

Expression of bacteriophage ϕ Ea1h lysozyme in *Escherichia coli* and its activity in growth inhibition of *Erwinia amylovora*

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A 3.3 kb fragment from *Erwinia amylovora* phage ϕ Ea1h in plasmid pJH94 was previously characterized and found to contain an exopolysaccharide depolymerase (*dpo*) gene and two additional ORFs encoding 178 and 119 amino acids. ORF178 (*lyz*) and ORF119 (*hol*) were found to overlap by 19 bp and they resembled genes encoding lysozymes and holins. In nucleotide sequence alignments, *lyz* had structurally conserved regions with residues important for lysozyme function. The *lyz* gene was cloned into an expression vector and expressed in *Escherichia coli*. Active lysozyme was detected only when *E. coli* cells with the *lyz* gene and a kanamycin-resistance cassette were grown in the presence of kanamycin. Growth of *Erw. amylovora* was inhibited after addition of enzyme exceeding a threshold for lysozyme to target cells. When immature pears were soaked in lysates of induced cells, symptoms such as ooze formation and necrosis were retarded or inhibited after inoculation with *Erw. amylovora*.

INTRODUCTION

Fire blight is a serious bacterial disease in pome fruit orchards; it is endemic in north-eastern America (Jock & Geider, 2004) and in the last century it spread to many countries of the northern hemisphere and to New Zealand (Bonn & van der Zwet, 2000). The causative agent *Erwinia amylovora* is often transmitted by insects (Hildebrand *et al.*, 2000), especially by bees when visiting flowers. To control fire blight, streptomycin and other antibiotics have been applied successfully (Psallidas & Tsiantos, 2000); however their use has been restricted in many countries. Bacteriophages have been used for identification of plant-associated bacteria, to determine capsulation of *Erw. amylovora* cells and also for disease control (Billing, 1960; Bernhard *et al.*, 1993; Schnabel & Jones, 2001); they have been isolated from the soil in the vicinity of diseased trees (Ritchie & Klos, 1977) and from diseased tissue of host plants (Okabe & Goto, 1963). A bacteriophage was applied to pear slices and it delayed symptom development after inoculation with *Erw. amylovora* (Erskine, 1973). Another potentially specific approach for interfering with growth of *Erw. amylovora* has been the proposed use of Serracin P, a phage-tail-like bacteriocin (Jabrane *et al.*, 2002). In orchards, successful control of fire blight with bacteriophages or bacteriocins has not yet been reported.

In a late stage of their life cycle, many bacteriophages express lysozyme and holin to lyse the host cells (Young, 1992). The

holin forms a pore in the host cell membrane to channel the lysozyme into the periplasm. The muramidase activity of lysozyme hydrolyses 1,4- β -linkages between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in the peptidoglycan layer of bacterial cell walls (Cooper, 1997). Lysozymes are classified into four families: chicken-, goose-, phage- and bacterial-type (Jolles & Jolles, 1984). The amino acid sequences within a family are related, but there are no clear sequence homologies among families (Weaver *et al.*, 1985).

In this study, we investigated the requirements for expression of the lysozyme gene cloned from *Erw. amylovora* phage ϕ Ea1h into *Escherichia coli*, and its ability to inhibit growth of *Erw. amylovora* in broth cultures and on agar plates. These features of lysozyme could be used for control of fire blight in orchards.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Media and growth conditions have been described previously (Kim & Geider, 2000). Antibiotics were ampicillin (Ap, 100 μ g ml⁻¹), chloramphenicol (Cm, 20 μ g ml⁻¹), kanamycin (Km, 20 μ g ml⁻¹) and streptomycin (Sm, 500 μ g ml⁻¹).

Cloning of the ϕ Ea1h lysozyme gene. Primer pairs were designed from the insert in plasmid pJH94 (GenBank accession no. AJ278614) to amplify the *lyz* gene without the ATG start codon for fusion into a His-tagged expression vector. The forward primer Lyz5 (5'-GCGCGGGATCCCTCTGTGAAGAAGGCTCT; a created *Bam*HI site is underlined) and the reverse primer Lyz3c (5'-GCGCGAAGC-TTCTACATATATTTTACGC; a created *Hind*III site is underlined) were applied with 2.5 U *Pfu* DNA polymerase (Stratagene) as

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Phenotype/relevant characteristics	Source or reference
Bacterial strains		
<i>E. coli</i>		
M15 (pREP4)	F ⁻ Str ^R Δ lacZ lacI ^q on pREP4 (Km ^R)	Qiagen
GI698	λ ⁻ lacI ^q lacPL8 ampC::P _{trp} cl	Invitrogen
<i>Erw. amylovora</i>		
Ea1/79	Isolated from <i>Cotoneaster</i> sp., Germany, 1979	Falkenstein <i>et al.</i> (1988)
Ea1/79Sm	Sm ^R strain of Ea1/79	
Plasmids		
pfdC1	fd <i>ori</i> , fd gene 2, Km ^R	Geider <i>et al.</i> (1995)
pJH94	A 3.3 kb insertion after a partial <i>Sau</i> 3A digest of DNA from ϕ Ea1h* in pUC8, Ap ^R	Hartung <i>et al.</i> (1988)
pREP4	lacI ^q Km ^R mini 15 <i>ori</i>	Qiagen
pREP-Cm	pREP4 with Cm ^R cassette	This study
pRK293	RKII (RP4) <i>ori</i> , Km ^R Tc ^R	Ditta <i>et al.</i> (1985)
pQE-30	6 \times His-tag ColE1 <i>ori</i> , Ap ^R	Qiagen
pQE-lyz1	0.53 kb <i>Bam</i> HI– <i>Hind</i> III fragment with ORF178 amplified from pJH94 and inserted into pQE-30, Ap ^R	This study

*Bacteriophage described by Ritchie & Klos (1977).

described previously (Kim & Geider, 2000). The amplified fragments were digested with *Bam*HI and *Hind*III, purified in an agarose gel and eluted with a gel extraction kit (QIAEX II; Qiagen). The 0.5 kb (*lyz*) PCR fragment was fused to the nucleotide sequence encoding the His tag in plasmid pQE-30, which was useful for detecting the protein with anti-His antibodies. The fusion was confirmed by sequence analysis, and the plasmid was transformed into *E. coli* strain M15(pREP4).

Preparation of cell lysates with soluble lysozyme. To express the cloned lysozyme gene in *E. coli* M15(pREP4, pQE-lyz1), the strain was cultured at 37 °C in 400 ml Luria Bertani (LB) medium with Ap (100 μ g ml⁻¹) and Km (20 μ g ml⁻¹), to an OD₆₀₀ of 0.5. The culture was induced with 1 mM IPTG and further incubated for 1 h without shaking, then centrifuged at 4000 g for 20 min, and the pellet was suspended in 2.5 ml buffer A [10 nM imidazole, 0.3 M NaCl, 50 nM NaH₂PO₄ (pH 8.0)], which is recommended for Ni columns (Qiagen). After sonication, a clear cell lysate was obtained by centrifugation at 10000 g for 20 min. The lysozyme activity was screened as a growth inhibition zone on a lawn of Ea1/79 after application of 10 μ l cell lysate. The protein concentration was measured by the Lowry method.

Lysozyme assays by inhibition of cell growth in culture. Lysozyme activity was assayed with *Erw. amylovora* Ea1/79Sm grown in LB medium overnight at 28 °C. The cells were diluted to 10⁵, 10⁴, 10³ and 10² c.f.u. ml⁻¹ and dispensed in triplicate into 96-well microtitre plates (200 μ l per well). The sonicated lysates of M15(pREP4, pQE-lyz1) were added at various concentrations to Ea1/79Sm dilutions, which were further incubated at 28 °C in LB medium with Sm for up to 3 days without shaking. For a negative control, protein from IPTG-induced M15(pREP4, pQE-30) lysate was applied. The bacterial growth rate was estimated by automatic OD₆₂₀ measurements using a Titertek Multiskan MCC/340 MKII (Flow Laboratories).

For survival assays, suspensions in Standard I broth (StI, Merck) were incubated in a microtitre plate for 0, 1, 2, or 4 h, and the reaction mixtures were plated on StI agar with 200 μ g Sm ml⁻¹.

Assays with pear slices. Immature pears (cv. Bartlett) were stored for 1–10 weeks in loosely sealed glass beakers at 6 °C. Slices of

approximately 5 mm thickness were cut and briefly immersed in cleared cell lysates, which had been obtained by sonication and subsequent centrifugation. The extracts were diluted in imidazole-containing buffer to a protein concentration of 500 μ g ml⁻¹. The soaked slices were air-dried in a laminar flow hood, and four slices were placed into a plastic box (5 cm diameter). With a pipette, 10 μ l volumes of dilutions of *Erw. amylovora* cultures were applied to the surface of the four slices in the box, which was then tightly closed and incubated at 26 °C. Necrosis and ooze formation were evaluated after 5 days with a scale from 0 (no symptoms) to 4 (dark brown slices and ooze in large drops or as a fluid layer on the surface).

Analysis of ϕ Ea1h lysozyme on Ni columns. Soluble extracts from M15(pREP4, pQE-lyz1) were loaded on an Ni-NTA agarose column (Qiagen). The column was washed with washing buffer (1 \times PBS with 0.5 M NaCl, 0.05% Tween 20, 5 mM imidazole, pH 8) and eluted with the same buffer containing 250 mM imidazole (pH 6.5). The columns were then treated with 0.1 M EDTA, and the matrix was further analysed for residual protein.

PAGE analysis and immunoblots. Fractions from each step of the purification procedure were separated by size in 14% polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970) and transferred to PVDF membranes (Millipore) in a Hoefer Mighty Small SE245 system as recommended by the manufacturer. The blots were treated by standard techniques, first using an anti-His antibody (Qiagen), and then an anti-mouse antibody conjugated with alkaline phosphatase. The colour detection of the immobilized antigen was done with NBT/BCIP (Pierce, Perbio Science).

RESULTS

Sequence analysis of ORF178 and ORF119

The fragment in plasmid pJH94 derived from a partial *Sau*3A digest was previously sequenced (GenBank accession no. AJ278614) and three ORFs were identified: one encoded an exopolysaccharide depolymerase (Kim & Geider,

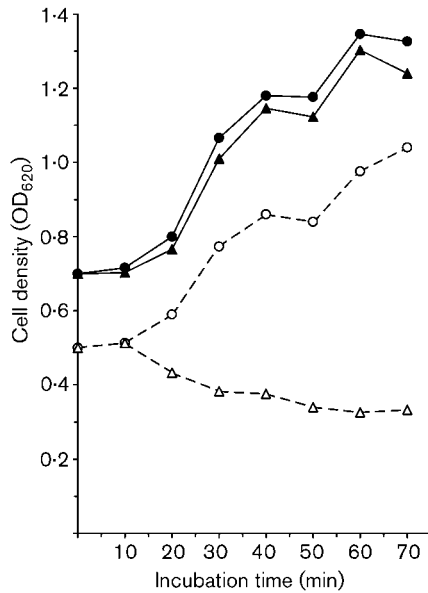


Fig. 3. Growth inhibition by induction of lysozyme expression in *E. coli*. Cultures of M15(pREP4) with pQE-30 (continuous line, solid symbols) or pQE-lyz1 (dashed line, open symbols) were grown at 37 °C to mid-exponential phase (OD_{620} , 0.5–0.7). Untreated cultures (●, ○) or cultures induced by addition of IPTG to a final concentration of 1 mM (▲, △) were monitored for growth. The experiments were repeated at least three times with similar results.

and the culture showed symptoms of cell lysis, which was in contrast to M15(pREP4, pQE-30), where no differences were found for the growth of non-induced and induced cultures. Supernatants of induced M15(pREP4, pQE-lyz1) cells and the cell pellet, resuspended in buffer A, were applied to the Ni column and analysed by Western blotting for lysozyme expression. No lysozyme was detected in the flowthrough, the wash or the elution fractions, including treatment with 0.1 M EDTA. However, a signal was detected by Western blot analysis after boiling the matrix in sample buffer. When aliquots of the column matrix were

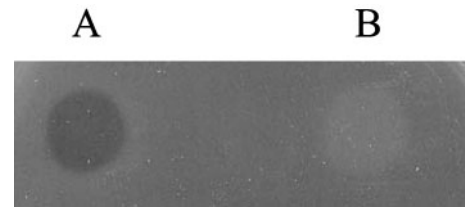


Fig. 4. Lysozyme activity on a lawn of Ea1/79Sm. Cell lysates (10 μ l with 120 μ g protein) in (A) are from the induced strain M15(pREP4, pQE-lyz1) after growth in LB with Ap and Km and those in (B) are from the induced strain M15(pREP4-Cm, pQE-lyz1) after growth in LB with Ap and Cm.

applied to Ea1/79 cells forming a lawn on agar, a growth inhibition zone was obtained due to the release of lysozyme (similar to the pattern in Fig. 4).

Requirements for cloning and enzyme expression

Host requirements for expression of the lysozyme gene were assayed by transfer of plasmids prepared from M15(pREP4, pQE-lyz1) into various *E. coli* strains. When selection was done with Ap only, all cells recovered contained the plasmids pREP4 and pQE-lyz1, indicating the need for a high level of *lac* repressor for cells with pQE-lyz1. Among several *E. coli* strains tested, only the commercial strains M15 and GI698 were successfully transformed with pQE-lyz1 and pREP4 for expression of lysozyme, which depended strictly on Km in the growth medium (Table 2). When M15(pREP4, pQE-lyz1) was grown in Ap without Km, the lysozyme was barely recovered in induced cell lysates, although plasmid pREP4 was not lost. When pREP4 was altered by replacing the Km-resistance gene with a Cm cassette, lysozyme expression was not detected after induction of a culture grown in Ap and Cm (Fig. 4B). Activity of M15(pREP4-Cm, pQE-lyz1) lysates was restored when the cells were transformed with another compatible plasmid carrying Km resistance, such as pfdC1 or pRK293 (Table 2).

Table 2. Bacterial strains and plasmids for expression of the lysozyme gene

Strain and plasmid	Relevant characteristics	Growth conditions and lysozyme activity
M15(pREP4, pQE-lyz1)	Ap ^R , pREP4 with Km ^R	LB with Ap and Km; active cell lysate
M15(pREP4, pQE-lyz1)	Ap ^R , pREP4 with Km ^R	LB with Ap, without Km; inactive cell lysate
M15(pREP4-Cm, pQE-lyz1)	Ap ^R , pREP4-Cm* with Cm ^R	LB with Ap and Cm, without Km; inactive cell lysate
GI698(pREP4, pQE-lyz1)	Ap ^R , pREP4 with Km ^R	LB with Ap and Km; active cell lysate
GI698(pREP4, pQE-lyz1)	Ap ^R , pREP4 with Km ^R	LB with Ap, without Km; inactive cell lysate
M15(pREP4-Cm, pQE-lyz1, pRK293)	Ap ^R , pREP4-Cm with Cm ^R pRK293 (low copy) with Km ^R	LB with Ap and Km; active cell lysate
M15(pREP4-Cm, pQE-lyz1, pfdC1)	Ap ^R , pREP4-Cm with Cm ^R pfdC1 (high copy) with Km ^R	LB with Ap and Km; active cell lysate

*Km^R of pREP4 replaced by Cm^R cassette.

Determination of lysozyme activity by cell growth

After induction with IPTG, cleared cell lysates were prepared from cultures of M15(pREP4, pQE-lyz1), and then tested for formation of inhibition zones on a lawn of Ea1/79 cells (Fig. 4A). The lysates showed haloes on the agar. A quantitative assay was developed by growth-inhibition experiments with cell cultures in the presence of lysozyme. Titration with a constant amount of protein of the cell lysate showed growth inhibition, which was dependent on the initial concentration of Ea1/79Sm. For 10^5 c.f.u. ml⁻¹, the optical density was approximately half that of the control after 48 h. An even greater growth inhibition was observed for 10^4 c.f.u. ml⁻¹ starting culture, and growth was completely inhibited when 10^3 c.f.u. ml⁻¹ or less was applied (Fig. 5). Control cell lysates did not affect growth of *Erw. amylovora*.

Increasing amounts of the cell lysate from induced *E. coli* M15(pREP4, pQE-lyz1) were added to 200 µl Ea1/79Sm culture, diluted to 10^5 c.f.u. ml⁻¹, and the effect on cell growth was measured by turbidity. Growth inhibition was visible for protein concentrations in the range of 5–30 µg, whereas the addition of control lysates had no effect on cell growth (Fig. 6). Addition of 30 µg protein from a

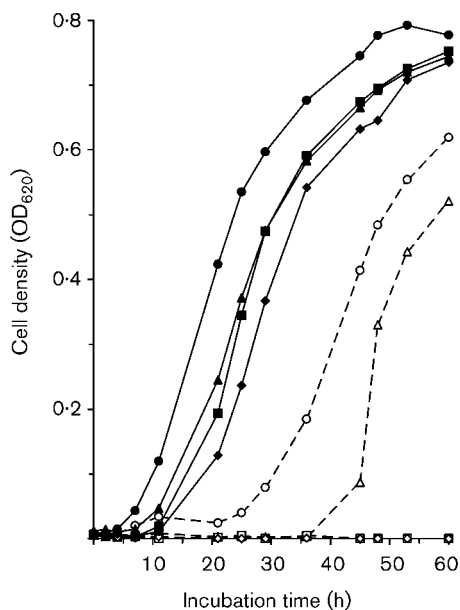


Fig. 5. Influence of lysozyme on the growth of *Erw. amylovora*. Aliquots with 25 µg protein of cleared *E. coli* cell lysates were added to 200 µl suspensions of *Erw. amylovora* strain Ea1/79Sm. Continuous line, solid symbols: supernatant from IPTG-induced *E. coli* M15(pREP4, pQE-30) was the control. Dashed line, open symbols: cell lysates of IPTG-induced M15(pREP4, pQE-lyz1). ●, ○, 10^5 c.f.u. ml⁻¹; ▲, △, 10^4 c.f.u. ml⁻¹; ■, □, 10^3 c.f.u. ml⁻¹; ◆, ◇, 10^2 c.f.u. ml⁻¹ of *Erw. amylovora* strain Ea1/79Sm. The experiments were repeated at least three times with similar results.

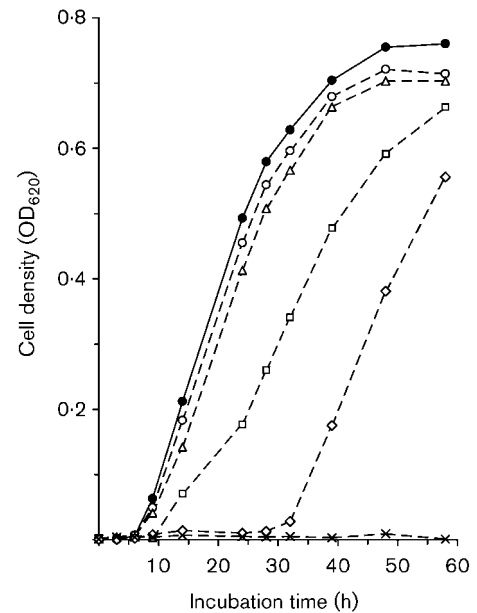


Fig. 6. Growth inhibition of *Erw. amylovora* cells at different lysozyme concentrations. A cell lysate from IPTG-induced M15(pREP4, pQE-lyz1) was diluted to protein concentrations of 5 (○), 11 (△), 16 (□), 22 (◇) and 27 µg (×) and added to 200 µl suspensions of *Erw. amylovora* strain Ea1/79Sm diluted in LB medium to 10^5 c.f.u. ml⁻¹. Cell lysate with 30 µg protein from IPTG-induced *E. coli* M15(pREP4, pQE-30) (●) was used as the control. The experiments were repeated at least three times with similar results.

M15(pREP4, pQE-lyz1) lysate completely abolished cell growth, and plating of culture aliquots after incubation for 72 h did not result in colony formation on agar (data not shown).

Incubation of Ea1/79Sm cells with extracts containing lysozyme resulted in a decrease of their viability (Table 3). After applying 36 µg protein for 4 h, a strong decrease in viability was achieved. A similar effect was observed for

Table 3. Effect of lysozyme on viability of *Erw. amylovora*

Various amounts of extract of induced M15(pREP4, pQE-lyz1) cells were added to 200 µl overnight cultures of 200 c.f.u. Ea1/79Sm. The control was an extract from M15(pREP4, pQE-30).

Soluble extract	Incubation time (h)			
	0	1	2	4
Control	+	+	+	+
18 µg	+	+	+	+
36 µg	+	+	+	-
72 µg	+	+	-	-

+, Bacteria recovered; -, no bacteria recovered.

72 µg protein and either 2 or 4 h incubation. We assume that the amount of lysozyme must exceed a threshold in order to inhibit the applied cells.

Virulence assays on pear slices treated with lysozyme-containing cell lysates

Slices from immature pears were soaked in cleared extracts of induced and sonicated cells from M15(pREP4, pQE-lyz1) or M15(pREP4, pQE-30). They were briefly dried, then inoculated with 10 µl diluted overnight cultures of Ea1/79 by applying tenfold dilutions from 5×10^4 to 5×10^1 c.f.u. per slice. For lysozyme-containing cell lysates, weak symptoms were found at the highest inoculation density, but none were found when lower amounts of *Erw. amylovora* cells were applied (results not shown). With cell lysates of M15(pREP4, pQE-30) as control, typical fire blight symptoms, such as ooze formation and necrosis, were observed. As for the cell-damaging effects of lysozyme in bacterial suspensions, the enzyme showed similar effects when applied to pear slices.

DISCUSSION

The expression of various lytic proteins has been attempted in transgenic plants for control of fire blight. The bacteriophage T4 lysozyme gene was introduced into potato to control soft rot caused by *Erwinia carotovora* (Düring *et al.*, 1993), and also into apple (Hanke *et al.*, 1998); a marginal reduction of symptom formation was described, but this might be surpassed by haemolytic proteins such as attacin (Reynoird *et al.*, 1999; Ko *et al.*, 2002) and cecropin (Mourgues *et al.*, 1998). The haemolytic protein genes are some of the defence mechanisms used by insects to attack microbial pathogens; their mode of action is disruption of membrane integrity (Engström *et al.*, 1984; Boman & Hultmark, 1987). The T4 lysozyme was combined with attacin to enhance fire blight resistance of transgenic apples but a synergistic effect was not observed (Ko *et al.*, 1999, 2002).

It has recently been suggested that attacin and cecropin might also affect mammals and might be harmful in transgenic food. Hen egg white contains a significant amount of lysozyme, which is a component of daily human nutrition. Viral lysozymes are produced in bacteria as tools for destruction of the host cell walls; they lack signal peptides and cannot be secreted into the periplasm. The pore-forming holins support lysozyme transport after simultaneous gene expression in the life cycle of bacteriophages. When the lysozyme is expressed in transgenic bacteria or supplied to cultured cells, the protein has been suggested to act via a membrane-disrupting activity (Düring *et al.*, 1999). Although the muramidase activity of lysozyme damage to the peptidoglycan layer is well documented, the protein, variants or even fragments, may also interact non-enzymically with the bacterial membrane (Düring *et al.*, 1999; Pellegrini *et al.*, 2000). This additional bactericidal activity could be an important feature of the

φEa1h lysozyme (H. Salm & K. Geider, unpublished). Bactericidal activities of proteins are quite common, and have also been suggested for lactoferrin (Zhang *et al.*, 1998); however, this effect might be different from the muramidase activity of lysozyme. In this study, we have cloned and partially characterized a lysozyme from *Erw. amylovora* phage φEa1h. Many elaborate lysozyme assays have been published (Saedi *et al.*, 1987; van de Guchte *et al.*, 1992; Pontarollo *et al.*, 1997; Wang & Chag, 1997). However, the outer membrane of Gram-negative bacteria prevents lysozyme from easily accessing the peptidoglycan layer. Therefore, in the above lysozyme assays, indicator cells were treated with EDTA or chloroform to facilitate invasion of lysozyme to the peptidoglycan layer, and the decrease in turbidity of the cell suspension was then determined. This application was not appropriate for *Erw. amylovora* because the EDTA-treated cells quickly lyse. Growth assays of broth cultures in microtitre plates and growth inhibition on a bacterial lawn were convenient tools to determine lysozyme activity in the present study.

Although the antibacterial activity was measured in lysates of induced M15(pREP4, pQE-lyz1) cells with several different assays, no signals were detected in Western blots with these protein fractions. Nevertheless, the matrix of the Ni column was positive in the immunological assay, and showed bactericidal activity on plates. Consequently, the amount of lysozyme in cell lysates was too low for protein detection in a Western blot, but sufficient for formation of inhibition zones on a bacterial cell lawn.

After induction of lysozyme expression, host cell growth was abolished and the bacteria were lysed. Since lysozyme seems to be toxic to host cells even at low levels, promoters for *lyz* expression must be tightly downregulated during cell growth. This was attempted with the *lac* repressor constitutively expressed from a plasmid. Even in this case, expression in *E. coli* was not always possible, and only two strains synthesized detectable amounts of lysozyme. This could be due to selection of the commercial strains for tolerance to foreign proteins expressed from cloned genes. Another requirement for *lyz* expression was host cell resistance to Km, and growth of the cells in the presence of the antibiotic. Km could act as an inhibitor of the enzyme by binding to φEa1h lysozyme. A similar effect was detected for hen egg white lysozyme activity and several aminoglycosidic antibiotics including Km A (Fernández-Sousa *et al.*, 1977). The inhibition effect was explained by the related structure of aminoglycosidic antibiotics and the saccharidic lysozyme substrate.

Large-scale production of cell extracts enriched for φEa1h lysozyme would allow attempts to protect orchards against fire blight by spraying. The gene could also be expressed in fire-blight host plants to release the protein after plant-cell damage by the pathogen. Adjacent to the *lyz* gene on the genome of bacteriophage φEa1h, an ORF encoding exopolysaccharide depolymerase was cloned (Vandenbergh *et al.*, 1985; Vandenbergh & Cole, 1986; Hartung *et al.*, 1988)

and further characterized (Kim & Geider, 2000). Expression of the exopolysaccharide depolymerase in plants was attempted in order to degrade the amylovoran capsules of *Erw. amylovora*, thus exposing the pathogen to plant defence reactions. First reports suggest a positive role of *dpo* expression in apples and pears, with reduced fire blight symptoms of selected cell lines (Hanke *et al.*, 2003; M. Malnoy, M. Faize, J. S. Venisse, K. Geider & E. Chevreau, unpublished). Coexpression of the depolymerase and the lysozyme of *Erw. amylovora* phage ϕ Ea1h in plant cells could result in synergistic effects of the two proteins in the control of colonization by the fire blight pathogen in plant tissue.

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