Folia Microbiol. **53** (2), 110–114 (2008) **http://www.biomed.cas.cz/mbu/folia/**

Phytase Activity and Its Regulation in a Rhizospheric Strain of *Serratia plymuthica*

E. SHEDOVA[a], V. LIPASOVA[a], G. VELIKODVORSKAYA[a], M. OVADIS[b], L. CHERNIN[b], I. KHMEL[a]*

[a]*Institute of Molecular Genetics, Russian Academy of Sciences, 123 182 Moscow, Russia*
[b]*The Otto Warburg Center for Biotechnology in Agriculture, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, 76100 Rehovot, Israel*

Received 19 June 2007
Revised version 10 December 2007

ABSTRACT. *Serratia plymuthica* strain IC1270 isolated from the rhizosphere, possessing antagonistic activity towards a wide range of plant-pathogenic fungi, is able to hydrolyze phytate. Phytase activity was found intracellularly, while no activity was detected in the culture liquid. Optimum activity was found at pH 4–5; it completely disappeared at pH > 7.0 and 2.5. Phytase production was practically absent in the exponential phase and reached a maximum in the late stationary phase. Mutations of genes *grrA* and *grrS*, encoding GacA/GacS-like 2-component global regulatory system, or in gene *rpoS* encoding the σ factor RpoS subunit of RNA polymerase, led to a deficiency in phytase production. Introduction into mutants of the respective wild-type genes cloned into the wide-range plasmid pJFF224-NX under the control of the bacteriophage T4 gene 32 promoter complemented this deficiency. This is the first report implicating the GacA/GacS global regulators and RpoS factor in phytase production in bacteria.

Abbreviations

Clm	chloramphenicol	Clm^r	chloramphenicol-resistant
Gen	gentamicin	Gen^r	gentamicin-resistant
Rif	rifampicin	Rif^r	rifampicin-resistant
Tet	tetracycline	Tet^r	tetracycline-resistant
PCR	polymerase chain reaction	PHY(s)	phytate(s)

Phytases (*myo*-inositol-hexakisphosphate phosphohydrolases, EC 3.1.3.8) catalyze the release of phosphate from PHY (*myo*-inositol hexakisphosphate). The enzymes participate in supplying inorganic phosphate to bacteria and plants and are widely used as an animal-feed additive to improve phosphate bioavailability and to reduce the loss of phosphate and divalent cations from PHY. The latter lower feed quality because PHYs bind proteins and minerals (Konietzny and Greiner 2004). The accumulation of PHYs in media causes significant environmental pollution. Therefore, phytases have become an important tool in animal production and in the remediation of industry-related environmental pollution (Pandey *et al.* 2001; Greiner and Konietzny 2006; Gargova *et al*. 2006; Gulati *et al.* 2007).

In some soil and plant-associated bacteria, phytases offer the ability to utilize PHYs as a source of phosphorus (Zinin *et al.* 2004). In bacteria, phytase is an inducible enzyme and its expression is subject to complex regulation, as a response to nutrient or energy limitations (Konietzny and Greiner 2004). The global regulators which are involved in the regulation of expression of a number of genes and operons belonging to different bacterial processes play a significant role in the control of bacterial gene expression. In Gram-negative bacteria, such global regulators include the two-component GacA/GacS regulatory system, which controls secondary metabolites and exoenzyme production in various species of *Pseudomonas*, *Pectobacterium carotovora* and *Serratia plymuthica*, among others (Chancey *et al.* 1999; Cui *et al.* 2001; Pernestig *et al.* 2001; Ovadis *et al.* 2004). This system acts *via* a phosphorylation–dephosphorylation cascade; GacS, a transmembrane sensor kinase, transfers a phosphoryl group to the GacA response regulator protein (Heeb and Haas 2001). In addition to the GacA/GasS-like regulatory system, the σ^S subunit of RNA polymerase plays a key role in the regulation of gene transcription during the transfer of cells to a stationary phase of growth and under different stresses, including starvation, osmotic stress, *etc.* (Hengge-Aronis 2002). Data on the role of global regulators in the regulation of phytase production are only available for *Raoultella terrigena* (Zamudio *et al.* 2002). Those authors showed a twofold decrease in phytase production in the stationary phase in an *rpoS*::*Tn*10 mutant.

*Corresponding author; e-mail khmel@img.ras.ru .

Production of phytases could be an advantage for biocontrol bacteria's ability to protect economically important crops against plant pathogens. A combination of plant-growth-promoting, antagonistic and phytase activities might lead to better performance of commercial biocontrol agents under field conditions. Several plant-associated isolates of *Serratia* species, mainly *S. marcescens* and *S. plymuthica*, have been described as biocontrol agents for fungal plant pathogens (Berg 2000; Kamensky *et al.* 2003; Ovadis *et al.* 2004). These isolates are known to produce a range of antifungal metabolites, including chitinolytic enzymes and antibiotics (Kalbe *et al.* 1996; Kamensky *et al.* 2003; Levenfors *et al.* 2004; Ovadis *et al.* 2004). However, to the best of our knowledge, only one study has described phytase production by a strain of *Serratia* (Mukesh *et al.* 2004).

The goal of this research was to investigate whether strains of *S. plymuthica* produce phytases and if so, whether the GacA/GacS-like and RpoS global regulators play a role in the regulation of phytase synthesis. Biocontrol strain IC1270 of *S. plymuthica* was chosen as the model organism. This strain has been shown to suppress the growth of a wide range of plant-pathogenic fungi due to its ability to produce chitinolytic and proteolytic enzymes, siderophores and the antibiotic pyrrolnitrin (Chernin *et al.* 1995; Ovadis *et al.* 2004). Recently, *grrA* and *grrS* genes (homologues of *gacA* and *gacS* genes of other Gram-negative bacteria), as well as the *rpoS* gene from this bacterium were cloned and sequenced and their null mutants obtained (Ovadis *et al.* 2004).

MATERIALS AND METHODS

Strains and culture conditions. The spontaneous Rif^r derivative of *Serratia plymuthica* strain IC1270, isolated from the rhizosphere of grape, and its Gen^r gene-replacement mutants IC1270 no. 20, no. 93, and no. 823 (deficient in genes *grrA*, *grrS* and *rpoS*, respectively) were described by Ovadis *et al.* (2004). *Escherichia coli* strains carrying pJFF224-NX and pJFF224-NX:*xylE*, both Clm^r (Frey 1992), were kindly supplied by Prof. J. Frey (*Institute of Veterinary Bacteriology*, Bern, Switzerland). Liquid or solid (1.5 %, *M*/*V*, agar) Luria broth were used as growth media (Miller 1992). *E. coli* strain XL1-Blue was from a laboratory collection (Tet^r; *Stratagen*, USA). Gen was used at a 20 μg/mL, Rif – 40 μg/mL, Clm – 25 μg/mL, Tet – 12.5 μg/mL.

DNA manipulations. Total genomic DNA isolation, restriction-enzyme digestion, agarose-gel electrophoresis, electroporation and PCR were generally performed according to standard procedures (Ausubel *et al.* 1994). Enzymes were purchased from *MBI Fermentas* (Lithuania) or *Promega* (USA) and used according to the manufacturers' directions. The following strategy was used for recloning of the regulatory genes *grrA* and *grrS*, encoding the two-component global regulation system, and *rpoS*, encoding the transcriptional σ^S factor, from strain IC1270. Each of these genes was recloned from previously described hybrid plasmids based on the pGEM-T Easy vector (Ovadis *et al.* 2004) into the wide-host-range shuttle vector pJFF224-XN, that is stably maintained in *E. coli* and many other bacteria (Frey 1992).

The desired genes were excised with *Eco*RI from the corresponding hybrid plasmids, recovered from the gel and religated with vector pJFF244-XN, which was also cut with *Eco*RI. The genes were inserted under the control of the strong promoter of phage T4 gene 32 (g32). The ligation mixtures were first electroporated into strain XL1-Blue competent cells. The corresponding recombinant clones were selected on Luria agar medium supplemented with Tet and Clm and tested by PCR for the presence of corresponding genes using specific primers against previously identified sequences of *S. plymuthica* IC1270 *grrA*, *grrS* and *rpoS* (*GenBank* accession no. AY057388, AY057389 and AY057391, respectively). The primers were:

grrA	5′-ACG TGA ACT GCA GAT AAT GCT GA-3′ – forward,
	5′-GGG CGC GTA GCG TAC AAT T-3′ – reverse;
grrS	5′-ATG ACC AAA TAC AGC TTG CTG G-3′ – forward,
	5′-TCA GCT TGT CGT TGG GCG A-3′ – reverse;
rpoS	5′-ATG AAC CAA AAT ACG CTG AAA GTT AA-3′ – forward,
	5′-TTA TTC GCG GAA CAG CGC-3′– reverse.

The PCR steps were as follows: (*1*) 3 min, 94 °C; (*2*) 2 min, 55 °C; (*3*) 2 min, 72 °C; (*4*) 30 s, 94 °C; (*5*) 30 s, 55 °C; (*6*) 1 min, 72 °C; (*7*) 30× steps *4*–*6*; (*8*) 10 min, 72 °C; (*9*) hold at 4 °C. Orientation of the structural parts of the cloned genes relative to the T4 g32 promoter was determined by restriction analyses using restriction enzyme *Pst*I, which has several restriction sites in all of the inserts but only one site in the vector. Finally, clones with insertions of genes *grrA*, *grrS* and *rpoS* in sense orientation towards the promoter were selected. The obtained hybrid plasmids pJFF224-NX/*grrA*, pJFF224-NX/*grrS* and pJFF224-NX/*rpoS*

were used for electroporation of competent cells of strain IC1270 mutants no. 20, no. 93 and no. 823, respectively, and the presence of the corresponding genes was confirmed by PCR with the above described primers.

Determination of pyrocatechol 2,3-oxygenase activity and plasmid stability. The ability of plasmid pJFF224-NX to express cloned genes was demonstrated by using the derivative of the pJFF224-NX plasmid carrying the reporter *xylE* gene encoding the enzyme pyrocatechol 2,3-oxygenase cloned under the control of phage T4 gene 32 promoter (Frey 1992). Strain IC1270 was transformed by electroporation with plasmid pJFF224-NX:*xylE*; *xylE* expression was detected by spraying colonies growing on 25 mg/mL Clm-supplemented Luria agar with pyrocatechol (Frey 1992). Colonies producing pyrocatechol 2,3-oxygenase turned yellow after 10–20 s.

Determination of phytase activity. Bacteria were grown with aeration at 6.7 Hz in 200 mL Luria broth at 30 °C, then spun down and washed in 0.89 % NaCl. The pellet was resuspended in 2 mL 50 mmol/L Tris-HCl buffer (pH 7.0) and sonicated at 4 °C. Phytase was measured using a modification of the diammonium molybdate method (Greiner *et al.* 1997; Idriss *et al*. 2002). Incubation mixtures (300 μL) contained 30 μL of cell extract and 120 μL of substrate containing 0.4 % dodecasodium phytate, 2.5 mmol/L $CaCl_2$, 50 mmol/L buffer and 150 μL H_2O. Glycine-HCl, citrate, Tris–maleate and Tris-HCl buffers (all 50 mmol/L) were used for the determination of phytase activity at pH 2.5–3.0, 4.0–5.0, 6.0, and 7.0–8.0, respectively. After a 1-h incubation at 30 °C, the reaction was stopped by adding 300 μL hexaammonium heptamolybdate–ammonium vanadate reagent solution. The samples were centrifuged (210 Hz, 1 min) and A_{415} was measured. Phytase activity was expressed in μkat per μg protein. Phosphate content was assayed with hexaammonium heptamolybdate–ammonium vanadate reagent as described for the phytase-activity assay. Protein content was measured according to Bradford (1976).

RESULTS AND DISCUSSION

We tried to detect phytase activity in *S. plymuthica* IC1270 and its *grrA* (IC1270 no. 20), *grrS* (IC1270 no. 93) and *rpoS* (IC1270 no. 823) mutants, deficient in the production of GrrA, GrrS and σ^S global regulators, respectively (Ovadis *et al.* 2004). Cells of strain IC1270 were shown to produce phytase(s). Optimum phytase activity was observed at pH 4.0–5.0; at pH 3.0 or 6.0, the activity drastically decreased to 5.0 and 6.3 % of the value at pH 4.0, respectively, and completely disappeared at pH 2.5 and ≥7 (Table I). The appearance of phytase activity in a narrow pH range suggests that the IC1270 cells produce only one enzyme responsible for this activity. Phytase production appeared at 36 h and reached a maximum at 40–44 h. No activity was detected in the culture liquid. Similar characteristics have been reported for phytases of bacteria belonging to various species of *Enterobacteriaceae*, including *Klebsiella terrigena* (Greiner *et al.* 1997), *Obesumbacterium proteus* (Zinin *et al.* 2004), *Enterobacter cloacae*, *Citrobacter freundii*, and *E. coli* (Zinin *et al.* 2003), *Citrobacter braakii* (Kim *et al.* 2003), and *Yersinia intermedia* (Huang *et al.* 2006).

In contrast to the wild-type strain, phytase activity was nearly undetectable in the *grrA*, *grrS* and *rpoS* mutants at pH 4.0–5.0, and did not appear at all under more acidic or basic conditions (Table I). None of these mutations influenced cell growth to any considerable extent in Luria broth medium, in agreement with Ovadis *et al.* (2004). On the other hand, the activity was almost completely restored when the mutant phenotype was complemented by the introduction of recombinant plasmids carrying the wild alleles of the *grrS* and *rpoS* genes cloned under the control of the *E. coli* bacteriophage T4 g32 promoter region into the wide-host-range expression vector pJFF224-NX, based on the replicon of plasmid RSF1010 (Frey 1992). In all three mutants complemented with the recombinant plasmids carrying the corresponding wild-type regulatory genes, phytase activity was found at the same level and pH optimum was the same as in the parental strain IC1270.

Activity of the g32 promoter was confirmed with plasmid pJFF224-NX carrying the reporter construct g32-*xylE* encoding pyrocatechol 2,3-oxygenase. The vector plasmid pJFF224-NX, the reporter plasmid pJFF224-NX:*xylE*, as well as the obtained hybrid plasmids carrying the global regulatory genes *grrA*, *grrS* or *rpoS* cloned under the g32 promoter into pJFF224-NX, were stably maintained in strain IC1270. Phytase production in the IC1270 strain depended on the activity of the GacA/GacS (GrrA/GrrS) global regulators of gene expression and σ^S subunit of RNA polymerase, adding phytase to the list of bacterial enzymes whose production depends on these regulators (Heeb and Haas 2001).

Bacterial phytases were shown to play an important role under conditions of phosphate starvation in plants. Hence, for example, the capacity of *Bacillus amyloliquefaciens* to produce phytases increases the plant-growth-promoting effect of these bacteria under conditions of phosphate deficiency in media (Idriss *et*

al. 2002). In these conditions, phytase production might be advantageous for biocontrol bacteria (including strain IC1270), because it enables the use of PHYs that are normally unavailable as a source of phosphate.

Table I. Phytase activity of the strain *S. plymuthica* IC1270 and its derivatives at various pH[a]

Strain IC1270 no.	Characteristics	pH	Phytase activity[b]
–	prototroph, Rif^r	2.5	<0.02
		3	0.80 ± 0.08
		4	16.1 ± 0.67
		5	5.75 ± 0.58
		6	1.02 ± 0.12
		7	<0.02
20	*grrA* mutant, Gen^r, Rif^r	2.5–8.0	<0.02
20/pJFF224-NX/*grr*A$^+$	20[c], Gen^r, Clm^r, Rif^r	2.5	<0.02
		3	0.97 ± 0.10
		4	14.2 ± 0.65
		5	5.30 ± 0.25
		6	0.87 ± 0.08
		7	<0.02
93	*grrS* mutant, Gen^r, Rif^r	2.5–8.0	<0.02
93/pJFF224-NX/*grr*S$^+$	93[d], Gen^r, Clm^r, Rif^r	2.5	<0.02
		3	1.03 ± 0.03
		4	14.7 ± 0.98
		5	5.03 ± 0.17
		6	0.98 ± 0.07
		7	<0.02
823	*rpoS* mutant, Gen^r, Rif^r	2.5–8.0	<0.02
823/pJFF224-NX/*rpo*S$^+$	823[e], Gen^r, Clm^r, Rif^r	2.5	<0.02
		3	0.90 ± 0.07
		4	15.4 ± 0.30
		5	6.48 ± 0.20
		6	0.92 ± 0.10
		7	<0.02

[a]The plasmid-free mutant strains were grown in Luria broth medium supplemented with Gen while strains carrying the recombinant plasmids were grown in medium supplemented with Gen and Clm for 44 h at 30 °C with aeration; means ± SE at $p < 0.05$ from three independent experiments.
[b]μkat per μg protein.
[c]Contains cloned *grrA*$^+$ gene in pJFF224-NX/*grrA*$^+$ plasmid.
[d]Contains cloned *grrS*$^+$ gene in pJFF224-NX/*grrS*$^+$ plasmid.
[e]Contains cloned *rpoS*$^+$ gene in pJFF224-NX/*rpoS*$^+$ plasmid.

This research was supported in part by the *Russian Foundation for Basic Research* (grant no. 06-04-48585) and by the *United States–Israel Binational Agricultural Research and Development Fund* (BARD), grant no. US-3789-05.

REFERENCES

AUSUBEL F.M., BRENDT R., KINGSTON R.E., MOORE D.D., SEIDMAN J.G., SMITH J.A., STRUHL K. (Eds): *Current Protocols in Molecular Biology*. John Wiley & Sons, New York 1994.

BERG G.: Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J.Appl.Microbiol.* **88**, 952–960 (2000).

BRADFORD M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. *Anal.Biochem.* **72**, 248–254 (1976).

CHANCEY S.T., WOOD D.W., PIERSON III L.S.: Two component transcriptional regulation of *N*-acyl-homoserine lactone production in *Pseudomonas aureofaciens*. *Appl.Environ.Microbiol.* **65**, 2294–2299 (1999).

CHERNIN L.S., ISMAILOV Z., HARAN S., CHET I.: Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ.Microbiol.* **61**, 1720–1726 (1995).

CUI Y., CHATTERJEE A., CHATTERJEE A.K.: Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory rsmB RNA, extracellular enzymes, and HarpinEcc. *Mol.Plant Microbe Interact*. **14**, 516–526 (2001).

FREY J.: Construction of a broad host range shuttle vector for gene cloning and expression in *Actinobacillus pleuropneumoniae* and other *Pasteurellaceae*. *Res.Microbiol.* **143**, 263–269 (1992).

GARGOVA S., SARIYSKA M., ANGELOV A., STOILOVA I.: *Aspergillus niger* pH 2.1 optimum acid phosphatase with high affinity for phytate. *Folia Microbiol*. **51**, 541–546 (2007).

GREINER R., HALLER E., KONIETZNY U., JANY K.D..: Purification and characterization of a phytase from *Klebsiella terrigena*. *Arch. Biochem.Biophys*. **341**, 201–206 (1997).

GREINER R., KONIETZNY U.: Phytase for food application. *Food Technol.Biotechnol.* **44**, 125–140 (2006).

GULATI H.K., CHADHA B.S., SAINI H.S.: Production of feed enzymes (phytase and plant cell wall hydrolyzing enzymes) by *Mucor indicus* MTCC 6333: purification and characterization of phytase. *Folia Microbiol*. **52**, 491–497 (2007).

HEEB S., HAAS D.: Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Plant Microbe Interact*. **14**, 1351–1363 (2001).

HENGGE-ARONIS R.: Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol.Molec.Biol.Rev.* **66**, 373–395 (2002).

HUANG H., LUO H., YANG P., MENG K., WANG Y., YUAN T., BAI Y., YAO B.: A novel phytase with preferable characteristics from *Yersinia intermedia. Biochem.Biophys.Res.Commun*. **350**, 884–889 (2006).

IDRISS E.E., MAKAREWICZ O., FAROUK A., ROSNER K., GREINER R., BOCHOW H., RICHTER T., BORRISS R.: Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiology* **148**, 2097–2109 (2002).

KALBE C., MARTEN P., BERG G.: Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. *Microbiol.Res.* **151**, 4400–4433 (1996).

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.* **35**, 323–331 (2003).

KIM H.W., KIM Y.O., LEE J.H., KIM K.K., KIM Y.J.: Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. *Biotechnol.Lett*. **25**, 1231–1234 (2003).

KONIETZNY U., GREINER R.: Bacterial phytases: potential application, *in vivo* function and regulation of its synthesis. *Brazil.J.Microbiol*. **35**, 11–18 (2004).

LEVENFORS J.J., HEDMAN R., THANING C., GERHARDSON B., WELCH C.J.: Broad-spectrum antifungal metabolites produced by the soil bacterium *Serratia plymuthica* A 153. *Soil Biol.Biochem.* **36**, 677–685 (2004).

MILLER J.H.: *A Short Course in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 1992.

MUKESH P., SUMA S., SINGARACHARYA M.A., LAKSHMIPATHI V.: Isolation of phytate-hydrolysing microbial strains from traditional waste water of rice fermentation and liquid cattle feeds. *World J.Microbiol.Biotechnol.* **20**, 531–534 (2004).

OVADIS M., LIU X., GAVRIEL S., ISMAILOV Z., CHET I., CHERNIN L.: The global regulator genes from biocontrol strain *Serratia plymuthica* IC1270: cloning, sequencing, and functional studies. *J.Bacteriol.* **186**, 4986–4993 (2004).

PANDEY A., SZAKACS G., SOCCOL C.R., RODRIGUEZ-LEON J.A., SOCCOL V.T.: Production, purification and properties of microbial phytases. *Biores.Technol.* **77**, 203–214 (2001).

PERNESTIG A.K., MELEFORS O., GEORGELIS D.: Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. *J.Biol.Chem.* **276**, 225–231 (2001).

ZAMUDIO M., GONZALEZ A., BASTARRACHEA F.: Regulation of *Raoultella terrigena* comb.nov. phytase expression. *Can.J.Microbiol*. **48**, 71–81 (2002).

ZININ N.V., SAMSONOV V.V., SAMSONOV V.V., BORSHCHEVSKAYA L.N., KANIKOVSKAYA A.A., GUDIMA M.YU., SINEOKII S.P.: Phytase activity of several bacteria groups. *Biotechnologia (Russia)* **2**, 3–10 (2003).

ZININ N.V., SERKINA A.V., GELFAND M.S., SHEVELEV A.B., SINEOKY S.P.: Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. *FEMS Microbiol.Lett.* **236**, 283–290 (2004).