigma$)	
	sucrose	polygalacturonic acid
SCRI1043	22±0.5	22±0.2
Δ*eca3749*	22±0.5	24±0.6
Δ*eca0852*	23±0.3	23±0.4

Possible effect of mutations in *eca3749* and *eca0852* loci on activity of key virulence factors, the pectate lyases [24], as well as mobility of microorganisms serving their virulence criteria [25-28], was analyzed by relevant testing systems. Extracellular pectate lyase activity of both mutant forms in cultures in vitro, when pectin was used as a sole source of carbon, had not differed from such in a wild type (Fig. 3). In analysis of swarming motility which ensures dis-

tribution of pectobacteria along plant tissues promoting the extension of soft rot area [25] we also had not found any differences between wild and mutant forms of *P. atrosepticum*.

In semi-liquid synthetic medium, whether containing sucrose or polygalacturonic acid, both mutant strains and wild forms moved with similar speed (Table 2). All it means that reduced virulence of strains mutant for *eca3749* and *eca0852* loci is not related to lower motility of microorganisms and lower destruction of homogalacturonan.

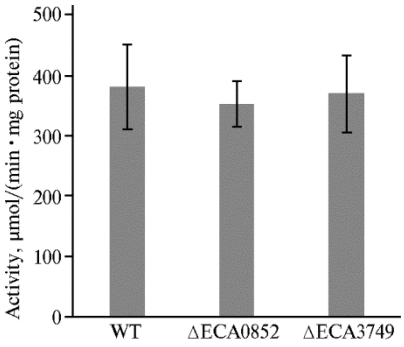

Fig. 3. Pectate lyase in supernatant of *Pectobacterium atrosepticum* SCRI1043 of wild-type (WT) **and mutant forms with inactivated loci *eca0852*** (inactivated ECA0852 enzyme) **and *eca3749*** (inactivated ECA3749 enzyme) **which encode rhamnagalacturonyl-hydrolase and galactanase, respectively**

Although RGU I a minor polymer in pectin fraction as compared to polygalacturonic acid [4], our tests denote the importance of its cleavage for development of soft rots caused by *P. atrosepticum*. Firstly, it could be related to RGU I modification ensuring formation of certain extracellular matrix for pectobacteria [13]. Secondly, regardless of that inactivation of target loci had not resulted in vitro to reduction of the microorganism capacity for cleavage of homogalacturonan (see Fig. 2), in in planta system where various types of pectin substances may represent domains for one molecule [29] inability to destruct RGU I may make certain homogalacturonan areas unavailable for pectobacterial enzymes.

Therefore, we had demonstrated that inactivation of catabolism enzyme genes of rhamnogalacturonan I (RGU I) reduces the capacity of pectobacteria for causing soft rot symptoms in plants. Herewith, it appears that destruction of side chains in such polymer largely contributes to host tissue maceration than backbone hydrolysis, as galactanase mutant is characterized by lesser virulence as compared to not only the parental wild type strain, but also to rhamnagalacturonyl-hydrolase mutant. Our findings allow referring RGU I degradation enzymes to virulence factors of phytopathogenic pectobacteria.

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