The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides

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ABSTRACT **Antimicrobial peptides (AMPs) have been shown in animal and human systems to be effective natural antibiotics. However, it is unclear how they convey protection; they often appear inactive when assayed under culture conditions applied to synthetic antibiotics. This inactivation has been associated with loss of function in physiological concentrations of NaCl or serum. In this study we show that the balance of host ionic conditions dictate microbial sensitivity to AMPs. Carbonate is identified as the critical ionic factor present in mammalian tissues that imparts the ability of AMPs such as cathelicidins and defensins to kill at physiological NaCl concentrations. After adapting to carbonate-containing solutions, global changes occur in *Staphylococcus aureus* and *Escherichia coli* structure and gene expression despite no change in growth rate. Our findings show that changes in cell wall thickness and Sigma factor B expression correspond to the increased susceptibility to the AMP LL-37. These observations provide new insight into the factors involved in enabling function of innate immune effector molecules, and suggest that discovery of new antimicrobials should specifically target pathogens as they exist in the host and not the distinctly different phenotype of bacteria grown in culture broth.—Dorschner, R. A., Lopez-Garcia, B., Peschel, A., Kraus, D., Morikawa, K., Nizet, V., Gallo, R. L. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. *FASEB J.* 20, 35–42 (2006)**

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Antimicrobial peptides (AMPs) are present throughout the animal and plant kingdoms, an observation that suggests these molecules have played an important role in the evolution of defense by multicellular organisms (1). The persistence of AMP activity stands in marked contrast to the human experience with design of effective pharmacologic antibiotics. In common clinical usage, the duration of effectiveness for synthetic antibiotics is very short due to the rapid evolution of various microbial strategies for resistance. Studies in animal models, however, have demonstrated that evolutionarily conserved gene-encoded antibiotics such as cathelicidins and defensins provide protection against microbial infection, while their absence results in increased risk of infectious disease (2–5). Clinical correlations suggest this immune function extends to humans, as patients with impaired epithelial AMP production (e.g., atopic dermatitis) are highly susceptible to secondary infection, while those with increased AMP production (e.g., psoriasis) are relatively protected (6).

It has been suggested that the study of AMPs may provide insight into design of more effective antibiotic therapies (1). To date this hope has yet to translate into reality. AMPs, such as the human cathelicidin LL-37, are highly membrane active. Their ability to kill bacteria correlates with charge and reflects an ability to disrupt the organized membrane structure of the target organism. A dependence on ionic interaction is confirmed when antimicrobial activity is evaluated in solutions containing salt. The addition of NaCl to microbial culture broth at concentrations as low as 50 mM suppresses the activity of LL-37 and many other AMPs (7, 8). These results led many investigators to conclude that AMPs can only kill microbes in solutions of low ionic strength or when the peptide is expressed at very high local concentrations, observations that contradict experience in several animal models.

An alternative explanation for the function of AMPs is that these peptides have evolved from simple antibiotics to act as signals of injury. Several examples now exist that show mammalian cells respond to AMPs with specific changes in gene expression. These responses include extracellular matrix synthesis (9), angiogenesis (10), leukocyte recruitment, and cytokine release (11). Surface receptors such as FPRL-1 (12) and CCR6 (13) may mediate the host cell response to AMPs. Thus, it is

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possible that the association between AMP expression and infection seen in animal models and human disease may be the result of their ability to augment a cellular immune defense program rather than direct microbicidal action.

This study sought to address the apparent contradictions regarding how AMPs can function as natural antibiotics. The direct bactericidal activity of AMPs was evaluated in vitro in an animal cell culture environment. Our findings show that when select bacteria are grown in mammalian ionic conditions, they respond with an altered gene expression profile that renders them susceptible to AMPs. These observations provide insight into the true parameters of AMP activity, and suggest a novel paradigm for evaluating the function of these peptides in innate immune defense.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used were *Staphylococcus aureus* Rosenbach ATCC 25923, *S. aureus* N315, *S. aureus* NKSB (SigB knockout in the N315 strain), methicillin-resistant *S. aureus* (MRSA) strains 81056 and 81025, *Salmonella enterica* serovar Dublin Lane, and *Escherichia coli* O29.

Antimicrobial assays

Bacteria were grown at 37°C in tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA) to stationary phase, diluted 1:100 into assay medium, and grown to early log phase. Assay medium contained 20% TSB, 10% fetal bovine serum (FBS), and 150 mM NaCl, unless otherwise noted, together with 70% of additional media or media components. The additional 70% volume used to complete the assay medium included minimal essential media (MEM) (Gibco, Grand Isle, NY, USA); the buffers from MEM, which are 27 mM $NaHCO_3$ and 1.1 mM NaH_2PO_4; 1 mM $NaH_2PO_4 \pm 50$ mM $NaHCO_3$. A 50 mM glycine-HCl buffer (Fisher, Tustin, CA, USA) was also used in place of 50 mM $NaHCO_3$ in **Fig. 1*c*** due to its similar buffering capacity. All solutions were adjusted to pH 7.4, unless otherwise noted in the figure legend. Antimicrobial peptides were added to 0.1 mL bacterial suspension containing $\sim 1 \times 10^6$ CFU/mL in a 96-well flat bottom plate (Costar, Corning, NY, USA). Plates were incubated at 37°C for the indicated times. Inhibition of bacterial growth was determined by comparing the change in turbidity at OD_{600} in the presence of peptide to that in the absence of peptide. Minimal inhibitory concentration (MIC) was defined as the concentration of peptide that resulted in no detectable bacterial growth. Bactericidal and bacteristatic activity was determined by plating serial dilutions of bacteria on tryptic soy agar and enumerating colony forming units after overnight incubation at 37°C. The minimal bactericidal concentration was determined as the minimum peptide concentration resulting in no surviving colonies. Statistical significance was determined using GraphPad Prism software (v. 21., GraphPad Software, San Diego, CA, USA). Means were calculated with standard deviation, then a 2-way ANOVA was applied with a Bonferroni post-test. P values <0.001 were considered significant.

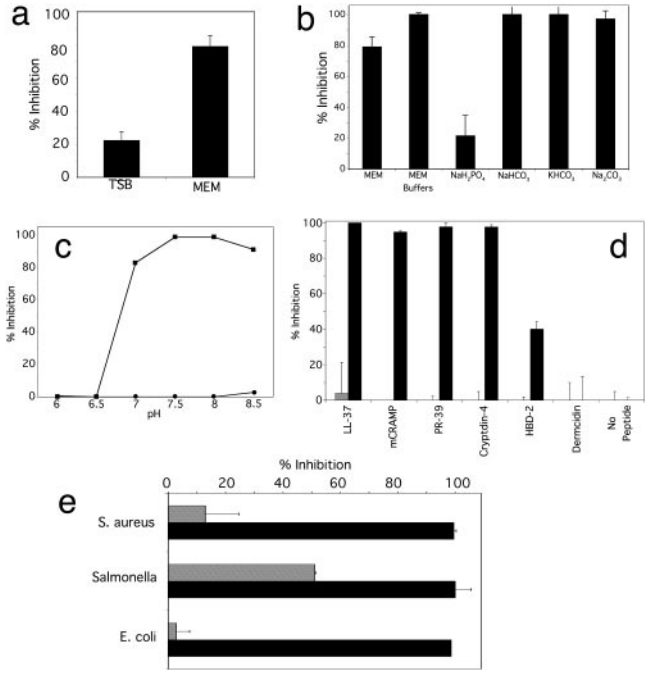

Figure 1. Carbonate-containing media increase the antimicrobial activity of LL-37. *a)* 32 μM LL-37 inhibited overnight growth of *S. aureus* Rosenbach poorly in the bacterial growth media TSB-containing 125 mM NaCl and 10%FBS, yet was effective in MEM with the same salt and serum concentrations. *b)* Serial deletion of MEM components revealed that $NaHCO_3$ was responsible for the enhancement of antimicrobial activity in the presence of salt and serum in MEM. The importance of carbonate was confirmed with other carbonate-containing compounds, including 50 mM $KHCO_3$ and Na_2CO_3, which similarly increased antimicrobial activity. pH in all media was 7.4. *c)* Carbonate itself, and not pH, was shown to be responsible for antimicrobial enhancement. Inhibition of *S. aureus* by 32 μM LL-37 overnight was measured at various pH in media containing 20% TSB, 10% FBS 1 mM NaH_2PO_4, and 150 mM NaCl, with either 50 mM $NaHCO_3$ (squares) or 50 mM Glycine-HCl buffer (circles). Only bicarbonate-containing media was able to confer increased antimicrobial activity to LL-37. *d)* Inhibition of *S. aureus* growth overnight was measured in the presence of 32 μM of an array of structurally diverse antimicrobial peptides. Antimicrobial activity was measured in 20% TSB, 1 mM NaH_2PO_4, with (black bars), or without (gray bars) 50 mM $NaHCO_3$at pH 7.4. Antimicrobial activity of human (LL-37) and murine (mCRAMP) cathelicidins, the linear porcine (PR-39) cathelicidin, and the β sheet murine (Cryptdin-4) and human (HBD-2) defensins, but not dermcidin, was enhanced by $NaHCO_3$. *e)* $NaHCO_3$ enhances antimicrobial activity against Gram negative and positive bacteria. *S. aureus* and *E. coli* were resistant to 16 μM mCRAMP without 50 mM $NaHCO_3$ (gray bars), but became susceptible in its presence (black bars) in 20% TSB, 1 mM NaH_2PO_4 at pH 7.4. *Salmonella dublin*'s susceptibility was also increased in the presence of $NaHCO_3$.

Reverse-transcriptase PCR and quantitative real-time PCR

Approximately 1×10^8 CFU of log phase bacteria were homogenized in 1 mL of TRIzol Reagent (Gibco, Grand Island, NY, USA) in a mini BeadBeater (Biospec Products, Bartlesville, OK, USA) with 0.1 mm glass beads on max power for 5 min and RNA was extracted per TRIzol manufacturer's directions. RT-PCR was performed on 1.5 μg RNA with Retroscript kit (Ambion, Austin, TX, USA). Briefly, first

strand synthesis combined RNA with 0.4 mM dNTPs and 4 μM decamers in 16 μL at 80°C for 3 min. Reaction was iced and 2 μL 10× RT-PCR buffer, 1 μL RNase inhibitor, and 1 μL mMLV RT added followed by incubation at 42°C for 1 h, then denatured at 94°C for 10 min. Real-time (RT) quantitative PCR was performed using an Applied Biosystems 7000 Sequence Detection System (Foster City, CA, USA). Primers were designed for the target genes *sigB* and *fliA*, as well as 16 s rRNA as an endogenous control. The sequences of the *sigB* primers are: F-AAAGATGGTTCAACTGTTACGCTATTAG, R-GGTCATCTTGTTGCCCCATAA. The sequences of the *fliA* primers are: F-GCGGCATTGGGTTACTTAATG, R-CGTTCCTTGTAGGGCGTCATA. The sequences of the 16 s primers are: F-GTTATCCGGAATTATTGGGCG, R-CCGGGCTTTCACATCAGACT. 5 μL RT reaction was used in SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with 100 nM primers. Thermal profile: 50°C 2 min, 95°C 10 min, 40 × (94°C 15 s, 60°C 1 min). Results were analyzed using the Comparative Ct Method, as described (14). Product specificity was confirmed by generating dissociation curves for the reactions.

Membrane permeability

E. coli strain ML-35p, which constitutively expresses a plasmid-encoded periplasmic β-lactamase and cytoplasmic β-galactosidase but lacks lactose permease, was used to measure inner and outer membrane permeability. After an overnight incubation at 37°C, the culture was adjusted to 2.5–5 × 10^7 CFU/mL (A_{600}=0.03) in buffer A (1 mM NaH_2PO_4, 20% TSB) or B (1 mM NaH_2PO_4, 20% TSB and 25 mM $NaHCO_3$).

To test permeabilization of the inner membrane, formation *o*-nitrophenol (ONP) as the hydrolytic product of β-galactosidase on the colorless substrate *o*-nitrophenyl-β-d-galactosidase (ONPG) was determined by an increase in absorbance at 420 nm (A_{420}). ONPG was prepared at a stock concentration of 3 mg/mL in 10 mM sodium phosphate buffer, pH 7.0 (NaPB). To study permeabilization of the outer membrane, we followed β-lactamase activity by measuring the decrease in A_{390} due to the substrate nitrocefin and increase of A_{486} due to the red product. Nitrocefin was prepared at a stock concentration of 500 μg/mL in NaPB with 0.05 % DMSO (DMSO).

The assay mixture contained 43 μL of bacteria from stock cultured in buffer A or B, with final concentrations of 4 μM LL-37, 0.3 mg/mL ONPG or 25 μg/mL nitrocefin. Samples were incubated at 37°C in sterile 96-well microtiter plates (Corning Inc., Corning, NY, USA) and the kinetics of β-galactosidase or β-lactamase activity calculated by measurement of the A_{420} or A_{390}/A_{486}, respectively, with a Spectra max PLUS 384 (Molecular Devices, Sunnyvale, CA, USA). To test bacterial growth, the A_{600} was also monitored.

As a positive control, 2.5–5 × 10^7 CFU/mL of *E. coli* ML-35p was permeabilized with 1 μg/mL of polymyxin B (Calbiochem, La Jolla, CA, USA), 1% Triton X-100 in buffer A or B.

Nuclear membrane permeabilization in *S. aureus* was also examined by fluorometric measurement. 74 μL of *S. aureus* cultured as above were dispensed onto each well of a sterile 96-well microtiter plate (Nunc, Roskilde, Denmark). 2 μL of 640 μM LL-37 or H_2O and 4 μL of 4 μM Sytox Green (Molecular Probes, Eugene, OR, USA) in 0.08% DMSO were added to each sample and the plates were incubated at 37°C in the dark. Fluorescence emission was measured with a microplate reader (Fluoroskan Ascent FL, Labsystems, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. 1 μg/mL polymyxin B, 1% Triton X-100 was used as a positive control.

Isolation and analysis of S. aureus membrane lipids

Bacteria were grown as described above for the antibacterial assay in the presence or absence of carbonate and disrupted using glass beads and a Disintegrator S (Biomatic GmbH, Rodgau, Germany). Lipids were isolated and analyzed as described recently (15). Briefly, polar lipids were extracted by the Bligh-Dyer procedure, vacuum dried, and dissolved in chloroform/methanol (2:1, by vol). Equal amounts of lipid extracts were spotted onto silica 60 F_254 HPTLC plates (Merck, Darmstadt, Germany) and developed with chloroform/methanol/water (65:25:4, by vol) in the first direction and chloroform/acetic acid/methanol/water (80:15:12:4, by vol) in the second direction. All lipids were visualized with molybdatophosporic acid spray (Merck) followed by charring at 120°C and treatment with ammonia vapor to improve the contrast. Phospholipids or amino group-containing lipids were selectively stained with Molybdenum Blue (Sigma) or ninhydrin spray (Merck), respectively. The lipid fatty acid composition was analyzed by gas chromatography as described recently (15).

Electron microscopy

Electron microscopy was performed on *S. aureus* Rosenbach grown to log phase in 20% TSB, 1 mM NaH_2PO_4, with or without addition of 25 mM $NaHCO_3$. 32 μM LL37 or an equal volume of water was added and the bacteria were cultured 4 h. Bacteria were pelleted at 1000 × *g* for 5 min at 4°C, fixed in Karnovsky's Solution 24 h at 4°C, and processed for EM on a Zeiss EM 10B Transmission Electron Microscope by the VA San Diego Microscopy Core Facility.

Microarray analysis

E. coli was grown, as described earlier, in the presence or absence of $NaHCO_3$ (buffer A or B) for 2 h. The samples for microarray analysis were prepared as described in Affymetrix Gene Chip Expression Analysis Procedure (Affymetrix, Santa Clara, CA, USA). Briefly, after centrifugation of bacteria, RNA extraction was carried out using RNeasy Mini kit (Qiagen, Germantown, MD, USA). Total RNA from four separate experiments was pooled for cDNA synthesis, RNA removed by hot-alkali treatment and cDNA was purified using a Qiaquick PCR Purification kit (Qiagen). cDNA was fragmented by DNase I treatment and terminally labeled using Enzo® BioArray™ Terminal Labeling Kit with Biotin-ddUTP (Affymetrix, Santa Clara, CA, USA). The target was hybridized onto probe arrays. Arrays were scanned at the UCSD-VA Gene Chip Core using an Affymetrix Genechip 3000 High Resolution Scanner.

Subsequent analysis was performed using GeneSpring software (Silicon Genetics, Redwood City, CA, USA). The raw data were normalized by housekeeping genes, spiked in controls, Total Intensity, Mean Centering, Lowess correction, Chen's Ratio Statistics, and filtered based on genes being present in at least one condition and having at least a 2-fold change.

RESULTS

The presence of carbonate increases peptide antimicrobial activity

Several human AMPs, such as cathelicidins and members of the defensin family of defense peptides, have been observed to lose antimicrobial activity in vitro with the

addition of physiological NaCl concentrations or plasma proteins (7, 8, 16). However, in animal model systems the expression of these gene products correlates with increased microbial resistance (4, 17, 18) (Lee et al., in press). To resolve the apparent contradiction between observations in animal systems and those measured in vitro, the antimicrobial activity of the human cathelicidin peptide, LL-37, against the pathogen *S. aureus* was evaluated in animal tissue culture media containing 10% serum and a physiological NaCl concentration. In contrast to the bacteriologic culture media TSB, LL-37 showed significant growth inhibition of *S. aureus* in MEM media with 10% serum (Fig. 1*a*).

To identify the factor in animal cell culture media responsible for increasing antimicrobial activity, the components of MEM were systematically tested. All media tested contained 20% TSB, 150 mM NaCl, and 10% FBS at pH 7.4. 20% TSB was chosen because it allowed robust bacterial growth in the absence of AMPs. The concentration of TSB did not affect the ability of LL-37 to kill *S. aureus*, as no difference in killing was observed when the assay was performed in MEM containing 0, 5, 10, or 20% TSB (data not shown). The presence of only the salts from MEM, $CaCl_2$, KCl, and $MgSO_4$ was not sufficient to enhance antimicrobial activity, nor were phenol red, amino acids or vitamins present in this medium (data not shown). Addition of the buffering components of MEM (27 mM $NaHCO_3$ and 1.1 mM NaH_2PO_4,) was the only treatment that showed effects similar to that of complete MEM (Fig. 1*b*). Further testing identified the carbonate anion as the active molecule (Fig. 1*b*). As a control, similar testing was performed using solutions buffered with either $NaHCO_3$ or glycine-HCl. No increase in antimicrobial activity was seen at any pH in glycine-based buffers (Fig. 1*c*), indicating the unique carbonate effect was not simply a reflection of pH stabilization. We also examined whether environmental CO_2 could diffuse into the media to potentiate killing. In the absence of carbonate, overnight growth and assay in a 5% CO_2 environment did not result in significantly enhanced antimicrobial activity.

Carbonate enhances the antimicrobial activity of a structurally diverse panel of peptides and renders diverse bacteria susceptible

To determine whether the observed carbonate-induced increase in antimicrobial activity applied to peptides other than LL-37, a structurally diverse panel of AMPs was tested (Fig. 1*d*). The human and murine cathelicidins LL-37 and mouse cathelin-related antimicrobial peptide (mCRAMP) are both α-helical, the porcine cathelicidin PR-39 is a proline/arginine-rich linear peptide, and murine α-defensin cryptdin-4 (19) and human β defensin 2 (hBD-2) (20) have β sheet structures. None of the peptides tested had significant activity in the presence of NaCl and serum without carbonate (MIC>64 μM), but in the presence of $NaHCO_3$ all showed significant inhibition of bacterial growth (i.e., LL-37 MIC=4 μM). Of the panel tested here, only the anionic human defense peptide dermcidin did not show increased antimicrobial activity in the presence of carbonate.

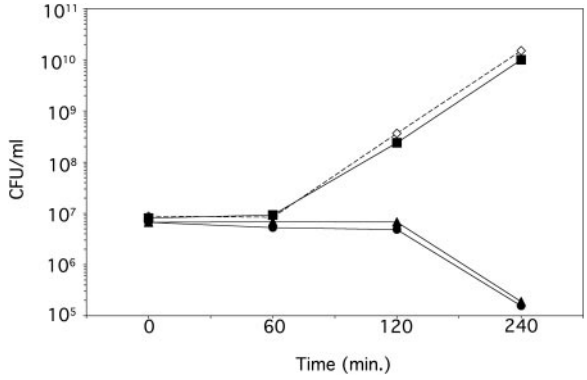

Figure 2. Preincubation of bacteria with $NaHCO_3$ is required for enhanced antimicrobial activity. *S. aureus* was grown to log phase in 20% TSB, 10% FBS, 1 mM NaH_2PO_4, 150 mM NaCl with or without 50 mM $NaHCO_3$, washed to remove $NaHCO_3$, and exposed to 12.8 μM LL-37 at time 0 h in media with or without $NaHCO_3$. Surviving bacteria were enumerated at various time points for 4 conditions. Open diamonds on a dashed line represent bacteria grown in $NaHCO_3$ before and after washing, but not exposed to LL-37. Squares represent bacteria cultured without $NaHCO_3$ both before and after washing and exposed to LL-37. Triangles represent bacteria grown in $NaHCO_3$ before and after washing and exposed to LL-37. Circles represent bacteria grown in $NaHCO_3$ before washing and assayed after washing without $NaHCO_3$ with LL-37. Exposure of the bacteria to $NaHCO_3$ is required for enhanced susceptibility, but this susceptibility persists when the $NaHCO_3$ is removed before exposure to the peptide.

Next, to compare the influence of carbonate on *S. aureus* with enhanced resistance to pharmacologic antibiotics, we tested two MRSA isolates, ATCC 81025 and 81056, and found both demonstrated an increase in susceptibility to LL-37 (MIC>32 μM in the absence of carbonate, and >98% inhibition of 81056 and 100% inhibition of 81025 at 8 μM with carbonate). A similar AMP-enhancing effect was seen with two important Gram-negative human pathogens. Enteroinvasive O29 *E. coli* were completely inhibited by 16 μM mCRAMP in the presence of carbonate but were resistant to cathelicidin without it. Similarly, *Salmonella dublin* Lane was inhibited ~50% without carbonate, while totally inhibited in its presence (Fig. 1*e*).

Carbonate acts on bacteria to render them susceptible

It has been reported that carbonate can affect the α-helical structure of LL-37 (21). This opens the possibility that the potentiating effects of carbonate could reflect structural changes in AMPs to somehow increase their potency, although the diversity of peptides tested seemed to diminish this possibility. To test whether the effects observed were due to action on the peptide or on the bacteria, we studied the ability of LL-37 to kill *S. aureus* as a function of the time of exposure to $NaHCO_3$

(**Fig. 2**). Bacteria were grown to log phase in the presence or absence of $NaHCO_3$, then washed free of media and resuspended in fresh media with or without $NaHCO_3$ and with or without LL-37. *S. aureus* grown initially without $NaHCO_3$ were not inhibited by LL-37 even if $NaHCO_3$ was added at the time of LL-37 exposure. In contrast, bacteria grown initially in the presence of $NaHCO_3$ were killed by LL-37 even if carbonate was removed prior to LL-37 addition. Separate experiments demonstrated that bacteria must grow for at least 2 h in carbonate-containing media to show increased susceptibility to LL-37 (data not shown).

To determine whether altered susceptibility to AMPs correlates with changes in membrane permeability, *S. aureus* and *E. coli* membrane permeability was examined directly. *S. aureus* cultured in carbonate exhibited an increase in permeability to Sytox green in response to LL-37 (**Fig. 3*a***). This effect was also observed in *E. coli* by measuring β-galactosidase release in response to Polymyxin B (Fig. 3*b*) or LL-37 (Fig. 3*c*). These data suggested that differences in the bacterial cell membrane or in membrane repair and synthesis mechanisms may make them more susceptible to permeabilization by cationic compounds. One important mechanism of bacterial AMP resistance reflects modification of cell wall lipids with cationic substitutions to decrease surface negative charge (15). Examination of membrane charge by binding of cytochrome *c* (22) and direct analysis of fatty acid and lipid composition of *S. aureus* showed no significant changes in response to growth in carbonate-containing media (data not shown). However, transmission electron microscopy of *S. aureus* treated with $NaHCO_3$ did show thinning of the cell wall (**Fig. 4*a, c***). Subsequent addition of LL-37 produced severe membrane blebbing, disintegration of the cell wall and widespread bacterial lysis (Fig. 4*d*).

Carbonate alters gene expression of key regulatory factors

Our data demonstrated that carbonate enhancement of bacterial AMP susceptibility occurred without alterations in membrane charge, but with potential structural alterations in the cell wall. The potential effects of carbonate on bacterial genes not previously associated with AMP sensitivity was explored by global transcriptional profiling. DNA microarray analysis of *E. coli* O29 cultured in the presence or absence of $NaHCO_3$ identified changes in the expression of several candidate genes (**Table 1**). Notably, > 30 flagellar genes, which have been implicated in virulence in *Salmonella* and stress response (23), were decreased >2-fold by culture in carbonate in *E. coli* O29. In fact, of the 10 genes with the largest decrease in expression shown in Table 1, five were flagellar genes. A large decrease in expression was also observed for *bar*A (24), which encodes a kinase that controls various virulence factors through the global regulator OmpR (25).

For confirmatory analysis we chose the *fli*A gene, which encodes a sigma factor that regulates many flagellar genes and can influence bacterial virulence and AMP sensitivity (23). Comparison of *fli*A to sigma factors in *S. aureus* revealed sequence similarities to the alternative sigma factor, *sig*B. SigB contributes to *S. aureus* virulence and is involved in a broad range of stress responses, including control of cell wall thickness and integrity (26, 27). Using quantitative RT-PCR, we verified a 5-fold decrease in *E. coli* *fli*A expression and a 10-fold decrease in *S. aureus sig*B upon growth in carbonate-containing media (**Fig. 5*a, b***). Thus, one important effect of carbonate upon the bacteria appears to be suppression of global regulatory genes involved in coordinating potential resistance phenotypes.

To directly test the hypothesis that a decrease in the expression of sigB in *S. aureus* could contribute to the increase sensitivity to LL-37 observed when cultured in carbonate, we evaluated *S. aureus* ΔsigB mutant (NKSB) for growth inhibition in response to LL-37. We found the ΔsigB mutant NKSB to be more susceptible to LL-37 during early growth periods from 2 to 8 h post-inoculum (Fig. 5*c*). Calculation of MIC in this period showed MIC of 16 μM for NKSB compared with wild-type parent strain MIC of >64 μM. However,

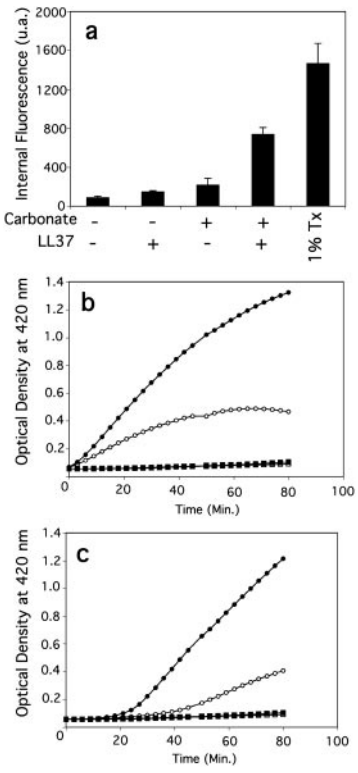

Figure 3. $NaHCO_3$ causes *S. aureus* and *E. coli* to become more susceptible to permeabilization. *S. aureus* or *E. coli* were grown to log phase with or without 25 mM $NaHCO_3$ and exposed to 4 μM LL-37 (*a, c*) or 1 μg/mL polymyxin B (*b*). Increases in *S. aureus* membrane permeability in response to carbonate and LL-37 was measured in panel *a* by uptake of the nuclear stain, Sytox Green. 1% Triton-X 100 (1%Tx) was used as a positive control. *E. coli* permeability was determined by measuring the release of β-galactosidase activity into the media in panels *b, c*. The membranes of *E. coli* cultured in $NaHCO_3$ (filled symbols, *b, c*) were more permeable after polymyxin B or LL-37 treatment (circles, *b, c*). Without peptides (squares, *b, c*), $NaHCO_3$ did not make the *E. coli* significantly more permeable.

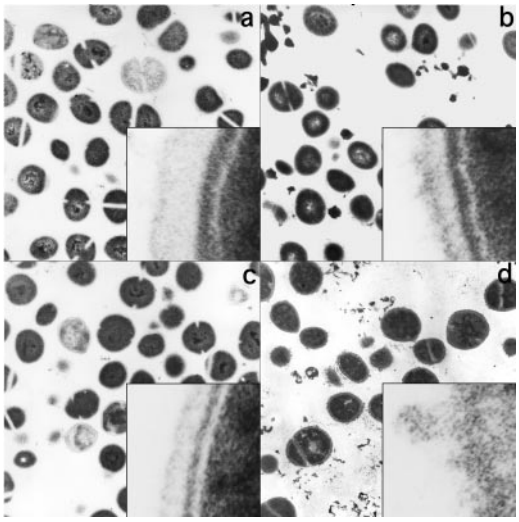

Figure 4. Electron microscopy reveals bacterial cell wall lysis by LL-37 and carbonate. *S. aureus* was grown for 4 h in 20% TSB, 1 mM NaH_2PO_4 with or without 25 mM $NaHCO_3$ or 32 μM LL-37. The presence of 25 mM $NaHCO_3$ alone (*c*) resulted in thinner but intact bacterial cell walls compared with untreated bacteria (*a*). 32 μM LL-37 caused a small amount of damage to bacterial cell walls in the absence of $NaHCO_3$ (*b*), but totally ablated them in its presence (*d*). Low magnification images are provided to verify similar cell numbers and demonstrate the general condition of the bacteria. High magnification images in the inset allow closer examination of the cell wall. Panel magnification, 33,000×. Inset magnification, 137,500×.

unlike *S. aureus* grown in carbonate-containing buffers, the ΔsigB mutant increase in sensitivity was transient.

DISCUSSION

The last decade has seen important advances in the discovery of antimicrobial peptides and proteins with lytic activity against pathogens including bacteria, viruses, fungi, and even cancer cells (28). Many of these molecules are thought to be integral mediators of host barrier defense, including the cathelicidins and defensins, whose family members have been described in most human epithelia (29). AMPs are either constitutively expressed at locations where initial interaction with potential invading microbes occurs or induced upon recognition of injury. The in vivo significance of several of these peptides has been demonstrated in animal models, yet many of these molecules appear to be inactive, or minimally active, when antimicrobial activity is directly evaluated in vitro using physiologic salt concentrations or serum (7) (21). This has lead to a debate as to the true relevance of these so-called "natural antibiotics" to immune defense. Here we demonstrate that bacterial susceptibility is significantly enhanced in the mammalian ionic environment in which AMPs have evolved their defense role.

Carbonate is a ubiquitous molecule in many microenvironments of the body, including blood, sweat, and gastrointestinal, urogenital, and respiratory tracts. Secretions containing carbonate at the concentrations evaluated in this study are generated from or bathe AMP-expressing tissues such as skin, gut, airway epithelia, and neutrophils (30–32). Our findings show that when grown in the presence of carbonate, both Gram-positive and Gram-negative bacteria may show dramatically increased AMP sensitivity. For example, the major human pathogen *S. aureus* has been described as resistant to mammalian AMPs, with an MIC to many cathelicidins and defensins of >32 μM (8). In mouse studies, the concentration of cathelicidin in wounds has been estimated as only between 3 and 10 μM (33). Our data now show that in the presence of carbonate at physiologic concentrations *S. aureus* is suppressed by the murine cathelicidin mCRAMP at 8 μM, within the effective concentration range found in vivo.

The AMP-enhancing effect of carbonate is due to alterations in bacterial susceptibility. Carbonate enhances activity of a broad range of antimicrobials including polymyxin and peptides with α-helical, β-sheet, and linear structures. Carbonate does not need to be present in the media at the same time as the AMP to produce increased bacterial susceptibility, suggesting its effects are indepen-

TABLE 1. *Culture in the presence of carbonate alters E. coli gene expression*[a]

Gene	Protein	Fold decrease
proM	tRNA-Pro	14.8
serT	tRNA-Ser	12.7
hdeA	Defense against acid stress	11.8
barA	Histidine kinase	7.4
fliD	Flagellar filament cap protein HAP2	7.4
fliZ	Structural component	7.3
flgL	Second hook-filament junction zone	6.6
cysC	Adenylsulfate kinase	6.2
fliA	σ^{28}	6.1
yhjH	New member of flagellar regulon	6.0

Gene	Protein	Fold increase
LAMCG_mRNA		39.5
yagL	DNA binding protein	15.7
yhhH	Hypothetical protein	9.5
elaA	Acyltransferase (predicted)	8.0
b2361	Hypothetical protein	5.4
nadB	L-aspartate oxidase	4.5
aceB	Malate synthase A	4.4
rfaJ	Lipopolysaccharide 1,2-glucosyltransferase	4.1
spy	Spheraplast protein Y (Cpx regulon member)	3.8
b3913	Hypothetical protein	3.7
betB	Betaine aldehyde dehydrogenase	3.6
nadA	Quinolinate synthetase	3.6

[a] Alterations in *E. coli* gene expression were determined in response to culture in 25 mM $NaHCO_3$ as described in Materials and Methods. These data represent the 10 genes with the largest fold increase or decrease in response to carbonate.

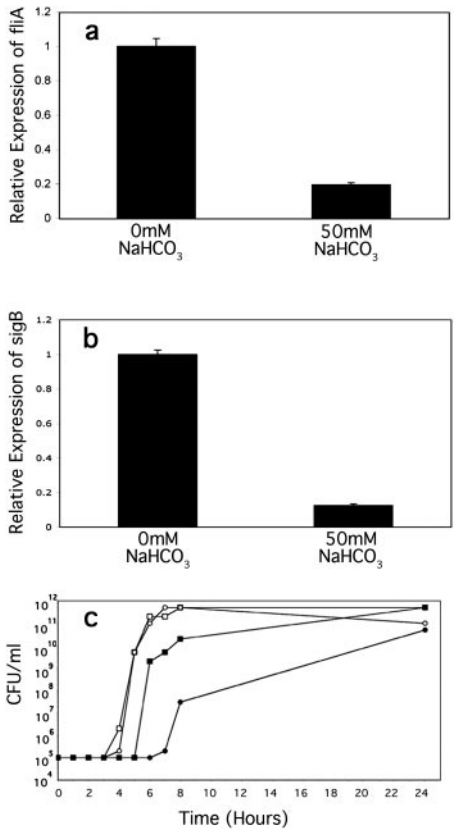

Figure 5. $NaHCO_3$ suppresses expression of the alternate sigma factors FliA in *E. coli* and SigB in *S. aureus*, which increases their susceptibility to AMPs. RNA was extracted from log phase *E. coli* (*a*) and *S. aureus* Rosenbach (*b*) cultured with or without 25 mM $NaHCO_3$ and expression levels determined via quantitative RT-PCR. Expression was normalized first to 16 s rRNA as an endogenous control, then to the bacteria grown without $NaHCO_3$ to determine a relative level of expression. *c)* S. Aureus N315 deficient in SigB, termed NKSB (circles), was inhibited by LL-37 to a greater degree than the parental strain (squares). Addition of 16 μM LL-37 (filled symbols) to NKSB resulted in a longer and larger inhibition of growth than it did on N315. Two-way ANOVA statistical analysis confirmed that the difference between both N315 and NKSB treated with LL-37 and their untreated controls was significant at $P < 0.001$ at 6 to 8 h. The difference between NKSB and N315 treated with LL-37 was not significant by 2-way ANOVA.

dent of any potential changes in the structural conformation of AMPs as previously reported. Culture of *S. aureus* and *E. coli* in the presence of carbonate increased the ability of LL-37 and polymyxin B to produce membrane permeabilization, indicating the anion has a deleterious effect on the bacteria's ability to resist external stresses that threaten its membrane integrity.

Bacterial growth in the presence of carbonate appears to produce significant changes in gene expression that correspond to the enhanced AMP sensitivity phenotype. In the Gram-negative *E. coli*, no difference in growth rate was observed upon carbonate treatment, but a global alteration in gene expression was seen with more than 300 gene transcripts altered >2-fold. Among the genes with significantly decreased expression was the global regulator *fli*A, as confirmed by quantitative RT-PCR analysis. Analysis of the Gram-positive *S. aureus* response to carbonate also showed marked changes in transcript abundance, including genes involved in virulence, stress response, and cell wall maintenance. Sharing homology to *fli*A, the *S. aureus* global regulatory gene *sig*B was also significantly suppressed when the Gram-positive pathogen was grown in the presence of carbonate.

In earlier investigations, deletion of *sig*B restored methicillin susceptibility in resistant strains of *S. aureus* (34), while overexpression of *sig*B increased cell wall thickness as well as resistance to cell wall antibiotics (27). Ultrastructural analysis of *S. aureus* grown in the presence of carbonate shows a decrease in cell wall thickness that corresponded to the enhanced susceptibility to AMP mediated lysis. Since SigB mutants of *S. aureus* are known to have decreased cell wall thickness, this suggests that functional phenotypic changes observed in our study in response to carbonate may be partly linked to *sig*B. However, the deletion of *sig*B did not confer the same level of susceptibility as culture of the parental strain in carbonate-containing media. Moreover, carbonate further enhanced the susceptibility of *sig*B-deficient bacteria to LL-37 (data not shown). This indicates that additional effects beyond suppression of *sig*B expression are involved in the AMP-enhancing properties of carbonate. Our data indicate that investigation of families of genes that are involved in the control of cell wall synthesis and repair may be fruitful in understanding microbial resistance systems and how native antimicrobial defense systems can function effectively.

The discovery that carbonate enhances microbial susceptibility to AMPs is important from many perspectives. First, it reconciles the apparent paradox described between the demonstrated in vivo innate immune function of AMPs and their lack of in vitro antimicrobial activity. The presence of carbonate in mammalian tissues may dictate alterations in bacterial gene expression and cell wall integrity that render them more susceptible to innate immune defense molecules. This phenomenon may also represent a mechanism to maintain susceptible microbial populations. AMPs are evolutionarily conserved, predicting their presence in the environment for millennia, yet bacteria have only developed incomplete resistance. The low level of carbonate in external reservoirs for microbial growth ensures that in these settings no survival advantage exists for the further development of AMP resistance.

These observations also provide an alternate paradigm for antimicrobial discovery and suggest that analysis of microbial susceptibility to compounds should be performed with organisms grown in an environment corresponding to the mammalian host for which they represent a potential pathogen. Potential new classes of effective antimicrobials may be discovered by this approach. Compounds that may have otherwise been deemed ineffective during in vitro screening may be effective in the animal. This approach would represent a major change in the continuing struggle for development of effective antibiotics in the setting of rapidly evolving antibiotic resistance, and would exploit the immune de-

fense strategy evolved by natural antibiotic peptides. FJ

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