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Antimicrobial activity of histidine-rich peptides is dependent on acidic conditions

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Abbreviations: AMP, antimicrobial peptide; GAG, glycosaminoglycan; hCAP-18, human cationic antimicrobial protein 18; HMWK, High-molecular weight kininogen; MALDI-TOF, matrix-assisted laser desorption/ ionization - time of flight; MES, 2-(morpholino)-ethanesulfonic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); TR, Texas red; Tris, trishydroxymethylaminomethane

Keywords: antimicrobial peptide; heparin-binding; histidine-rich glycoprotein; pH

Abstract

Synthetic peptides composed of multiples of the consensus heparin-binding Cardin and Weintraub sequences AKKARA and ARKKAAKA are antimicrobial. Replacement of lysine and arginine by histidine in these peptides completely abrogates their antimicrobial and heparin-binding activities at neutral pH. However, the antibacterial activity against Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) as well as the fungus *Candida albicans,* was restored at acidic conditions (pH 5.5). Fluorescence microscopy and FACS analysis showed that the binding of the histidine-rich peptides to *E. coli* and *Candida* was significantly enhanced at pH 5.5. Likewise, fluorescence studies for assessment of membrane permeation as well as electron microscopy analysis of peptide-treated bacteria, paired with studies of peptide effects on liposomes, demonstrated that the peptides induce membrane lysis only at acidic pH. No discernible hemolysis was noted for the histidine-rich peptides. Similar pH-dependent antimicrobial activities were demonstrated for peptides derived from histidine-rich and heparin-binding regions of human kininogen and histidine-rich glycoprotein. The results demonstrate that the presence of an acidic environment is an important regulator of the activity of histidine-rich antimicrobial peptides.

1. Introduction

Antimicrobial peptides (AMPs), originally isolated from human leukocyte extracts in the 1960´s [1], belong to a diverse and evolutionary old group molecules that are important effectors of innate immunity. AMPs participate in the first line of defense against invading microorganisms in both invertebrates and vertebrates [2-4]. In contrast to adaptive immune systems, AMPs mediate a rapid and non-specific response against both Gram-positive and Gram-negative bacteria, as well as fungi. AMPs are generally characterized by a net positive charge and an amphipathic structure, having clusters of hydrophobic and cationic amino acids spatially organized in sectors of the molecules [3, 5, 6]. Today, nearly 900 AMP sequences from both vertebrates and invertebrates are known (see http://www.bbcm.univ.trieste.it/~tossi/search.htm).

It is well established that peptide binding and thus, interaction with bacterial membranes is required for AMP-mediated killing of microbes (for reviews see [3, 6, 7]. However, the modes of action of AMPs on their targets are complex, and can be divided into membrane and non-membrane disruptive mechanisms. Some AMPs cause a significant membrane destabilisation and permeabilisation, as exemplified by recent high resolution and mechanistic studies on the antimicrobial peptides G15 of granulysin [8], the magainin-derived MSI-78 and MSI-594 [9, 10], LL-37 [11], and tachyplesin-variant peptides [12]. Considering the human AMP LL-37, recent studies indicate a toroidal-pore mechanism of lipid layer disruption. Furthermore, hydrophobic interactions between LL-37 and the hydrophobic acyl chains are as important for the ability of this peptide to disrupt lipid bilayers as its electrostatic interactions with the polar headgroups [11, 13]. Other AMPs display different modes of action. For example, buforin II enters bacterial cells and interferes with intracellular processes, while the porcine peptide PR-39 arrests DNA synthesis [4], and histatins may

translocate through fungal membranes and bind mitochondria, where they may induce cell death by non-lytic ATP-release. Notably, the membrane effects of histatins have been found to be less pronounced when compared with LL-37, only leading to efflux of nucleotides through fungal membranes [14]. It is now clear that many of AMPs are multifunctional, linking the innate and adaptive immune systems. They not only act directly on microbes, but may also be involved in angiogenesis and apoptosis and display chemotactic effects against monocytes, mast cells and T cells [15]. Conversely, it has been shown that human C3a and related peptides, generated upon complement activation, are antimicrobial [16]. Furthermore, proteolytic degradation of human kininogen yields AMPs derived from domains 4 [17], 5 [18] as well as domain 3 [19].

Apart from their antimicrobial and immunoregulatory activities cationic AMPs interact with negatively charged glycosaminoglycans (GAGs) like heparin. Some pathogenic bacterial species are able to release GAGs during infection, which may facilitate invasion into host tisssues by inactivating AMPs [20]. Conversely, we have shown that heparin-binding motifs of many endogenous proteins exhibit antimicrobial effects, including peptides derived from laminin, fibronectin, histidine-rich glycoprotein, domain 5 of high molecular weight kininogen and others [18, 21, 22]. In analogy, model peptides, composed of multiples of consensus heparin-binding sequences: $(ARKKAAKA)_3$ and $(AKKARA)_4$ (denoted as ARK24 and AKK24, respectively) [23], exert direct antimicrobial effect against Gram-positive and Gram-negative bacteria [18, 24]. Replacement of all basic amino acids in the ARK24 and AKK24 peptides by histidines yielded the histidine-rich peptides (AHH24:1 and AHH24:2, respectively) exhibiting no antimicrobial activities at neutral pH. As previously reported, addition of Zn^{2+} restored the antimicrobial activity of the AHH peptides [25]. In conjunction with this, peptides derived from human histidine-rich glycoprotein displayed a similar zinc-dependent mode of action [21]. Considering that Zn^{2+} imposes a positive charge on histidine-

rich peptide sequences (at neutral pH), leading to enhanced antimicrobial effects of these peptides, we decided to examine whether low pH, leading to protonation of histidines, thus yielding positively charged peptides, could affect the antibacterial properties of histidine-rich peptide sequences. Here, we demonstrate that antimicrobial activity of histidine-rich consensus peptides as well as a range of other histidine-rich peptides of endogenous origin is indeed induced at acidic pH.

2. Materials and methods

2.1. Peptides

The synthetic peptides (for sequences see Table 1), LL-37, AKK24 $(AKKARA)_4$, ARK24 $(ARKKAAKA)_3$, AHH24:1 $(AHHAHA)_4$, AHH24:2 $(AHHHAAHA)_3$, KHN20, GHG20, GHG21, GGH20, HKH20, GKH17, GHH20, Histatin 5, and Texas Red-conjugated peptides: TR-LL-37, TR-AHH24:1 and TR-AHH24:2 were all obtained from Innovagen AB (Lund, Sweden). The peptides were all of >95% (in most cases >98%) purity as indicated by HPLC and MALDI-TOF analysis (Voyager, Applied Biosystems). The primary source of impurity is peptides with one or several amino acids deleted. Overall, this minuteness and type of impurities is not expected to affect the interpretation of the data.

2.2. Microorganisms

Bacillus subtilis ATCC 6633, *Candida albicans* ATCC 90028, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were obtained from The American Type Culture Collection (ATCC, Rockville, MD, USA).

2.3. Radial diffusion assay (RDA)

Radial diffusion assay (RDA) was performed essentially as described earlier [26]. Bacteria were grown to midlogarithmic phase in 10 ml of full-strength (3% wt/vol) trypticase soy broth (TSB) (Becton, Dickinson and Company, Sparks, MD, USA). Fungi (*C. albicans)* were grown to midlogarithmic phase in 10 ml of full-strength (3% wt/vol) YPD (Sigma-Aldrich Inc. St. Louis, USA). The microorganisms were washed with doubly distilled (dd)H_2O. 4 x 10^6 bacterial or fungal colony forming units (cfu) was added to 5 ml of the underlay agarose gel containing (0.03% (wt/vol) TSB/1% (wt/vol) low electroendosmosis type (EEO) agarose (Sigma), and 0.02% (vol/vol) Tween 20 (Sigma). Four different underlay gels were used, each based on different buffers (10 mM Tris, pH 7.4, 10 mM MES, pH 5.5, 10 mM PIPES, pH 7.4, 10 mM acetate buffer, pH 5.5). The underlay gel was poured into an 85-mm Petri dish. After agarose solidification, wells of 4 mm in diameter were punched, and 6 μl of peptide solution was added to each well. LL-37 solution was used as a positive control, while the respective buffers were used as negative controls. Plates were incubated at 37°C for bacteria (28°C for fungi) for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 5 ml of molten overlay (6% TSB/1% low-EEO agarose in distilled H_20). Antimicrobial activity of a peptide was then visualized as a zone of clearance around each well after 18-24 h of incubation at 37°C for bacteria or 28°C for fungi. Peptides were tested in concentrations of 100 μM.

2.4. Heparin-binding assay

Peptides were tested for heparin-binding abilities. The radioiodination of heparin (from porcine intestinal mucosa, Sigma-Aldrich, St Louis MO, USA) was performed as previously described [20]. 2 and 5 μg of AKK24, ARK24, AHH24:1 and AHH24:2 were applied onto nitrocellulose membranes (Hybond^{TM}-C, Amersham Biosciences, Little Chalfont, UK) using a slot-blot apparatus. Membranes were then incubated with I^{125}-radiolabelled heparin (~10 $\mu g \cdot mL^{-1}$, 0.4 x 10^6 $cpm \cdot \mu g^{-1}$) for 1 h at room temperature in different buffers. The membranes were finally washed for 3 x 10 min in 10mM Tris, pH 7.4. A Bas 2000 radio-imaging system (Fujifilm, Tokyo, Japan) was used for visualization of radioactivity.

2.5. Fluorescence microscopy

For assessement of binding of fluorescently labelled peptides to microbial cells, *E. coli* 25922 bacteria and *C. albicans* 90028 were grown at 37º C and 28º C, respectively, in Todd-Hewitt (TH) medium (Becton, Dickinson and Company, Sparks, MD, USA) to mid-logarithmic phase, washed twice with ddH_2O and resuspended in ddH_2O. 5 μl of 1% bacterial or fungal suspension (1-2 x 10^7 CFU) were then added to 1 ml of 10 mM buffer (either Tris, pH 7.4 or MES, pH 5.5). 200 μl of the suspension were incubated on ice for 5 min with 2 μg of Texas red labeled peptide. Subsequently, microorganisms were washed twice with the buffer (10 mM Tris, pH 7.4, or 10 mM MES, pH 5.5) and fixed with 4% paraformaldehyde, first on ice for 15 minutes and then in room temperature for 45 minutes. Fixed microorganisms were then applied onto poly(L-lysine)-coated cover glasses, incubated for 30 min at room temperature and finally mounted on a slide using DakoCytomation fluorescent

mounting medium (DakoCytomation, Carpinteria, CA, USA). For study of membrane permeabilisation using the impermeant probe FITC, *E. coli* ATCC 25922 bacteria were grown to mid-logarithmic phase in TSB medium. The bacteria were washed and resuspended in either 10 mM Tris, pH 7.4, 10 mM glucose or 10 mM MES, pH 5.5, 10 mM glucose to yield a suspension of 1 x 10^7 cfu/ml. 100 µl of the bacterial suspension was incubated with 30 µM of the respective peptides at 30°C for 30 min. Microorganisms were then immobilized on poly (L-lysine)–coated glass slides by incubation for 45 min at 30°C, followed by addition onto the slides of 200 µl of FITC (6 µg/ml) in the appropriate buffers and incubated for 30 min at 30°C. The slides were washed and bacteria were fixed by incubation, first on ice for 15 min, then in room temperature for 45 min in 4% paraformaldehyde. The glass slides were subsequently mounted on slides using Prolong Gold antifade reagent mounting medium (Invitrogen). For fluorescence analysis, bacteria and fungi were visualized using a Nikon Eclipse TE300 (Nikon, Melville, NY) inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera (Hamamatsu, Bridgewater, MJ) and a Plan Apochromat ×100 objective (Olympus, Orangeburg, NY). Differential interference contrast (Nomarski) imaging was used for visualization of the microbes themselves.

2.6. Flow cytometry

E. coli 25922 was grown to midlogarithmic phase in Todd-Hewitt (TH) medium (Becton, Dickinson and Company, Sparks, MD, USA), washed once with buffer (either 10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5) and resuspended in buffer to obtain 2x10^9 cfu/ml bacterial suspension. 10 µl of such bacterial suspension (2x10^7 cfu) was added to 2 ml of buffer. 500 µl of this suspension (5x10^6 cfu) was transferred to an eppendorf tube and 2 µl of

fluorescent labelled peptide solution (2 mg/ml) was added. The samples were incubated on ice for 5 minutes, and then washed with proper buffer (10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5). Bacteria were fixed with 4% paraformaldehyde, first on ice for 15 minutes and then at room temperature for 45 minutes. A FACSCalibur flow cytometer (Becton, Dickinson and Company) was used with a gate setting for the intact bacterial cells population. Data acquisition and processing from 20000 cells was analyzed in the logarithmic mode.

2.7. Viable count assay

C. albicans 90028 and *E. coli* ATCC 25922 were grown to the mid-log phase in Todd-Hewitt (TH) medium (Becton, Dickinson and Company, Sparks, MD, USA), washed once with ddH2O and resuspended in buffer (either 10 mM Tris, pH 7.4 or 10 mM MES, pH 5.5) and then diluted to obtain $2x10^6$ cfu/ml suspension. *C. albicans* ($1x10^5$ cfu) was incubated for 2 h at 28º C in presence of 60 μM peptides (LL-37, AHH24:1, AHH24:2, KHN20, GHH20, GGH20, GHG20). Afterwards, serial dilutions of the incubation mixtures in proper buffer (Tris, MES) were plated on Todd-Hewitt agar followed by overnight incubation at 28º C. Next day, the number of cfu was determined. Survival of *C. albicans* in the absence of peptide in the corresponding buffer, using the same conditions, was used as a positive control, and taken to be 100%. Significance was determined using Kruskall-Wallis one way ANOVA analysis (Sigmastat, SPSS, Chicago, IL). For determination of effects of salt concentration on peptide activity (Fig. 1B), 2 x 10^6 cfu/ml of *E. coli* bacteria were incubated in 50 μl with either AHH24:1 or AHH24:2 at 6 and 60 μM in 10 mM MES buffer pH 5.5, 5 mM glucose in presence 0, 25, 50, 100, and 150 mM NaCl.

2.8. Hemolysis assay

EDTA-blood was centrifuged at 800 g for 10 min, whereafter plasma and buffy coat were removed. The erythrocytes were then washed three times and resuspended in 10 mM Tris, 0.15 M NaCl, pH 7.4, or 10 mM MES, 0.15 M NaCl, pH 5.5 (in both cases yielding a 5% suspension), after which they were then incubated with end-over-end rotation for 1 hour at 37°C in the presence of peptides (60 μM). 2% Triton X-100 (Sigma-Aldrich) served as positive control. The samples were then centrifuged at 800 g for 10 min. The absorbance of hemoglobin release was measured at λ 550 nm and is in the plot expressed as % of Triton X-100 induced hemolysis.

2.9. Negative staining and transmission electron microscopy

E. coli bacteria were grown in TH medium at 37ºC to mid-logarithmic phase. The bacteria were washed in 10 mM Tris, 5 mM glucose, pH 7.4, and resuspended in the same buffer. *E. coli* or *P. aeruginosa* (1 x 10^8 bacteria) were incubated with AHH24:1 or AKK24 (all at 30 μM) for two hours in a total volume of 50 μl in 10 mM Tris, 5 mM glucose, pH 7.4, or in 10 mM MES, 5 mM glucose, pH 5.5. Ten μl of the bacterial suspensions were then adsorbed onto carbon-coated copper grids for 1 min, washed briefly on two drops of water, and negatively stained on two drops of 0.75 % uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera.

2. 10. Liposome preparation and leakage assay

Dry lipid films were prepared by dissolving dioleoylphosphatidylcholine (DOPC) (1,2-dioleoyl-sn-Glycero-3-phoshocholine, >99% purity, Avanti Polar Lipids, Alabaster, AL) (30 mol%), dioleoylphoshatidic acid (DOPA) (1,2-dioleoyl-*sn*-Glycero-3-Phosphate, monosodium salt, >99% purity, Avanti Polar Lipids, Alabaster, AL) (30 mol%), and cholesterol (>99% purity, Sigma, St Louis, MO) (40 mol%), and then removing the solvent by evaporation under vacuum overnight. Subsequently, buffer (10 mM Tris, pH 7.4 or 10 mM acetate buffer pH 6.0) was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St Louis, MO). After hydration, the lipid mixture was subjected to eight freeze-thaw cycles consisting of freezing in liquid nitrogen and heating to 60°C. Unilamellar liposomes, of about Ø140 nm were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22°C. Untrapped CF was then removed by two gel filtrations (Sephadex G-50) at 22°C, with Tris buffer as eluent.

CF release was determined by monitoring the emitted fluorescence at 520 nm from liposome dispersions (10 mM lipid in 10 mM Tris). An absolute leakage scale was obtained by disrupting the liposomes at the end of the experiment through addition of 0.8 mM Triton X100 (Sigma, St Louis, MO), causing 100% release and dequenching of CF. Although calcein is frequently used for pH-dependent liposome leakage studies, the high charge of this dye has been noted to influence its leakage behavior in the presence of highly cationic peptides [27]. Instead, CF was used as a leakage marker at both pH 6.0 and 7.4. In order to avoid problems related to the pH-dependent fluorescence of CF, pH was adjusted back to 7.4 after protein/peptide-induced leakage at pH 6.0, but prior to disruption of the liposomes by Triton addition, thus allowing the limiting leakage at pH 6.0 to be correctly measured. In

separate experiments, it was confirmed that pH-cycling between 6.0 and 7.4 in the absence of protein/peptide does not result in any measurable CF leakage. Throughout, a SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, NJ) was used for the liposome leakage assay. Measurements were performed at 37 °C.

2.11. CD-spectroscopy

The CD spectra of the peptides in solution were measured on a Jasco J-810 Spectropolarimeter (Jasco, U.K.). The measurements were performed at 37°C in a 10 mm quartz cuvet under stirring at a peptide concentration of 10 μM. The effect on peptide secondary structure of liposomes at a lipid concentration of 100 μM was monitored in the range 200-250 nm. The fraction of the peptide in α-helical conformation, X_α, was calculated from

$$X_\alpha = (A - A_c)/(A_\alpha - A_c)$$

where A is the recorded CD signal at 225 nm, and A_α and A_c are the CD signal at 225 nm for a reference peptide in 100% α-helix and 100% random coil conformation, respectively. 100% α-helix and 100% random coil references were obtained from 0.133 mM (monomer concentration) poly-L-lysine in 0.1 M NaOH and 0.1 M HCl, respectively. To account for the instrumental differences between measurements the background value (detected at 250 nm, where no peptide signal is present) was subtracted. Signals from the bulk solution were also corrected for.

3. Results

3.1. Antimicrobial activities of histidine-rich peptides in neutral and acidic pH

Peptides comprising multiples of AKKARA and ARKKAAKA (AKK24 and ARK24, respectively, see Table 1), as well as their histidine-rich derivatives (denoted as AHH24:1 and AHH24:2, respectively), were tested for antimicrobial activities against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), and fungi (*Candida albicans*) in radial diffusion assays (RDA) at low salt conditions at neutral and acidic pH. In order to exclude influence of buffering ions on activity, four different buffers were used – two neutral (Tris and PIPES) and two acidic (MES and Acetate). At pH 7.4, AHH24:1 and AHH24:2 exerted no or little antimicrobial activity (at 100 μM) (Fig. 1). In contrast, the peptides exerted antimicrobial effects in acidic pH, i.e., below pKa of the histidine groups (pKa=6.5 for the isolated amino acid), whereas the corresponding R/K-containing peptides AKK24 and ARK24 displayed largely pH-independent microbial killing in this pH range. Similarly, the antimicrobial activities of LL-37, a "classical" AMP used here as a benchmark, was not significantly affected by the change of pH. As seen in Fig. 1, the bactericidal activities observed were consistent for three of the four tested buffers. All active peptides showed similar efficiencies in the two neutral buffers (Tris and PIPES). In acidic conditions, where AHH24:1 and AHH24:2 both carry a net charge of +9, while ARK24 and AKK24 carries a net charge of +12, the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were particularly sensitive to ARK24, AKK24 and the two AHH24 peptides (both buffers), whereas the Gram-positive *S. aureus* and *B. subtilis* were only affected using MES buffer. Furthermore, the

acetate buffer potentiated the antimicrobial activity of LL-37 against *P. aeruginosa* and *E. coli* (Fig. 1). It is well-known that activities of AMPs are dependent of the microenvironment. For example, various chemokines, defensins, as well as LL-37 are partly, or completely, antagonized by high salt conditions or the presence of plasma proteins *in vitro* [28, 29]. As shown in Fig. 1B, the antibacterial activity of 6 μM AHH24:1 peptide against *E. coli* was partly inhibited at increasing salt concentrations, and higher concentrations of the peptide (60 μM) were required for efficient killing. Similar results were obtained with the AHH24:2 peptide (not shown). Ca^{2+} and Mg^{2+} at physiological concentrations (2 mM) did not affect the antibacterial activity of the AHH peptides against *E. coli* (at pH 5.5) (not shown). In contrast to the pH-dependent bactericidal activity of AHH24:1 and AHH24:2, no pH-dependence was observed in terms of hemolysis, which most likely is an effect of the higher ionic strength used for the hemolysis experiments. Specifically, none of the peptides displayed any significant hemolytic activity at 60 μM (Figure 1C).

3.2. Enhanced antimicrobial activity at low pH correlates with enhanced binding to heparin and bacterial/fungal membranes

Having demonstrated activation of histidine-rich peptides containing the prototypic Cardin motifs at reduced pH, we examined their capability to interact with heparin as well as microbial cell envelopes. As assessed by the heparin-binding assay, antimicrobial activity corresponded well with an ability to bind to the negatively charged glycosaminoglycan. Hence, unlike AKK24 and ARK24, which interacted with heparin in a pH-independent mode, the two AHH24 peptides bound to heparin only at acidic pH (Fig. 2). We next tested the capability of the peptides to bind to bacteria and fungi. As shown in Fig. 3, binding of LL-37 (carrying a net charge of +6 at both pH 7.4 and 5.5) to *Escherichia coli* was not affected by

pH in this pH range, whereas AHH24:1 and AHH24:2 interacted efficiently with bacterial cells only at pH 5.5 (Fig. 3, panels 1-6). Similarly, the two AHH24 peptides showed high affinity for *Candida albicans* fungal cells only at acidic pH (Fig. 3, panels 7-12). Also similar to the bacterial results, LL-37 bound to *C. albicans* in both Tris and MES buffer. The binding of all the tested peptides was completely blocked by addition of heparin (50 μg/ml) (not shown). Flow cytometry was utilized to confirm the results obtained by fluorescence microscopy. As demonstrated in Fig. 4, binding of Texas Red-labelled LL-37 to bacterial membranes did not show significant pH-dependence. In contrast to this, a significant increase of the fraction of bacterial cells which had bound the two AHH24 peptides in MES buffer when compared to Tris buffer was observed (Fig. 4). Taken together, these results indicate that acidic pH potentiates interactions between the AHH24 peptides and negatively charged bacteria as well as heparin.

3.3. Permeabilisation of bacteria

To examine whether the histidine-rich peptides disrupt bacterial plasma membranes, *E. coli* and *P. aeruginosa* was incubated with the peptide AHH24:1 as well as the lysine-containing variant (AKK24) at concentrations yielding complete bacterial killing (30 μM), and analysed by electron microscopy (Figure 5). The peptides caused marked changes. *E. coli* bacteria displayed residual cell walls, reminiscent of bacterial "ghosts" with cell envelopes devoid of their cytoplasmic contents, and intracellular material was found extracellularly. Similar results were obtained using the significant pathogen *P. aeruginosa.* The AHH peptide exclusively caused these changes at acidic pH, whereas the AKK24 peptide was also active at pH 7.4, findings compatible with the antibacterial data (Fig. 1A). Notably, treatment of *E. coli* with other potent AMPs, such as temporin L [30] or lipopeptides [31, 32] at neutral pH,

yielded very similar results as those observed here with the AHH peptide at acidic pH. These data suggest that the studied peptides act on bacterial membranes, however they do not demonstrate the exact mechanistic events following peptide addition to bacteria, as secondary metabolic effects on bacteria also may trigger bacterial death and membrane destabilization. Analogous results were also obtained using the impermeant dye FITC. Thus, whereas the AKK24 peptide was active at acidic and neutral conditions, the histidine-rich counterpart only permeabilised *E. coli* at pH 5.5 (Fig. 6).

3.4. CD spectroscopy and permeabilisation of liposomes

CD spectroscopy was utilised for detection of possible structural changes in the AHH peptide. As shown in Fig. 7A, AHH24 displayed essentially a random coil conformation in buffer solution, and, more importantly, no conformational changes associated with binding to acidic liposomes. Addition of LPS yielded significant dichroism, however, the spectrum differed from result obtained with LPS alone. The change in CD spectrum for AHH24 in presence of LPS was different when compared with results observed for "typical" helical peptides, such as magainin and melittin derivatives [9]. In principle, these changes in CD spectra may be due to both peptide and LPS. However, given the lack of induction of ordered secondary structures by the negatively charged liposomes, it seems likely that conformational changes in LPS is primarily shown in the CD spectra. In analogy to the results from the antimicrobial assays using bacteria and fungi, as well as the EM analysis, AHH24 displayed little or no membrane-disruptive effect at pH 7.4, but permeabilised membranes at lower pH (Fig. 7B). Kinetic analysis showed that ~80% of the maximal release occurred within 5-10 minutes (Fig. 7C). In contrast to this, AKK24 displayed significant membrane-disruptive effects already at pH 7.4,

which was not further enhanced when lowering pH (Fig. 7B and C). At low pH, the liposome leakage effects observed for AHH24 and AKK24 were quantitatively comparable. In this context, it should be emphasized however, that the purpose of using liposomes in these experiments was not to mimic bacterial membranes but merely to qualitatively monitor more general membrane disruptive effects of these peptides.

3.5. Endogenous histidine-rich sequences demonstrate similar pH-dependent mode of action

Finally, we examined whether the histidine-rich peptides: histatin 5 and peptides derived from two endogenous proteins having histidine-rich regions; i.e., human high molecular weight kininogen and histidine-rich glycoprotein (Table 1), display a similar pH dependence. *C. albicans* was selected for the further analysis since this organism appeared to be most sensitive to the AHH24 peptides. Indeed, the results showed that some of the endogenous sequences display an analogous, pH-dependent antifungal activity. Thus, we found that KHN20, GHG20, GGH20, and GHH20 all show significant activity against *C. albicans* in MES pH 5.5 but not in Tris pH 7.4 (Fig. 8A and 8B, indicated by black arrows). Similar pH-dependent effects were demonstrated using the above-mentioned peptides (KHN20, GHG20, GGH20 and GHH20) in viable count assays using 60 μM of the respective peptide (Fig. 8C).

3.6. Gain in antimicrobial activity of peptides correlates with pH-dependent changes of their net charge

Finally, we tried to correlate the net charge of peptides at a given pH with their antimicrobial activities. The net charge of all tested peptides at both neutral (7.4) and acidic

(5.5) pH was estimated by using the isoeletric plot application from JaMBW 1.1 (http://www.bioinformatics.org/JaMBW//) (see Table 1). The analysis showed that peptides displaying antifungal activity at both pH 7.4 and pH 5.5 all carry a high net positive charge at neutral pH, ranging from +5.3 for histatin 5, to +12.0 for AKK24/ARK24. Thus, the R and K content of histatin 5, HKH20 and GKH17 is likely *sufficient* for antimicrobial effects at neutral pH in this group of AMPs. In the next group of peptides, displaying *no* antimicrobial activity at neutral pH, the net positive charge of these peptides at pH 7.4 ranged from –1.8 (GHG21) to +2.3 (GGH20). In this group of peptides, a gain in net positive charge at pH 5.5 (ranging from +4.6 to +10.8) correlated well with an increase in antifungal activity (Table 1). The only peptide with no activity at both neutral and acidic pH, GHG21, displays net charge values of –1.8 and +1.0 at neutral and acidic pH, respectively, likely insufficient for bacterial binding at acidic conditions.

4. Discussion

The main finding in this report is that acidic pH influences the antimicrobial activity of a range of histidine-rich peptides of non-natural as well as endogenous origin. The replacement of lysine and arginine residues in the AMPs AKK24 and ARK24 (Table 1) by histidines completely abolished the antimicrobial capacity of these peptides. Histidines, having a pKa of approximately 6.5 for the isolated amino acids, are largely unprotonated and uncharged at physiological pH but become protonated and cationic at acidic pH. Thus, by imposing a positive charge on the AHH24 peptides through lowering of pH, a restored antibacterial activity of these motif peptides is observed. Notably, this gain in activity is paralleled by an increased affinity of these peptides for heparin. The demonstration that low pH potentiated the antimicrobial activity of a set of peptides from the heparin binding regions of domain 5 of

human kininogen as well as histidine-rich glycoprotein, further substantiates the findings with the prototypic AHH-peptides. In this context, acidic pH seems functionally similar to Zn^{2+}, which also impose a positive charge on histidine-rich peptides, including the AHH24 variants, leading to enhanced antimicrobial activities [33]. The results reinforce the view that peptide charge is an important determinant for peptide activity, corroborating with previous findings that outer membrane permeabilisation (of *E. coli*) is dependent of electrostatic interactions [9].

The cell walls of Gram-positive organisms comprise a relatively thick peptidoglycan layer containing teichoic or teichuronic acids, whereas Gram-negative bacteria have a cell wall rich in acidic lipids such as LPS as well as a central peptidoglycan layer. Similarly, β-glucan, chitin, mannoprotein and a blend of other cell wall proteins and polysaccharides contribute to a negative surface potential of fungal surfaces. In either case, the microbial cell wall presents a highly electronegative surface. Furthermore, most bacterial plasma membranes are rich in anionic phospholipids, including phosphatidylglycerol, phosphatidylserine, and cardiolipin. These electronegative constituents are often oriented toward the inner leaflet of the plasma membrane, and confer a net negative charge to the membrane, and particularly toward its inner aspect [3]. All these factors likely contribute to AMPs exhibiting different activity spectra on bacterial and fungal membranes, as well as toxicity on eukaryotic cells. It has, however, become increasingly clear that AMP selectivity may also depend on factors such as AMP oligomerisation and preassembly (in solution and membrane) [34]. Thus, the finding that the AHH-peptides displayed no hemolytic activities, while retaining an antibacterial effect in presence of physiological salt concentrations is compatible with this general view of AMP action. Although the purpose of this work was not to study the exact mode of action of the AHH-peptides on bacterial inner and outer membranes, or their possible oligomerisation, it is of note that the AHH peptides did not

assume a helical conformation in presence of acidic (PA) liposomes. This observation suggests that helicity is not a strict prerequisite for antibacterial action, but rather corresponds to hemolytic activity [5, 12]. In this context, it should be mentioned that the designed AHH peptides comprised alanine, which is of low hydrophobicity, while the endogenous peptides derived from kininogen (eg. KHN20 and GGH20) as well as histidine-rich glycoprotein (GHH20) contain mostly polar and charged residues. Interestingly, recent studies utilizing ellipsometry, CD, fluorescence spectroscopy, and z-potential measurements on the peptide HKH20 [22], showed that the peptide displays primarily random coil conformation in buffer and at lipid bilayers, having lipid interactions dominated by electrostatics [27].

In general, the AHH peptides displayed a higher activity against Gram-negative bacteria compared to Gram-positive bacteria. The mechanistic reason for this remains to be elucidated, although obviously, subtle but specific differences in bacterial cell walls (see above) must underlie these differences. In this context, it is interesting to note that Bosshart *et al*. found that poly-L-histidine, as well as the the 25-mer peptide $(GHHPH)_5$ of histidine-rich glycoprotein was shown to bind and neutralise LPS-induced host proinflammatory response [35]. Although the focus in that work was on endotoxin-neutralization, it is interesting to note that LPS binding by these peptides is enhanced at low pH, thus corresponding to our findings. On the other hand, surface components of Gram-positive bacteria such as proteins, cross-linked peptidoglycan and polyanionic teichoic acids (TAs) could also display different specificities for histidine rich AMPs. For example, it has been reported that D-alanylation of WTA and LTA results in lower net polyanionic charge of teichoic acids, which confers higher resistance to cationic peptides in Gram-positive species. The fact that the D-alanyl ester content in LTA and WTA of *S. aureus* and *B. subtilis* relates to the pH of the growth medium, could constitute yet other underlying resistance mechanisms [36]. In acetate buffer (pH 5.5), the Cardin and Weintraub peptides (AKK24, ARK24 as well as the H-rich variants) displayed

no activity against *S. aureus and B. subtilis*, which contrasted, with their high activity on *E. coli* and *P. aeruginosa.* Clearly, environmental factors (ions, salt), as well as structural prerequisites of the target organisms, determine the activity of a given AMP. For example, as shown by Dorschner et al., the ionic environment does alter antimicrobial effects of variety of structurally diverse peptides by modifying bacterial gene expression, thus rendering bacteria more susceptible. [37]. Notably, the presence of HCO_3^- results in decreased bacterial cell wall thickness. The results with *C. albicans* demonstrated that a number of endogenous, histidine-rich sequences might show a similar pH-dependence as that demonstrated for the consensus AHH-peptides. It was noted that some peptides showed a diminished antimicrobial activity in MES buffer at pH 5.5, even though their net charge increased (eg. HKH20, AKK24, ARK24 as well as histatin 5). Reasons for this contradictory result could involve either pH-induced changes in the fungal cell envelope or electrostatic limitations for peptide adsorption at very high peptide charge densities [27]. Interestingly, these results correlate well with the observation that most R and K-rich AMPs of natural origin have a net charge of +4 to +7, and that a net charge above +8 to +9 is often detrimental for antimicrobial activity [5].

Also previous studies have shown that some AMPs, e.g., clavanins [38] and LAH-peptides [39], are potentiated at acidic pH. The LAH peptides, however, are active in both neutral and acidic conditions, and display distinct mechanisms of action, depending on pH. In contrast to clavanins and LAH4-family peptides, which show lower but still detectable activity at neutral pH, the herein described AHH-peptides are devoid of activity at neutral pH. Furthermore, their low hydrophobicity separates the from the LAH-peptides, which are reported to interact with the lipidic moieties and assume a transmembrane orientation [39]. Furthermore, Makovitzki and Shai investigated the pH dependence of lipopeptides with dodecanoic acid-modified 12-mer peptides of the type LXXLLXXLLXXL, where X is H, K or R [40]. While the K- and R-containing peptides displayed little pH dependence, an H-

containing one showed pronounced pH dependence. This H-containing peptide did not display any antifungal properties at pH 7.4 (contrary to the K- and R-containing peptides), but was active at pH 5.5. Analogously, this H-containing peptide induced liposome leakage at the lower pH but not at the higher, an effect related to a higher peptide adsorption at lipid membranes at the lower pH. These results clearly demonstrate the importance of electrostatic interactions both for peptide adsorption and resulting lipid membrane rupture, and are in agreement with the reports mentioned above in which the peptide charge density was varied by composition changes rather than by pH.

Although it was not explicitly addressed in the present study, it is tempting to speculate about the biological significance of the phenomenon of pH activation. pH-regulated ”switches” can be responsible for restricting antimicrobial activity of histidine-rich peptides to certain compartments of cellular environment (i.e., acidified phagosome) or certain tissues and organs. Both physiological and pathological mechanisms are responsible for generating a low pH *in vivo*. From a clinical point of view, it is well established that acidic pH plays a role in skin defence against fungal and bacterial infections (healthy skin has a normal pH of about 5). It has been shown that skin disorders are associated with increase in cutaneous pH, which predisposes skin to secondary bacterial infections [41]. For example, the skin of atopic dermatitis patients is characterized by significantly higher pH when compared to healthy controls [42]. Likewise, maintaining an acidic pH in vagina (pH between 3.8 – 4.2) by *Lactobacillus* species prevents bacterial and fungal infections [43]. For example, eradication of Lactobacilli after prolonged antibiotic treatment predisposes women to secondary infections and *Candida* vaginitis. Of relevance are also recent findings showing that hemoglobin-derived hemocidins (HbB115–146), constituting a novel antimicrobial defence mechanism in the female urogenital tract generated during menstruation, display strongly enhanced activity at acidic pH [44]. Notably, the net charge of hemocidin is +2, but due to 4

histidines, it increases to +6 at acidic pH. Altogether, these observations suggest AMP activation at low pH, and AMP inactivation at neutral pH, may be of clinical importance. Clearly, however, the extent of which these observations are related to activation of histidine-rich AMPs needs further study.

Acknowledgements

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Table 1. Peptides investigated in the study. The activity values indicate the zone of inhibition (using *C. albicans*) in RDA. Mean values and SD are presented (n=3). The net charge at the indicated pH, and the content (in %) of Lys and Arg, and His residues are indicated. Net charge was estimated using the isoeletric plot application from JaMBW 1.1 (http://www.bioinformatics.org/JaMBW//)

Peptide name	Peptide sequence	Net charge		Activity against *C. albicans* in RDA		K, R [%]	H [%]
		TRIS pH 7.4	MES pH 5.5	TRIS pH 7.4	MES pH 5.5		
AKK24	AKKARAAKKARAAKKARAAKKARA	+ 12.0	+12.0	6.9 ± 0.6	4.3 ± 0.19	50.0	0.0
AHH24:1	AHHAHAAHHAHAAHHAHAAHHAHA	+ 0.4	+ 9.1	0.0 ± 0.0	2.4 ± 0.02	0.0	50.0
ARK24	ARKKAAKAARKKAAKAARKKAAKA	+ 12.0	+12.0	7.9 ± 0.9	4.4 ± 0.37	50.0	0.0
AHH24:2	AHHHAAHAAHHHAAHAAHHHAAHA	+ 0.4	+ 9.1	0.0 ± 0.0	3.7 ± 0.45	0.0	50.0
GHH20	GHHPHGHHPHGHHPHGHHPH	+ 0.4	+ 8.4	0.0 ± 0.0	3.9 ± 0.12	0.0	60.0
KHN20	KHNLGHGHKHERDQGHGHQR	+ 1.2	+ 5.6	0.0 ± 0.0	3.9 ± 0.40	20.0	30.0
GHG20	GHGLGHGHEQQHGLGHGHKF	+ 0.3	+ 4.6	0.0 ± 0.0	2.1 ± 0.29	5.0	30.0
GHG21	GHGHKFKLDDDLEHQGGHVLD	- 1.8	+ 1.0	0.0 ± 0.0	0.0 ± 0.00	9.5	19.0
GGH20	GGHVLDHGHKHKHGHGHGKH	+ 2.3	+ 7.3	2.1 ± 0.2	4.6 ± 0.47	15.0	40.0
HKH20	HKHGHGHGKHKNKGKKNGKH	+ 7.1	+ 10.8	5.6 ± 0.7	3.4 ± 0.30	35.0	30.0
GKH17	GKHKNKGKKNGKHNGWK	+ 6.1	+ 7.5	5.8 ± 0.3	3.2 ± 0.25	41.2	11.8
histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	+ 5.3	+ 10.2	7.2 ± 0.2	5.3 ± 0.28	29.2	29.2
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+ 6.0	+ 6.1	4.3 ± 0.3	4.3 ± 0.01	29.7	0.0

Figure legends

Fig. 1. (A) Low pH-induced antibacterial effects of histidine-rich peptides. Peptides were tested in RDA in four different buffers against both Gram-negative (*E. coli*, *P. aeruginosa*) and Gram-positive (*S. aureus*, *B. subtilis*) bacteria as well as against fungi (*C. albicans*). $4x10^6$ cfu was inoculated in 0.1% TSB agarose gel. Each 4-mm diameter well was loaded with 6 µl of a 100 µM peptide solution in the indicated buffer. Buffers without peptides were used as negative controls. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 37°C (bacteria) or at 28°C (fungi) for 18-24 h. Zones of clearance were measured and the diameter of a well containing buffer only was subtracted from the observed zone of clearance. Graphs are representative of at least two independent experiments performed in triplicates. (B) Effects of salt on peptide activity. In viable count assays 2×10^6 cfu/ml of *E. coli* bacteria were incubated in 50 µl with peptides at the indicated concentrations in 10 mM MES buffer pH 5.5, 5 mM glucose in presence of the indicated NaCl concentrations. Error bars indicate standard deviation. (C) Analysis of hemolytic effects of AHH and AKK and ARK peptides. The cells were incubated with peptides at the indicated concentrations (x-axis). 2% Triton X-100 (Sigma-Aldrich) served as positive control. The absorbance of hemoglobin release was measured at λ 550 nm and is expressed as % of Triton X-100 induced hemolysis (y-axis) (n=3, mean values and SD is indicated).

Fig. 2. Heparin-binding properties of histidine-rich peptides. 2 and 5 µg of AKK24, AHH24:1, ARK24 and AHH24:2 peptides were applied onto nitrocellulose membranes, followed by incubation with iodinated (^{125}I) heparin in either Tris pH 7.4, MES pH 5.5, PIPES, pH 7.4 or acetate pH 5.5 (all at 10 mM). Radioactivity of bound heparin was visualized using a phosphorimager system.

Fig. 3. Low pH-induced binding of histidine-rich peptides to bacterial and fungal cells. *E. coli* 25922 (1-6) or *C. albicans* 90028 (7-12) were incubated with Texas Red-labelled peptides in either 10 mM Tris, Tris pH 7.4 (panels 1-3 and 7-9) or 10 mM MES, pH 5.5 (panels 4-6 and 10-12). The upper images in each row are Nomarski Differential Interference Contrast images, wheras the lower images show the red fluorescence of bacteria. 1,4,7,10; shows results with LL-37, 2,5,8,11; AHH24:1, and 3,6,9,12; AHH24:2.

Fig. 4. Flow cytometry analysis of low pH-induced binding of histidine-rich peptides to bacterial membranes. *E . coli* was incubated with Texas Red-labelled peptides: LL-37, AHH24:1 and AHH24:2 in Tris pH 7.4 and in MES pH 5.5. Histograms show red fluorescence intensity of gated, homogenous populations of bacterial cells, given in arbitrary units. Percentages of positive cells are indicated.

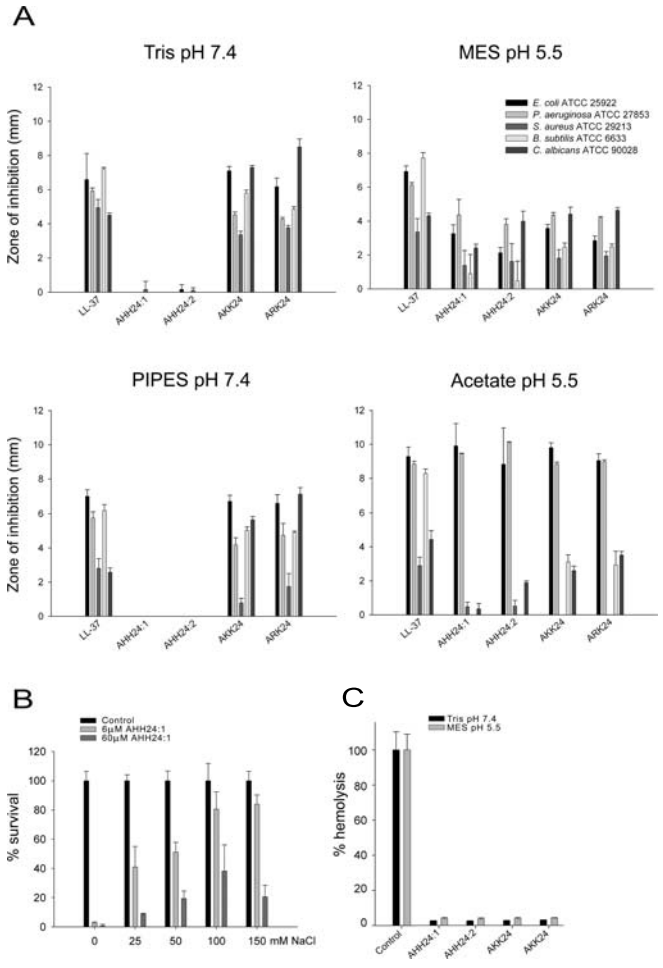
Fig. 5. Electron microscopy analysis of peptide effects at neutral and acidic pH. *E. coli* and *P. aeruginosa* were incubated for 2 h at 37ºC with 30 μM of the respective peptides and the indicated buffers and analysed with electron microscopy. Scale bar represents 1 μm.

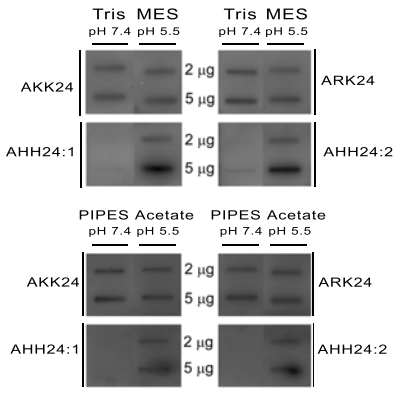
Fig. 6 Bacterial permeabilisation by AHH24:1 and AKK24 peptides at neutral and acidic pH. *E. coli* ATCC 25922 was incubated for 2 h at 37ºC with 30 μM of the respective peptides (indicated in the figure) and permeabilisation was assessed by the addition of FITC.

Fig. 7. (A) CD spectra of AHH24:1 in Tris buffer and in presence of acidic lipsomes or LPS. For control, CD spectra for buffer and LPS alone are presented. (●) AHH24:1, (○) AHH24:1 + PA liposomes, (Δ) LPS, (▼) AHH24:1 + LPS. The graphs for the AHH peptide alone and in presence of liposomes overlap. (B) Effect of pH on AHH24- and AKK24-induced leakage

induction in negatively charged liposomes. (C) Representative time-resolved leakage experiments.

Fig. 8. Low pH-induced antimicrobial effects of endogenous histidine-rich motifs. Peptides derived from histidine-rich domain of high molecular weight kininogen (KHN20, GHG21, GHG21, GGH20, HKH20, GKH17), histatin 5, and a peptide of histidine-rich glycoprotein (GHH20) was tested in RDA in Tris, pH 7.4 (A) and MES pH, 5.5 (B) against *C. albicans*. $4x10^6$ cfu was inoculated in 0.1% TSB agarose gel. Each 4-mm diameter well was loaded with 6 µl of a 100 µM peptide solution in the indicated buffer. The zones of clearance correspond to the inhibitory effect of each peptide after incubation at 28°C for 18-24 h. A negative control containing buffer (10 mM Tris, pH 7.4 and 10 mM MES, pH 5.5, in A and B, respectively) was included in the well at the left side of the plate. This clear zone corresponds to the 4 mm well. Arrows indicate peptides activated in acidic pH. (C) Antifungal effects of consensus and endogenous histidine-rich motif peptides demonstrated by viable count assay. $1x10^5$ cfu of *C. albicans* was incubated with 60 µM of the indicated peptides in 50 µl of 10 mM Tris, pH 7.4 (black bars) or 10 mM MES buffer pH 5.5 (grey bars). Error bars indicate standard deviation. For LL-37 in Tris buffer complete killing was observed.


A
Tris pH 7.4
MES pH 5.5
PIPES pH 7.4
Acetate pH 5.5
Zone of inhibition (mm)
E. coli ATCC 25922
P. aeruginosa ATCC 27853
S. aureus ATCC 29213
B. subtilis ATCC 6633
C. albicans ATCC 90028
LL-37
AHH24:1
AHH24:2
AKK24
ARK24
B
Control
6μM AHH24:1
60μM AHH24:1
% survival
0
25
50
100
150 mM NaCl
C
Tris pH 7.4
MES pH 5.5
% hemolysis
Control


Tris
MES
pH 7.4
pH 5.5
Tris
MES
pH 7.4
pH 5.5
AKK24
2 µg
5 µg
ARK24
AHH24:1
2 µg
5 µg
AHH24:2
PIPES
Acetate
pH 7.4
pH 5.5
PIPES
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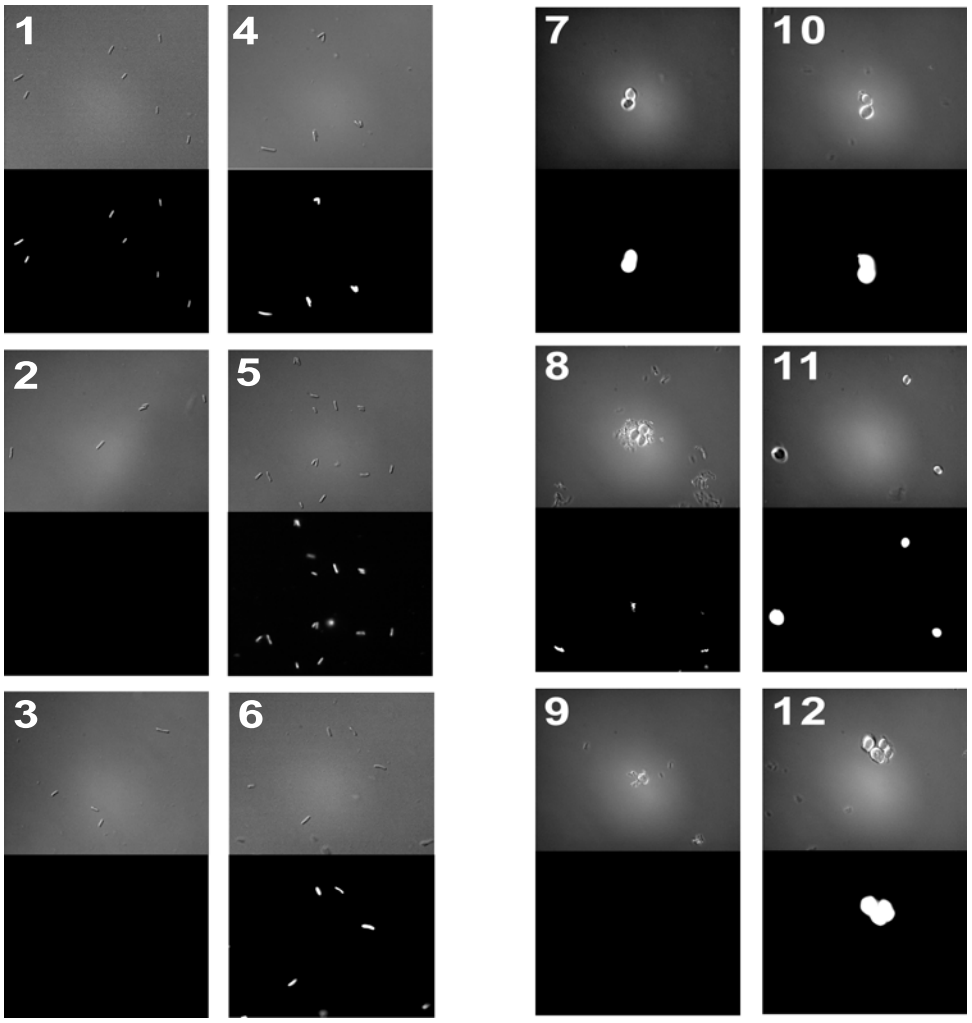
5 µg
ARK24
AHH24:1
2 µg
5 µg
AHH24:2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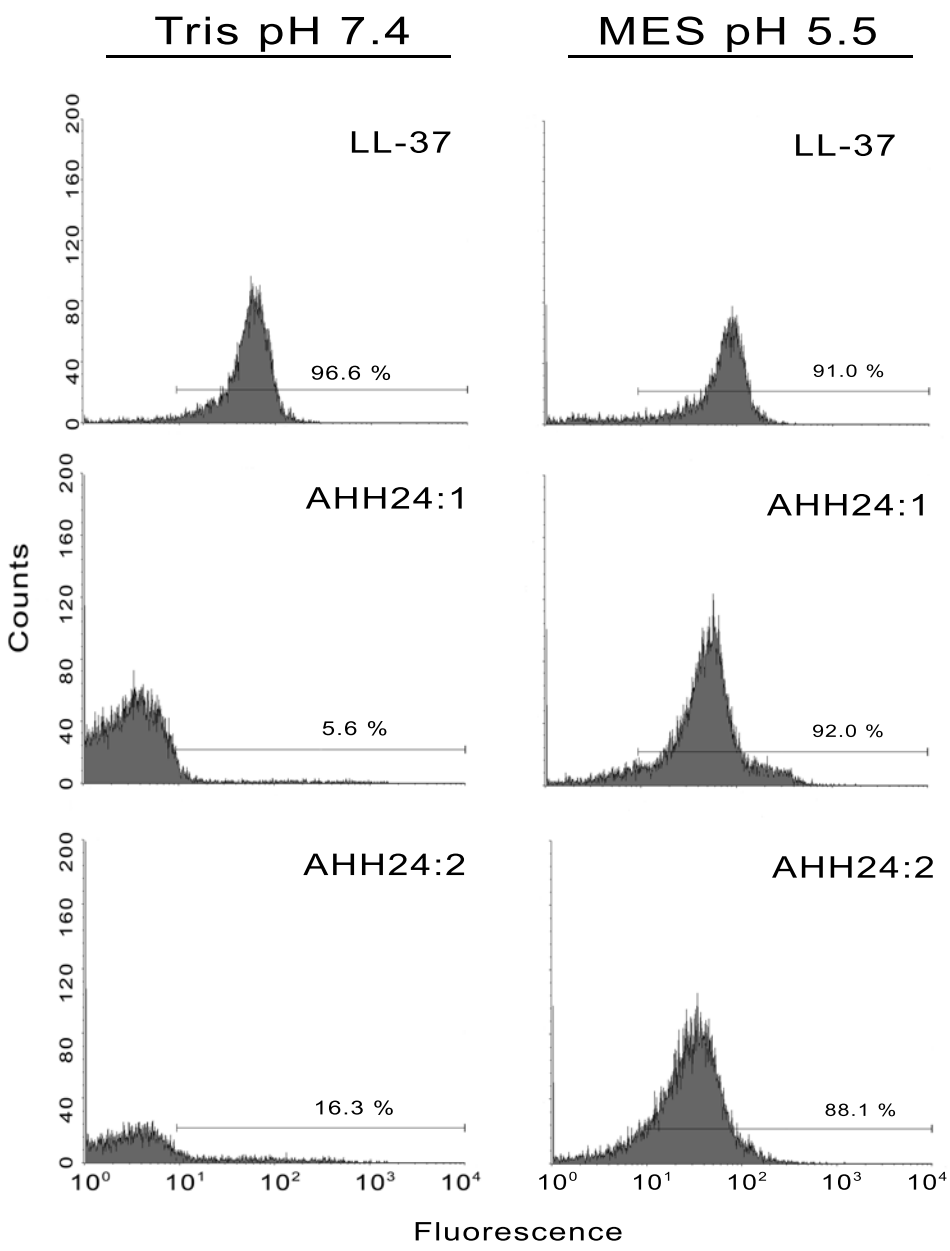
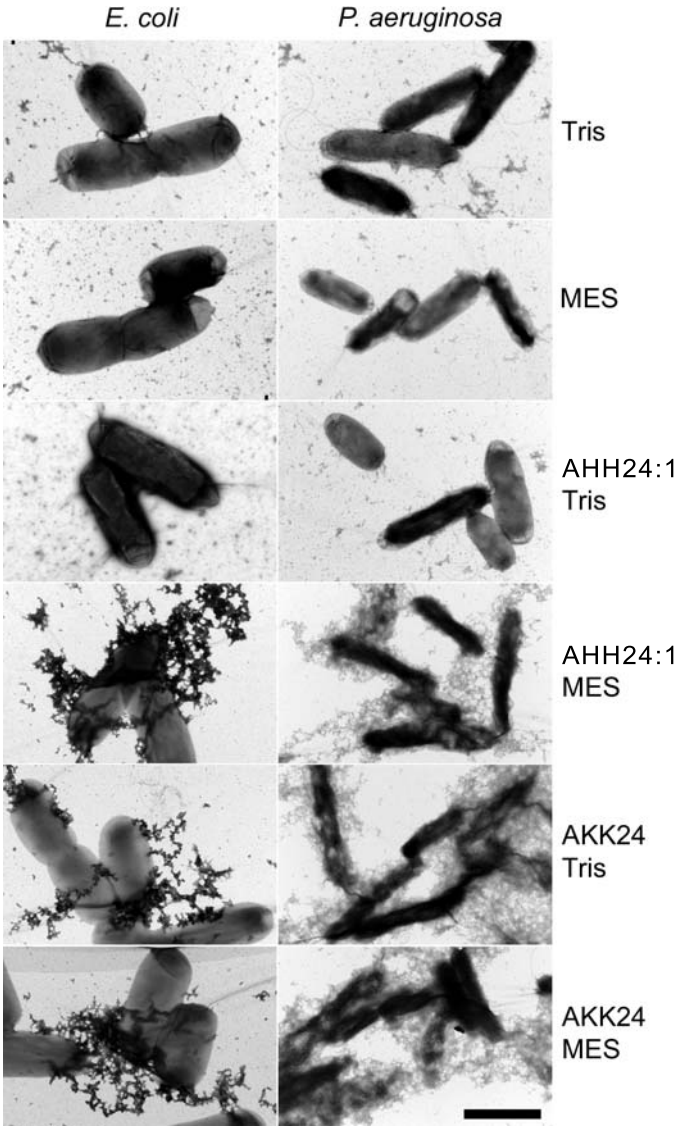
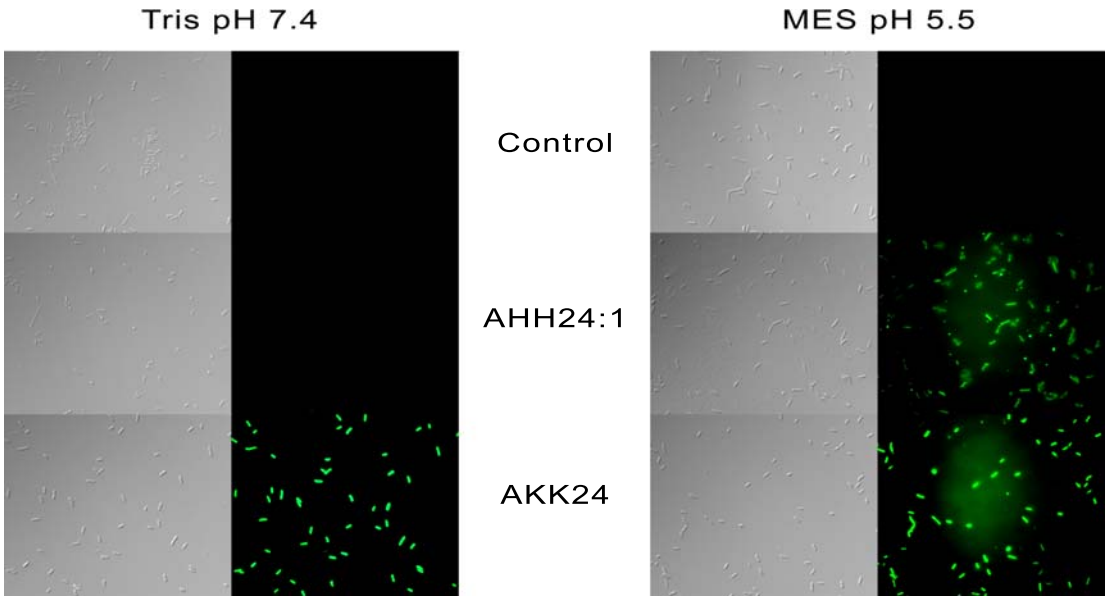
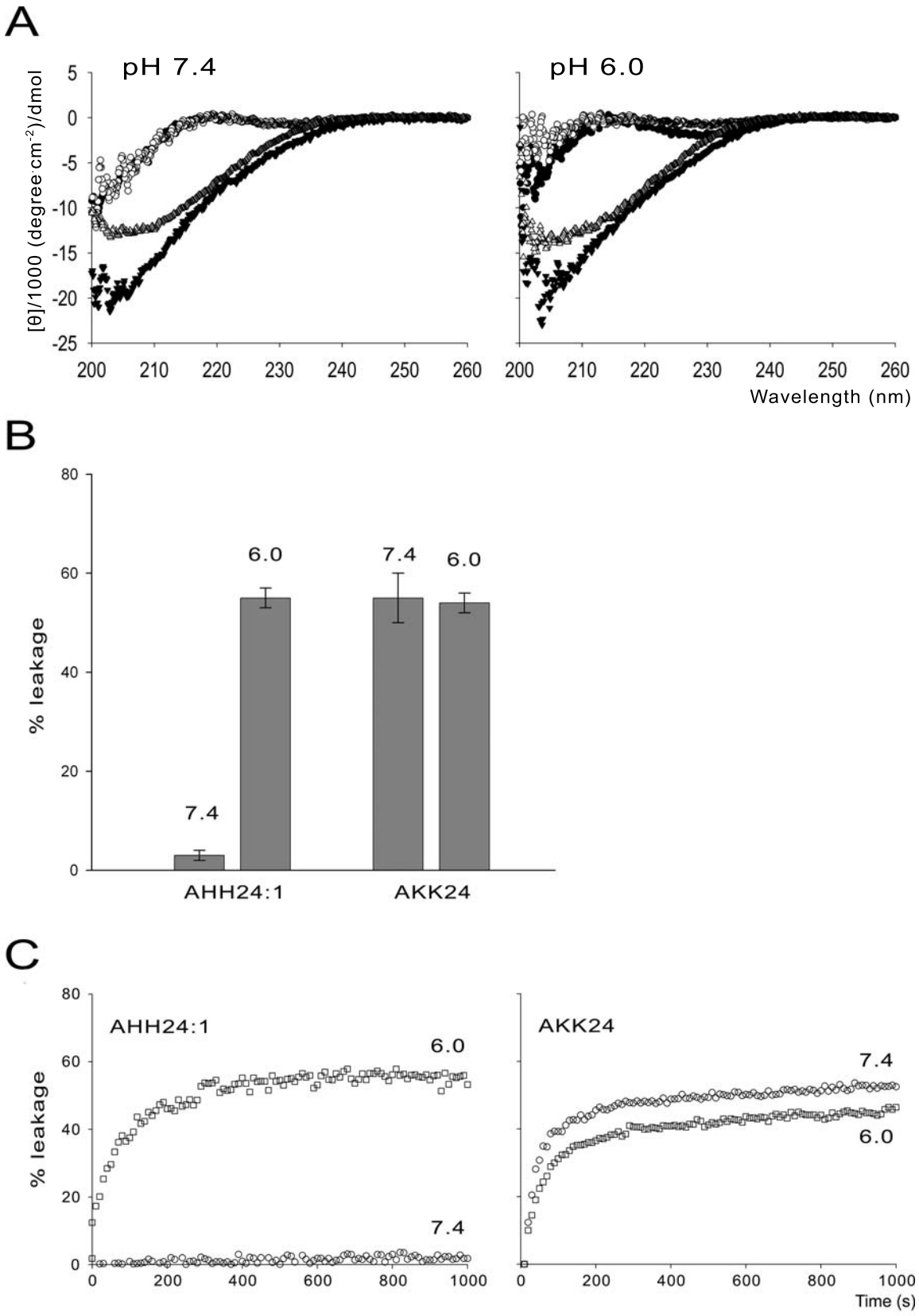
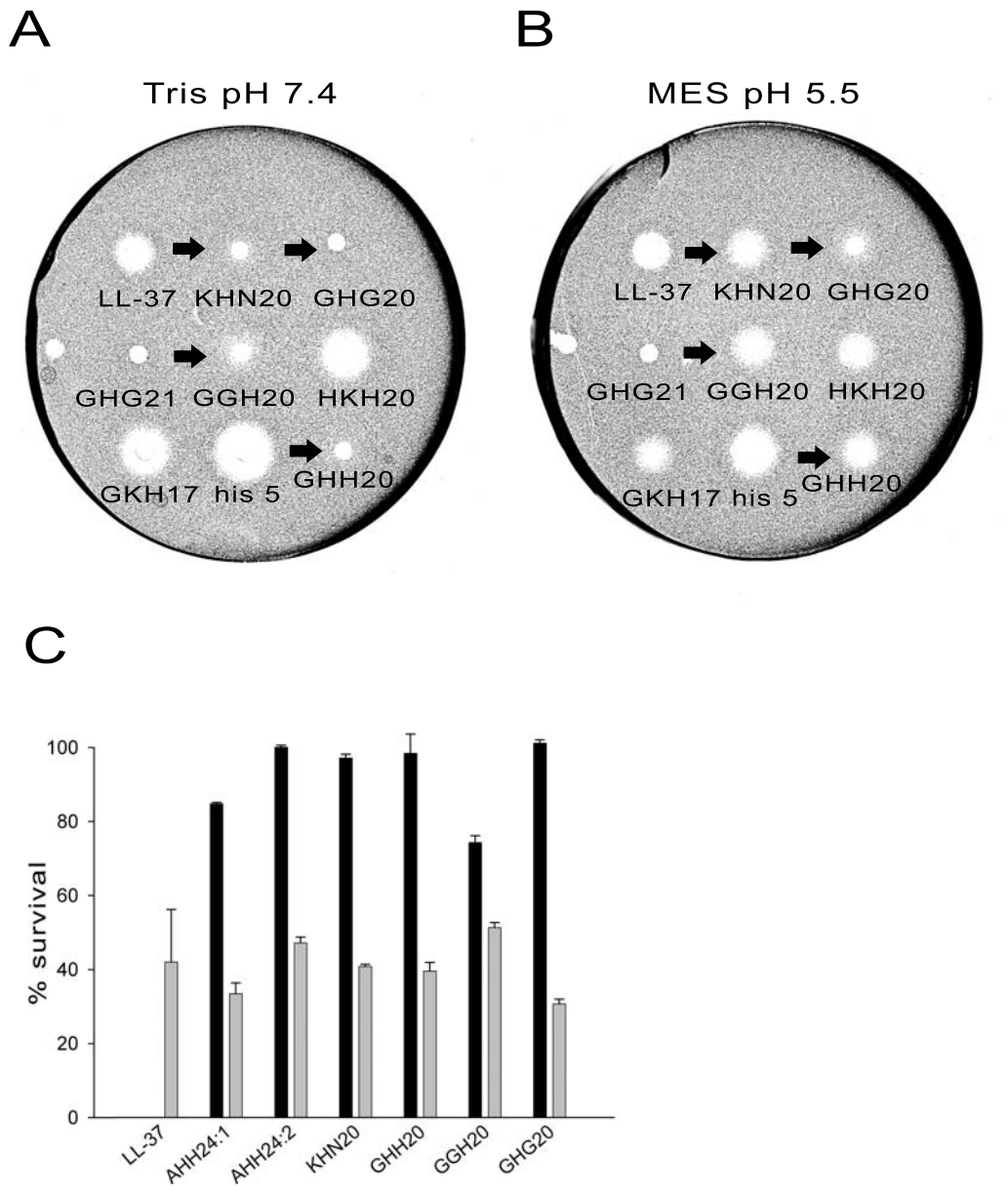

Tris pH 7.4
MES pH 5.5
LL-37
LL-37
96.6 %
91.0 %
AHH24:1
AHH24:1
5.6 %
92.0 %
AHH24:2
AHH24:2
16.3 %
88.1 %
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
Fluorescence


E. coli
P. aeruginosa
Tris
MES
AHH24:1
Tris
AHH24:1
MES
AKK24
Tris
AKK24
MES


Tris pH 7.4
MES pH 5.5
Control
AHH24:1
AKK24


A
pH 7.4
pH 6.0
[θ]/1000 (degree·cm-2)/dmol
5
0
-5
-10
-15
-20
-25
200
210
220
230
240
250
260
Wavelength (nm)
B
% leakage
80
60
40
20
0
6.0
7.4
6.0
7.4
AHH24:1
AKK24
C
AHH24:1
AKK24
% leakage
6.0
7.4
7.4
6.0
0
200
400
600
800
1000
Time (s)


A
Tris pH 7.4
LL-37 KHN20 GHG20
GHG21 GGH20 HKH20
GKH17 his 5
GHH20
B
MES pH 5.5
LL-37 KHN20 GHG20
GHG21 GGH20 HKH20
GKH17 his 5
GHH20
C
% survival
100
80
60
40
20
0
LL-37
AHH24:1
AHH24:2
KHN20
GHH20
GGH20
GHG20