CMLS, Cell. Mol. Life Sci. 61 (2004) 1229–1237
1420-682X/04/101229-09
DOI 10.1007/s00018-004-4066-3

CMLS Cellular and Molecular Life Sciences

Research Article

Periplasmic lysozyme inhibitor contributes to lysozyme resistance in *Escherichia coli*

D. Deckers, B. Masschalck, A. Aertsen, L. Callewaert, C. G. M. Van Tiggelen, M. Atanassova and C. W. Michiels *

Laboratory of Food Microbiology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, 3001 Leuven (Belgium), Fax: +32 16 321960, e-mail: Chris.Michiels@agr.kuleuven.ac.be

Received 12 February 2004; received after revision 11 March 2004; accepted 16 March 2004

Abstract. The product of the *Escherichia coli* ORFan gene *ykfE* was recently shown to be a strong inhibitor of C-type lysozyme in vitro. The gene was correspondingly renamed *ivy* (inhibitor of vertebrate lysozyme), but its biological function in *E. coli* remains unknown. In this work, we investigated the role of Ivy in the resistance of *E. coli* to the bactericidal effect of lysozyme in the presence of outer-membrane-permeabilizing treatments. Both in the presence of lactoferrin (3.0 mg/ml) and under high hydrostatic pressure (250 MPa), the lysozyme resistance of *E.* coli MG1655 was decreased by knock-out of Ivy, and increased by overexpression of Ivy. However, knock-out of Ivy did not increase the lysozyme sensitivity of an *E. coli* MG1655 mutant previously described to be resistant to lysozyme under high pressure. These results indicate that Ivy is one of several factors that affect lysozyme resistance in *E. coli*, and suggest a possible function for Ivy as a host interaction factor in commensal and pathogenic *E. coli*.

Key words. Lysozyme; lysozyme inhibitor; *Escherichia coli*; defense mechanism; Ivy.

Lysozyme is the common name of a group of enzymes that can hydrolyze the bacterial cell wall polymer peptidoglycan by virtue of an N-acetylmuramoylhydrolase activity (E.C. 3.2.1.17). Lysozymes are commonly found in the major taxa of prokaryotes and eukaryotes, including the bacteria themselves, and also in bacteriophages, but they may have different roles in different organisms. For example, animal and plant lysozymes are important players in the defense against bacterial invaders [1–5], phage lysozymes play a role in phage penetration into, and/or release from the host cell [6, 7], while some bacterial lysozymes, called autolysins, allow controlled hydrolysis of the cell wall at sites of cell growth or cell division [8, 9]. The widespread natural occurrence of lysozyme, even in the human body, and its specificity against bacteria (peptidoglycan occurs exclusively but almost universally in the phylum of the Bacteria), has fueled the development of applications of this enzyme as an antibacterial agent in foods, pharmaceuticals and cosmetics, and even for treating bacterial infections [10, 11]. One of the major limitations of lysozyme in this type of application is its poor efficiency against some bacteria. In particular, Gram-negative bacteria are generally insensitive to lysozyme because their peptidoglycan is protected by the surrounding outer membrane layer. However, two different approaches have proven useful to improve the efficiency of lysozyme against Gram-negative bacteria [12]. First, lysozyme can be structurally modified such that it can cross the outer membrane, probably by a mechanism related to self-promoted uptake [13, 14]. This has been accomplished by conjugation with a variety of molecules including fatty acids [15, 16], and also by extension of the polypeptide with a stretch of hydrophobic amino acids using a recombinant genetic approach [17]. Alternatively,

* Corresponding author.

the barrier function of the outer membrane can be disrupted to allow passage of (unmodified) lysozyme. This can be achieved by substances that complex or displace the stabilizing Ca^{2+} and Mg^{2+} ions from the outer leaflet of the outer membrane, such as EDTA or the polycation lactoferrin [2, 18–20], but also by some physical treatments, in particular high pressure [21–23]. Nevertheless, for example under high pressure, not all bacteria in a population appear to become sensitive to lysozyme [24, 25]. Studies with *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* outer-membrane mutants suggested that variations in outer membrane composition in bacterial populations may explain these differences in lysozyme susceptibility under high pressure, but other factors may be involved as well, such as variations in peptidoglycan structure, or the presence of different levels of lysozyme inhibitors [26].

The existence of a lysozyme inhibitor in *Escherichia coli* has been discovered only recently. When the *E. coli* orphan gene *ykfE* was fused to a His tag and overexpressed to investigate its gene product, lysozyme, used to lyse the cells, was co-purified with the YkfE protein [27]. YkfE was subsequently found not only to bind specifically and strongly to lysozyme, but also to inhibit its enzymatic activity. Since inhibition was specific for C-type lysozymes (including besides hen egg white, also human lysozyme), *ykf*E was renamed *ivy* (inhibitor of vertebrate lysozyme). It was the first well-characterised proteinaceous lysozyme inhibitor to be described. Open reading frames with various levels of homology to the *E. coli ivy* gene exist in a number of other proteobacteria belonging to the genera *Shigella, Yersinia, Pseudomonas, Chromobacterium* and *Burkholderia* [unpublished results], but their lysozyme-inhibiting activity has not been demonstrated. In view of the widespread occurrence of lysozyme and its important role in antibacterial defense in animals including humans, a hypothetical function of lysozyme inhibitors in commensal or pathogenic strains of these Gram-negative bacteria could be to provide an extra layer of defense against the host lysozyme, when the outer membrane becomes permeabilized by components of the innate defense such as lactoferrin and defensins. Other possible functions include protection against bacteriophages, or regulation of autolysin activity during cell growth and cell division. A contribution of lysozyme inhibitors to bacterial lysozyme resistance would also have important consequences for the development of applications of lysozyme in food, pharmaceuticals, cosmetics and medicine. In this work, we investigated the role of Ivy in lysozyme resistance of *E. coli*, using Ivy knock-out and overexpression strains, and using high-pressure treatment or lactoferrin to permeabilize the outer membrane.

Material and methods

Construction of an arabinose-inducible Ivy overexpressor strain

A PCR- (Platinum *Pfx* DNA polymerase; Invitrogen, Merelbeke, Belgium) amplified fragment containing *araC* and the arabinose-inducible P_{BAD} promoter of the *araBAD* operon [28] of *E. coli* MG1655 was cloned upstream of promoterless *gfp* in pFPV25 [29] cut with *Eco*RI and *Xba*I. The *gfp* gene was subsequently removed by cleavage with *Xba*I and *Hin*dIII and replaced by the MG1655 *ivy* gene, amplified with the primers: ivyF (5′-gatctctagacgccaggctttaggagg-3′) and ivyR (5′-gatcaagcttcggagccgaaaggctcc-3′) and cut with the same enzymes. All restriction enzymes were purchased from Roche Diagnostics Belgium (Vilvoorde, Belgium). The resulting construct contains the *ivy* gene under control of the P_{BAD} promoter and was designated pAA410. This plasmid was transformed into *E. coli* MG1655 and the resulting strain was designated as *E. coli* MG1655 (pAA410).

Construction of *ivy* knock-out mutants

E. coli TE2680, containing a chromosomal *ivy*::Kan knock-out was kindly donated by the laboratory of J. M. Claverie (CNRS Institute of Structural Biology and Microbiology, Marseille, France). From this strain, a ± 1.7-kb *ivy*::Kan fragment was obtained by PCR, using primers ivyF and ivyR, and transformed to MG1655 containing the pKD46 plasmid which strongly enhances the rate of allelic exchange with linear double-stranded DNA fragments [30]. A Kan-resistant transformant was purified and Kan resistance was transduced by P1*vir* [31] to MG1655 and LMM1010 (a high-pressure-resistant derivative of MG1655, isolated by Hauben et al. [22]. Replacement of *ivy* by the *ivy*::Kan allele was confirmed by PCR and the resulting strains were designated as *E. coli* MG1655 *ivy*::Kan and LMM1010 *ivy*::Kan.

Growth conditions

Luria Bertani (LB, 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) agar master plates were inoculated from –80 °C stocks and contained ampicillin (100 µg/ml) for MG1655 (pAA410) and kanamycin (50 µg/ml) for MG1655 *ivy*::Kan and LMM1010 *ivy*::Kan. All antibiotics were purchased from Sigma-Aldrich (Bornem, Belgium). Cultures for lysozyme sensitivity experiments were grown overnight at 37 °C in tryptone soy broth (TSB; Oxoid, Basingstoke, U. K.) without antibiotics, diluted 1/100 in fresh TSB and incubated further at 37 °C. At an optical density (OD_{600}) of 0.4, 0.2% (w/v) L-(+)-arabinose (Fluka, Buchs, Switzerland) was added and growth was continued at 37 °C until an OD_{600} around 0.9 (early stationary phase, approx. 5×10^8 CFU/ml). Although arabinose served only to induce *ivy* expression

from plasmid pAA410, it was also added to cultures of strains not carrying this plasmid to ensure identical culture conditions for all strains in an experiment. Addition of arabinose was omitted in experiments not involving pAA410.

Lysozyme inhibition assay

The production of Ivy by the different *E. coli* strains was analyzed by measuring the inhibition of lysozyme enzymatic activity by *E. coli* cell extracts and by periplasmic fractions (see below for preparation). Cell extracts were made by sonication (2 × 5 min, amplitude 40%, pulse 5 s on/5 s off; Sonics & Materials SMC, Danbury, Conn.) of early stationary phase cells (5×10^8 CFU/ml) in potassium phosphate buffer (10 mM, pH 7.0), followed by centrifugation (24,000 g, 30 min) to remove cell debris. The extracts were stored at –20 °C. The inhibition of lysozyme activity was measured using an adapted lysozyme activity assay of Weisner [32], which is based on the decrease in turbidity of a culture of lyophilized *Micrococcus lysodeikticus*. First, freeze-dried *M. lysodeikticus* ATCC 4698 (two different batches were used, Sigma, St. Louis, Mo.) were resuspended at 0.5 mg/ml in the cell extracts or in different dilutions of the periplasmic fractions of *E. coli* or in potassium phosphate buffer (10 mM, pH 7.0) for the control. Thirty microliters of 10 µg/ml hen egg white lysozyme (66,000 U/mg; Fluka) in potassium phosphate buffer (10 mM, pH 7.0) was added to 270 µl of *M. lysodeikticus* cell suspension (final working concentration 1.0 µg/ml lysozyme), and cell lysis was followed during 4 h at 20 °C as the decrease in optical density (OD_{600}) using a Bioscreen C Microbiology Reader (Labsystems Oy, Helsinki, Finland). Inhibition was expressed as the percent reduction in the initial enzymatic reaction rate (decrease in OD_{600}/h).

Isolation of periplasmic proteins by osmotic shock

An *E. coli* culture grown for 21 h in 50 ml LB broth at 37 °C, was diluted 100-fold in 350 ml LB broth and induced with 0.2% arabinose when the OD_{600} reached 0.5. After 4 h of further incubation, 200 ml of the culture was harvested by centrifugation (10 min, 2900 g, room temperature). The cell pellet was resuspended in 25 ml 30 mM Tris-HCl (pH 8) with 20% (w/v) sucrose. After addition of 2.5 ml 10 mM EDTA and 10 min shaking at room temperature, the sample was centrifuged (10 min, 6350 g, 4 °C), and the cell pellet resuspended in 25 ml ice-cold 5 mM $MgSO_4$, and shaken for 10 min in an ice-waterbath. This suspension was centrifuged again (10 min at 16,800 g, 4 °C), and the supernatant, corresponding to the periplasmic fraction, was stored at –20 °C until further analysis.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was conducted according to the Laemmli method [33], with a 12% separating gel and a 4% stacking gel. Samples of the periplasmic protein extracts were mixed with an equal volume of denaturation buffer containing β-mercaptoethanol and SDS and boiled for 3 min in a water bath. Fifty microliters of the samples was loaded per lane, and flanked by a lane with molecular-weight markers (low-molecular-weight calibration kit, Amersham Biosciences, Uppsala, Sweden). After electrophoresis, gels were stained overnight with Coomassie blue R 250 [300 ml/l ethanol, 50 ml/l acetic acid and 5 mg/l Coomassie blue R 250 (Sigma-Aldrich)] followed by a series of destaining steps in a solution with 300 ml/l ethanol and 50 ml/l acetic acid. For higher sensitivity, gels were subsequently fully destained and silver-stained by the procedure of Heukenshoven and Dernick [34].

Amino acid sequencing by mass spectrometry

A protein band suspected to correspond to Ivy was sampled from a Coomassie-stained gel and trypsin-digested according to the method of Shevchenko et al. [35]. Tryptic digests were then analyzed by electrospray tandem mass spectrometry on a LCQ Classic (ThermoFinnigan, San Jose, Calif.) 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.

Sensitivity of *E. coli* to lysozyme under high pressure

Cultures were grown to early stationary phase and induced with arabinose as described above, except for the experiment involving strain LMM1010, in which growth was for 21 h (stationary phase, approx. 1×10^9 CFU/ml) because this strain was reported to be resistant to lysozyme under high pressure when it was grown under these conditions [21]. The cells were harvested by centrifugation (3800 g, 5 min) and resuspended in the same volume of potassium phosphate buffer (10 mM, pH 7.0). Although the pH of phosphate buffer is more pressure dependent than that of some other buffers [36], we preferred to use phosphate buffer because it is widely used in bacterial inactivation studies and is not harmful to *E. coli*. Portions of this cell suspension (270 µl), supplemented with 30 µl of lysozyme (final concentration 1.0 or 10.0 µg/ml) or 30 µl of buffer for the controls, were sealed in a sterile polyethylene pouch after exclusion of air bubbles and subjected at 20 °C to high-pressure treatment at 250 or 600 MPa in an 8-ml high-pressure vessel (Resato, Roden, The Netherlands), which was thermostatically controlled with a water jacket. Compression and decompression were achieved with a manual spindle pump at a rate of approximately 100 MPa per minute. Under these conditions, the adiabatic temperature increase in the vessel increased to 38 °C upon pressurization to 600 MPa. The

high-pressure transmission fluid was TP1, a mixture of glycols (Van Meeuwen, Weesp, The Netherlands). After decompression, the samples were aseptically removed from the pouches, serially diluted in sterile potassium phosphate buffer (10 mM, pH 7.0) and, along with untreated control cultures, plated on tryptone soy agar (Oxoid) with a spiral plater (Spiral Systems Inc., Cincinnati, Ohio). The plated volume was 50.0 µl, and hence the lower detection limit was 20 CFU/ml. Colonies were allowed to develop for 24 h at 37 °C. Inactivation was expressed as a logarithmic viability reduction factor, $\log_{10} N_o/N$, where N_o and N are the colony counts of control and treated samples, respectively.

Sensitivity of *E. coli* to lysozyme in the presence of lactoferrin

Cultures were grown as described above, harvested by centrifugation (3800 g, 5 min) and resuspended in the same volume of potassium phosphate buffer (10 mM, pH 7.0) without and with lactoferrin (gift from Morinaga Milk Industries, Kanagawa, Japan; 3.0 mg/ml final concentration) and/or lysozyme (50 µg/ml). After 7 h of incubation at 20 °C, the number of survivors was enumerated using the same plate count procedure as for pressure-treated cell suspensions. Inactivation was expressed as a viability reduction factor, N_o/N, where N_o and N are the colony counts of control and treated samples, respectively.

Statistical analysis

All data shown are averages ± standard deviations (SD) from three experiments with three independent cultures of each strain. The possible effect of high pressure/lactoferrin and lysozyme was investigated using a two-factor ANOVA model. The main effects of high pressure/lactoferrin and lysozyme as well as their interaction were included in the model. Following significant main effects or interactions, post hoc comparisons of treatment means were carried out using a Bonferroni multiple-comparison procedure with a 5% family significance level [37]. The SAS software (SAS version 8.2; SAS Institute Inc., Cary, N. C.) was used throughout all analyses.

Results

Overexpression and deletion of Ivy in *E. coli* MG1655

Ivy knock-out and overexpression strains [respectively MG1655 *ivy*::Kan and MG1655 (pAA410)] were constructed as described in Materials and methods, and subjected to a rapid evaluation by comparing the lysozyme inhibition activity of their sonicated cell extracts with those obtained from wild-type strain MG1655. Figure 1 A shows the progression of *M. lysodeikticus* lysis by lysozyme in the presence of these cell extracts. The presence of cell extracts increased the initial OD_{600} by 0.05. The rate of cell lysis by lysozyme (0.50 OD_{600}/h in the absence of *E. coli* cell extracts) was reduced by 74% by cell extract from the Ivy knock-out strain (0.13 OD_{600}/h), by 84% by the wild-type extract (0.08 OD_{600}/h) and completely inhibited by the extract from the Ivy overexpressor strain.

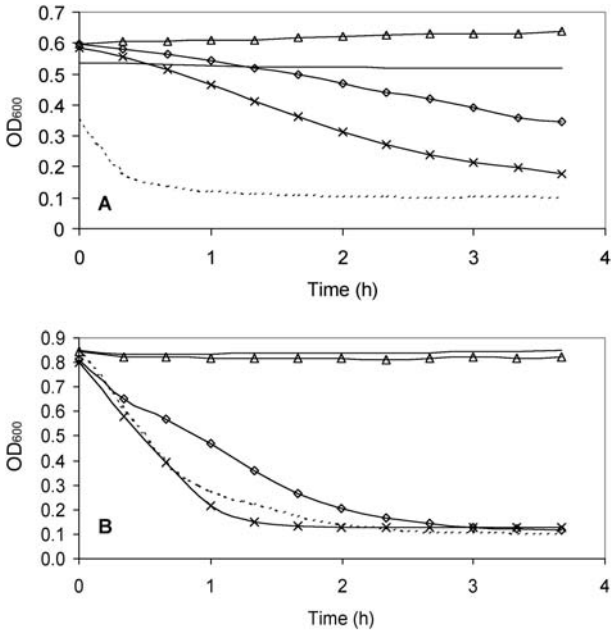

Figure 1. Lysis (expressed as OD_{600} against time) of *M. lysodeikticus* cell suspension (0.5 mg/ml) by 1.0 µg/ml lysozyme in the absence (dotted line) and presence of undiluted sonicated cell extracts of *E. coli* MG1655 (diamonds), MG1655 (pAA410) (triangles) and MG1655 *ivy*::Kan (crosses) (*A*) and periplasmic fractions of twofold-diluted *E. coli* MG1655 (diamonds), twofold-diluted MG1655 (pAA410) (triangles) and undiluted MG1655 *ivy*::Kan (crosses) (*B*). The control sample (full line) consisted of phosphate buffer instead of lysozyme solution added to *M. lysodeikticus*. The differences in initial optical density between *A* and *B* was caused by the use of different batches of *M. lysodeikticus.*

These results indicated that knock-out and overexpression of Ivy had been successful and, therefore, the production of Ivy protein in the three strains was further studied in periplasmic cell fractions, because Ivy is a periplasmic protein [27]. Figure 1 B shows the progression of *M. lysodeikticus* lysis by lysozyme in the presence of these periplasmic fractions. The rate of cell lysis by lysozyme (0.58 OD_{600}/h) was not reduced by the periplasmic fraction from the Ivy knock-out strain (0.59 OD_{600}/h), was reduced by 41% by the twofold-diluted wild-type periplasmic extract (0.34 OD_{600}/h) and completely inhibited by the twofold-diluted periplasmic fraction from the Ivy overexpressor strain and by the undiluted sample from the wild-type (latter data not shown). Lysozyme inhibitory activity of wild-type and overexpressor strain periplasmic extracts became undetectable upon 4-fold and 27-fold dilution respectively, indicating

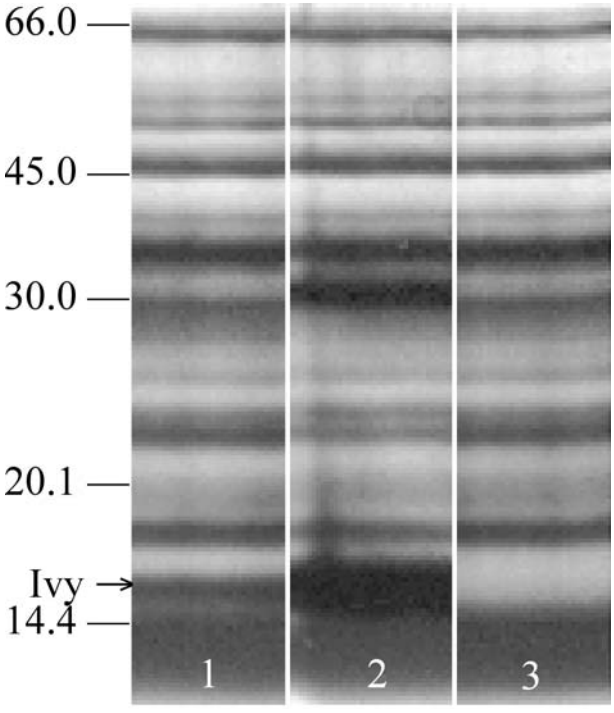

Figure 2. Silver-stained SDS-PAGE gel of periplasmic fractions of *E. coli* MG1655 (lane 1), Ivy overexpressor strain MG1655 (pAA410) (lane 2) and MG1655 *ivy*::Kan (lane 3). The position of Ivy is indicated by an arrow and the positions of the molecular-weight markers by lines.

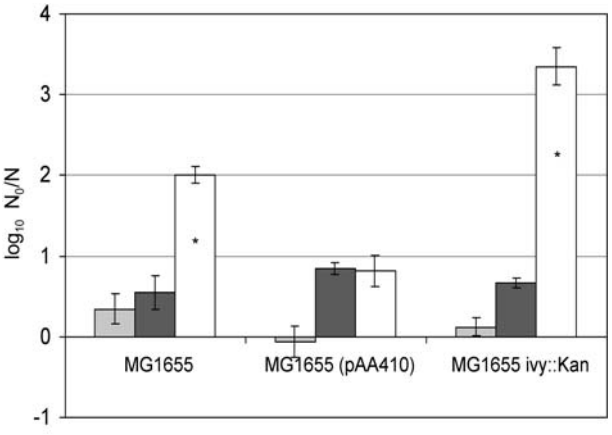

Figure 3. Inactivation ($\log_{10}N_0/N$) of *E. coli* MG1655, MG1655 (pAA410) and MG1655 *ivy*::Kan at atmospheric pressure (3 h) in the presence of lysozyme (hatched bars), and under high pressure (250 MPa, 15 min) in the absence (black bars) and presence (open bars) of lysozyme (1.0 µg/ml). Mean values ± SD (error bars) are shown. Lysozyme treatments resulting in significant differences ($p < 0.05$) compared to the same treatments without lysozyme are marked with an asterisk.

that the overexpressor strain contained about 7-fold-higher levels of Ivy in its periplasm. These results were confirmed by a silver-stained SDS-PAGE gel (fig. 2), where a band corresponding to the molecular weight of Ivy (15.0 kDa) was present in the periplasmic fractions of MG1655 wild-type and of the Ivy overexpressor MG1655 (pAA410) (highest intensity in the latter), while the same band was completely lacking in Ivy knock-out strain MG1655 *ivy*::Kan. The same pattern was observed on Coomassie-stained gels, but the Ivy band in the wild-type was only very weak (data not shown). Tandem mass spectrometry analysis identified the 15-kDa band as mature Ivy protein with high confidence by finding 92.2% amino acid sequence coverage. The band of 31.2 kDa, which is only present in MG1655 (pAA410) (fig. 2), most likely corresponds to β-lactamase encoded by the *bla* gene of the pAA410 plasmid, since the molecular weight matches and the same band was also seen in periplasmic cell fractions of *E. coli* MG1655 containing a plasmid with the same *bla* gene but lacking the *ivy* gene and the arabinose promoter (data not shown).

Since no other changes in periplasmic protein profile were observed, and since knock-out or overexpression of Ivy did not affect the growth of the *E. coli* strains or their morphology in phase contrast microscopy (data not shown), we can reasonably assume that overexpression or knock-out of Ivy does not have a major impact on cell viability and physiology. Consequently, the constructed strains can be used to assess the role of Ivy in cellular resistance to lysozyme. Lysozyme up to 10 mg/ml did not inhibit the growth in LB broth of any of the strains in the absence of an outer-membrane-permeabilizing treatment (data not shown). Therefore, in the next experiments, we tested the lysozyme sensitivity of the strains using either hydrostatic pressure or lactoferrin to permeabilize the outer membrane.

Role of Ivy in protection of *E. coli* MG1655 against lysozyme under high pressure

Suspensions of early stationary phase wild-type, *ivy* knock-out and *ivy* overexpression strains that had been induced with arabinose were treated with and without 1.0 or 10.0 µg/ml lysozyme at ambient pressure (3 h at 20 °C) and under high pressure (15 min, 250 MPa at 20 °C), and survivors were enumerated. At ambient pressure, none of the strains was significantly sensitive to lysozyme, indicating that the outer membrane was an effective lysozyme barrier in all strains under these conditions. High pressure alone caused only a low level of inactivation for all the strains (0.5–0.9 log units), but for 1.0 µg/ml lysozyme, it induced lysozyme sensitivity in an Ivy-dependent manner (fig. 3): the Ivy knock-out strain was strongly sensitized (2.6 log extra inactivation compared to high pressure alone), the wild-type strain partly (1.4 log units extra inactivation) and the Ivy overexpressor strain not at all. However, at a level of 10.0 µg/ml lysozyme, all three strains became equally sensitive to lysozyme under high pressure (data not shown). These results showed that the sensitivity of *E. coli* to lysozyme depended on the periplasmic Ivy content of the cells.

Role of Ivy in protection of *E. coli* MG1655 against lysozyme in the presence of lactoferrin

Suspensions of early stationary phase wild-type, *ivy* knock-out and *ivy* overexpression strains that had been induced with arabinose were treated with 3.0 mg/ml lactoferrin and/or 50 µg/ml lysozyme, and survivors were enumerated (fig. 4). The levels of inactivation achieved in this experiment were lower than in the high-pressure experiment, probably because lactoferrin is a weaker outer-membrane permeabilizer than high pressure, at least for our *E. coli* strains. That is also the reason why a higher lysozyme concentration was chosen in this experiment. Nevertheless, sensitization to lysozyme by lactoferrin was again Ivy dependent: strong sensitization occurred in the Ivy knock-out strain, intermediate sensitization in the wild-type strain and no sensitization in the Ivy over-expressor. Treatments with either of the antimicrobial proteins alone had little effect on any of the strains ($1.7 < N_0/N < 3.7$).

Contribution of Ivy in lysozyme resistance of *E. coli* LMM1010

E. coli LMM1010 is a mutant of *E. coli* MG1655 that was originally selected for high-pressure resistance [22], but which was later found to also have increased lysozyme resistance under high pressure [25], unlike two other pressure-resistant mutants that were isolated with the same approach. The finding that Ivy affects lysozyme resistance in *E. coli* now raised the question whether the lysozyme resistance of strain LMM1010 could be due to overexpression of Ivy. This was investigated by subjecting *E. coli* LMM1010 and its *ivy* knock-out mutant to treatments with high pressure and lysozyme (fig. 5), and with lactoferrin and lysozyme (fig. 6), in the same way as described above for MG1655 and its derivative strains. Strain LMM1010 was more resistant to high pressure than MG1655 and was not sensitized to lysozyme under high pressure as expected, even at 600 MPa. However, knock-out of Ivy did not render LMM1010 sensitive to lysozyme at 250 or 600 MPa. Similar results were obtained with lactoferrin as a permeabilizer: neither the parental strain LMM1010 nor its Ivy knock-out derivative were sensitized to lysozyme by lactoferrin.

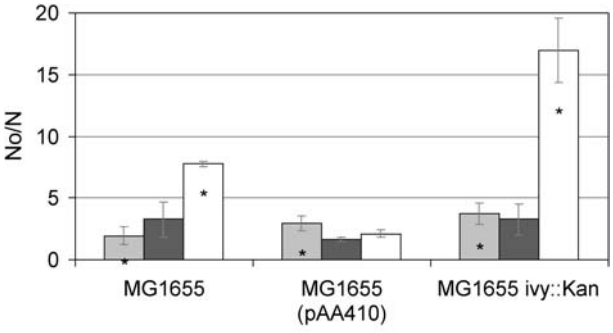

Figure 4. Inactivation (N_o/N) of *E. coli* MG1655, MG1655 *ivy*::Kan and MG1655 (pAA410) after 7 h of incubation with 50 µg/ml lysozyme (hatched bars), 3.0 mg/ml lactoferrin (black bars) and lysozyme and lactoferrin together (open bars). Mean values ± SD (error bars) are shown. Lysozyme treatments resulting in significant differences ($p < 0.05$) compared to the same treatments without lysozyme are marked with an asterisk.

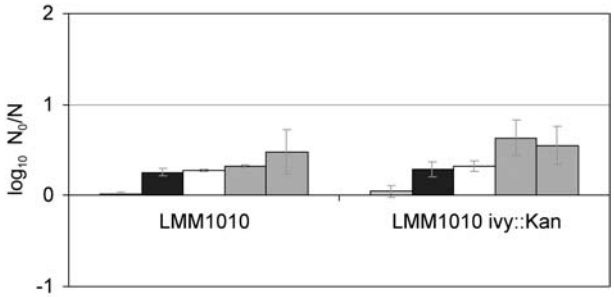

Figure 5. Inactivation ($\log_{10}N_0/N$) of *E. coli* LMM1010 and LMM1010 *ivy*::Kan in the presence of 1.0 µg/ml lysozyme at, from left to right, atmospheric pressure (3 h), 250 MPa (15 min) in the absence and in the presence of lysozyme, and at 600 MPa (15 min) in the absence and in the presence of lysozyme. Mean values ± SD (error bars) are shown. No significant differences ($p > 0.05$) were observed between treatments with and without lysozyme.

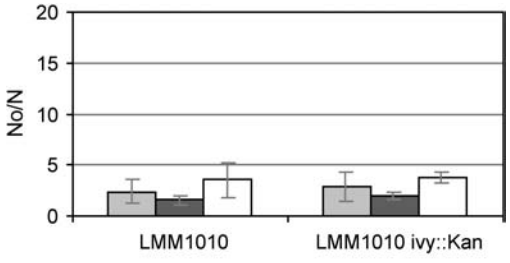

Figure 6. Inactivation (N_o/N) of *E. coli* LMM1010 and LMM1010 *ivy*::Kan after 7 h of incubation with 50 µg/ml lysozyme (hatched bars), 3.0 mg/ml lactoferrin (black bars) and both lysozyme and lactoferrin (open bars). Mean values ± SD (error bars) are shown. No significant differences ($p > 0.05$) were observed between treatments with and without lysozyme.

Discussion

The discovery that the *E. coli* orphan gene *ykfE* encodes a strong and specific lysozyme inhibitor [27] has raised speculation about the cellular and/or ecological role of this protein. In our study, we investigated the possible role of Ivy in the defense of *E.* coli against exogenous lysozyme. Since lysozymes are important components of the antibacterial defense systems of many plants and animals, a lysozyme inhibitor could enhance the survival of commensal or pathogenic bacteria in their host. If this were indeed the case, one would expect to find lysozyme inhibitors to be widely distributed in bacteria. However, a

BLAST search in the GenBank (http://www.ncbi.nlm.nih.gov/) and TIGR (http://www.tigr.org/) databases revealed putative Ivy orthologs only in a few other Gram-negative bacteria: *P. aeruginosa* PAO1 (two sequences with 47 and 44% similarity), *Burkholderia mallei* (45% similarity), *Yersinia pestis* KIM and CO92 (both 51% similarity), *Shigella flexneri* 2a (99% similarity), *Chromobacterium violaceum* ATCC12472 (44% similarity) and *E. coli* 0157:H7 and CFT073 (both 100% similarity). On the other hand, the limited distribution of Ivy does not necessarily imply that less well conserved lysozyme inhibitors are absent in other bacteria. Secreted proteinaceous lysozyme inhibitors, different from Ivy in molecular weight or composition, have indeed been reported earlier, but they have been poorly characterized: a putative lipoprotein was reported in *Bacillus subtilis* [38, 39], two proteins in *P. aeruginosa* [40] and a glycoprotein in *Enterobacter cloacae* [41]. We have constructed an *E. coli* MG1655 derivative that overexpresses Ivy from an arabinose-inducible promoter and one in which the *ivy* gene was knocked out by insertion of a Kan resistance cassette. These strains overproduced Ivy or failed to produce it, respectively, as shown by the inhibition of lysozyme activity by cellular extracts (fig. 1A) and by periplasmic fractions (fig. 1B) and by the levels of Ivy in periplasmic fractions (fig. 2), but their growth was otherwise unaffected (data not shown).

Using these Ivy knock-out and overexpressor strains, we were able to demonstrate that Ivy, at wild-type level, provides some protection against certain levels of lysozyme when the outer membrane is permeabilized by high pressure or by lactoferrin, and that an increased or decreased production of Ivy resulted in an increased and decreased lysozyme resistance, respectively (figs 3, 4). Although our study was conducted with hen egg white lysozyme, human lysozyme belongs to the same structural class of lysozymes called C-type lysozymes and was previously shown also to be inhibited by Ivy [27]. Therefore, our findings suggest the existence of a novel interaction mechanism that may play a role in many bacteria-host interactions. Although the functionality of Ivy in a real in vivo situation where *E. coli* interacts with a vertebrate host is not yet proven, the concentrations of lysozyme that we used (1 and 50 µg/ml for the high-pressure and lactoferrin experiments, respectively) were within the range of naturally occurring concentrations. For example, the concentration of human lysozyme ranges from 0.77 µg/ml in mixed respiratory secretions to 60 µg/ml in preterm human colostrum [42, 43]. Furthermore, although we had to use a higher than natural concentration of lactoferrin to accomplish outer membrane permeabilization, in vivo, multiple components are known to act together in a synergistic manner, such as lactoferrin, bactericidal permeability-inducing protein, cathelicidins and defensins [44]. With respect to the pathogens in which *ivy* orthologs have been identified, of interest will be to investigate whether these orthologs are functional and contribute to virulence.

Several lines of evidence suggest that lysozyme possesses a bactericidal mode of action that is independent of its enzymatic activity and that does not cause cell lysis. This non-enzymatic inactivation mechanism has been attributed to cytoplasmic membrane destabilization due to the cationic properties of lysozyme, resulting in cell leakage, or to the induction of autolysins [45–47]. Therefore, an interesting question to be resolved in future work is whether this non-enzymatic mode of action is also inhibited by Ivy. Although our results provide evidence for a role of Ivy in the lysozyme resistance of *E. coli*, other functions of Ivy cannot be ruled out at this point. One possible function could be the regulation of autolysin activity, because some bacterial autolysins have muramidase activity, and because some *E. coli* autolysins such as the lytic transglycosylases Slt70, MltC and MltD are structurally related to type C lysozymes [48]. However, in view of the important role of autolysins in cellular growth and division, one would expect loss of this control function by knock-out of the *ivy* gene to have a major effect on cell growth or morphology, and this was not the case (data not shown). On the other hand, if control of autolysin function is so important, the cell may dispose of redundant inhibitor systems, and this could explain the lack of a phenotype related to cell growth or division.

We also demonstrated that the lysozyme resistance of an MG1655 mutant with enhanced resistance to lysozyme under high pressure [25] is not due to overproduction of Ivy, because an Ivy knock-out derivative of this strain did not become lysozyme sensitive. This illustrates that Ivy is not the only factor that confers lysozyme resistance in *E. coli*. Alterations in outer-membrane composition, for example, may affect the passage of lysozyme through this barrier, and thus lysozyme sensitivity. This was demonstrated earlier for *Salmonella* Typhimurium rough and deep-rough mutants under high pressure [26].

In conclusion, the finding that Ivy confers lysozyme resistance in *E. coli* may be of broad ecological importance because it highlights Ivy, and lysozyme inhibitors in general, as a totally novel type of factor that can contribute to the fitness of bacterial populations when they are challenged with lysozymes produced by plants, animals or perhaps even bacteriophages. This finding will also be relevant in the development of applications that make use of the antibacterial properties of lysozyme.

Acknowledgements. B. M. and A. M. have postdoctoral fellowships from the Research Fund K. U. Leuven, and D. D. and A. A., doctoral fellowships from the Flemish Institute for the Promotion of Scientific Technological Research (IWT). This research was conducted in the framework of research project GOA/03/10 financed by the Research Fund K. U. Leuven. The authors wish to thank C. Abergel and J. M. Claverie for the Ivy knock-out *E. coli* TE2680 strain, J.

Robben from the Biomedical Research Institute (BIOMED) of the Limburgs Universitair Centrum for the mass spectrometry analysis and B. De Ketelaere (Laboratory for Agricultural Machinery and Processing, K.U.Leuven) for the statistical analysis.

1 Beintema J. J. and Terwisscha van Scheltinga A. C. (1996) Plant lysozymes. In: Lysozymes: Model Enzyme in Biochemistry and Biology, pp. 75–86. Jollès P. (ed.), Birkhaüser, Basel
2 Ellison R. T. and Giehl T. J. (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme. J. Clin. Invest. **88:** 1080–1091
3 Hancock R. E. W. and Scott M. G. (2000) The role of antimicrobial peptides in animal defense. Proc. Natl. Acad. Sci. USA **97:** 8856–8861
4 Ohno N. and Morrison D. C. (1989) Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. Eur. J. Biochem. **186:** 629–636
5 Taylor P. W. (1983) Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. Microbiol. Rev. **47:** 46–83
6 Rydman P. S. and Bamford D. H. (2002) Phage enzymes digest peptidoglycan to deliver DNA. ASM News **68:** 330–335
7 Young R. (1992) Bacteriophage lysis: mechanism and regulation. Microbiol. Rev. **56:** 430–481
8 Höltje J.-V. (1995) From growth to autolysis: the murein hydrolases of *Escherichia coli*. Arch. Microbiol. **164:** 243–254
9 Shockman G. D. and Höltje J.-V. (1994) Microbial peptidoglycan (murein) hydrolases. In: Bacterial Cell Wall: New Comprehensive Biochemistry, vol. 27, pp. 131–160, Ghuysen J. M. and Hakenbeck R. (eds), Elsevier, Amsterdam
10 Losso J. N., Nakai S. and Charter E. A. (2000) Lysozyme. In: Natural Food Antimicrobial Systems, pp. 185–210, Naidu A. S. (ed.), CRC Press LLC, Boca Raton, Fla
11 Proctor V. A. and Cunningham F. E. (1988) The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. **26:** 359–395
12 Masschalck B. and Michiels C. M. (2003) Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. **29:** 191–214
13 Hancock R. E. W. (1984) Alterations in outer membrane permeability. Annu. Rev. Microbiol. **38:** 237–294
14 Hancock R. E. W. (1997) Peptide antibiotics. Lancet. **349:** 418–422
15 Ibrahim H. R., Kato A. and Kobayashi K. (1991) Antimicrobial effects of lysozyme against gram-negative bacteria due to covalent binding of palmitic acid. J. Agric. Food Chem. **39:** 2077–2082
16 Ibrahim H. R., Kobayashi K. and Kato A. (1993) Length of hydrocarbon chain and antimicrobial action to gram-negative bacteria of fatty acylated lysozyme. J. Agric. Food Chem. **41:** 1164– 1168
17 Arima H., Ibrahim H. R., Kinoshita T. and Kato A. (1997) Bactericidal action of lysozymes attached with various sizes of hydrophobic peptides to the C-terminal using genetic modification. FEBS Lett. **415:** 114–118
18 Facon M. J. and Skura B. J. (1996) Antibacterial activity of lactoferricin, lysozyme and EDTA against *Salmonella enteritidis*. Int. Dairy J. **6:** 303–313
19 Payne K. D., Oliver S. P. and Davidson P. M. (1994). Comparison of EDTA and Apo-lactoferrin with lysozyme on the growth of foodborne pathogenic and spoilage bacteria. J. Food Prot. **57:** 62–65
20 Vaara M. (1992) Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:** 395–411
21 García-Graells C., Masschalck B. and Michiels C. (1999) Inactivation of *Escherichia coli* in milk by high hydrostatic pressure treatment in combination with antimicrobial peptides. J. Food Prot. **62:** 1248–1254
22 Hauben K., Wuytack E., Soontjens C. and Michiels C. (1996) High pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. J. Food Prot. **59:** 350–355
23 Masschalck B., Van Houdt R. and Michiels C. W. (2001) High pressure increases bacteriricidal activity and spectrum of lactoferrin, lactoferricin and nisin. Int. J. Food Microbiol. **64:** 325–332
24 Hauben K., Barlett D., Soontjes C., Cornelis K., Wuytack E. Y. and Michiels C. W. (1997) *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. Appl. Environ. Microbiol. **63:** 945–955
25 Masschalck B., Garcia-Graells C., Van Haver E. and Michiels C. W. (2000). Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin under high pressure. Innovat. Food Sci. New Emerg. Technol. **1:** 39–47
26 Masschalck B., Deckers D. and Michiels C. W. (2003) Sensitization of outer membrane mutants of *Salmonella typhimurium* and *Pseudomonas aeruginosa* to antimicrobial peptides under high pressure. J. Food Prot. **66:** 1360–1367
27 Monchois V., Abergel C., Sturgis J., Jeudy S. and Claverie J. M. (2001) *Escherichia coli ykfE* ORFan gene encodes a potent inhibitor of C-type lysozyme. J. Biol. Chem. **276:** 18437–18441
28 Guzman L.-Z., Belin D., Carson M. J. and Beckwith J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:** 4121–4130
29 Valdivia R. H. and Falkow S. (1996) Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22:** 367–378
30 Datsenko K. A. and Wanner B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:** 6640–6645
31 Miller J. H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
32 Weisner B. (1984) Lysozyme. In: Methods of Enzymatic Analysis, vol. 4, pp. 189–194, Bergmayer H. R. (ed.), Verlag Chemie, Weinheim
33 Leammli M. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
34 Heukeshoven J. and Dernick R. (1988) Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. Electrophoresis **9:** 28–32
35 Shevchenko A., Wilm M., Vorm O. and Mann M. (1996) Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem. **68:** 850–858
36 Kitamura Y. and Itoh T. (1987) Reaction volume of protonic ionization for buffering agents: prediction of pressure dependence of pH and pOH. J. Solut. Chem. **16:** 715–725
37 Neter J., Kutner M. H., Nachtsheim C. J. and Wasserman W. (1996) Applied Linear Statistical Models, Irwin, Boston
38 Murao S., Kato M., Wang S.-L., Hoshino M. and Arai M. (1990) Isolation and characterization of a novel hen egg white lysozyme inhibitor from *Bacillus subtilis* I-139. Agric. Biol. Chem. **54:** 1129–1136
39 Wang S.-L., Murao S. and Arai M. (1990) Some properties of hen egg white lysozyme inhibitor from *Bacillus subtilis* I-139. Agric. Biol. Chem. **54:** 2447–2448
40 Wang S.-L., Shieh S.-T. and Pai C.-S. (1995) Production, purification and characterization of two proteinaceous hen-egg-white lysozyme inhibitors from *Pseudomonas aeruginosa* M-1001. Proc. Natl. Sci. Council B Life Sci. **19:** 166–175
41 Wang S.-L., Pai C.-S. and Sieh S.-T. (1997) Production, purification and characterization of the hen egg-white lysozyme inhibitor from *Enterobacter cloacae* M-1002. J. Chin. Chem. Soc. **44:** 349–355

42 Thompson A. B., Bohling T., Payvandi F. and Rennard S. I. (1990) Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. J. Lab. Clin. Med. **115:** 148–158
43 Marthur N. B., Dwarkadas A. M., Sharma V. K., Saha K. and Jain N. (1990) Anti-infective factors in preterm human colostrum. Acta Paediatr. Scand. **79:** 1039–1044
44 Ganz T. and Weiss J. **(**1997) Antimicrobial peptides of phagocytes and epithelia. Semin. Hematol. **34:** 343–354
45 Laible N. J. and Germaine G. R. (1985) Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect. Immun. **48:** 720–728
46 Masschalck B., Deckers D. and Michiels C. W. (2002) Lytic and non-lytic mechanism of inactivation of gram-positive bacteria by lysozyme under atmospheric and high hydrostatic pressure. J. Food Prot. **65:** 1916–1923
47 Ibrahim H. R., Higashiguchi S., Sugimoto Y. and Aoki T. (1997) Role of divalent cations in the novel bactericidal activity of the partially unfolded lysozyme. J. Agric. Food Chem. **45:** 89–94
48 Dijkstra A. J. (1997) The Lytic Transglycosylase Family of *Escherichia coli*, Ph. D. thesis, University of Groningen, The Netherlands