Cell. Mol. Life Sci. (2010) 67:1177–1188
DOI 10.1007/s00018-009-0241-x

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Lysozyme inhibitor conferring bacterial tolerance to invertebrate type lysozyme

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Received: 29 October 2009 / Revised: 30 November 2009 / Accepted: 18 December 2009 / Published online: 5 January 2010

Abstract Invertebrate (I-) type lysozymes, like all other known lysozymes, are dedicated to the hydrolysis of peptidoglycan, the major bacterial cell wall polymer, thereby contributing to the innate immune system and/or digestive system of invertebrate organisms. Bacteria on the other hand have developed several protective strategies against lysozymes, including the production of periplasmic and/or membrane-bound lysozyme inhibitors. The latter have until now only been described for chicken (C-) type lysozymes. We here report the discovery, purification, identification and characterization of the first bacterial specific I-type lysozyme inhibitor from *Aeromonas hydrophila*, which we designate PliI (*p*eriplasmic *l*ysozyme *i*nhibitor of the *I*-type lysozyme). PliI has homologs in several proteobacterial genera and contributes to I-type lysozyme tolerance in *A. hydrophila* in the presence of an outer membrane permeabilizer. These and previous findings on C-type lysozyme inhibitors suggest that bacterial lysozyme inhibitors may have an important function, for example, in bacteria-host interactions.

Keywords *Tapes japonica* lysozyme · Lysozyme inhibitor · *Aeromonas hydrophila* · Peptidoglycan hydrolysis · Invertebrates

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Introduction

Lysozymes (EC 3.2.1.17) are a widely distributed and diverse family of enzymes that specifically cleave the glycosidic bond between the *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues in the glycan moiety of peptidoglycan, an essential and unique cross-linked bacterial cell wall polymer that forms a sacculus around the entire bacterial cell and allows it to resist the internal turgor pressure. The major function of lysozymes in most organisms is to provide antibacterial protection, because hydrolysis of peptidoglycan will compromise the integrity of the cell wall sacculus and cause cell lysis. Within the animal kingdom the major lysozyme structural types that can be distinguished are the chicken (C) type, the goose (G) type and the invertebrate (I) type. C-type and G-type lysozymes, or at least the genes encoding them, occur in all vertebrates, while invertebrate Metazoa typically have I-type lysozymes. In addition, some invertebrates also have a C-type (Arthropoda) or a G-type (Mollusca) lysozyme. The existence of an invertebrate-specific lysozyme was first proposed in 1975 by Jollès and Jollès [1] based on their study of a lysozyme isolated from the starfish, *Asterias rubens*. Later studies have found similar lysozymes in a wide range of invertebrate organisms including molluscs, annelids, echinoderms, nematodes and arthropods, and confirmed the existence of a typical I-type lysozyme family [2]. The lysozyme from the marine bivalve *Tapes japonica* (TjL) was the first I-type lysozyme to have its three-dimensional structure resolved [3]. Based on topological similarities with C-type lysozymes and supported by site-specific mutagenesis, the Glu-18 and Asp-30 were identified as the catalytic residues in TjL, as earlier proposed by Bachali et al. [4] based on the conservation of these residues in most I-type lysozyme sequences. Besides their role in the

innate immunity of invertebrate organisms [2], the I-type lysozymes have been proposed to additionally fulfill a digestive function in marine bivalves, for which bacteria constitute an important feeding source [2, 5]. Furthermore, peptidoglycan fragments are increasingly recognized as mediators in various microbial and bacteria-host interactions [6, 7], and lysozymes emerge as potential modulators of such interactions by virtue of their enzymatic activity [8].

A highly specific and only recently discovered anti-lysozyme strategy of bacteria is the production of periplasmic or membrane-bound proteinaceous lysozyme inhibitors. Currently, two families of bacterial C-type lysozyme inhibitors have been described: the Ivy (*i*nhibitor of *v*ertebrate l*y*sozyme) family, periplasmic proteins which are found in *Escherichia coli* and a limited number of other—exclusively proteobacterial—genera [9], and the PliC/MliC family, comprising respectively periplasmic and membrane-bound proteins, which also exist mainly in the Proteobacteria, but with a much wider distribution [10]. Using knockout mutants, representatives of both inhibitor families were shown to contribute to bacterial lysozyme tolerance [10, 11]. Evidence for a role of these lysozyme inhibitors in bacteria-host interactions is accumulating. An indirect indication is that the production of Ivy and MliC in *E. coli* is under control of the Rcs regulon, which is required for virulence in several pathogenic *Enterobacteriaceae* members and is induced by cell wall stress provoked by antibiotics or lysozymes [12]. In addition, MliC is one of the genes that is induced during residence of *Salmonella* in macrophages, where lysozyme is produced to help kill and digest phagocytosed bacteria [13]. More direct evidence is the finding that Ivy allows *E. coli* to survive and even grow in human saliva, since knockout of *ivy* resulted in a rapid elimination of the bacteria. This effect of Ivy was not seen when the lysozyme was first selectively removed by passage of the saliva over an immobilized Ivy affinity matrix, thus demonstrating unequivocally that the effect of Ivy in this experiment was due to its neutralization of the salivary lysozyme [14]. Meanwhile the spatial structure of Ivy and MliC and their complex with hen egg white lysozyme have been elucidated by X-ray crystallography [15, 16] providing insight into the interaction of these inhibitors with C-type lysozymes.

Although G- and I-type lysozymes have been less studied than their C-type counterparts, their phylogenetic distribution, enzymatic and bactericidal activities suggest an equally important role in antibacterial defense and possibly in other functions. Following the hypothesis discussed above that the Ivy and MliC/PliC family of inhibitors have evolved in bacteria as an answer to C-type lysozymes, we anticipated that specific inhibitors against other lysozyme types, like I-type lysozyme, were also likely to exist in bacteria. In the current work, we report the discovery of the first family of periplasmic bacterial I-type lysozyme inhibitors (PliI), and we demonstrate that PliI from *Aeromonas hydrophila* contributes to lysozyme tolerance.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacteria included in the screening for I-type lysozyme inhibitors and their growth conditions are listed in Table 1. Overnight cultures of these bacteria were obtained by inoculating a single colony in either Luria-Bertani broth, nutrient broth or brain heart infusion according to Table 1. Cultures intended for the isolation of periplasmic proteins were obtained by a 1/100 dilution of an overnight culture in 40 ml fresh medium and further incubation for 21 h, whereupon periplasmic proteins were extracted as described further below.

Other bacterial strains and plasmids used in this study are listed in Table 2. Antibiotics (Sigma-Aldrich, Bornem, Belgium) were added to the growth medium whenever appropriate in the following final concentrations: tetracyclin (Tc) 5 μg/ml; ampicillin (Amp) 100 μg/ml, kanamycin (Km) 50 μg/ml and chloramphenicol (Cm) 30 μg/ml.

Recombinant production of *Tapes japonica* lysozyme (TjL)

TjL was recombinantly produced using *Pichia pastoris* YJT46 as described by Takeshita et al. [17] with some modifications as follows. A single colony was inoculated from a YPD (10 g/l yeast extract, 20 g/l bacteriological peptone, 20 g/l glucose) master plate in 4 ml YPD broth and grown overnight at 30°C with continuous shaking (200 rpm). Subsequently, this overnight culture was diluted 1/100 in BMGY-medium [1% yeast extract, 2% bacteriological peptone, 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5}% biotin, 100 mM potassium phosphate (pH 6.0) and 1.0% glycerol] and grown at 30°C in a baffled Erlenmeyer flask with continuous shaking (100 rpm) until the culture reached an OD_{600nm} of at least 2.0. Subsequently, the cells were pelleted by centrifugation (2,900×*g*, 10 min) and resuspended in BMMY (BMGY with 0.75% methanol instead of 1% glycerol) to an OD_{600nm} of 1.0. The culture was further incubated in a baffled Erlenmeyer flask at 20°C with continuous shaking (100 rpm). Methanol (0.75%, v/v) was added each 24 h. After 72 h the culture was centrifuged (2,900×*g*, 10 min), the supernatant filtered (0.45 μm pore size) and stored at 4°C until further use.

Table 1 Bacteria, growth conditions and I-type lysozyme inhibitory activity in periplasmic extracts

Strain	Growth medium	Growth temperature (°C)	Inhibition (%) of I-type lysozyme
Aeromonas hydrophila ATCC7966	NB	30	92
Serratia marcescens SS-1 LMM-11-72	LB	30	98
Enterobacter aerogenes ATCC13048	NB	30	0
Erwinia carotovora subsp. *carotovora* BCCM/LMG2457	NB	37	0
Pseudomonas aeruginosa PAO1-H103 LMM-05-55	NB	28	0
Escherichia coli MG1655	LB	37	0
Salmonella Enteritidis ATCC13076	LB	37	0
Proteus mirabilis LMM-20-10	NB	37	44
Klebsiella pneumoniae ATCC13883	NB	30	0
Cupriavidus metallidurans CH34 ATCC 34123	LB	37	0
Shigella flexneri BCCM/LMG10472	NB	37	0
Vibrio harveyi ATCC BAA-1116	LB + 1,5% NaCl	37	0
Yersinia enterocolitica ATCC9610	BHI	30	0
Citrobacter freundii ATCC8090	NB	37	29

LB Luria-Bertani (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl); *NB* nutrient broth (Oxoid, Basingstoke, UK); *BHI* brain heart infusion (Oxoid, Basingstoke, UK); *ATCC* American type culture collection; *BCCM*^{TM}*/LMG* Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology, University Ghent; *LMM* Collection Labo Levensmiddelenmicrobiologie, Katholieke Universiteit Leuven

Table 2 Other strains and plasmids used

Strain or plasmid	Description	References or source
Yersinia pseudotuberculosis IP32953	Wild type	[21]
Aeromonas hydrophila ATCC7966 *ΔpliI::aph*	*pliI* gene replaced by *aph* gene cassette from pKD4; Km^R	This study
Escherichia coli S17-1 *λ*pir	*pro thi hsdR*$^-$ *hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn7 *λ*pir	[44]
Escherichia coli BL21 (DE3)	Expression host for pET series vectors, containing IPTG-inducible T7 RNA polymerase gene	Novagen, Merck Biosciences, Darmstadt, Germany
Pichia pastoris YJT46	Contains *tjl* gene of *Tapes japonica* under P_{AOX} control, methanol inducible	[17]
pFAJ1702	Broad host range plasmid Tc^R, Amp^R	[45]
pFAJ1702-*pliI*	*pliI* from *A. hydrophila* ATCC7966 with its own promotor cloned in pFAJ1702; Tc^R, Amp^R	This study
pET26b(+)	Expression vector	Novagen, Merck Biosciences, Darmstadt, Germany
pET26b(+) (P_{T7}-*pliI*)	*pliI* from *A. hydrophila* under control of P_{T7} promotor in pET26b(+); Km^R	This study
pDS132	Suicide plasmid for *A. hydrophila*, mobilizable from *E. coli* S17-1; containing *sacB* for counterselection with sucrose; Cm^R	[46]
pDS132 (Δ *pliI::aph*)	pDS132 containing *aph* cassette flanked by regions upstream and downstream from *pliI*; Km^R, Cm^R	This study
pKD4	Template vector containing *aph* gene flanked by FRT sites, Km^R, Amp^R	[47]

Lysozyme (inhibition) activity assay

Lysozyme activity of TjL was measured using a turbidity assay described by Callewaert et al. [18]. One lysozyme unit was defined as the amount of enzyme that caused a decrease in OD_{600nm} of 0.001/min in 1 ml of a *Micrococcus luteus* (Sigma–Aldrich, Bornem, Belgium) cell suspension [0.8 mg/ml in 10 mM potassium phosphate buffer (PPB), pH 7.0] at 30°C using a Bioscreen C Microbiology Reader (Labsystems Oy, Helsinki, Finland). Lysozyme inhibitory activity of periplasmic extract, isolated by cold osmotic shock as described by Callewaert et al. [18], or purified inhibitor was measured using the same assay and was expressed as an inhibition percentage (I %) calculated as

follows: $I\,(\%) = ((L_0 - \mathrm{L}) - (R_0 - R))*100/((L_0 - L) - (B_0 - B))$ with $L_0 - L$, $R_0 - R$ and $B_0 - B$, representing the OD_{600nm} decrease over a period of 2 h respectively in the presence of lysozyme but with buffer instead of the inhibitor sample, in the presence of lysozyme and the inhibitor sample, and in the presence of the inhibitor sample, but with buffer instead of lysozyme. One inhibitory unit (IU) is defined as the amount of inhibitor causing a 50% decrease of lysozyme activity in the above assay.

Purification of *Tapes japonica* lysozyme and construction of a TjL affinity column

Pichia pastoris YJT46 culture supernatant was concentrated approximately ten-fold by centrifugation (2,000×*g*; 2 h; 20°C) using a Vivacell 100 ultrafiltration unit with a molecular weight cutoff of 5 kDa (Sartorius Stedim Biotech S.A., Aubagne Cedex, France) and subsequently diafiltrated by diluting the concentrate in the same ultrafiltration unit to the initial volume with 50 mM potassium phosphate buffer (PPB), pH 7.0, and recentrifugating for another 2 h. Concentrated and diafiltrated culture supernatant from several runs was combined and loaded onto a CM-Sepharose column linked to an ÄKTA-FPLC system (GE Healthcare Life Sciences, Uppsala, Sweden). After washing with 50 mM PPB, pH 7.0, a linear gradient of 1 M KCl in the same buffer was applied, and fractions showing lysozyme activity were pooled and concentrated by centrifugation (2,000×*g*; 2 h, 4°C) using a Vivacell 100 ultrafiltration unit that was passivated by overnight treatment with Tween20 (5%). TjL intended for immobilization on NHS-activated Sepharose was subsequently diafiltrated with 100 mM MOPS buffer, pH 7.0, lyophilized and stored at 4°C until further use. TjL intended for inhibitory activity assays was diafiltrated with 10 mM PPB, pH 7.0, dialyzed once more against the same buffer, sterilized using a 0.22-µm syringe filter and finally stored at 4°C.

A TjL-affinity column was constructed by immobilizing purified TjL (37 mg) on NHS-activated Sepharose™ 4 Fast Flow matrix (15 ml) (GE Healthcare Life Sciences, Uppsala, Sweden), according to the manufacturer's guidelines. Finally, the TjL matrix was packed in a XK 16/20 column (GE Healthcare), resulting in a TJL-affinity column with a volume of 12 ml.

Purification and identification of I-type lysozyme inhibitor of *A. hydrophila*

Fifty milliliters of cell and particle free periplasmic extract of *A. hydrophila* ATCC 7966 was loaded onto the TjL-affinity column connected to an ÄKTA-FPLC system. After a wash step with 0.1 M Tris-HCl pH 7.0 buffer, bound proteins were eluted using a linear gradient of 0.1 M Tris, pH 12.0, in the washing buffer and collected in 3-ml fractions to which 300 µl of neutralization buffer (1.0 M Tris-HCl, pH 7.0) was immediately added. One-milliliter portions of these fractions were removed and, after addition of bovine serum albumin (BSA) to a final concentration of 0.5 mg/ml, dialyzed against 10 mM PPB, pH 7.0, for activity testing, while the remaining 2 ml (without BSA added) was dialyzed against water, lyophilized and analyzed for purity by SDS-PAGE (4% concentrating and 15% separating gel). A protein band was sampled from the Coomassie-stained gel and trypsin-digested according to the method of Shevchenko et al. [19]. Tryptic digests were analyzed by LC–ESI MS/MS on a LCQ Classic (Thermo Electron, San Jose, CA) ion trap MS equipped with a nano-LC column switching system as described by Dumont et al. [20]. MS/MS data were searched against the bacteria division of the GenBank non-redundant protein database (2613988 sequences) using MASCOT version 2.2 (Matrix Sciences, London, UK). The MASCOT mass tolerances for parent and fragment ions were 3 and 1 Da, respectively. Carbamidomethylation of Cys and oxidation of Met, Trp and His were set as fixed and variable modifications, respectively. Maximally one missed cleavage was allowed, and the neutral loss of water and ammonia from b- and y-ions was taken into consideration. Scaffold (version Scaffold_2_06_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications by Mascot.

Cloning, recombinant expression and purification of I-type lysozyme inhibitor (PliI)

The *A. hydrophila* ATCC7966 gene encoding the PliI protein (AHA_1205; NCBI RefSeq: YP_855746) was amplified without its signal peptide and stop codon using Phusion DNA polymerase (Finnzymes, Espoo, Finland) with the primers NcoI_AHA_1205-sign_pET_FW (5′-AAAACCATGGCGGACGGGTTCTTCAAACA-3′) and XhoI_AHA1205-stop_pET_RV (5′-AAAACTCGAGGTCGCGCGGGGTCTTGAG-3′). After digestion with *Nco*I and *Xho*I (underlined recognition sites in primers), the resulting fragment was ligated into pET26b(+) (Novagen, Merck Biosciences, Darmstadt, Germany), providing an N-terminal signal peptide for periplasmic localization and a C-terminal His_6-tag, and transformed to *E. coli* BL21(DE3). PliI-His_6 was expressed by growing this strain in LB at 37°C to mid-exponential phase ($OD_{600nm} = 0.3$), addition of 1 mM IPTG (Acros Organics, Geel, Belgium) and further overnight growth at 30°C. PliI-His_6 was subsequently purified from a periplasmic extract of this culture using Ni-Sepharose™ affinity chromatography with a HisTrap™ HP column (GE Healthcare) according to the manufacturer's guidelines and using an ÄKTA-FPLC

system. In a similar way the *pliI* homolog of *Yersinia pseudotuberculosis* IP32953 [21] (YPTB3534; NCBI RefSeq: YP_072017) was cloned, expressed and purified. Protein concentrations were determined using a BCA protein assay kit (Novabiochem, Merck Biosciences, Darmstadt, Germany).

Surface plasmon resonance analysis

Inhibition specificity and affinity constants for binding between lysozymes and inhibitors were assessed by surface plasmon resonance (SPR) analysis using a Biacore 3000 analytical system (Biacore AB, Uppsala, Sweden). Hen egg white lysozyme (HEWL, Fluka Biochemica, Bornem, Belgium), Salmon goose type lysozyme (SalG) [22] and TjL were used as representatives of the C-, G- and I-type lysozymes, respectively. Random amine coupling of lysozymes was carried out by injecting the proteins (5 µg/ml) in 10 mM sodium acetate, pH 4.5, following preactivation of the carboxymethylated dextran matrix (CM5 sensor chip, GE Healthcare Life Sciences, Uppsala, Sweden) using *N*-hydroxysuccinimide (NHS)/1-ethyl-3-[3-(dimethylaminopropyl)] carbodiimide hydrochloride (EDC). After coupling of the proteins, the residual NHS esters were deactivated by the injection of ethanolamine (1.0 M, pH 8.5). Each lysozyme was covalently coupled to approximately 1,200 resonance units (RU) with 1 RU corresponding to 1 pg of bound protein/mm^2. Lysozyme inhibitors were diluted in HBS-EP buffer (Biacore) to concentrations between 5 and 750 nM and injected at a flow rate of 30 µl/min. Association and dissociation data were both collected for 6 min. After each cycle, the chip was regenerated with 10 µl of 0.1 M NaOH (PliI) or with 10 µl of 0.5 M NaOH (PliC, Ivy). Analysis of the association and dissociation phases was performed using Biaevaluation version 3.1 software (Langmuir binding, local fit) (Biacore). Data obtained from a parallel flow cell to which a nonrelated monoclonal antibody against human plasminogen activator inhibitor 1 (MA-31C9) was coupled served as blank sensorgrams for substraction of changes in the bulk refractive index.

Analysis of contribution of PliI to I-type lysozyme tolerance in *A. hydrophila*

A strain deficient in PliI production was made by replacing *pliI* with an *aph* gene providing kanamycin resistance. First, a *ΔpliI::aph* gene replacement cassette was constructed by ligation of the 687-bp upstream and 657-bp downstream genomic sequence flanking the *pliI* gene to the *aph* gene from pKD4 using multiple PCR amplification rounds as described by Erova et al. [23]. The cassette was then ligated into the *λpir*-dependent suicide vector pDS132, and the resulting construct was conjugated from *E. coli* S17-1 *λpir* to *A. hydrophila* ATCC7966. Exconjugants growing on LBNS (LB without NaCl) plates with Amp, Km and 10% sucrose were selected, and gene replacement in these strains was confirmed by PCR.

For genetic complementation, *pliI* with its own promotor was amplified using primers AHA_1205_Fw (5′-AGGCTCTAGATAACAAGGGTCAAACCGATG-3′) and AHA_1205_Rv (5′-CGGGGCGTGGTGATGGGGATG-3′) and ligated into the broad host range vector pFAJ1702, resulting in pFAJ170-*pliI*, which was subsequently conjugated into *A. hydrophila ΔpliI::aph*.

To analyze the role of PliI in *A. hydrophila* lysozyme tolerance, overnight stationary cultures of *A. hydrophila* wild-type (pFAJ1702), *A. hydrophila ΔpliI::aph* (pFAJ1702) and *A. hydrophila ΔpliI::aph* (pFAJ1702-*pliI*) were diluted (1/1,000) in fresh NB with Tc and grown to late exponential phase (approximately 3 h). The cells were harvested by centrifugation (3,000×*g*; 5 min; room temperature), washed three times with 10 mM Tris-HCl, pH 7.0, and finally resuspended in 1/3 of the original volume of the same buffer. Next, 100 µl of each suspension (in 5-fold) was mixed with 100 µl lactoferrin (3 mg/ml) and 100 µl TjL (300 µg/ml) or Tris-HCl, pH 7.0, for controls, and incubated at 30°C with continuous shaking (200 rpm). Colony counts were determined at the start and after 24 h by plating on NB agar with Tc.

Results

Screening for possible I-type lysozyme inhibitors

In a lysozyme inhibition assay, performed as described in "Materials and methods," the known bacterial C-type lysozyme inhibitors Ivy and PliC were found unable to inhibit the I-type lysozyme of *T. japonica*. Based on the hypothesis that the Ivy and MliC/PliC families of inhibitors have evolved in bacteria as an answer to C-type lysozymes in animal hosts, we anticipated that specific inhibitors against other lysozyme types, like I-type lysozyme, were also likely to exist in bacteria. Therefore, a screening for I-type lysozyme inhibitors was conducted in periplasmic extracts of 14 strains of bacteria belonging to different genera of the Proteobacteria in which C-type inhibitors of the Ivy and MliC/PliC type are widely distributed (Table 1). Crude *Pichia pastoris* YJT46 culture supernatant was used as a source of TjL (15.3 U/ml) in this screening. Only the extracts from *A. hydrophila* and *S. marcescens* showed a high (>90%) and reproducible level of inhibition, whereas moderate activity was observed in extracts of *Proteus mirabilis* (44%) and no significant inhibitory activity (<30%) in all other extracts (see

Table 1). The clear inhibitory activity in *A. hydrophila* (see Fig. 1) and *S. marcescens* indicated the potential presence of a novel I-type lysozyme inhibitor. In view of the availability of the complete genome sequence of *A. hydrophila* ATCC7966 [24], this strain was chosen for isolation and identification of the putative inhibitor.

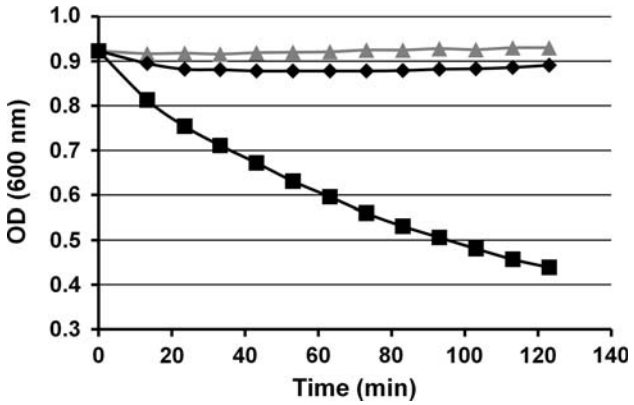

Fig. 1 Detection of I-type lysozyme inhibitory activity in the periplasmic extract of *Aeromonas hydrophila.* TjL activity measured as the time-dependent decrease in OD_{600nm} of a *Micrococcus lysodeikticus* cell suspension in absence (*squares*) or presence (*diamonds*) of periplasmic extract of *Aeromonas hydrophila*. Control samples (*triangles*) consisted of *Micrococcus* cell suspension with periplasmic extract and phosphate buffer (10 mM, pH 7.0) instead of TjL

Purification and identification of a potential I-type lysozyme inhibitor from *Aeromonas hydrophila*

Thirty-seven milligrams of purified TjL was obtained from in total 3 l of *Pichia pastoris* YJT46 culture supernatant, with a purification factor of 92 and a total recovery of 71%. An affinity matrix was made by immobilizing the purified TjL (612 ± 170 U/ml) on NHS-activated Sepharose 4 Fast Flow resin. During loading of periplasmic extract (50 ml) of *A. hydrophila* ATCC 7966 and washing of the TjL column, no inhibitory activity could be observed in the flow-through fractions, indicating that the inhibitor was effectively bound on the column. Subsequent elution of the column with an alkaline pH gradient yielded a single UV (280 nm) absorption peak in the eluate, which completely matched the presence of inhibitory activity in the collected fractions (see Fig. 2). Subsequent SDS-PAGE analysis of these active fractions revealed a single protein band after Coomassie staining, with an estimated molecular mass between 14 and 18 kDa (see inset Fig. 2). A sample from this band was trypsin digested and subjected to LC-ESI tandem mass spectrometry analysis. Subsequent analysis of the obtained MS/MS data by Mascot search resulted in the identification of the peptides GEPASTGSYDVR and LYSGANPQFP LDQFIDGK with a probability of 95% according to Scaffold. These peptides were assigned to be part (99.8% probability, 21% coverage) of a 15-kDa *A. hydrophila* ATCC7966 hypothetical protein of unknown function (locus tag AHA_1205; NCBI RefSeq: YP_855746) (see

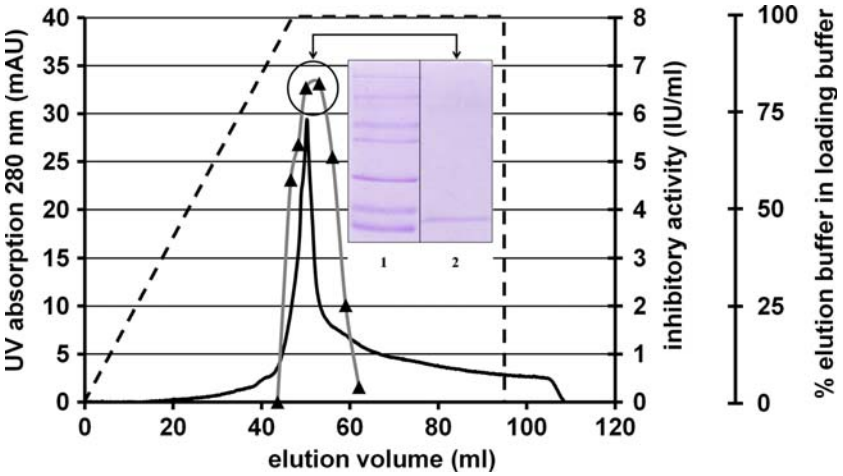

Fig. 2 TjL-affinity chromatography-based purification of the potential inhibitor of I-type lysozyme from the periplasmic extract of *Aeromonas hydrophila.* Elution profile of the purification of the I-type lysozyme inhibitor of *Aeromonas hydrophila* from TjL-affinity column recorded using an ÄKTA-FPLC system. Elution was obtained by building up a linear gradient of 0.1 M Tris, pH 12 (elution buffer), in 0.1 M Tris-HCl, pH 7.0 (binding buffer), followed by prolonged elution with elution buffer exclusively (*broken lines*). During elution, protein concentration in the elution fraction was recorded as the UV absorption at 280 nm (*straight line*) and expressed on the left *Y*-axis as milli-absorption units (mAU). Inhibitory activity in the elution fractions (*filled triangle*) was measured by an inhibitory assay and expressed as IU/ml. The inset picture shows a Coomassie-stained SDS-PAGE gel with protein molecular weight markers (molecular weights (kDa) from top to bottom: 116, 66.2, 45, 35, 25, 18.4 and 14.4 in *lane 1* and a pool of the two peak fractions (*circle*) in *lane 2*

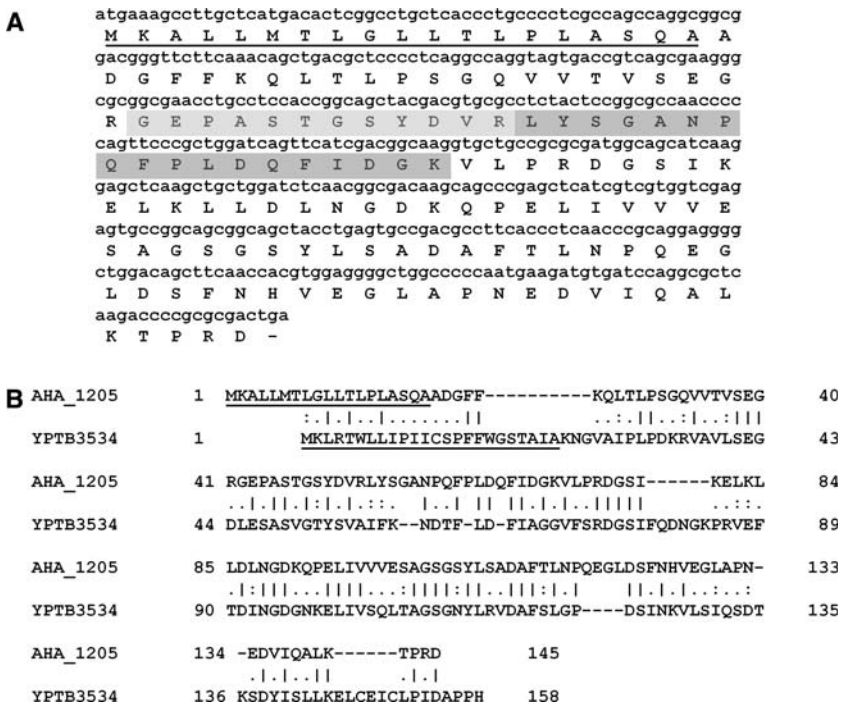

Fig. 3 Nucleotide and corresponding amino acid sequence of AHA_1205 and pairwise alignment with its homologue from *Yersinia pseudotuberculosis*. **a** Nucleotide and corresponding amino acid sequence of AHA_1205. The signal peptide (predicted by Signal P 3.0) is *underlined*, while the identified peptides by tandem mass spectrometry are highlighted by *two grey boxes*. **b** Pairwise amino acid sequence alignment of AHA_1205 and its homologue from *Y. pseudotuberculosis* (YPTB3534). *Identical residues* in the alignment are marked with "|", while conserved and semi-conserved substitutions with ":" and ".", respectively. The signal peptide is *underlined*

Fig. 3). This identification was further supported by: the presence of a 19-residue signal peptide (predicted by Signal P 3.0; http://www.cbs.dtu.dk/services/SignalP/) [25], which is in accordance with the periplasmic localization from which the unknown protein was originally extracted (see Fig. 3), the accordance of the molecular weight of the identified protein with the observed molecular weight on SDS-PAGE (see inset Fig. 2) and the identification within the right bacterial taxon. The identified protein, provided with a C-terminal His_6 tag, was recombinantly expressed in *E. coli* BL21(DE3) and purified by Ni-Sepharose™ Affinity Chromatography, yielding a pure AHA_1205-His solution (0.63 mg/ml) that showed strong inhibitory activity against TjL (4×10^3 IU/ml). This result confirmed that AHA_1205 is indeed an I-type lysozyme inhibitor, and the protein was further designated as PliI (*p*eriplasmic *l*ysozyme *i*nhibitor of the *I*-type lysozyme).

Like the other known (C-type) lysozyme inhibitors, PliI is also a small anionic protein (*p*I: 4.51; according to ProtParam http://www.expasy.org/tools/protparam.html), targeted to the periplasm (or to the outer membrane but facing the periplasm in the case of MliC). It shows low overall sequence similarity to Ivy from *Escherichia coli* MG1655 (23.5%), PliC from *Salmonella* Typhimurium LT2 (4.5%) and MliC from *Pseudomonas aeruginosa* PAO1 (21.5%) (alignments performed with mature sequences without signal peptide using EMBOSS pairwise alignment algorithms using default parameters [26]).

Distribution of PliI relatives

A tBlastn [27] search against the translated non-redundant NCBI nucleotide database indicated that homologs of *pliI* are not widespread, but exist in at least ten proteobacterial genera. In most cases, homologs are found in all the species and strains of these genera for which a genome sequence is available. A notable exception is the genus *Yersinia*, where *pliI* homologs exist only in the species *Y. pseudotuberculosis* and *Y. pestis*, but not in *Y. enterocolitica*. Heterologous expression and purification of the PliI homolog of *Y. pseudotuberculosis* IP32953 (YPTB3534; NCBI RefSeq: YP_072017) (46.9% identity according to a global pairwise alignment using EMBOSS align; see Fig. 3) in a similar way to PliI from *A. hydrophila* proved it to be an active I-type lysozyme inhibitor (5.1×10^2 IU/ml) as well.

Specificity and affinity of PliI for I-type lysozyme

The ability of purified PliI-His_6 (previously referred to as AHA_1205-His) to inhibit I-type, C-type and G-type lysozymes was investigated using the earlier described *M. lysodeikticus* lysozyme assay. TjL, HEWL and SGTL were used at concentrations of 70, 80 and 120 U/ml respectively, because these caused a similar decrease in OD_{600nm} of 0.305 ± 0.058 OD units within 2 h, and PliI was added at different molar ratios. The results presented in Fig. 4 clearly show that TjL is fully inhibited at equimolar concentrations of PliI, while HEWL and SalG are not even

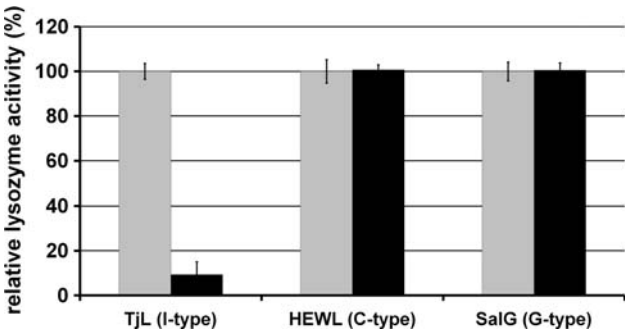

Fig. 4 Measuring PliI inhibitory activity against different lysozyme types. Relative lysozyme activity (%) of I-type lysozyme (TjL), C-type lysozyme (HEWL) and G-type lysozyme (SalG) in the absence (*grey bars*) or presence (*black bars*) of PliI. For TjL, equimolar amounts of lysozyme and inhibitor were used, whereas for HEWL and SalG a 5-fold molar excess of PliI was used

partly inhibited at five-fold molar excess of PliI, indicating that PliI is a specific I-type lysozyme inhibitor. A more quantitative study of the interaction was performed by SPR analysis. The three lysozymes, together with an unrelated monoclonal antibody as a negative control, were immobilized on the four channels of a sensor chip (CM5), and the interaction with each of the three inhibitors (PliI, PliC and Ivy), applied in different concentrations, was analyzed. PliI was confirmed to have a high specificity for the I-type lysozyme, characterized by a very high affinity constant (K_A 2.15×10^{10} ($\pm 6.14 \times 10^9$) (1/M)), indicating a very strong interaction as result of a high association rate constant (k_a 6.64×10^5 (1/Ms)) and a low dissociation rate constant (k_d 3.29×10^{-5} (1/s)). Moreover, neither Ivy nor PliC were able to bind TjL, confirming earlier observations.

Contribution of PliI to I-type lysozyme tolerance in *A. hydrophila*

To investigate the in vivo contribution of PliI to bacterial I-type lysozyme tolerance, a *pliI* deletion knock-out strain (*A. hydrophila* ATCC 7966 *ΔpliI::aph*) and a complemented knock-out strain (*A. hydrophila* ATCC7966 *ΔpliI::aph* pFAJ1702-*pliI*) were constructed. In order to rule out any potential influence of the presence of the pFAJ1702 replicon in the complemented knock-out strain, also the wild-type and knock-out strains were equipped with an empty pFAJ1702. Verification of the inhibitory activity of PliI in their crude periplasmic extract, in comparison to the *A. hydrophila* wild-type strain, confirmed the expected absence (*ΔpliI::aph*) and presence (complemented *ΔpliI::aph*) of inhibitory activity, as shown in Table 3. For the in vivo sensitivity test, late exponential phase *A. hydrophila* wild-type, *ΔpliI::aph* and complemented *ΔpliI::aph* cell suspensions were treated with 1 mg/ml lactoferrin and a combination of 1 mg/ml lactoferrin and 100 μg/ml TjL. As can be seen from Table 3, all strains survived the treatments well, showing even some weak growth after 24 h, except for the uncomplemented *ΔpliI::aph* strain, which was 44-fold ($p < 0.001$) inactivated by the lactoferrin-lysozyme combination. From these results it can be concluded that the periplasmic lysozyme inhibitor PliI contributes to lysozyme tolerance in *A. hydrophila* in the presence of outer membrane permeabilizing compounds.

Discussion

In this work we report for the first time the discovery of a specific inhibitor against an invertebrate (I)-type lysozyme. This inhibitor, produced by the gram-negative bacterium *Aeromonas hydrophila* and named PliI (*p*eriplasmic *l*ysozyme *i*nhibitor of *I*-type lysozyme), has a high affinity for I-type lysozyme, but does not bind or inhibit vertebrate C- or G-type lysozymes. Using an *A. hydrophila pliI* deletion strain and genetically complemented deletion strain, we demonstrated that PliI improves the survival of the bacteria when they are challenged with I-type lysozyme in the presence of the outer membrane permeabilizing compound lactoferrin. This combined treatment is representative for the environment that bacteria encounter when infecting an animal host, since both vertebrate and invertebrate animals secrete complex mixtures of antimicrobial peptides and proteins [28], several of which have outer membrane permeabilizing properties [29].

Table 3 Inactivation of *A. hydrophila* strains by *Tapes japonica* lysozyme

Strain	Inhibitory activity (IU/ml) in periplasmic extract	Viability reduction factor (N0/N24)[a]	
		1 mg/ml lactoferrin	1 mg/ml lactoferrin and 100 μg/ml TjL
A. hydrophila wild type	5.67 ± 1.14	0.634 ± 0. 423	0.342 ± 0.167
A. hydrophila ΔpliI::aph	0	0.323 ± 0.149	44.3 ± 7.85
A. hydrophila ΔpliI ::aph pFAJ1702-*pliI*	4.04 ± 0.145	0.171 ± 0.0473	1.14 ± 0.366

[a] Inactivation was expressed by the viability reduction factor, with N_0 the initial colony count and N_{24} the colony count after 24 h of incubation at 30°C. Mean values were calculated from five replicate treatments

Relatives of PliI are not widespread in the bacterial world, but exist in about a dozen proteobacterial genera, including *Aeromonas*, *Herminiimonas*, *Janthinobacterium*, *Laribacter*, *Burkholderia*, *Yersinia*, *Shewanella*, *Bordetella*, *Ralstonia*, *Cupriavidus* and *Sulfurovum.* Also the previously described C-type lysozyme inhibitor families Ivy and PliC/MliC are found almost exclusively in the Proteobacteria. Since our screening revealed clear inhibitory activity in the periplasmic extract of *Serratia marcescens* SS1, it was unexpected that no PliI homologue is present in the genome sequence of *Serratia marcescens* Db11 (http://www.sanger.ac.uk/Projects/S_marcescens/). This indicates that either a *pliI* gene is present in strain SS1 but not in strain Db11, or that strain SS1 produces a different I-type inhibitor, which has yet to be identified.

Interestingly, for several of the bacteria containing a PliI homologue a relationship to invertebrate organisms has been documented. *Aeromonas* spp. are ubiquitous in aquatic environments (fresh, brackish and marine water) and have been isolated from various aquatic invertebrates such as mussels and oysters [30–33]. Some *Aeromonas* species closely associate with these animals, and this is sometimes reflected in their names, as for *Aeromonas bivalvium* sp. nov. [34] and *Aeromonas molluscorum* sp. nov. [35]. Another *Aeromonas* species, *A. veronii* biovar sobria, together with an uncultivable bacterium related to *Rikenella*, lives in an exclusive symbiotic relationship in the digestive tract of the medical leech (*Hirudo medicinalis*) [36]. In this niche, the bacteria are exposed to the enzyme destabilase, which is named after its blood clot solubilizing activity, but which is in fact an I-type lysozyme based on its enzymatic activity and amino acid sequence [37]. After the identification of PliI in *A. hydrophila*, we also analyzed periplasmic extracts from *A. bivalvium* 66, *A. molluscorum* 849 and *A. veronii* biovar Sobria 391 Hm 21, and found I-type lysozyme inhibitory activity in each of them (data not shown). Also some *Burkholderia* species have been identified as endosymbionts, living in the midgut crypts of the Broad-Headed Bugs *Riptortus clavatus* and *Leptocorisa chinensis* [38, 39]. One of the best-known and particularly relevant specific interactions of bacteria with an invertebrate host is that of *Yersinia pestis* with the flea. *Y. pestis* is transmitted from rats or other rodents to humans by blood-sucking fleas, and produces several proteins that help it survive in the flea digestive tract, such as the hemin storage system (Hms) and the *Yersinia* murine toxin (Ymt) [40]. Since insects have an I-type lysozyme gene homolog, *Y. pestis* PliI could represent an additional adaptation for survival in the flea digestive tract. This *Y. pestis* PliI is identical to the *Y. pseudotuberculosis* IP32953 homologue for which we demonstrated inhibitory activity in this work. Finally, *Sulfurovum* sp. have been found to colonize the gill surfaces of the deep-sea hydrothermal vent shrimp *Alvinocaris longirostris* [41]. Of course, more direct experimental evidence will be necessary to find out whether PliI really plays a role in these interactions. In the case of the C-type inhibitors Ivy and PliC/MliC, there is already some evidence for a contribution to the colonization of vertebrate hosts [10, 14]. Besides PliI, *A. hydrophila* also encodes a specific G-type lysozyme inhibitor (unpublished observations) as well as the known C-type lysozyme inhibitors Ivy and MliC. This, in combination with the fact that *Aeromonas* spp. cause infections in both invertebrate and vertebrate hosts [24], makes bacteria of this genus attractive models for studying the influence of bacterial lysozyme inhibitors in the interaction with both vertebrate and invertebrate hosts. From an applications point of view, and specifically with regard to pathogenic bacteria, lysozyme inhibitors may represent an interesting novel target for antibacterial drug development.

Recently, Yum and coworkers [16] revealed the structural basis for the recognition of HEWL by MliC from *P. aeruginosa* and found it to be based on the insertion of two conserved regions of MliC into the active site of HEWL (see Fig. 5, part a). Within these conserved regions, Ser89 and Lys103 were respectively proposed to form a hydrogen bond with Asp52 and an ionic bond with Asp52 and Glu35, the active site residues of HEWL. Additionally, the MliC conserved residues Tyr 92 and Thr102 are buried in a shallow pocket of MliC found to accommodate a loop of HEWL and forming hydrogen bonds with Thr47 of HEWL. As a result, MliC forms a double key-lock type of interaction with HEWL. We used GLAM2 (Gapped Local Alignment of Motifs [42]) to look for the presence of the two conserved MliC regions in PliI and the other currently known lysozyme inhibitors PliC and Ivy, and found that both regions are present in PliC, only region 1 is present in PliI, and neither can be found in Ivy (Fig. 5 part a, b). The latter inhibitor is known to have a different mechanism of interaction with C-type lysozymes [15]. This distribution of motifs involved in the interaction with lysozymes suggests that PliC may interact with C-type lysozymes (e.g., HEWL) in a similar way as MliC. PliI, on the other hand, may depend only on region 1 to bind with I-type lysozymes. The key residues identified within this region in MliC (Ser89 and Tyr92) are strongly conserved among PliI homologues (Fig. 5a). Whether and how these residues are involved in the interaction of PliI with I-type lysozymes and whether other regions or residues in PliI are involved in lysozyme binding and confer I-type lysozyme specificity will be investigated by site-specific mutagenesis and by the determination of the spatial structure of PliI and its lysozyme complex by X-ray crystallography. The finding that MliC, PliC and PliI share a common conserved motif may indicate a common ancestral origin for these inhibitors,

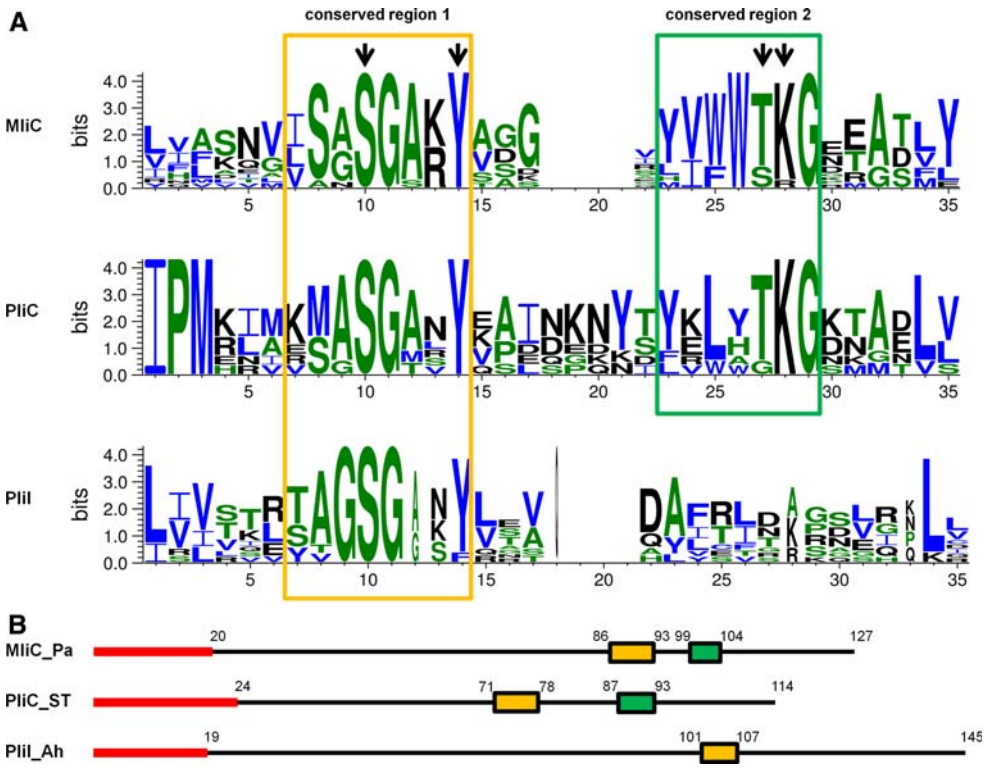

Fig. 5 Representation of the common conserved regions in MliC, PliC and PliI. **a** Sequence logos built with weblogo 3.0 [48], showing conserved motifs detected using GLAM2 [42], in a set of 13 MliC, 10 PliI and 7 PliC homologues. Homologues were obtained by tblastn search in the NCBI database, starting with MliC from *Pseudomonas aeruginosa* PAO1, PliC from *Salmonella* Typhimurium LT2 and PliI from *Aeromonas hydrophila* ATCC7966, and retaining one sequence for every different genus harboring a homologue with an *E*-value of at least 8.0 e^{-4}. Signal peptides as predicted by Signal 3.0 [25] or Dolop [49, 50] were omitted from the motif search. Conserved residues shown to be involved in the interaction of *P. aeruginosa* MliC and HEWL by Yum et al. [16] are indicated by *black arrows*. Amino acids are colored by *hydrophobicity*: *hydrophobic, blue*; *neutral, green*; *hydrophilic, black*. **b** Schematic representation of the distribution the conserved regions 1 (*orange*) and 2 (*green*) along the protein sequence of MliC (*Pseudomonas aeruginosa* PAO1), PliC (*Salmonella* Typhimurium LT2) and PliI (*Aeromonas hydrophila* ATCC7966), respectively. The signal peptides are shown in *red*, and *numbers* represent amino acid positions

similar to the common origin that has been proposed for C- and I-type lysozymes [4, 43]. As such, the different lysozyme types and their corresponding inhibitors may represent an interesting example of parallel evolution.

Acknowledgments J.M.V.H. holds a doctoral, A.A. and L.C. a postdoctoral fellowship from the Research Foundation-Flanders (F.W.O.-Vlaanderen). L.V. was supported by a doctoral fellowship from the Flemish Institute for the Promotion of Scientific Technological Research (IWT). This work was further financially supported by Research Grants from FWO-Vlaanderen (G.0308.05 and G.0363.08) and by the Research Fund K.U.Leuven (research project GOA/03/10). The authors wish to thank: T. Iomoto (Sojo University, Ikeda Kumamoto, Japan) for Pichia pastoris YJT46, Elisabeth Carniel (Insitut Pasteur, Paris Cedex, France) for Yersinia pseudotuberculosis IP32953, Inge W. Nilsen (NOFIMA, Tromsø, Norway) for Salmon goose type lysozyme (SalG), Dominique Schneider (Université Joseph FOURIER, Saint Martin d'Heres, France) for plasmid pDS132, Joerg Graf (Department of Molecular and Cellular Biology, University of Connecticut, USA) for Aeromonas veronii biovar Sobria 391 Hm 21, and J. Robben (Department of Chemistry, K.U. Leuven) for the mass spectrometry analysis.

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