Interaction of Viral and Bacterial Lysozymes with *Erwinia amylovora* and Their Inhibition by a Bacterial Protein

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Abstract

Viral lysozymes can interact with *Erwinia amylovora*, and their genes may control fire blight when expressed in the pathogen. We have investigated lysozymes from *E. amylovora* phages and from genomes of several *Erwinia* species. With chloroform-treated cells as substrate, the highest lytic activity was found for viral lysozymes. Fusion proteins with the ϕEa1h lysozyme, such as addition of a His-tag reduced lysis efficiency significantly. For large fusions at the N- or C-terminus lytic activity was completely lost. When a protein fused to glutathione S-transferase was cleaved off with a specific protease, the activity of the viral lysozyme was restored. Intact lysozyme from *E. amylovora* phage ϕEa1h had a similar lytic activity as *E. coli* phage T4 lysozyme. Viable cells of *E. amylovora* were not inhibited by external addition of lysozyme from sonicated cell extracts in assays with ϕEa1h lysozyme and T4 lysozyme. The cellular expression of a viral lysozyme gene can be toxic for a bacterial host. The gene was therefore expressed in yeast as safe environment for its secretion. Lysozyme-specific antibodies detected the protein in culture supernatants of transgenic yeast. When *E. amylovora* was transformed with a viral lysozyme gene, the cells were killed after induction by IPTG. Transfer of a *lyz*-gene into *E. amylovora* can thus strongly reduce the viability of the pathogen. The ϕEa1h lysozyme was inhibited by a cellular protein from *E. amylovora* related to an inhibitor for vertebrate lysozymes of *E. coli*. The lytic activity of lysozyme from phage ϕEa104 and ϕEa116 was not affected by the enzyme inhibitor.

INTRODUCTION

Bacteriophages possess viral lysozymes (muramidases), which hydrolyze the β-1,4 glycosidic bond between MurNAc and GlucNAc of the peptidoglycan leading at the end of the phage life cycle to lysis of the host cells. Most phages depend on a holin/lysozyme system for lysis, in which the hydrophobic holin proteins trigger lysis (Young et al., 2000). The c-type and g-type lysozymes can be inhibited by a protein family called inhibitors of vertebrate lysozyme (Ivy), while viral lysozymes are only weakly inhibited (Callewaert et al., 2005; Monchois et al., 2001). In this work, the effects of several lysozymes from *E. amylovora* phages and from three *Erwinia* species, on *E. amylovora* cells were investigated. Fusion proteins of viral ϕEa1h lysozyme were not as active as the intact enzyme (Kim and Geider, 2000). In mating experiments, a *lyz*-gene on broad-host range plasmids was transferred to *E. amylovora* and the activity of *ivy*-genes from *E. amylovora* and *E. pyrifoliae* genomes was determined.

RESULTS AND DISCUSSION

Effect of Viral Lysozymes on *E. amylovora* Cells

Several lysozymes from *E. amylovora* phages were cloned in various vectors and tested for their toxic effect when expressed in *E. amylovora*. The colony number obtained on solid medium was drastically reduced when lysozyme expression was induced by IPTG. In liquid cultures a growth inhibiting effect was observed.

Gram-negative cells are insensitive to lysozyme when applied from outside due to

an outer membrane, which forms a barrier for large molecules (Benz, 1988). When Gram-negative bacteria are pre-treated with EDTA or chloroform they can be lyzed by external addition of lysozyme (Loessner, 2005). In assays with chloroform-treated cells we measured the effect of the phage lysozymes expressed in *E. coli*. As native protein the ϕEa1h lysozyme reduced the optical density more efficiently than an N-terminal or C-terminal fusion. Lysozymes from other *E. amylovora* phages were comparable to the ϕEa1h lysozyme in their activity. Dilution series of cell extracts with lysozyme at low concentrations still affected chloroform-treated cells.

Boiling and large fusion proteins at the N-terminus or C-terminus abolished lytic activity. When fused to glutathione S-transferase, the ϕEa1h lysozyme was inactive. Treatment with a specific protease to cleave the fusion protein restored full lysozyme activity. Expression of ϕEa1h lysozyme was not toxic to *Pichia pastoris*. The protein was secreted by the yeast and culture supernatants reduced the optical density of *E. amylovora* in the chloroform-cell assay.

Effect of Chromosomal Lysozymes on *E. amylovora* Cells

Chromosomal lysozymes were detected in the genomes of several *Erwinia* strains. Some of them were related to each other, while others differed and were more similar to ϕEa1h lysozyme. The chromosomal lysozymes were cloned and then expressed in *E. amylovora*. The effect observed was weaker in comparison to viral lysozymes. The fusion of a His-tag to these and to the viral lysozymes reduced lytic activity on chloroform-treated cells.

Ivy Proteins of *Erwinia* Strains

In the genomes of *E. amylovora* and *E. pyrifoliae* a gene coding for Ivy was detected and cloned. Its activity was similar to the Ivy proteins previously detected in *E. coli* (Monchois et al., 2001). Very recently, it was reported that only Gram-negatives without any O-acetylation of GlucNAc residues in the peptidoglycan possess lytic transglycosylases, are inhibited by Ivy proteins and also express *ivy* genes. It was assumed that the inhibitory effect on lysozyme is an additional positive feature for the bacteria and that the true Ivy targets are the lytic transglycosylases (Clarke et al., 2010). The ϕEa1h lysozyme was inhibited by *Erwinia* Ivy proteins, as was the positive control of egg white lysozyme. Other viral lysozymes tested were not inhibited.

Transfer of *lyz*-carrying Plasmids to *E. amylovora*

Transfer of toxin genes into other bacteria by conjugation can be of commercial interest and might be a tool to control certain pathogens (Filutowicz et al., 2008). It has been proposed as an alternative to the application of antibiotics.

The lysozyme genes of two bacteriophages were subcloned into a broad-host range vector and transferred into *E. amylovora*. Expression of both constructs was toxic to the pathogen. We conducted matings with the broad-host range plasmid RP4 and various plasmid donors. Two avirulent *E. amylovora* strains showed a promising high transfer rate to *E. amylovora* recipients. They were chosen as donors for a bacteriophage-gene inserted in broad-host range vectors. While the empty vector could be transferred to the *E. amylovora* recipient, matings with the lysozyme donor did not result in transconjugants, indicating a high toxicity of the construct to the recipient.

CONCLUSION

Lysozymes derived from bacteriophages inhibit the fire blight pathogen, when expressed inside *E. amylovora*. Treatment of the Gram-negative bacteria with chloroform rendered them sensitive to exogenous lysozyme. The viral lysozymes tested did not differ in their lytic effect. Different protein fusions to the N-terminus or C-terminus of the lysozymes resulted in loss of activity when the fusions exceeded a certain size. Lysozymes derived from genomic genes of several *Erwinia* strains also reduced the optical density in the chloroform-cell assay. ϕEa1h lysozyme was over-expressed in

yeast, and its activity against chloroform-treated cells was shown. It was inhibited by Ivy proteins encoded in the genome of two *Erwinia* strains, while two other viral lysozymes were not inhibited. The transfer of a ϕEa1h lysozyme expression construct reduced the viability of *Erwinia* cells after conjugation with the fire blight pathogen.

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