Role of the Lysozyme Inhibitor Ivy in Growth or Survival of *Escherichia coli* and *Pseudomonas aeruginosa* Bacteria in Hen Egg White and in Human Saliva and Breast Milk[▽]

Daphne Deckers, Dietrich Vanlint, Lien Callewaert, Abram Aertsen, and Chris W. Michiels*

Laboratory of Food Microbiology and Leuven Food Science and Nutrition Research Center (LForCe),
Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, B-3001 Leuven, Belgium

Received 12 March 2008/Accepted 20 May 2008

Ivy is a lysozyme inhibitor that protects *Escherichia coli* against lysozyme-mediated cell wall hydrolysis when the outer membrane is permeabilized by mutation or by chemical or physical stress. In the current work, we have investigated whether Ivy is necessary for the survival or growth of *E. coli* MG1655 and *Pseudomonas aeruginosa* PAO1 in hen egg white and in human saliva and breast milk, which are naturally rich in lysozyme and in membrane-permeabilizing components. Wild-type *E. coli* was able to grow in saliva and breast milk but showed partial inactivation in egg white. The knockout of Ivy did not affect growth in breast milk but slightly increased sensitivity to egg white and caused hypersensitivity to saliva, resulting in the complete inactivation of 10^4 CFU ml⁻¹ of bacteria within less than 5 hours. The depletion of lysozyme from saliva completely restored the ability of the *ivy* mutant to grow like the parental strain. *P. aeruginosa*, in contrast, showed growth in all three substrates, which was not affected by the knockout of Ivy production. These results indicate that lysozyme inhibitors like Ivy promote bacterial survival or growth in particular lysozyme-rich secretions and suggest that they may promote the bacterial colonization of specific niches in the animal host.

Lysozymes are a heterogeneous group of enzymes that hydrolyze the β -(1,4)-glycosidic bond between the alternating *N*-acetylmuramic acid and *N*-acetylglucosamine residues in peptidoglycan, a heteropolysaccharide uniquely found in the cell walls of bacteria. Hydrolysis of this structural polymer results in rapid cell lysis in a hypo-osmotic environment. Lysozymes occur in all major taxa of eukaryotes and are divided into different types based on differences in catalytic, immunological, and structural characteristics. The major lysozyme type in vertebrates is c-type, with hen egg white lysozyme (HEWL) being the most well-known representative (Carbohydrate-Active Enzymes database; <http://www.cazy.org/>) (10). In animals and humans, lysozyme acts as a protective barrier against colonization and invasion by bacterial pathogens. The enzyme occurs in mammalian body fluids such as saliva, tears, serum, breast milk, and urine (20, 34) and is produced by epithelial cell layers of the respiratory and intestinal tract (15, 29, 31, 37) and by the lysosomal granules of macrophages and neutrophils (16). On gram-negative bacteria, lysozyme acts in concert with other antimicrobial compounds, such as lactoferrin, defensins, and cathelicidins, and with the complement system that disrupts the outer membrane and allows access of lysozyme to the peptidoglycan (2, 3, 13).

Bacteria, for their part, have developed mechanisms that confer lysozyme resistance. The shielding action of the outer membrane in gram-negative bacteria can be considered as

such, but a more specific mechanism is the production of chemical variants of peptidoglycan that are resistant to lysozyme. The O acetylation of the *N*-acetylmuramic acid C-6 hydroxyl group is a common modification, for example, in *Staphylococcus aureus*, that precludes the binding of lysozyme to peptidoglycan by sterical hindrance (4, 9). Bera et al. (4) observed that the gene encoding the O-acetyltransferase (*oatA*) is widely distributed, mainly among pathogenic strains, suggesting the importance of OatA as a virulence factor of these bacteria. The deacetylation of *N*-acetylglucosamine residues in *Bacillus* spp. and *Streptococcus pneumoniae* peptidoglycans is also a well-known modification that confers resistance to lysozyme and may be implicated in the virulence of these bacteria (35, 36, 38).

Besides these chemical modifications of the peptidoglycan structure itself, bacteria can also evade the bacteriolytic action of lysozyme by the production of lysozyme inhibitors. Although many inhibitors of a wide range of polysaccharide hydrolases have been discovered during recent years, the first specific proteinaceous inhibitor of lysozyme was only recently reported for *Escherichia coli* (30). This inhibitor was termed Ivy (inhibitor of vertebrate lysozyme) because of its specificity against vertebrate (c-type) lysozymes. Ivy homologs are found in a limited number of other proteobacteria (1), but recently we have identified a novel type of bacterial lysozyme inhibitor that is different from Ivy and that is more widely distributed among gram-negative bacteria (5). This finding suggests that lysozyme inhibitors are functionally well conserved in bacteria and may play a role in interactions with vertebrate hosts. It was already shown that knockout of the *ivy* gene increased the lysozyme sensitivity of *E. coli* considerably when the outer membrane was destabilized by high-pressure treatment or high doses of

* Corresponding author. Mailing address: Laboratory of Food Microbiology, Kasteelpark Arenberg 22, B-3001 Leuven, Belgium. Phone: 32-16-321578. Fax: 32-16-321960. E-mail: Chris.Michiels@biw.kuleuven.be.

[▽] Published ahead of print on 30 May 2008.

lactoferrin (11) or by mutation (1). However, the antibacterial efficacy of lysozyme in a natural environment depends on the presence of several other compounds which may act synergistically or antagonistically. Therefore, the objective of the present study was to investigate whether Ivy affects the persistence or growth of *E. coli* MG1655 and *Pseudomonas aeruginosa* PAO1 in three different lysozyme-rich fluids: hen egg white, human saliva, and human breast milk.

MATERIALS AND METHODS

Strains. *Escherichia coli* MG1655, *Pseudomonas aeruginosa* PAO1, and their corresponding Ivy knockout mutants, MG1655 *ivy::Kan* (11) and PAO1 *lux_68_C1* (further designated as PAO1 *ivy::Tet*) (26), respectively, were used throughout this study. Luria-Bertani (LB) agar master plates were inoculated from -80°C glycerol stocks and contained kanamycin ($50\text{ }\mu\text{g ml}^{-1}$) (Sigma-Aldrich, Bornem, Belgium) for MG1655 *ivy::Kan* and tetracycline ($20\text{ }\mu\text{g ml}^{-1}$) (Sigma-Aldrich) for PAO1 *ivy::Tet*. All liquid cultures were grown aerobically overnight at 37°C in LB broth with the appropriate antibiotics.

Cloning, expression, and purification of the *Pseudomonas aeruginosa* Ivy protein. The PCR product of the *ivy* open reading frame PA3902 of *P. aeruginosa* PAO1 (GeneID 878927; Entrez Gene; <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>), amplified using Platinum *Pfx* DNA polymerase (Invitrogen, Merelbeke, Belgium) with the primers *ivyF* (5'-CGTAGGATCCAACGGAGTATCCAGACTG-3') and *ivyR* (5'-CGTAGGATCCCTTCCAGTTCGGATCGCT-3') (Eurogentec, Seraing Belgium), was cut with BamHI (Roche Diagnostics Belgium, Vilvoorde, Belgium) and ligated into the pQE-EC vector (which was kindly donated by Kirsten Hertveldt of the Division of Gene Technology, Katholieke Universiteit Leuven, Belgium) to create an in-frame fusion with a C-terminal E-tag and His₆ tag. The resulting construct contains the *ivy* gene under control of the phage T5 promoter and two *lac* operator sequences and was designated pIvy_{pa}. This plasmid was transformed into the Ivy knockout strain of *E. coli* MG1655, and the resulting strain was designated as *E. coli* MG1655 *ivy::Kan*(pIvy_{pa}).

Cultures for recombinant Ivy_{pa} expression were grown overnight at 37°C in Superbroth medium composed of 35 g liter^{-1} tryptone, 20 g liter^{-1} yeast extract, 5 g liter^{-1} NaCl and containing ampicillin ($100\text{ }\mu\text{g ml}^{-1}$) (Sigma-Aldrich), diluted 1/100 in 200 ml fresh Superbroth medium plus ampicillin, and incubated further at 37°C . When the optical density at 600 nm (OD_{600}) reached 0.4, Ivy_{pa} production was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma-Aldrich) for 4 h at 37°C . Cells were harvested at an OD_{600} of approximately 0.9 by centrifugation ($3,800 \times g$ for 5 min) and resuspended in 5 ml of 50 mM sodium phosphate-300 mM NaCl buffer (pH 8.0) containing 10 mM imidazole. Cell extracts were made by sonication (two times for 5 min, amplitude 40%, pulse 5 s on/5 s off) (Vibra-Cell 600; Sonics & Materials SMC, Danbury, Connecticut), followed by centrifugation ($24,000 \times g$ for 10 min) to remove cell debris. Two milliliters of the cleared lysate was applied to an Ni-nitrilotriacetic acid spin column (Qiagen, Venlo, The Netherlands), which was subsequently washed three times with $600\text{ }\mu\text{l}$ of washing buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl, 20 mM imidazole), and eluted with $600\text{ }\mu\text{l}$ of elution buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl, 250 mM imidazole). After dialysis against sodium phosphate buffer (10 mM [pH 7.0]) was used, the purified protein was stored at -20°C , and a sample was analyzed by conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the procedure of Laemmli (24), with a 12% separating gel and a 4% stacking gel. The identity of Ivy_{pa} was confirmed by electrospray tandem mass spectrometry of a trypsin-digested sample taken from a Coomassie blue-stained SDS-PAGE gel.

Lysozyme inhibition assay of the *Pseudomonas aeruginosa* Ivy_{pa} protein. Freeze-dried *Micrococcus luteus* (formerly *Micrococcus lysodeikticus*) ATCC 4698 (Sigma-Aldrich) were resuspended at 0.5 mg ml^{-1} in $270\text{ }\mu\text{l}$ of the purified *E. coli* MG1655 *ivy::Kan*(pIvy_{pa}) preparations or mock preparations from *E. coli* *ivy::Kan* containing the empty pQE-EC vector obtained by following the same procedure as described above. Thirty microliters of $10\text{ }\mu\text{g ml}^{-1}$ hen egg white lysozyme ($66,000\text{ U mg}^{-1}$) (Fluka, Buchs, Switzerland) in sodium phosphate buffer (10 mM [pH 7.0]) or $30\text{ }\mu\text{l}$ sodium phosphate buffer (10 mM [pH 7.0]) as a control was added to $270\text{ }\mu\text{l}$ of the *M. luteus* cell suspension, and cell lysis was followed for 2 h at 25°C and measured as the decrease in OD_{600} using a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland).

Quantification of bacterial survival in egg white, saliva, and breast milk. Overnight cultures of *E. coli* MG1655, MG1655 *ivy::Kan*, *P. aeruginosa* PAO1, and PAO1 *ivy::Tet* were diluted 1/100 in fresh LB and further incubated for 5 h

at 37°C until the early stationary phase ($\pm 10^9\text{ CFU ml}^{-1}$). The cells were harvested by centrifugation ($3,800 \times g$ for 5 min) and resuspended in the same volume of 10 mM potassium phosphate buffer (pH 7.0).

Organic eggs purchased from a local supermarket (Colruyt, Belgium) were disinfected with 70% ethanol, dried in a laminar flow cabinet, and aseptically broken to separate the egg white. Egg white from 10 eggs was pooled, diluted by the addition of 25% (vol) 10 mM potassium phosphate buffer (pH 7.0), and homogenized for 30 s at 230 rpm in a Stomacher apparatus model 400 circulator (Led Techno, Eksel, Belgium). The dilution of egg white was necessary to allow homogeneous mixing with bacterial suspensions and to facilitate handling. Twenty milliliters of diluted egg white was then inoculated to a final concentration of 10^6 CFU ml^{-1} . After a 24-h incubation at 30°C , the surviving bacteria were enumerated by plating appropriate dilutions of the bacterial suspensions in 10 mM potassium phosphate buffer (pH 7.0) on LB agar plates. Colonies were allowed to develop for 24 h at 37°C . Inactivation was expressed as a viability reduction factor, N_0/N , where N_0 and N are the colony counts at the beginning of the experiment and after the overnight incubation at 30°C , respectively.

Whole, nonstimulated saliva was collected on ice from healthy volunteers in the laboratory. The saliva was clarified by centrifugation for 10 min at $12,000 \times g$, sterilized by filtration through a $0.20\text{-}\mu\text{m}$ filter, pooled, and stored in small amounts at -20°C until use. Because the wild-type strains of *E. coli* and *P. aeruginosa* were able to grow in saliva (see Results), early stationary-phase cells from the wild-type strains and their corresponding Ivy knockouts were diluted in saliva to a final concentration of only 10^4 CFU ml^{-1} . Their growth/inactivation were followed at 37°C by plating samples at regular intervals as described above.

Human milk samples were pooled from two donors in their third and fourth lactation month, respectively, and immediately stored at -20°C . The pooled milk samples were centrifuged for 10 min at $10,000 \times g$ to remove most of the fat and cells. The defatted milk was sterilized by filtration through a $0.20\text{-}\mu\text{m}$ filter and used immediately. As with those in saliva, the wild-type strains were able to grow in human milk. The inoculation level was therefore set at 10^4 CFU ml^{-1} , and the growth/inactivation of the bacteria at 37°C was recorded in the same way as described above.

Selective removal of lysozyme from saliva by cold adsorption with *Micrococcus luteus*. The selective removal of lysozyme from human saliva by cold adsorption with *M. luteus* ATCC 4698 was done following a method described by Germaine and Tellefson (19). A 1.5-ml *M. luteus* cell suspension (0.5 mg ml^{-1} in potassium phosphate buffer, 10 mM [pH 7.0]) was centrifuged at $3,800 \times g$ for 5 min, and the pellet was put on ice for 30 min and resuspended in 1 ml of ice-cold clarified saliva. After a 10-min incubation on ice, *M. luteus* cells were removed by centrifugation ($12,000 \times g$ for 10 min). This procedure was repeated until lysozyme activity in the saliva became undetectable.

Selective removal of lysozyme from saliva by affinity chromatography using Ivy as a ligand. Ivy was purified from *E. coli* MG1655 carrying the Ivy overexpression plasmid pAA410 by using affinity chromatography with HEWL as a ligand, as described by Callewaert et al. (6). The protein concentration was calculated based on the absorption at 280 nm using the predicted extinction coefficient of Ivy (www.expasy.org/tools/protparam.html). The purity of the samples was confirmed by conventional SDS-PAGE (24), with a 12% separating gel and a 4% stacking gel and Coomassie blue staining (Sigma-Aldrich).

About 8 mg of Ivy was immobilized on 2 ml *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow resin (Amersham Pharmacia Biotech, Uppsala, Sweden) following the instructions of the manufacturer, and the resin was packed in a column. Six milliliters of clarified saliva was applied to the column after it had been preequilibrated with 2 column volumes of 0.1 M Tris-hydroxymethyl aminomethane-HCl buffer (pH 7.0) and allowed to drain by gravity. The saliva flowing through the column was collected and was subjected to additional passages over the column after the column had been regenerated with 2 column volumes 0.1 M Tris-hydroxymethyl aminomethane buffer adjusted to pH 12.0 with NaOH to remove the bound lysozyme.

Assay for lysozyme activity in egg white, saliva, and breast milk. Freeze-dried *M. luteus* cells were suspended to a final OD_{600} of approximately 0.7 (0.5 mg ml^{-1}) in potassium phosphate buffer (10 mM [pH 7.0]). The cells were sedimented by centrifugation ($3,800 \times g$ for 5 min) and resuspended in the same volume of sample fluid. Cell lysis was followed for 2 h at 25°C and measured as the decrease in OD_{600} using a Bioscreen C microbiology reader. Lysozyme activity was expressed in units of activity, with one unit corresponding to a decrease in absorbance of 0.001/min at pH 7.0 and 25°C .

Statistical analysis. All data shown are averages $\pm$ standard deviations from three experiments with three independent cultures of each strain. *P* values were estimated using the Student *t* test with a two-sample unequal variance.

FIG. 1. Lysis by $1.0 \mu\text{g ml}^{-1}$ lysozyme (expressed as OD_{600} against time) of *M. luteus* cells (0.5 mg ml^{-1}) suspended in potassium phosphate buffer (O) in undiluted purified cell lysates of *E. coli* MG1655 *ivy::Kan* pQE-EC ($\times$) or in undiluted purified cell lysates of MG1655 *ivy::Kan*(pIvy_{Pa}) ($\square$). The control sample (dashed line) consisted of phosphate buffer instead of lysozyme solution added to *M. luteus*.

RESULTS

Purification and lysozyme inhibition activity measurements of Ivy_{Pa}. SDS-PAGE analysis of purified Ivy_{Pa} produced recombinantly in *E. coli*, showed a single protein band of the expected molecular weight after Coomassie blue staining (data not shown). Tandem mass spectrometry confirmed the identity of this protein as Ivy_{Pa}. The lysozyme inhibitory activity of Ivy_{Pa} was evaluated by an *M. luteus* lysis test and compared to the activity of the mock preparations from *E. coli* *ivy::Kan* containing the empty pQE-EC vector (Fig. 1). The rate of *M. luteus* lysis over 1 h in the absence of cell extract (ΔOD_{600} of 0.345) was reduced by 84% by Ivy_{Pa} (ΔOD_{600} of 0.060) and was not reduced by the preparations of *E. coli* *ivy::Kan* pQE-EC (ΔOD_{600} of 0.347). These data show that the Ivy_{Pa} protein has lysozyme inhibitory activity and is expressed in an active form in *E. coli*, confirming recent findings of Abergel et al. (1), who performed an extensive evolutionary and structural study of the Ivy protein family, including Ivy from *P. aeruginosa*.

Growth/inactivation in egg white. We determined the change in CFU ml^{-1} of *E. coli* MG1655, *P. aeruginosa* PAO1, and their corresponding Ivy knockout strains after incubation in egg white for 24 h. The lysozyme concentration of the egg white was typically around $50 \times 10^3 \text{ U ml}^{-1}$. The experiment was repeated with three different batches of egg white, using independent bacterial suspensions. While the numbers of *E. coli* decreased, those of *P. aeruginosa* slightly increased during the experiments (Table 1). The reduction (N_0/N) of *E. coli* numbers was two- to almost fourfold higher for the *ivy* deletion strain than for the wild type. In contrast, the plate counts of

FIG. 2. Growth curves of *E. coli* MG1655 ($\square$) and MG1655 *ivy::Kan* ($\times$) in saliva (unbroken line) and lysozyme-depleted saliva (dotted line) using cold adsorption with *M. luteus* (A) or affinity chromatography with purified Ivy (B). (C) Growth curves of *P. aeruginosa* PAO1 ($\square$) and PAO1 *ivy::Tet* ($\times$) in saliva (unbroken line) and lysozyme-depleted saliva (dotted line) using cold adsorption with *M. luteus*. Mean values and standard deviations of the results from three independent experiments are shown.

both the wild type and the Ivy knockout of *P. aeruginosa* increased slightly, indicating growth, but there was no significant difference between both strains.

Growth/inactivation in saliva. Preliminary experiments showed that the wild-type strains of *E. coli* and *P. aeruginosa* were both able to grow in clarified saliva. To investigate the role of Ivy in this ability to grow, inoculation was therefore done at a relatively low density, i.e., 10^4 CFU ml^{-1} . For *E. coli*, after 3 h, we already observed complete inactivation (>3 log units) of the Ivy knockout strain, whereas the wild-type counts had not changed during this period (Fig. 2A). Between 3 and 24 h of incubation, the counts of the Ivy knockout strain remained below the detection limit (20 CFU ml^{-1}), but the wild

TABLE 1. Inactivation of 10^6 CFU ml^{-1} of *E. coli* MG1655, *P. aeruginosa* PAO1, and their corresponding Ivy knockout strains after a 24-h incubation at 30°C in egg white^a

Expt.	Inactivation of <i>E. coli</i>		<i>P</i> value	Inactivation of <i>P. aeruginosa</i>		<i>P</i> value
	Wild type	Ivy knockout		Wild type	Ivy knockout	
1	32.1 ± 3.8	85.2 ± 14.9	0.01	0.08 ± 0.12	0.07 ± 0.07	0.98
2	50.2 ± 20.8	100.3 ± 22.3	0.05	0.33 ± 0.06	0.52 ± 0.18	0.15
3	10.2 ± 2.4	36.4 ± 8.9	0.008	0.14 ± 0.05	0.12 ± 0.03	0.64

^a Mean N_0/N values $\pm$ standard deviations of the results from three independent experiments using three independent cultures and different batches of egg white are shown. *P* values express the statistical significance of differences between wild-type and Ivy mutant strains.

FIG. 3. Lysis (expressed as the decrease of OD₆₀₀ in time) was determined for a *M. luteus* cell suspension (0.5 mg ml⁻¹) in saliva (○) or in lysozyme-depleted salivary supernatants after one (□), two (×), three (◇), or four (△) cycles of cold adsorption with *M. luteus* (A) and in lysozyme-depleted salivary supernatants after one (□), two (×), three (◇), or four (△) cycles of affinity chromatography with purified Ivy (B). The control sample (unbroken line) consisted of *M. luteus* suspension in potassium phosphate buffer (10 mM [pH 7.0]). Representative data of three independent experiments are shown.

type reached 10⁷ CFU ml⁻¹ after 24 h, indicating that Ivy is indispensable for the survival and growth of *E. coli* in saliva.

To more specifically demonstrate that the difference in survival between the wild type and the Ivy knockout of *E. coli* in saliva is due to the lysozyme sensitivity of the latter strain and not to adventitious sensitivity to another component in saliva, we repeated the same experiment with lysozyme-depleted saliva. We first used a relatively simple method to remove lysozyme from saliva based on the adsorption of lysozyme to *M. luteus* cells. Two cycles of adsorption were sufficient to reduce the lysozyme activity (466 U ml⁻¹) to undetectable levels (<6.0 U ml⁻¹) (Fig. 3A). Growth of the wild-type *E. coli* strain was strongly enhanced in this lysozyme-depleted saliva, and interestingly, the Ivy knockout strain grew equally as well as the wild type, both strains reaching 10⁶ CFU ml⁻¹ in 6 h (Fig. 2A). Adsorption on *M. luteus* was reported to cause a small change in the total protein content of saliva (19) and may therefore have removed components other than lysozyme. To more selectively remove lysozyme, we used affinity chromatography using immobilized pure Ivy protein. The passage of saliva over this affinity matrix gradually removed lysozyme, and after 4 cycles, all detectable lysozyme activity had disappeared (Fig. 3B). The challenge of the wild-type and Ivy-deficient *E. coli* in the lysozyme-depleted saliva obtained in this way yielded results comparable to when lysozyme was depleted by *M. luteus* adsorption (Fig. 2B).

Similar experiments were performed with *P. aeruginosa*

FIG. 4. Growth curves of *E. coli* MG1655 (□) and MG1655 *ivy::Kan* (×) (A) and of *P. aeruginosa* PAO1 (□) and PAO1 *ivy::Tet* (×) (B) in breast milk. Mean values and standard deviations of the results from three independent experiments are shown.

PAO1 and PAO1 *ivy::Tet*. Both the wild type and the Ivy knockout strain showed an increase in plate count of 1 log unit during the first 5 h of incubation in saliva and even reached 10⁸ CFU ml⁻¹ after 24 h (Fig. 2C). In addition, no differences were observed in the growth of either *P. aeruginosa* strain in lysozyme-depleted saliva compared to those in undepleted saliva, indicating that *P. aeruginosa* is not affected by lysozyme in saliva (Fig. 2C).

Growth/inactivation in breast milk. The lysozyme content of the (defatted) breast milk was 11 × 10³ U ml⁻¹. Both *E. coli* and *P. aeruginosa* reached about 10⁸ CFU ml⁻¹ in breast milk after 24 h, and inactivation of the *ivy* gene had no influence on growth (Fig. 4). Remarkably, both the wild-type and the Ivy-deficient *E. coli* strains showed a 1-log decrease in counts during the first hours of the experiment, but growth commenced after about 3 h. In *P. aeruginosa*, growth was initiated after a lag phase of about 4 h, and there was also no difference between the wild type and the Ivy knockout strain.

DISCUSSION

In this work, we investigated whether the lysozyme inhibitor Ivy can help bacteria to colonize an animal host by studying the effect of Ivy production on the survival or growth of *E. coli* and *P. aeruginosa* in human milk and saliva and in hen egg white, all of which are known to contain significant amounts of lysozyme. Ivy was the first highly specific bacterial lysozyme inhibitor to be discovered (30), but the recent description of an additional family of lysozyme inhibitors suggests that these molecules are functionally well conserved among gram-negative bacteria and may have an important function in bacteria-host interactions (5).

In egg white, wild-type *E. coli* was partially inactivated after

24 h, but the production of Ivy significantly enhanced the survival capacity. Although the effect of Ivy was not very large, this finding indicates that inactivation of *E. coli* in egg white is at least partly due to the action of lysozyme. Since *E. coli* MG1655 is not normally sensitive to HEWL, it can be inferred that egg white contains accessory components that sensitize the bacteria for lysozyme by permeabilization of the outer membrane. One of the components that may be responsible for this is ovotransferrin (13, 14, 22). As opposed to *E. coli*, *P. aeruginosa* was able to grow in egg white, a feature that may be related to the fact that this species and some related fluorescent pseudomonads are common spoilage organisms of fresh eggs. However, this ability was not due to the production of Ivy because an Ivy knockout mutant of this strain grew equally as well as the wild-type strain. Therefore, lysozyme does not seem to affect *P. aeruginosa* in egg white, possibly because the outer membrane of *P. aeruginosa* is not permeabilized in egg white and does not allow the passage of lysozyme. An alternative explanation is the existence of a second lysozyme inhibitor in *P. aeruginosa*, which was recently demonstrated, and it will be interesting to further investigate the functional redundancy of these inhibitors (5).

Salivary lysozyme is known to be important to oral health. Although oral colonization by gram-negative bacteria does not occur readily in healthy individuals, it is quite common when the cleansing effect of saliva production is blocked, such as in patients undergoing mechanical ventilation (7) and in tube-fed patients (25) but also in the elderly, for which the mechanical clearance is often reduced (32). A shift to a gram-negative oral microflora can lead to progressive oropharyngeal and intestinal colonization with gram-negative pathogens, including *E. coli*, *P. aeruginosa*, or *Klebsiella pneumoniae*, and may contribute to the development of pneumonia and heart disease (12, 17, 18, 23). Moreover, nosocomial pneumonia is considered to be one of the most common causes of mortality in mechanically ventilated patients (7). Therefore, we evaluated the role of Ivy on the persistence of *E. coli* and *P. aeruginosa* in saliva. Inactivation of *ivy* in *E. coli* resulted in the rapid and complete eradication of the bacteria in saliva, while the parental Ivy-producing strain was able to grow. This dramatic effect of Ivy was much stronger than the effect seen in egg white, in spite of the fact that the lysozyme concentration in saliva was about 100-fold lower than it was in egg white. The removal of lysozyme from saliva by two different methods did not only enhance the growth of the wild-type strains but fully restored a wild-type growth rate in the Ivy-deficient *E. coli*. In the same context, Shelburne et al. (33) discovered that the virulence factor Sic (streptococcal inhibitor of complement), which also has lysozyme inhibitory activity, is essential for the growth of group A streptococci in human saliva. These bacteria cause a wide range of diseases in humans, and their major site of entry into the human body is the oropharynx. However, the positive effect of Sic on the growth of these bacteria in saliva could not be unequivocally ascribed to lysozyme inhibition, since Sic is known to bind also to other antimicrobial components present in saliva, such as β -defensins. The specific removal of lysozyme from saliva by means of Ivy affinity chromatography as described in this work would be an appropriate approach to provide more detailed insight into the molecular strategies used by group A streptococci and other bacteria to colonize the

oral cavity and the oropharynx. Given the high specificity of Ivy for lysozyme, this technique is expected to remove lysozyme much more selectively than the conventional adsorption of lysozyme by *Micrococcus luteus*. *P. aeruginosa* was also able to grow in saliva but, unlike *E. coli*, did not require Ivy since wild-type and *ivy* mutant strains grew equally well. Furthermore, lysozyme depletion did not enhance growth of either strain. The behavior of *P. aeruginosa* in saliva is thus similar to its behavior in egg white.

In (clarified) breast milk, both *E. coli* and *P. aeruginosa* showed growth, and there was no effect of the knockout of Ivy production. The breast milk contained about 20 times more lysozyme than the saliva (11,000 U ml⁻¹ compared to 466 U ml⁻¹). Furthermore, breast milk also contains much more lactoferrin than saliva, typical levels being 1 to 2 g liter⁻¹ and 10 mg liter⁻¹, respectively (27). Earlier experiments performed with bacterial suspensions in buffer demonstrated that mg liter⁻¹ concentrations of lactoferrin are sufficient to permeabilize the outer membrane of *E. coli* MG1655 for lysozyme and to induce lysozyme-mediated killing at lysozyme concentrations as low as 50 mg liter⁻¹ (11). The absence of any killing of both the wild type and the Ivy knockout *E. coli* strain in breast milk in the current work therefore illustrates that the lactoferrin-lysozyme cooperative interaction strongly depends on the environment and highlights the importance of studies in real matrices. The combined activity of lactoferrin and lysozyme was demonstrated to be suppressed by bivalent cations such as calcium and by osmolarities exceeding 100 mosmol liter⁻¹ (13). The large calcium content and osmolarity of breast milk (approximately 250 mg liter⁻¹ and 300 mosmol liter⁻¹, respectively) compared to that of saliva (approximately 50 mg liter⁻¹ and 38 mosmol liter⁻¹) may therefore explain the lack of anti-gram-negative activity in breast milk. Although lysozyme had no direct antibacterial activity against the tested bacteria in breast milk, this does not exclude an antibacterial effect on other bacteria, for example, gram-positive bacteria. In addition, breast milk lysozyme may modulate the gut microflora, either directly or after being processed by pepsin in the stomach into various antibacterial peptides (8, 21, 28).

In contrast to *E. coli*, neither a *P. aeruginosa* wild type nor its Ivy-deficient mutant was sensitive to egg white, human saliva, or breast milk, indicating that the outer membrane of this organism is not permeabilized in these environments. Therefore, it was not possible to unmask the phenotypic effects of Ivy in this organism. However, in view of the strongly different effects of saliva, breast milk, and egg white on *E. coli* seen in this work, it would be interesting to conduct additional tests with *P. aeruginosa* in some other lysozyme-rich fluids with relevance to typical *P. aeruginosa* infections, such as tears, blister fluids, or exudates of skin burns or (cystic fibrosis) lungs.

In conclusion, we demonstrated that the lysozyme inhibitor Ivy is required for the ability of some bacteria to survive and/or grow in lysozyme-rich fluids of vertebrate animals. Because lysozymes are widely distributed components of the innate defense of animals against bacterial invaders, this work supports the notion that Ivy and bacterial lysozyme inhibitors in general have evolved as virulence factors of pathogenic bacteria or colonization factors of commensal bacteria which provide protection against host lysozymes. It will be interesting to further investigate the role of Ivy in bacterial pathogenesis and

to search for additional bacterial lysozyme inhibitors. Furthermore, this work opens perspectives for the development of a new type of antibacterial therapeutic that binds to and neutralizes lysozyme inhibitors, thereby safeguarding the antibacterial activity of lysozyme. Additionally, this work illustrates how bacterial mutants defective in lysozyme inhibitor production can be used elegantly to improve our understanding of the role of lysozyme in antibacterial defense in vivo. This approach for the first time allows us to distinguish the effects of lysozyme from those of other antibacterial compounds.

ACKNOWLEDGMENTS

D.D. and L.C. have a doctoral fellowship from the Flemish Institute for the Promotion of Scientific Technological Research (IWT). A.A. is a postdoctoral fellow of the Research Foundation (FWO-Vlaanderen, Belgium). This project was conducted in the framework of research projects GOA/03/10 and G.0308.05, funded by the K.U. Leuven Research Fund and the Research Foundation Flanders (FWO-Vlaanderen), respectively.

We thank J. Robben from the Biomedical Research Institute (BIOMED) of the Limburgs Universitair Centrum for the mass spectrometry analysis.

REFERENCES

- Abergel, C., V. Monchois, D. Byrbe, S. Chenivresse, F. Lembo, J.-C. Lazzeroni, and J.-M. Claverie. 2007. Structure and evolution of the Ivy protein family, unexpected lysozyme inhibitors in Gram-negative bacteria. *Proc. Natl. Acad. Sci. USA* **104**:6394–6399.
- Bals, R., X. Wang, Z. Wu, T. Freemann, V. Bafna, M. Zasloff, and J. M. Wilson. 1998. Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J. Clin. Invest.* **102**:874–880.
- Bals, R., X. Wang, M. Zasloff, and J. M. Wilson. 1998. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. USA* **95**:9541–9546.
- Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Götz. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.* **55**:778–787.
- Callewaert, L., A. Aertsen, D. Deckers, K. G. A. Vanoirbeek, L. Vanderkelen, J. M. Van Herreweghe, B. Masschalck, D. Nakimbugwe, J. Robben, and C. W. Michiels. 2008. A new family of lysozyme inhibitors contributing to lysozyme tolerance in gram-negative bacteria. *PLoS Pathog.* **4**:e1000019.
- Callewaert, L., B. Masschalck, D. Deckers, D. Nakimbugwe, M. Atanassova, B. Aertsen, and C. W. Michiels. 2005. Purification of Ivy, a lysozyme inhibitor from *Escherichia coli*, and characterisation of its specificity for various lysozymes. *Enzyme Microb. Technol.* **37**:205–211.
- Cardenosa Cendrero, J. A., J. Sole-Violan, A. B. Benitez, J. N. Catalán, J. A. Fernández, P. S. Santana, and F. Rodríguez de Castro. 1999. Role of different routes of tracheal colonization in the development of pneumonia in patients receiving mechanical ventilation. *Chest* **116**:462–470.
- Chierici, R., S. Fanaro, D. Saccomandi, and V. Vigi. 2003. Advances in the modulation of microbial ecology of the gut in early infancy. *Acta Paediatr. Suppl.* **91**:56–63.
- Clarke, A., and C. Dupont. 1992. *O*-acetylated peptidoglycan: its occurrence, pathological significance, and biosynthesis. *Can. J. Microbiol.* **38**:85–91.
- Coutinho, P. M., and B. Henrissat. 1999. Carbohydrate-active enzymes: an integrated database approach, p. 3–12. In H. J. Gilbert, G. Davies, B. Henrissat, and B. Svensson (ed.), *Recent advances in carbohydrate bioengineering*. The Royal Society of Chemistry, Cambridge, United Kingdom.
- Deckers, D., B. Masschalck, A. Aertsen, L. Callewaert, C. G. M. Van Tiggele, M. Atanassova, and C. W. Michiels. 2004. Periplasmic lysozyme inhibitor contributes to lysozyme resistance in *Escherichia coli*. *Cell. Mol. Life Sci.* **61**:1229–1237.
- de Jonge, E., M. J. Schultz, L. Spanjaard, P. M. M. Bossuyt, M. B. Vroom, J. Dankert, and J. Kesecioglu. 2003. Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. *Lancet* **362**:1011–1016.
- Ellison, R. T., III, and T. J. Giehl. 1991. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest.* **88**:1080–1091.
- Ellison, R. T., III, F. M. LaForce, T. Giehl, D. S. Boose, and B. E. Dunn. 1990. Lactoferrin and transferrin damage of the gram-negative outer membrane is modulated by Ca^{2+} and Mg^{2+} . *J. Gen. Microbiol.* **136**:1437–1446.
- Franken, C., C. J. M. Meijer, and J. H. Dijkman. 1989. Tissue distribution of antileukoprotease and lysozyme in humans. *J. Histochem. Cytochem.* **37**:493–498.
- Ganz, T., and J. Weiss. 1997. Antimicrobial peptides of phagocytes and epithelia. *Semin. Hematol.* **34**:343–354.
- Garcia, R. 2005. A review of the possible role of oral and dental colonization on the occurrence of health care-associated pneumonia: underappreciated risk and a call for interventions. *Am. J. Infect. Control* **33**:527–541.
- Gaynes, R., J. R. Edwards, and National Nosocomial Infections Surveillance System. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* **41**:848–854.
- Germaine, G. R., and L. M. Tellefson. 1979. Simple and rapid procedure for the selective removal of lysozyme from human saliva. *Infect. Immun.* **26**:991–995.
- Grossowicz, N., and M. Ariel. 1983. Methods for determination of lysozyme activity. *Methods Biochem. Anal.* **29**:435–446.
- Ibrahim, H. R., D. Inazaki, A. Abdou, T. Aoki, and M. Kim. 2005. Processing of lysozyme at distinct loops by pepsin: a novel action for generating multiple antimicrobial peptide motifs in the newborn stomach. *Biochim. Biophys. Acta* **1726**:102–114.
- Ibrahim, H. R., Y. Sugimoto, and T. Aoki. 2000. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim. Biophys. Acta* **1523**:196–205.
- Johanson, W. G., A. K. Pierce, and J. P. Sanford. 1969. Changing pharyngeal bacterial flora of hospitalized patients. Emergence of gram-negative bacilli. *N. Engl. J. Med.* **281**:1137–1140.
- Laemmli, M. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Leibovitz, A., M. Dan, J. Zinger, Y. Carmeli, B. Hahot, and R. Segal. 2003. *Pseudomonas aeruginosa* and the oropharyngeal ecosystem of tube-fed patients. *Emerg. Infect. Dis.* **9**:956–959.
- Levenza, S., R. K. Falsafi, G. Winsor, W. J. Gooderham, J. B. McPhee, F. S. Brinkman, and R. E. W. Hancock. 2005. Construction of a mini-Tn5-*luxC-DABE* mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. *Genome Res.* **15**:583–589.
- Lovelady, C. A., C. P. Hunter, and C. Geigerman. 2003. Effect of exercise on immunologic factors in breast milk. *Pediatrics* **111**:148–152.
- Maga, A. E., R. L. Walker, G. B. Anderson, and J. D. Murray. 2006. Consumption of milk from transgenic goats expressing human lysozyme in the mammary gland results in the modulation of intestinal microflora. *Transgenic Res.* **15**:515–519.
- Mason, D. Y., and C. R. Taylor. 1975. The distribution of muramidase (lysozyme) in human tissues. *J. Clin. Pathol.* **28**:124–132.
- Monchois, V., C. Aberel, J. Sturgis, S. Jedy, and J. M. Claverie. 2001. *Escherichia coli* *ykfE* ORF gene encodes a potent inhibitor of C-type lysozyme. *J. Biol. Chem.* **276**:18437–18441.
- Rogan, M. P., P. Geraghty, C. M. Greene, S. J. O'Neill, C. C. Taggart, and N. G. McElvaney. 2006. Antimicrobial proteins and polypeptides in pulmonary innate defence. *Respir. Res.* **7**:29–39.
- Senpuku, H., A. Sogame, E. Inoshita, Y. Tsuha, H. Miyazaki, and N. Hanada. 2003. Systemic diseases in association with microbial species in oral biofilm from elderly requiring care. *Gerontology* **49**:301–309.
- Shelburne, S. A., III, C. Ganville, M. Tokuyama, I. Sitkiewics, P. Patel, and J. M. Musser. 2005. Growth characteristics of and virulence factor production by group A *Streptococcus* during cultivation in human saliva. *Infect. Immun.* **73**:4723–4731.
- Taylor, P. W. 1983. Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol. Rev.* **47**:46–83.
- Vollmer, W., and A. Tomasz. 2002. Peptidoglycan *N*-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. *Infect. Immun.* **70**:7176–7178.
- Vollmer, W., and A. Tomasz. 2000. The *pgdA* gene encodes for a peptidoglycan *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. *J. Biol. Chem.* **275**:20496–20501.
- Wehkamp, J., H. Chu, B. Shen, R. W. Feathers, R. J. Kays, S. K. Lee, and C. L. Bevins. 2006. Paneth cell antimicrobial peptides: topographical distribution and quantification in human gastrointestinal tissues. *FEBS Lett.* **580**:5344–5350.
- Zipperle, G. F., J. W. Ezzell, and R. J. Doyle. 1984. Glucosamine substitution and muramidase susceptibility in *Bacillus anthracis*. *Can. J. Microbiol.* **30**:553–559.