A New Family of Lysozyme Inhibitors Contributing to Lysozyme Tolerance in Gram-Negative Bacteria

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Abstract

Lysozymes are ancient and important components of the innate immune system of animals that hydrolyze peptidoglycan, the major bacterial cell wall polymer. Bacteria engaging in commensal or pathogenic interactions with an animal host have evolved various strategies to evade this bactericidal enzyme, one recently proposed strategy being the production of lysozyme inhibitors. We here report the discovery of a novel family of bacterial lysozyme inhibitors with widespread homologs in gram-negative bacteria. First, a lysozyme inhibitor was isolated by affinity chromatography from a periplasmic extract of Salmonella Enteritidis, identified by mass spectrometry and correspondingly designated as PliC (periplasmic lysozyme inhibitor of c-type lysozyme). A pliC knock-out mutant no longer produced lysozyme inhibitory activity and showed increased lysozyme sensitivity in the presence of the outer membrane permeabilizing protein lactoferrin. PliC lacks similarity with the previously described Escherichia coli lysozyme inhibitor Ivy, but is related to a group of proteins with a common conserved COG3895 domain, some of them predicted to be lipoproteins. No function has yet been assigned to these proteins, although they are widely spread among the Proteobacteria. We demonstrate that at least two representatives of this group, MliC (membrane bound lysozyme inhibitor of c-type lysozyme) of E. coli and Pseudomonas aeruginosa, also possess lysozyme inhibitory activity and confer increased lysozyme tolerance upon expression in E. coli. Interestingly, mliC of Salmonella Typhi was picked up earlier in a screen for genes induced during residence in macrophages, and knockout of mliC was shown to reduce macrophage survival of S. Typhi. Based on these observations, we suggest that the COG3895 domain is a common feature of a novel and widespread family of bacterial lysozyme inhibitors in gram-negative bacteria that may function as colonization or virulence factors in bacteria interacting with an animal host.

Introduction

Lysozymes (EC 3.2.1.17) hydrolyse the β-(1,4) glycosidic bond between N-acetylmuramic acid and N-acetylgalcosamine in peptidoglycan, the major cell wall polymer in the Bacteria. Peptidoglycan forms a network that surrounds the entire bacterial cell, and its hydrolysis by lysozyme renders bacteria sensitive to lysis driven by turbor pressure. Lysozymes are implicated in defensive and offensive bactericidal systems in a wide range of taxonomically diverse organisms including fungi, protozoa, plants, invertebrate and vertebrate animals and even bacteriophages, indicating their evolutionary success as bactericidal tools. Most gram-negative bacteria are not susceptible to the action of lysozyme alone because their outer membrane prevents access of the enzyme to the peptidoglycan layer. However, this barrier has been overcome in the innate immune systems of animals by the production of accessory antibacterial proteins which permeabilize the outer membrane, such as lactoferrin. In addition, some natural lysozymes as well as chemically or genetically modified hen egg white lysozyme (HEWL) have been reported to be active against gram-negative bacteria even in the absence of such permeabilizers [1–4].

In view of the widespread occurrence and effectiveness of lysozymes as antibacterial agents, it is not surprising that bacteria have in turn evolved mechanisms to evade or subvert this threat. A bacterial lysozyme resistance mechanism that has been known for long is peptidoglycan modification. Examples are the de-N-acetylation of N-acetylglucosamine in Bacillus subtilis vegetative cells [5], and O-acetylation of the C-6 hydroxyl group of N-acetylglucosamine residues in Staphylococcus aureus and several other...
New Family of Lysozyme Inhibitors

Author Summary

Lysozyme is an ancient bactericidal enzyme that is part of the antibacterial defense system of vertebrate and invertebrate animals. Bacteria colonizing or infecting an animal host have developed various ways to overcome lysozyme action, a recently proposed mechanism being the production of lysozyme inhibitors. However, the only high-affinity bacterial lysozyme inhibitor known thus far is produced only in few bacteria, and this raised questions about their wider relevance in bacteria–host interactions. We here report the discovery of a novel and distinct family of bacterial lysozyme inhibitors that is widely distributed among the Proteobacteria, including several major pathogens. The family comprises periplasmic as well as membrane-bound inhibitors, and both types contribute to lysozyme tolerance of bacterial cells, as we experimentally demonstrate for the periplasmic inhibitor from Salmonella Typhimurium and the membrane-bound inhibitors from Escherichia coli and Pseudomonas aeruginosa. Interestingly, a gene encoding one of the newly identified inhibitors has been previously found to promote macrophage survival of Salmonella Typhi. The widespread occurrence of lysozyme inhibitors in bacteria is likely to reflect their functional importance in a wide range of bacteria–host interactions. As such, they are also attractive novel targets for antibacterial drug development.

Results

Isolation and identification of a HEWL-inhibitor from S. Enteritidis

In previous work we tested the sensitivity of cell walls of different gram-negative bacteria against several lysozymes [12]. To remove the outer membranes from these cells and make their cell walls accessible to lysozyme, we applied an extraction with chloroform-saturated butanol. A side observation in this work was that this procedure also allowed efficient extraction of the periplasmic lysozyme inhibitor Ivy from E. coli cells since extracts from the wildtype strain showed inhibitory activity against HEWL, while those from the Ivy− strain did not. Interestingly, extracts from S. Typhimurium also showed HEWL inhibition, although S. Typhimurium does not contain an Ivy homolog, nor do any of the other Salmonella serotypes from which a genome sequence is available. This observation was extended to extracts of S. Enteritidis (data not shown). Since we previously purified Ivy by a single HEWL affinity chromatography step to more than 95% purity starting from a periplasmic extract of E. coli overexpressing Ivy from a plasmid [13], we used the same approach and the same matrix (HEWL coupled to N-hydroxysuccinimide-activated Sepharose 4 Fast Flow resin) to isolate the putative lysozyme inhibitor from wildtype S. Enteritidis. When the periplasmic extract obtained from S. Enteritidis (inhibitory activity of 11.6 IU/ml) was passed over the affinity column, the flow-through fraction did not longer show HEWL inhibitory activity. The elution of the bound proteins, with their corresponding inhibitory activity, is shown in Figure 1. Two peaks of 27 and 20 milli absorption units were detected at elution volumes of respectively 19 ml and 27 ml, the latter coinciding with a single peak of HEWL inhibitory activity (67 IU/ml). SDS-PAGE analysis of this active fraction showed only a single band after Coomassie or silver staining (Figure 1). Material recovered from a Coomassie band was subjected to trypsin digestion and tandem mass spectrometry analysis allowing to identify with high confidence peptides (MASGANYEAIKD, MASGANYEAIKNYTKY, TAELVEGDDK and TAELEVGDSDKPVLSNCILAN) corresponding to fragments of the predicted product of the SEN1802 open reading frame in the genome sequence of S. Enteritidis PT4 (Wellcome Trust Sanger Institute, Cambridge UK; http://www.sanger.ac.uk/). A SEN1802 homolog is present in S. Typhimurium LT2 and all other sequenced Salmonella genomes (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). The function of this gene product is unknown but it carries a predicted N-terminal signal peptide of 24 amino acids for Sec-dependent transport to the periplasm. This prediction is in good agreement with our isolation of the protein from the periplasmic cell fraction and with its supposed activity as a lysozyme inhibitor. SEN1802 has two cysteines in its amino acid sequence for possible disulfide bridge formation, a calculated pI of 4.76 and a predicted molecular weight of 9981 Da (for 90 amino acid residues) after cleavage of the signal peptide. This is less than our molecular...
weight estimation from gel migration (14.4 kDa), but such a deviation is not uncommon for acidic proteins and has been ascribed to poor binding of SDS [14]. Because of its HEWL inhibitory activity, we named the protein as PliC (periplasmic lysozyme inhibitor of ε-type lysozyme).

Overexpression and knock-out of pliC in S. Enteritidis

To investigate the function of PliC in S. Enteritidis, a PliC knock-out (S. Enteritidis pliC) and PliC overexpression strain (S. Enteritidis pliC (pAA510)) were constructed. The level of PliC production by these strains in comparison to the wildtype strain was evaluated by analyzing the lysozyme inhibitory activity of crude periplasmic protein extracts (Figure 2). Knock-out of PliC resulted in a strong reduction of inhibitory activity in extracts of S. Enteritidis pliC (4.3 IU/ml) compared to wildtype extracts (29.0 IU/ml). Since the open reading frame downstream of pliC has an opposite orientation, this loss of inhibitory activity cannot be due to a polar effect of the knock-out. Introduction of the pAA510 plasmid in S. Enteritidis pliC rescued lysozyme inhibitory activity (176.5 IU/ml when grown in the presence of 0.2% arabinose to induce the cloned pliC gene. These results confirm that the lysozyme inhibitory activity in the periplasmic extracts can be ascribed to the PliC protein. It should be remarked that the inhibitory activity of the wildtype extract in this experiment was higher than in the extract used for chromatographical purification (29.0 IU/ml versus 11.6 IU/ml). This is due to variability of yield between different osmotic shock treatments (data not shown). However, the yields of samples that were simultaneously processed in a single osmotic shock treatment were reproducible for a particular strain.

PliC protects S. Enteritidis against HEWL in the presence of lactoferrin

Suspensions of late exponential phase wildtype, pliC knock-out and pliC overexpression cells induced with arabinose were treated with 3.0 mg/ml lactoferrin, 100 µg/ml lysozyme, or a combination of both, and survivors were enumerated after 24 h (Figure 3). Most cells survived these treatments very well (inactivation levels not exceeding twofold), except for S. Enteritidis pliC cells in the presence of the lactoferrin - lysozyme mixture, which showed almost 15-fold inactivation. Lactoferrin is known to sensitize gram-negative bacteria to lysozyme and other antibacterial peptides by assisting their penetration through the outer membrane. Although the sensitizing action did not suffice to kill the wildtype S. Enteritidis under the conditions of our experiment, the fact that
the pliC knock-out was sensitized demonstrates that natural levels of PliC were sufficient to protect S. Enteritidis cells against lysozyme.

Distribution of PliC relatives
An iterative search for sequences similar to the mature PliC protein using Psi-Blast [15] revealed besides the homologs in other Salmonella serotypes, similarity to proteins containing the conserved domain COG3895 (Clusters of Orthologous Groups, [16] http://www.ncbi.nlm.nih.gov/COG/). Proteins harboring this domain are widespread among members of the Proteobacteria, except the α-Proteobacteria. Representatives are found in at least 52 different genera of the 155 completely sequenced genomes of all bacteria, the two predicted MliC proteins share only 32% (over 53 amino acids) and 27% (over 65 amino acids) identity with PliC, and 30% identity (over 70 amino acids) with each other (Figure 4).

Because of this relatively large distance and because a 3-D structure is available for MliC of E. coli [YdhA, [21]], MliC from E. coli and PliC were chosen as representatives to further investigate the lysozyme inhibitory activity of the lipoprotein subgroup within the COG3895 group of proteins.

In vitro HEWL-inhibitory activity of MliC proteins
mliC from P. aeruginosa and E. coli were cloned under control of an arabinose inducible promoter (pAA520 and pAA530 respectively) in an E. coli ivy mliC background, to avoid interference from endogenous E. coli inhibitors. Lysozyme inhibitory activity was measured in the periplasmic extracts and membrane fractions of the overexpression strains after induction and compared to that of the control strain E. coli ivy mliC without overexpression plasmid. No significant differences in lysozyme inhibitory activity were found in the periplasmic protein extracts (data not shown). On the other hand, while only 6.3 IU/ml inhibitory activity was detected in the membrane fraction of E. coli ivy mliC, much higher levels of inhibitory activity were measured in the extracts upon induction of MliC expression from P. aeruginosa (67.6 IU/ml) or MliC from E. coli (40.7 IU/ml) (Figure 5). Therefore, we can conclude that both MliC of P. aeruginosa and MliC of E. coli are HEWL-inhibitors.

It can also be seen in Figure 5, that knock-out of mliC in E. coli had almost no influence on the level of inhibitory activity of the membrane extracts (6.7 versus 6.3 IU/ml, for an ivy and an ivy mliC strain respectively). This is in line with earlier reports that mliC transcripts of E. coli are not detected under normal laboratory growth conditions [22].

Figure 3. Sensitivity of S. Enteritidis strains to lysozyme in the presence of lactoferrin. Inactivation (N/N) of S. Enteritidis pliC, S. Enteritidis and S. Enteritidis pliC (pAA510) after 24 h of incubation with 10 mM Tris-HCl pH 7.0 (□), 100 μg/ml lysozyme ( ■), 3.0 mg/ml lactoferrin ( ●) and lysozyme and lactoferrin together ( ■). Mean values ± standard deviations (error bars) are shown (n = 4). Lysozyme treatments resulting in significant differences (p<0.01) compared to the same treatments without lysozyme are marked with an asterisk.

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Figure 4. Amino acid sequence alignment of new HEWL inhibitors. Amino acid sequence alignment (http://www.ebi.ac.uk/clustalw/ [37]) of MliC from P. aeruginosa ( = PA0867 from P. aeruginosa PA01), MliC from E. coli ( = YdhA from E. coli MG1655) (both proteins containing the COG3895 domain) and PliC from S. Enteritidis ( = SEN1802 from S. Enteritidis). Residues that are identical in all sequences in the alignment are marked with “*” in the bottom row, conserved and semi-conserved substitutions with “:” and “.” respectively. The lipobox of the lipoproteins is underlined, while cysteine residues of the mature protein are highlighted in grey.
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Expression of the novel lysozyme inhibitors suppresses growth inhibition of E. coli by HEWL

To investigate the actual contribution of the inhibitors to bacterial HEWL resilience, E. coli ivy mliC was rendered sensitive to HEWL by introducing a tolA mutation that increases its outer membrane permeability. The resulting triple mutant was subsequently transformed with different plasmids that enable arabinose induced expression of either Ivy from E. coli (pAA410), PliC from S. Enteritidis (pAA510), MliC from P. aeruginosa (pAA520), and MliC from E. coli (pAA530). Next, we compared the growth inhibition by HEWL of these strains in the absence and in the presence of arabinose in the medium. At a HEWL concentration of 25 μg/ml, significant differences in optical density (OD₆₀₀) and in plate counts (CFU/ml) of the cultures were observed upon induction of each inhibitor (Figure 6). Overexpression of Ivy, PliC of S. Enteritidis, MliC of P. aeruginosa or MliC from E. coli increased bacterial growth after 8 hours respectively 9, 7, 7 and 5-fold. A control construct (pAA100) containing the gene for green fluorescent protein (gfp) in the same vector and E. coli background, showed no significant differences in optical density or plate counts upon induction (data not shown). These results demonstrate that besides Ivy, also at least three members of the newly identified family of lysozyme inhibitors can effectively protect bacterial cells against lysozyme when expressed at appropriate levels.

Discussion

In this work, we have identified a novel class of lysozyme inhibitors different from Ivy, the lysozyme inhibitor discovered...
earlier in *E. coli* [10]. These novel inhibitors belong to a large family of proteobacterial predicted periplasmic proteins or lipoproteins which share a common COG3895 structural motif with unknown function. We demonstrated lysozyme inhibitory activity for one periplasmic (PlIC from *S. Enteritidis,* as well as for two lipoprotein members of this family (MiIC from *P. aeruginosa* and from *E. coli*). Although no function had hitherto been assigned to any of the COG3895 proteins the 3-D solution structure of MiIC from *E. coli* has been recently resolved, featuring an 8-stranded β-barrel, stabilized by a disulfide bond [21]. At the 3-D level, there is no resemblance with Ivy, which adopts a central β-sheet made of 5 antiparallel β-strands flanked on the convex side by two short helices and on the concave side by an amphipathic helix [18]. The Cys residues engaging in the disulfide bond in MiIC from *E. coli* are conserved in both PlIC from *S. Enteritidis* and MiIC from *P. aeruginosa,* and in the majority of COG3895 proteins, suggesting that they may be important for preserving conformational stability.

The existence and possible function of lysozyme inhibitors in bacteria has not received much attention thus far. To our knowledge, a systematic screen for bacterial lysozyme inhibitors has not yet been conducted. This is surprising, given the important role of lysozymes in antibacterial defense in all major eukaryotic lineages, and the extensively documented existence of inhibitors of various other glycosyl hydrolases. Particularly plants produce a wide range of such inhibitors, for example against polygalacturonases, xylanases, z-amylases and β-glucanases, to thwart microbial attack. Therefore, the discovery in this work of a novel class of bacterial lysozyme inhibitors and the wide distribution of homologs of these inhibitors in the Proteobacteria may be indicative for their functional importance, for example in bacteria-host interactions. The location of the bacterial lysozyme inhibitors either in the periplasm (Ivy and PlIC from *S. Enteritidis,* or anchored to the luminal face of the outer membrane (MiIC from *E. coli* and *P. aeruginosa*) is also consistent with a role in protecting peptidoglycan from hydrolysis by exogenous lysozymes. In at least one instance more direct evidence for a role in host interaction exists. In *Salmonella* Typhi, expression of the mIC homolog was induced in cells residing within macrophages and knockout of mIC reduced macrophage survival [23]. Macrophages are known to produce a battery of antibacterial peptides including lysozyme and membrane permeabilizers, and hence the production of one or more lysozyme inhibitors by intracellular pathogens like *S. Typhi* makes sense from this point of view. The observed increased lysozyme sensitivity of an *S. Enteritidis* pliC knockout in the presence of 3.0 mg/ml of the outer membrane permeabilizing protein lactoferrin (Figure 3) provides a relevant indication in this context. Lactoferrin concentrations in this range occur in secretions like tears, airway mucus or colostrum [24,25,26]. Moreover, Ivy and all three new HEWL-inhibitors identified in this study suppressed growth inhibition by HEWL when overexpressed in an *E. coli* MG1655 tolA ivy mIC strain (Figure 6).

The genomic context of the newly identified lysozyme inhibitor genes also provides some interesting clues about their possible function. Immediately upstream of *pliC* of *S. Typhimurium* are the genes *pagC, pagD* and *msgA,* which play a role in macrophage survival of *S. Typhimurium.* Furthermore, transcriptome analysis has revealed that expression of *pliC* is controlled by ShyA, the transcriptional activator that controls expression of *pagC* and *pagD* and that is necessary for virulence [27]. Based on its low GC content, the region encompassing *pagC* and a number of its immediate upstream genes was suggested to be acquired by lateral gene transfer, as is often the case for virulence genes [28]. The *pliC* gene, which is immediately downstream of *pagC,* also has a markedly lower GC content (42.0%) than the average of the LT2 chromosome (52.2%), and thus probably is an integral part of this acquired genome fragment. Interestingly, the *mliC* gene is located downstream of *yfdA* in all sequenced *Salmonella* strains. Furthermore, both in *E. coli* and in *Salmonella,* mliC or its homolog are adjacent to *yihD,* an open reading frame recently renamed to *anmk* because it encodes an anhydro N-acetyl muramic acid kinase involved in recycling of murine [29]. This allows speculation on a possible role of MiIC in murein recycling, for example by controlling excessive hydrolysis of the murein backbone by lytic transglycosylases. However, at present we do not know whether the latter enzymes are inhibited by MiIC or any of the other COG3895 proteins.

**C-type lysozymes** (e.g. HEWL or human lysozyme) are the major lysozymes produced by most vertebrates. In addition, all vertebrates have genes encoding g-type lysozyme. While the importance of the latter is not clear in man, it is the dominant type in some birds and it also occurs in fish species. A third type of lysozyme, called i-type, is characteristic for invertebrate animals such as arthropods, molluscs, nematodes etc. [30]. Neither PlIC from *S. Enteritidis,* nor MiIC from *E. coli* or *P. aeruginosa* have inhibitory activity against g-type lysozyme from goose egg white (data not shown). Ivy, in contrast, is active against goose egg white lysozyme [13] but not against g-type lysozyme from the urochordate *Oikopleura dioica* and i-type lysozyme from the scallop *Chlamys islandica* [31]. Given the existence and widespread occurrence of two types of c-type-specific lysozyme inhibitors in Proteobacteria, we anticipate that additional inhibitor classes specific against other types of lysozymes are likely to be produced in bacteria. Screening of crude periplasmic extracts of a diverse range of bacteria for inhibitory activity against these g- and i-type lysozymes seems to corroborate this assumption (unpublished results), but definitive confirmation will have to await isolation and identification of the putative inhibitors.

The possible effect of bacterial lysozyme inhibitors in bacterial pathogenesis may even extend beyond neutralizing the direct antibacterial effect of lysozyme. Peptidoglycan has recently emerged as a powerful effector of the innate immune system through interaction with specific host receptors. The actual elicitor molecules are specific muropeptide fragments derived from peptidoglycan by bacterial and/or host lytic enzymes [32,33]. This system of pattern recognition is believed to allow the host to distinguish pathogenic from non-pathogenic bacteria and to maintain its immune functions at an appropriate level. Malfunctioning of this system has been linked to chronic immune-related diseases such as inflammatory bowel disease and Crohn’s disease. By interfering with the fragmentation of peptidoglycan by host lysozymes, bacterial lysozyme inhibitors can be anticipated to influence this system, and thus to play a potential role in these immune related pathologies. Provided that their role in bacterial pathogenesis can be further substantiated, bacterial lysozyme inhibitors may constitute an attractive new target for the development of anti-inflammatory and/or immunomodulating drugs.

**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. Construction of mutants and plasmids is discussed in Text
Purification of HEWL-inhibiting proteins

For the purification of PliC, 500 ml cultures of *S. Enteritidis* ATCC 13076 were grown on a rotary shaker to stationary phase (21 h, shaking at 200 rpm) in LB at 37°C. Periplasmic cell extracts were then prepared by a gentle cold osmotic shock procedure as described earlier [13], and stored at −20°C until further analysis. Lysozyme binding inhibitors were isolated from this periplasmic cell fraction on an A¨ KTA-FPLC platform (Amersham Pharmacia Biotech, Upsalla, Sweden) by affinity chromatography using immobilized HEWL as a ligand as described earlier for the Ivy binding inhibitors [13], except that 100 ml of crude extract was loaded rather than 25 ml, and fractions of 5.0 ml rather than 2.0 ml were collected. The fractions were collected in tubes containing 300 μl of 1.0 M Tris-HCl pH 8.0 to neutralize the high pH of the elution buffer (pH 12.0), and bovine serum albumin (BSA, Sigma-Aldrich) was added to a final concentration of 0.5 mg/ml to stabilize the purified protein unless the samples were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions were then desalted by dialysis against 10 mM potassium phosphate buffer pH 7.0 (12 kDa cut off, Sigma-Aldrich) and stored at −20°C until further use.

After purification, protein purity was checked with SDS-PAGE, conducted according to [34] with a 15% separating gel and a 4% stacking gel. Samples were prepared by boiling for 3 min in the presence of 1% β-mercaptoethanol and 1% SDS. Gels were stained with Coomassie blue R 250 (Sigma-Aldrich), and, if higher sensitivity was desired, destained and subsequently silver-stained following the procedure of [35].

For the isolation of MliC of *P. aeruginosa* or *E. coli*, cultures of *E. coli* ivy mliC harboring plasmid pAA520 or pAA530 respectively, were grown overnight at 37°C in LB with ampicillin (100 μg/ml, Sigma-Aldrich), diluted 1/100 in fresh LB without antibiotics, induced with 0.2% (w/v) L-arabinose after 4 hours of growth, and further incubated at 37°C until stationary phase. Portions of 200 ml were subsequently harvested, resuspended in 10 ml 10 mM Tris-HCl pH 8 and lysed by three cycles of freezing and thawing followed by sonication (3×3 min, amplitude 40%, pulses 5 s on/5 s off). These suspensions were centrifuged for 1 hour at 100,000 × g (4°C). The resulting pellet was resuspended in 10 ml 10 mM Tris-HCl buffer (pH 6.8) supplemented with 1.0 M NaCl, and sedimented again as described above. The membrane-bound proteins were then extracted using 2% Triton X-100 in a 10 mM Tris-HCl buffer (pH 6.8) supplemented with 10 mM MgCl₂ and 150 mM NaCl and separated from insoluble material by centrifugation (1 hour at 100,000 × g, 4°C).

**Protein identification by mass spectrometry**

Active fractions containing the purified inhibitor protein were lyophilized, redissolved and subjected to SDS-PAGE and Coomassie staining. A gel fragment from the band corresponding to the inhibitor was trypsin-digested according to the method of [36], and the digests were then analyzed by electrospray tandem mass spectrometry on a LCQ Classic (ThermoFinnigan, San Jose, California) ion trap mass spectrometer equipped with a nano-liquid
chromatography column switching system and a nanoelectrospray device. Tandem mass spectrometry data were searched using MASCOT (Matrix Sciences, London, U.K.) and SEQUEST (ThermoFinnigan) against the GenBank non-redundant protein database.

Determination of HEWL inhibitory activity
Freeze-dried *M. lysodeikticus* ATCC46900 cells (Sigma-Aldrich) were resuspended at 0.5 mg/ml either in appropriate dilutions of the bacterial crude extracts, purified column fractions or in potassium phosphate buffer (10 mM, pH 7.0) with 0.5 mg/ml Bovine Serum Albumine (BSA) for the controls. Thirty μl of 66 U/ml HEWL (Hen Egg White Lysozyme; Fluka, 66,000 U/mg protein) in potassium phosphate buffer (10 mM, pH 7.0) was then added to 270 μl of these suspensions and cell lysis was followed during 2 h at 25°C as the decrease in optical density (OD600) using a Bioscreen C Microbiology Reader (Labsystems Oy, Helsinki, Finland). In the absence of inhibitor, this procedure resulted in a linear OD600 decrease of 0.27 ± 0.04 over 2 h. The percentage inhibition \(I\) for each column fraction was calculated as:

\[
I = \frac{L_{0} - L}{R_{0} - R} \times 100 \%
\]

with \(L_{0} - L\), \(R_{0} - R\) and \(B_{0} - B\) representing the OD600 decrease over a period of 2 h of the *M. lysodeikticus* suspensions respectively in the presence of lysozyme but with buffer instead of a bacterial extract/column fraction, in the presence of the bacterial extract/column fraction and lysozyme, and in the presence of the bacterial extract/column fraction but with buffer instead of lysozyme. Inhibitory activity was expressed in inhibitory units, with one unit being the amount of inhibitor that is needed to decrease the lysozyme activity by 50% under the above assay conditions.

Sensitivity of *S. Enteritidis* to lysozyme in the presence of lactoferrin
*S. Enteritidis*, *S. Enteritidis* pliC and *S. Enteritidis* pliC (pAA510) cultures were grown overnight in LB with ampicillin and/or chloramphenicol when appropriate, diluted 1/100 in fresh LB without antibiotics, induced with 0.2% (w/v) L-(+)-arabinose (Fluka, Buchs, Switzerland) and incubated further. Arabinose served only to induce pliC expression from plasmid pAA510, but was also added to cultures of strains not carrying this plasmid to ensure identical culture conditions for all strains in the experiment. At an optical density (OD600) of 0.6 (5.108 cells/ml of these suspensions and cell lysis was followed during 2 h at 25°C as the decrease in optical density (OD600) using a Multiscan RC (Thermo Scientific, Zellik, Belgium). After 0 h the viable cell number was enumerated by plating on LB agar.

Lysozyme growth inhibition in vivo
Precultures of *E. coli* MG1655 tolu ivy pliC harboring plasmid pAA410, pAA510, pAA520, or pAA530 were grown overnight in LB broth containing ampicillin, kanamycin and chloramphenicol. Subsequently, cultures were diluted (1/100) in duplicate in fresh LB containing ampicillin, and after three hours of growth (exponential phase), either H2O or 0.02% L-(+)-arabinose was added, resulting in control and induced precipitates respectively. These cultures were further grown to stationary phase to allow inhibitor expression. Subsequently, test tubes containing 4 ml LB with ampicillin, and either water or 0.02% L-(+)-arabinose and 25 μg/ml HEWL were inoculated (1/100) with the control and induced *E. coli* precultures respectively. These cultures were grown at 37°C during 10 hours. Each hour the OD600 was determined using a Multiscan RC (Thermo Scientific, Zellik, Belgium). After 0 h the viable cell number was enumerated by plating on LB agar.

List of genED numbers
From *E. coli* MG1655: ivy (before ybfE); 946530 (Gene Entrez), pliC (before ybfA); 946611 (Gene Entrez), toluA: 946625 (Gene Entrez); *P. aeruginosa* mliC (before PA0667): 882238 (Gene Entrez); *Salmonella Enteritidis*: pliC: SEN1802 [http://www.sanger.ac.uk/].

Supporting Information
Text S1 The construction of the *S. Enteritidis* pliC knockout mutant, the *E. coli* ivy mliC mutant, the *E. coli* toluA ivy mliC mutant and the construction of the plasmids pAA510, pAA520 and pAA530 is described. Found at: doi:10.1371/journal.ppat.1000019.s001 (0.04 MB DOC)

Author Contributions
Conceived and designed the experiments: LC BM CWM. Performed the experiments: LC AA CWM. Contributed reagents/materials/analysis tools: LD DD NJR. Wrote the paper: LC AA CWM.

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Lysozyme growth inhibition in vivo
Precultures of *E. coli* MG1655 tolu ivy mliC harboring plasmid pAA410, pAA510, pAA520, or pAA530 were grown overnight in LB broth containing ampicillin, kanamycin and chloramphenicol. Subsequently, cultures were diluted (1/100) in duplicate in fresh LB containing ampicillin, and after three hours of growth (exponential phase), either H2O or 0.02% L-(+)-arabinose was added, resulting in control and induced precipitates respectively. These cultures were further grown to stationary phase to allow inhibitor expression. Subsequently, test tubes containing 4 ml LB with ampicillin, and either water or 0.02% L-(+)-arabinose and 25 μg/ml HEWL were inoculated (1/100) with the control and induced *E. coli* precultures respectively. These cultures were grown at 37°C during 10 hours. Each hour the OD600 was determined using a Multiscan RC (Thermo Scientific, Zellik, Belgium). After 0 h the viable cell number was enumerated by plating on LB agar.

List of genED numbers
From *E. coli* MG1655: ivy (before ybfE); 946530 (Gene Entrez), mliC (before ybfA); 946611 (Gene Entrez), toluA: 946625 (Gene Entrez); *P. aeruginosa* mliC (before PA0667): 882238 (Gene Entrez); *Salmonella Enteritidis*: pliC: SEN1802 [http://www.sanger.ac.uk/].

Supporting Information
Text S1 The construction of the *S. Enteritidis* pliC knockout mutant, the *E. coli* ivy mliC mutant, the *E. coli* toluA ivy mliC mutant and the construction of the plasmids pAA510, pAA520 and pAA530 is described. Found at: doi:10.1371/journal.ppat.1000019.s001 (0.04 MB DOC)

Author Contributions
Conceived and designed the experiments: LC BM CWM. Performed the experiments: LC AA DD KGAV LV JMH JR. Analyzed the data: LC AA BM CWM. Contributed reagents/materials/analysis tools: LD DD NJR. Wrote the paper: LC AA CWM.

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