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Role of YadA in Resistance to Killing of *Yersinia enterocolitica* by Antimicrobial Polypeptides of Human Granulocytes

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The virulence plasmid pYVe of *Yersinia enterocolitica* codes for the production of the outer membrane protein YadA and the secretion of several proteins, called Yops, which may protect this bacterium against killing by human granulocytes. Granulocytes kill ingested microorganisms by oxygen-dependent and oxygen-independent mechanisms, the latter including antimicrobial polypeptides. The aim of this study was to determine whether virulent (pYVe$^+$) *Y. enterocolitica* and plasmid-cured avirulent (pYVe$^-$) *Y. enterocolitica* differ in susceptibility to antimicrobial polypeptides extracted from granules of human granulocytes. The acetic acid granule extract contained several polypeptides with antimicrobial activity against *Y. enterocolitica* as determined by gel overlay and radial diffusion assays. Two of these polypeptides were identified as lysozyme and defensins. pYVe$^+$ *Y. enterocolitica* was less susceptible than pYVe$^-$ *Y. enterocolitica* to the antimicrobial activity of granule extract, lysozyme, and defensins as determined in a suspension assay, which indicated that the pYVe plasmid mediates a reduced susceptibility to these polypeptides. The role of YadA in the resistance to antimicrobial polypeptides was analyzed by using mutants of *Y. enterocolitica* that specifically lack or express YadA. The results demonstrated that YadA conferred resistance to the killing of *Y. enterocolitica* by the granule extract. Together, these results indicate that the plasmid-encoded factor YadA contributes to the resistance of *Y. enterocolitica* to the killing by antimicrobial polypeptides of human granulocytes.

Opsonized virulent *Yersinia enterocolitica* strains resist phagocytosis and killing by human granulocytes, whereas avirulent strains are readily phagocytosed and killed (2, 24, 38). The ability of virulent *Y. enterocolitica* to avoid mammalian host defense mechanisms is correlated with the presence of a 70-kb plasmid, designated pYVe (plasmid involved in yersinial virulence) (for reviews, see references 4 and 29). The pYVe plasmid codes for several thermodependent properties, including calcium dependence for growth, the production of at least two outer membrane proteins called *Yersinia* adhesin A (YadA) and *Yersinia* lipoprotein A (YlpA), and the secretion of 11 proteins, referred to as Yops (6).

The killing of microorganisms by granulocytes is mediated by oxygen-dependent and oxygen-independent mechanisms (9). Oxygen-dependent killing is mediated by reactive oxygen intermediates, which are produced upon contact with the microorganisms. Nonoxidative antimicrobial mechanisms include acidification of the phagosome, deprivation of nutrients, and killing by antimicrobial polypeptides. Antimicrobial polypeptides present in azurophilic granules of human granulocytes include bactericidal/permeability-increasing protein, cathepsin G, elastase, proteinase 3, azurocidin, lysozyme, and defensins. Other granules, called specific granules, contain lactoferrin and lysozyme. The antimicrobial polypeptides are released into the phagolysosome through fusion of cytoplasmic granules with the phagosome. During this process, these polypeptides are also released extracellularly.

The mechanisms by which virulent *Y. enterocolitica* is protected from killing by human granulocytes are not well known. Apart from the plasmid-encoded inhibition of phagocytosis of *Y. enterocolitica* by human granulocytes (2, 24, 38), plasmid-encoded differences in susceptibility to the oxidative and nonoxidative killing by human granulocytes may also exist. There are indications that the oxidative burst of human granulocytes, measured by the chemiluminescence response to *Y. enterocolitica*, is inhibited by plasmid-bearing yersiniae (2, 25).

We wanted to gain insight into the susceptibility of *Y. enterocolitica* to the nonoxidative killing by human granulocytes. Therefore, we studied whether granules of human granulocytes contain polypeptides with antimicrobial activity against *Y. enterocolitica*, quantified differences in the antimicrobial activities of these polypeptides between the virulent and plasmid-cured avirulent isogenic strains of *Y. enterocolitica*, and determined whether plasmid-encoded factors are responsible for the observed differences.

MATERIALS AND METHODS

Microorganisms and growth conditions. Studies were carried out with the virulent strain (pYVe$^+$) and the isogenic plasmid-cured avirulent (pYVe$^-$) strain of *Y. enterocolitica* W22703, which is a restriction mutant (Res$^-$ Mod$^+$) of the O:9 strain W227. In addition, various mutants of *Y. enterocolitica* W22703 (kindly provided by G. Cornelis, Microbial Pathogenesis Unit, Université Catholique de Louvain, Brussels, Belgium) were used (Table 1). All strains were stored in 35% (vol/vol) glycerol at −70°C. Every 2 weeks, they were plated onto blood agar and stored at 4°C. Bacteria were brought to the stationary growth phase by overnight incubation in 50 ml of brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) at 25°C. *Y. enterocolitica* (10^8 CFU) from an overnight culture was inoculated into 25 ml of fresh brain heart infusion broth supplemented with 20 mM sodium oxalate and incubated with shaking for 180 min at 37°C to induce the expression of the *yop* regulon (14). Microorganisms were harvested by centrifugation at 1,200 × *g* for 10 min at 20°C, washed once with 10 mM sodium phosphate buffer (NaPB; pH 7.4), resuspended in 5 ml of NaPB, and stored at 4°C until use. The bacterial suspension was adjusted to contain the indicated number of CFU per milliliter, as determined by measuring the optical density at 620 nm by spectrophotometry (optical density of 0.2 represents 5×10^7 CFU/ml). The presence or absence of YadA on the outer membranes of cells of the various strains was checked by immunofluorescence after incubation of the bacterial suspension with rabbit anti-YadA antiserum, which was obtained by immunization with electroeluted YadA (kindly provided by J. Heesemann, Institut für Hygiene und Mikrobiologie der Universität Würzburg, Würzburg, Germany) (13, 33) followed by fluorescein isothiocyanate-labelled swine anti-rabbit immunoglobulin G (Nordic Immunological Laborato-

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TABLE 1. *Y. enterocolitica* W22703 mutants used

Plasmid carried by strain	Description	Phenotype		Reference or source
		YadA	Yops	
None (pYVe$^-$)	Plasmid-cured strain	−	−	
pYV227 (pYVe$^+$)	Virulence plasmid	+	+	5
pBC7 (pYVe$^+$)	pYV227*yadA*::pBC9	−	+	3
pMS12 (pYVe$^-$)	pSelect-1, P_{tac}-*yadA*	+	−	M. P. Sory and G. Cornelis

ries, Tilburg, The Netherlands). YadA was not expressed by either pYVe$^-$ *Y. enterocolitica* or *Y. enterocolitica* W22703(pBC7) but was present on all other mutant strains (data not shown).

Preparation of acetic acid granule extract. Granulocytes were isolated from buffy coat blood (Red Cross Bloodbank, Leiden, The Netherlands) from healthy donors, using Ficoll-Isopaque (ρ = 1.077 g/ml; Pharmacia, Uppsala, Sweden), dextran sedimentation with Plasmasteril (Fresenius AG, Bad Homburg, Germany), and hypotonic lysis in distilled water to remove residual erythrocytes. The cells were resuspended in sucrose buffer (0.34 M sucrose, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 10 mM NaPB [pH 7.4]) at a concentration of 10^8/ml and disrupted by nitrogen cavitation for 20 min at 350 lb/in^2 at 4°C. Next, the suspension was collected and centrifuged at 200 × *g* for 10 min at 4°C to sediment intact cells, nuclei, and cellular debris. The supernatant was removed and centrifuged at 27,000 × *g* for 20 min at 4°C. The granule-rich pellet was recovered, suspended in 5% (vol/vol) acetic acid, sonicated on ice, and extracted overnight in 5% (vol/vol) acetic acid under slow rotation at 4°C (7). The acid-insoluble residue was removed by centrifugation at 27,000 × *g* for 20 min at 4°C, and the supernatant was dialyzed overnight in Spectrapor 3 tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) against 5% (vol/vol) acetic acid. The protein content of the acetic acid granule extract (referred to herein as granule extract) was measured by the bicinchoninic acid (BCA) method (Pierce, Rockford, Ill.), using bovine serum albumin as the standard (concentration range, 50 to 250 μg of protein per ml).

Fractionation of granule extract. The granule extract of 2×10^9 granulocytes was fractionated by gel filtration on a column of Bio-Gel P-60 (2.5 by 90 cm; Bio-Rad Laboratories, Richmond, Calif.) that was equilibrated in 5% (vol/vol) acetic acid and run in the same buffer. The protein content of each of these fractions was measured by the BCA method. The antimicrobial activity of each fraction against pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* organisms was determined by a radial diffusion assay as described below.

Characterization of lysozyme. Fractions containing lysozyme activity were identified by demonstrating lysis of heat-killed *Micrococcus lysodeikticus* in a radial diffusion assay (32). The antimicrobial activity of purchased lysozyme, purified from human colostrum (enzymatic activity, 100,000 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) (referred to herein as lysozyme), against pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* organisms was determined by a suspension assay as described below. The protein content of the lysozyme preparation was measured by the BCA method. The purity of this preparation was analyzed by tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), which showed the presence of a single band with an estimated molecular mass of 14 kDa (data not shown).

Isolation and characterization of defensins. The low-molecular-weight fractions containing antimicrobial activity against *Y. enterocolitica* that were eluted after the fractions with lysozyme activity from the Bio-Gel P-60 column were analyzed for the presence of defensins by an enzyme-linked immunofluorescence assay (ELISA) using a polyclonal rabbit antiserum against human defensins HNP1 to HNP3 (a generous gift from Tomas Ganz, Will Rogers Institute Pulmonary Research Laboratory, UCLA School of Medicine, Los Angeles, Calif.). Defensin-containing fractions were pooled and concentrated by vacuum centrifugation (Speedvac concentrator SVC 200 H; Savant Instrument Inc., Farmingdale, N.Y.). The purity of this material was analyzed by SDS-PAGE (31).

Assays to determine antimicrobial activity. The gel overlay assay was performed as described previously (23). In short, granule extract of 5×10^6 granulocytes was subjected to PAGE using 12.5% acid-urea polyacrylamide gels (acid-urea PAGE) (23). To locate the zones of bacterial clearing that correspond to those of lysozyme and defensins present in the granule extract, 1 μg of lysozyme and 5 μg of defensins were run in the same gel as the granule extract. Next, the gels were washed in 10 mM NaPB (pH 7.4) and incubated at 37°C on top of a 1-mm-thick underlayer of agar containing 10^5 pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* cells per ml. The underlayer of agar consisted of 9 mM NaPB (pH 6.5), 1 mM citrate, 1% (vol/vol) Trypticase soy broth (Oxoid), and 1% (wt/vol) agarose. After 3 h of diffusion of the proteins into the underlayer of agar, the polyacrylamide gel was removed, a nutrient-rich agar was added on top of the underlayer of agar, and the plate was incubated for 18 h at 37°C to allow growth of the bacteria. The location of substances with antimicrobial activity against *Y. enterocolitica* in the gels was indicated by clear, bacterium-free bands in the agar.

The radial diffusion assay was performed as described previously (23). Samples of 5 μl of each fraction of the granule extract were evaporated by vacuum centrifugation, dissolved in 0.01% (vol/vol) acetic acid, and added to wells 3 mm in diameter made in an underlayer of agar containing 10^5 pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* cells per ml. After incubation for 3 h at 37°C, a nutrient-rich agar was added on top of the underlayer of agar and the plate was incubated for 18 h at 37°C to allow growth of the bacteria. The presence of antimicrobial activity in the samples was indicated by the presence of a clear bacterium-free zone around the wells. The diameter of this clear zone was measured and expressed in millimeters after subtraction of the diameter of the central well.

In the suspension assay, 10^6 bacteria per ml were incubated at 37°C with different concentrations of the granule extract, lysozyme, or defensins. Lysozyme was dialyzed against 0.01% (vol/vol) acetic acid before use. All preparations were evaporated by vacuum centrifugation and dissolved in medium that consisted of 10 mM NaPB, 1 mM citrate, and 1% (vol/vol) Trypticase soy broth at pH 7.4 in a final volume of 100 μl. At the start and after 3 h of incubation, a 25-μl sample of the suspension was diluted in 975 μl of phosphate-buffered saline to prevent carryover of antimicrobial polypeptide, and 10-fold dilutions were prepared. The numbers of CFU were determined by plating six times 10 μl of each dilution onto blood agar.

Calculations and statistical analysis. Growth of *Y. enterocolitica* was calculated as the difference between the logarithms of the numbers of CFU after 3 h of incubation in the absence of antimicrobial polypeptides and at time zero. Killing of *Y. enterocolitica* was expressed as the difference between the logarithms of the numbers of CFU after 3 h of incubation in the presence of antimicrobial polypeptides and at time zero. The antimicrobial effect on bacterial numbers of a given concentration of antimicrobial polypeptides was calculated as the difference between the logarithms of the numbers of CFU in the presence and absence of the antimicrobial polypeptides after 3 h of incubation.

With the procedures used, the limit of detection in the suspension assay would be 667 CFU/ml. Therefore, when no bacteria were detected on the blood agar, the number of CFU was set at 667/ml. Statistical analysis for the interaction between outgrowth and killing and the presence or absence on pYVe was performed with factorial analysis of variance with the Systat software package. Statistical analysis for comparison of the antimicrobial effects on various strains of *Y. enterocolitica* was performed with nonparametric analysis by the Kruskal-Wallis method.

RESULTS

Antimicrobial activity of the granule extract. To identify polypeptides present in the granule extract with antimicrobial activity against pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* organisms, the granule extract was separated by acid-urea PAGE and analyzed in the gel overlay assay. The granule extract contained several polypeptides with antimicrobial activity against *Y. enterocolitica*, as indicated by the presence of several bands of bacterial clearing in the agar containing either strain of *Y. enterocolitica* (Fig. 1). In the agar containing pYVe$^+$ *Y. enterocolitica*, four bands of bacterial clearing were observed. The agar with pYVe$^-$ *Y. enterocolitica* contained larger and additional bands of bacterial clearing compared with the agar with pYVe$^+$ *Y. enterocolitica*. When defensins and lysozyme were run in the same gel, these polypeptides gave a band of bacterial clearing with electrophoretic mobility corresponding to the bands of the granule extract at the cathodic side (Fig. 1).

Antimicrobial activities of the fractions of the granule extract. To identify which fractions of the granule extract displayed antimicrobial activity against *Y. enterocolitica*, the granule extract was fractionated by gel filtration. The protein content of each fraction was determined, and the antimicrobial activity of each fraction against pYVe$^+$ or pYVe$^-$ *Y. enterocolitica* organisms was studied in the radial diffusion assay. Fractions 8 to 44 and 110 to 154 contained substantial amounts of protein (Fig. 2A); antimicrobial activity against both strains of *Y. enterocolitica* was observed in fractions 8 to 78 and 94 to 154 (Fig. 2B).

Further analysis demonstrated that fractions 20 to 78 contained lysozyme activity, as determined by lysis of *M. lysodeikticus* (Fig. 2C). The presence of human defensins in fractions 94 to 154 was demonstrated by ELISA using a rabbit antiserum to human defensins HNP1 to HNP3 (data not shown). These

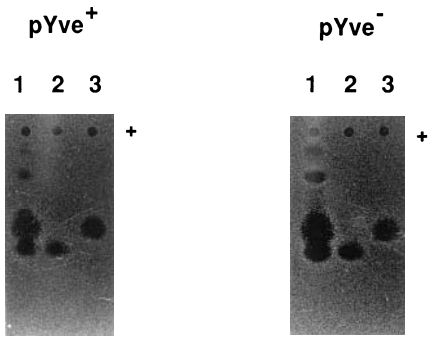

FIG. 1. Identification of antimicrobial activities of polypeptides present in granule extract of human granulocytes against *Y. enterocolitica* by gel overlay assay. Acetic acid granule extract (lane 1), lysozyme (Sigma) (lane 2), or defensins (lane 3) were separated by acid-urea PAGE. Gels were then incubated on top of an agar containing the virulent (pYVe$^+$) or the plasmid-cured isogenic (pYVe$^-$) strain of *Y. enterocolitica* W22703. The locations of substances with antimicrobial activity in the gel are indicated by clear bacterium-free bands in the agar.

fractions were pooled and concentrated. SDS-PAGE of this material demonstrated the presence of a single band with an estimated molecular mass of 4 kDa, corresponding to that of defensins (data not shown).

Comparison of antimicrobial activities of granule extract, lysozyme, and defensins against pYVe$^+$ and pYVe$^-$ *Y. enterocolitica*. To quantify differences in antibacterial effects against pYVe$^+$ and pYVe$^-$ *Y. enterocolitica*, the antimicrobial activities of granule extract, lysozyme, and defensins were studied in the suspension assay. With increasing concentrations of granule extract, lysozyme, or defensins, larger antimicrobial effects were observed for both strains (Fig. 3). The reduction in numbers of viable pYVe$^+$ *Y. enterocolitica* was significantly less than that of pYVe$^-$ *Y. enterocolitica*. Only at the highest concentrations of lysozyme did the numbers of viable pYVe$^+$ *Y. enterocolitica* drop below the level of detection of the assay, as indicated by the absence of colonies on the blood agar; for pYVe$^-$ *Y. enterocolitica*, this occurred at much lower concentrations of lysozyme and also in the presence of high concentrations of granule extract or defensins. These results indicate that the virulence plasmid pYVe mediates reduced susceptibility of *Y. enterocolitica* to the antimicrobial effects of antimicrobial polypeptides of human granulocytes.

In nutrient-free medium, *Y. enterocolitica* did not grow, and no antimicrobial activity against either strain was observed during incubation with granule extract, lysozyme, or defensins (data not shown). During 3 h of incubation in the absence of antimicrobial polypeptides, growth ($\pm$ standard error) of pYVe$^+$ *Y. enterocolitica* (0.132 ± 0.022 log CFU ml^{-1}) was significantly ($P = 0.0004$) less than that of pYVe$^-$ *Y. enterocolitica* (0.323 ± 0.047 log CFU ml^{-1}). To determine whether the observed differences in antimicrobial effect of the granule extract were due to differences in growth between pYVe$^+$ and pYVe$^-$ *Y. enterocolitica*, growth in the absence of antimicrobial polypeptides, calculated from the various experiments, was plotted against killing of *Y. enterocolitica* in the presence of 100 μg of granule extract per ml. At a similar growth of pYVe$^+$ and pYVe$^-$ *Y. enterocolitica*, killing of pYVe$^+$ *Y. enterocolitica* was significantly ($P < 0.001$) less than that of pYVe$^-$ *Y. enterocolitica* (Fig. 4). Similar differences were found in the presence of 25, 50, and 200 μg of granule extract per ml, 50 and 100 μg of lysozyme per ml, or 50, 100, 200, and 400 μg of defensins per ml (data not shown).

Role of YadA in the resistance of *Y. enterocolitica* to the antimicrobial activity of the granule extract. To investigate the role of the expression of YadA in reducing the susceptibility of pYVe$^+$ *Y. enterocolitica* to the antimicrobial activity of the granule extract, a mutant strain unable to express YadA on the outer membrane was used. Incubation of *Y. enterocolitica* W22703(pBC7) YadA$^-$ Yops$^+$ in the presence of 200 μg of granule extract per ml resulted in a significantly larger decrease

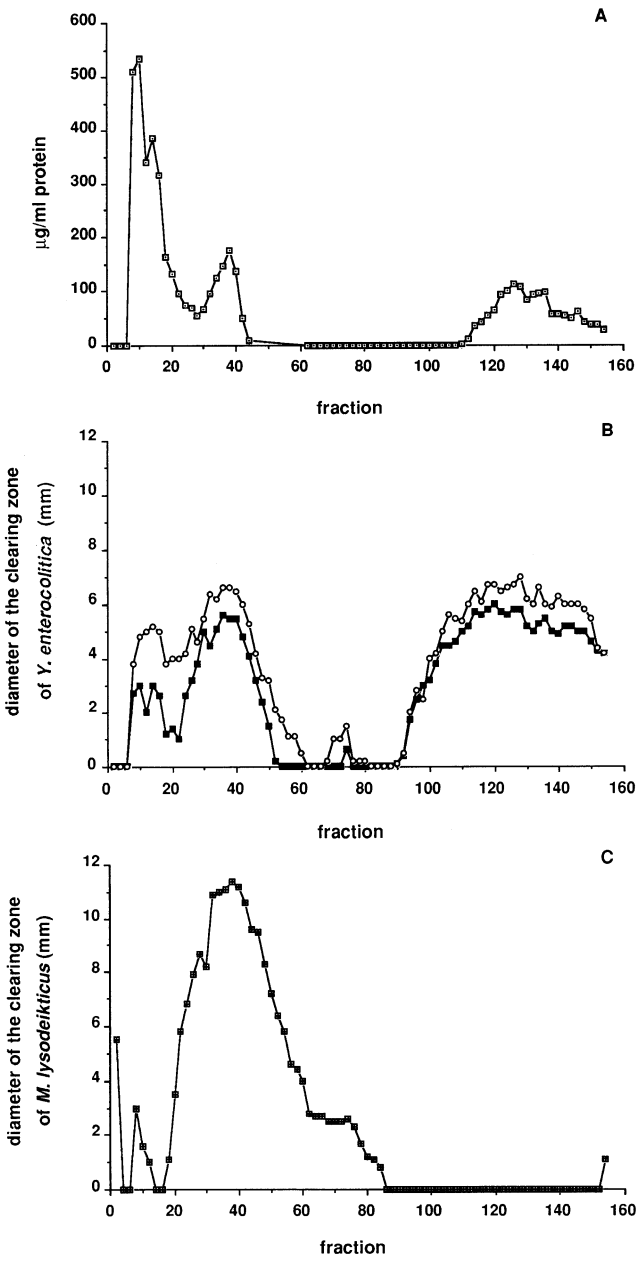

FIG. 2. Protein contents, antimicrobial activities, and lysozyme activities of various fractions of the granule extract against *Y. enterocolitica*. (A) Protein contents of the various fractions after gel filtration of granule extract, assessed by the BCA method. (B) Antimicrobial activities against *Y. enterocolitica* as determined in the radial diffusion assay. Samples of each fraction of the granule extract were added to wells in the agar containing pYVe$^+$ (■) or pYVe$^-$ (○) *Y. enterocolitica*. The diameter of the bacterium-free zone around each well was expressed in millimeters. (C) Lysozyme activities in the fractions as determined by lysis of *M. lysodeikticus*. Results are from a representative experiment (number of experiments, ≥2).

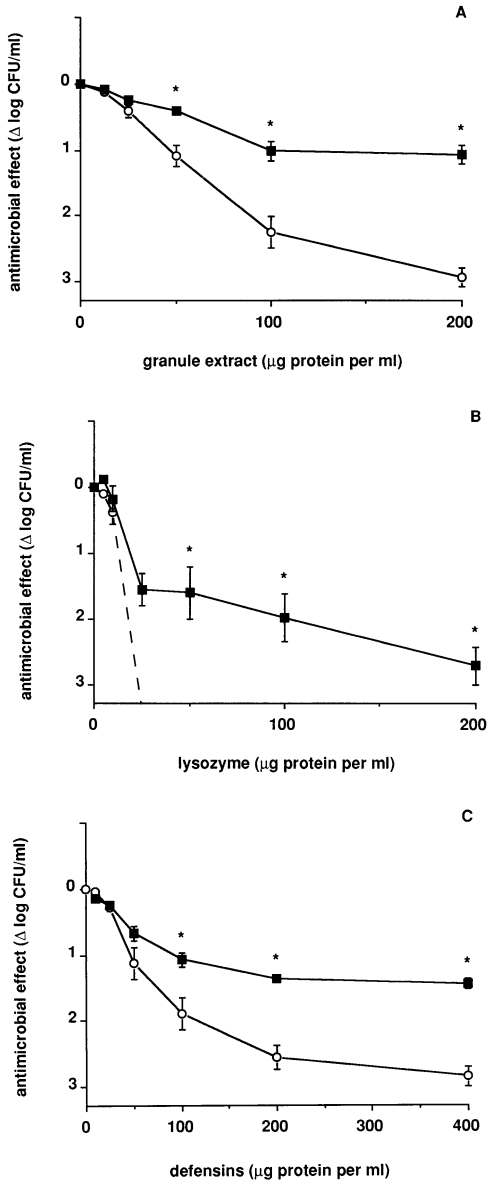

FIG. 3. Antimicrobial effects of granule extract, lysozyme, or defensins on *Y. enterocolitica* determined by suspension assay. pYVe$^+$ (■) or pYVe$^-$ (○) *Y. enterocolitica* was incubated for 3 h with different concentrations of antimicrobial polypeptides. The antimicrobial effect of a given concentration of polypeptides on bacterial numbers was expressed as the difference between the logarithms of the numbers of CFU in the presence and absence of the antimicrobial polypeptides. The number of CFU below the level of detection was set at 667/ml. Note that the detection limit of the assay is indicated by the intersection of the *x* axis with the *y* axis. Values are the means ± standard errors of at least three independent experiments. Significant differences ($P < 0.05$) are indicated by asterisks. (A) Antimicrobial effect of acetic acid granule extract. (B) Antimicrobial effect of lysozyme. For concentrations of 50 μg of lysozyme per ml or more, no bacterial growth of pYVe$^-$ *Y. enterocolitica* was observed (dashed line). (C) Antimicrobial effect of defensins.

in bacterial numbers than found for pYVe$^+$ *Y. enterocolitica* (Fig. 5). Since the *yadA* mutation in *Y. enterocolitica* W22703(pBC7) could be polar and affect the expression of uncharacterized downstream genes, we tried to confirm the role of YadA by using a pYVe$^-$ *Y. enterocolitica* strain complemented with plasmid pMS12, which contains an amplified *yadA* gene cloned under control of the *lac* promoter (2). The

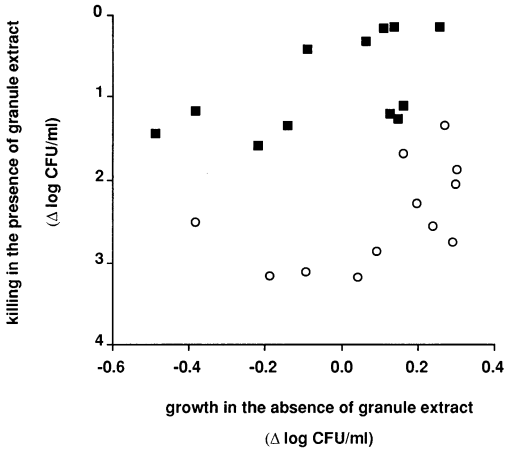

FIG. 4. Killing of pYVe$^+$ and pYVe$^-$ *Y. enterocolitica* in the presence of granule extract in relation to growth in the absence of granule extract. Growth of pYVe$^+$ (■) or pYVe$^-$ (○) *Y. enterocolitica* during 3 h of incubation in the absence of granule extract was plotted against the corresponding killing in the presence of 100 μg of granule extract per ml. A negative value for growth indicates a decrease in the numbers of bacteria during 3 h of incubation. At a similar growth, the killing of pYVe$^+$ *Y. enterocolitica* was significantly ($P < 0.001$) less than that of pYVe$^-$ *Y. enterocolitica.*

antimicrobial effect of 200 μg of granule extract per ml against *Y. enterocolitica* W22703(pMS12) YadA$^+$ was significantly lower than that found for pYVe$^-$ *Y. enterocolitica*, which does not express YadA. Together, these results demonstrate that the expression of YadA on the outer membrane plays a role in the reduced susceptibility of pYVe$^+$ *Y. enterocolitica* to the antimicrobial activity of the granule extract from human granulocytes. However, YadA does not protect *Y. enterocolitica* entirely against the antimicrobial activity of the granule extract. In contrast to what was observed with the granule extract, the antimicrobial effect of lysozyme and defensins against *Y. enterocolitica* W22703(pMS12) YadA$^+$ was not significantly different from that against pYVe$^-$ *Y. enterocolitica* (data not shown).

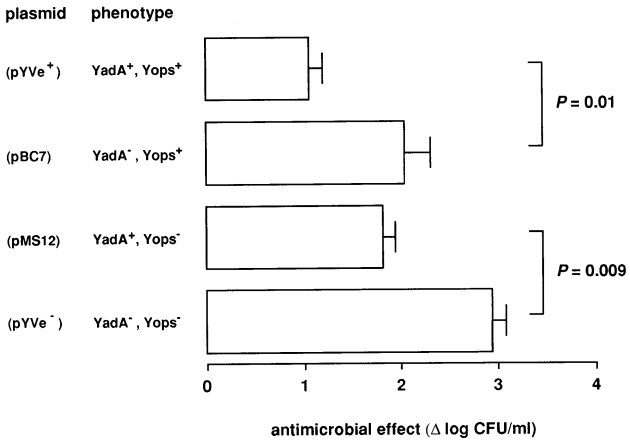

FIG. 5. Antimicrobial effect of acetic acid granule extract on various mutants of *Y. enterocolitica* W22703. Bacteria were incubated for 3 h with 200 μg of granule extract per ml. The antimicrobial effect was expressed as the difference between the logarithms of the numbers of CFU in the presence and absence of the antimicrobial polypeptide. Values are the means ± standard errors of at least three independent experiments.

DISCUSSION

The main conclusion of this study is that the expression of YadA on the outer membrane plays a role in the resistance of *Y. enterocolitica* to the antimicrobial activity of polypeptides of human granulocytes. This conclusion is supported by the following observations. pYVe$^+$ *Y. enterocolitica* is less susceptible to the antimicrobial activity of granule extract, defensins, and lysozyme than the isogenic plasmid-cured strain. A *Y. enterocolitica* mutant unable to express YadA is more susceptible to the antimicrobial activity of the granule extract than pYVe$^+$ *Y. enterocolitica*. Finally, complementation of pYVe$^-$ *Y. enterocolitica* with a plasmid coding only for the expression of YadA on the outer membrane partly protects the bacteria against the antimicrobial activity of the granule extract.

The acetic acid granule extract of human granulocytes contains several polypeptides with antimicrobial activity against *Y. enterocolitica*, as determined by gel overlay and radial diffusion assays. The low- and medium-molecular-weight fractions of the granule extract with antimicrobial activity against *Y. enterocolitica* contain defensins and lysozyme. The identities of polypeptides present in the high-molecular-weight fractions of the granule extract which have antimicrobial activity against *Y. enterocolitica* were not determined in detail. Which polypeptides present in the granule extract contribute most to the antimicrobial effect against *Y. enterocolitica* is difficult to assess from the radial diffusion assay, because large polypeptides diffuse slowly through the agar, resulting in smaller diameters of bacterial clearing compared with smaller polypeptides with equal antimicrobial activity against *Y. enterocolitica*. We chose to quantify the antimicrobial activity of lysozyme and defensins because these polypeptides are abundantly present in the granules of human granulocytes.

Human granulocytes contain between 150 and 300 μg of lysozyme (9) and about 580 μg of defensins per 10^8 cells (for a review, see reference 11). Lysozyme is a small enzyme which hydrolyzes glycosidic bonds present in bacterial wall peptidoglycan (35). Within the physiological pH range, lysozyme is a highly cationic molecule, a property which confers antimicrobial activity independent of its enzymatic action (20). Defensins are cationic, low-molecular-weight polypeptides which form anion-specific channels in lipid bilayers and exhibit antibacterial activity in vitro (for reviews, see references 12 and 22). The permeabilization of the outer and inner membranes of bacteria by human defensins depends on active bacterial metabolism and bacterial growth (21). In the present study, we demonstrated that lysozyme and defensins exhibit antimicrobial activity against *Y. enterocolitica* and that pYVe$^+$ *Y. enterocolitica* is less susceptible to their antimicrobial activity than pYVe$^-$ *Y. enterocolitica.*

In the absence of growth, no antimicrobial effect against *Y. enterocolitica* was observed during incubation with granule extract, lysozyme, or defensins. Since growth of pYVe$^+$ *Y. enterocolitica*, but not of pYVe$^-$ *Y. enterocolitica*, is restricted at 37°C by the low calcium concentration of the medium, the observed differences in antimicrobial effect of the granule extract, lysozyme, and defensins could be due to differences in growth rates of the two strains. However, when growth and killing of pYVe$^+$ and pYVe$^-$ *Y. enterocolitica* in the presence of the same concentration of antimicrobial polypeptides were compared, we observed that at a similar growth, the killing of pYVe$^+$ *Y. enterocolitica* was significantly less than that of pYVe$^-$ *Y. enterocolitica*. This finding indicates that independent of differences in bacterial growth, plasmid-encoded factors play a role in the reduced susceptibility of pYVe$^+$ *Y. enterocolitica* to the antimicrobial activity of these polypeptides.

A very likely candidate for this property is YadA. This plasmid-encoded outer membrane protein is maximally expressed at 37°C and forms a polymeric fibrillar matrix covering the outer membrane of *Y. enterocolitica* (17). YadA increases the negative cell surface charge (18) and has distinct hydrophobic domains (19, 26) which play a role in the attachment to host cells (15) and to the extracellular matrix proteins fibronectin, laminin, and collagen (10, 30, 33, 36, 37). In addition, YadA contributes to the resistance to the bactericidal action of human complement (1, 3, 27). The results of the present study indicate that YadA is also involved in the reduced susceptibility of pYVe$^+$ *Y. enterocolitica* to antimicrobial polypeptides present in the granule extract of human granulocytes. A *Y. enterocolitica* mutant unable to express YadA is more susceptible to the antimicrobial activity of the granule extract, whereas complementation of pYVe$^-$ *Y. enterocolitica* with a plasmid coding only for the expression of YadA on the outer membrane partly protects the bacteria against the antimicrobial activity of the granule extract. Other plasmid-encoded factors may also play a role since YadA does not protect *Y. enterocolitica* entirely against the antimicrobial effect of granule extract, and YadA does not reduce the antimicrobial activity of lysozyme or defensins against *Y. enterocolitica.*

The present observations on the antimicrobial activity of defensins and lysozyme against yersiniae may also be relevant to bacteria ingested by cells other than granulocytes. Monocytes and macrophages also contain lysozyme (9). In addition, lysozyme and defensin-related antimicrobial polypeptides, called cryptdins, are present in Paneth cells of the small intestine (8, 16, 34). These specialized epithelial cells can phagocytoze and have secretory capacities. The reduced susceptibility of pYVe$^+$ *Y. enterocolitica* to these antimicrobial polypeptides may add to the ability to colonize the mucosal barrier of the gut.

Previously it has been shown that opsonized virulent *Y. enterocolitica* adheres to the surface of the granulocyte and is poorly ingested by these cells, a property determined by the secretion of Yops (38). There are indications that the virulence plasmid codes for the inhibition of the respiratory burst of granulocytes in response to *Y. enterocolitica* (2, 25). The results of the present study indicate that this plasmid also codes for a reduced susceptibility of *Y. enterocolitica* to antimicrobial polypeptides present in the human granulocyte. We suppose that virulent *Y. enterocolitica*, when adherent to the granulocyte or localized within the forming phagosome, can resist nonoxidative killing by antimicrobial polypeptides which are released to the surface of the granulocyte or into the phagosome. Together, these properties enhance the potentiality of virulent *Y. enterocolitica* to persist in the host.

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