BBA - Proteins and Proteomics 1865 (2017) 1500–1511

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Review

Recent progress on the application of ^{2}H solid-state NMR to probe the interaction of antimicrobial peptides with intact bacteria

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ARTICLE INFO

Keywords:
Gram-positive and Gram-negative bacteria
Escherichia coli
Bacillus subtilis
In-cell NMR
Membrane interactions
Action mechanism

ABSTRACT

Discoveries relating to innate immunity and antimicrobial peptides (AMPs) granted Bruce Beutler and Jules Hoffmann a Nobel prize in medicine in 2011, and opened up new avenues for the development of therapies against infections, and even cancers. The mechanisms by which AMPs interact with, and ultimately disrupt, bacterial cell membranes is still, to a large extent, incompletely understood. Up until recently, this mechanism was studied using model lipid membranes that failed to reproduce the complexity of molecular interactions present in real cells comprising lipids but also membrane proteins, a cell wall containing peptidoglycan or lipopolysaccharides, and other molecules. In this review, we focus on recent attempts to study, at the molecular level, the interaction between cationic AMPs and intact bacteria, by ^{2}H solid-state NMR. Specifically-labeled lipids allow us to focus on the interaction of AMPs with the heart of the bacterial membrane, and measure the lipid order and its variation upon interaction with various peptides. We will review the important parameters to consider in such a study, and summarize the results obtained in the past 5 years on various peptides, in particular aurein 1.2, caerin 1.1, MSI-78 and CA(1-8)M(1-10). This article is part of a Special Issue entitled: Biophysics in Canada, edited by Lewis Kay, John Baenziger, Albert Berghuis and Peter Tieleman.

1. Introduction

Antimicrobial peptides (AMPs) have received a great deal of attention due to their potential to help solve the crisis of antibiotic resistance to conventional small molecule drugs [1–8]. Applications of AMPs to cancer treatment are also being explored [9–11]. A wide variety of organisms, from bacteria to humans, produce AMPs as part of their innate immune defense systems [12]. In addition to natural AMP sequences, such as caerin 1.1 and aurein 1.2 from Australian tree frogs [13,14], a number of synthetic sequences have been generated, including MSI-78 [15] and CAME [16,17]. Many AMPs exhibit a degree of specificity and can kill pathogens at concentrations that do not harm host cells. Much of this specificity is thought to relate to the cationic charge that most AMPs possess. AMPs are generally small, positively charged, have a substantial hydrophobic content and can form amphipathic structures [12]. Most AMPs are largely unstructured in solution, and fold upon membrane binding. A variety of structures have been observed in membrane-bound AMPs, including α-helical and β-sheet type structures. In this review, we focus on cationic, α-helical AMPs.

The amphipathicity of AMP structures confers a propensity to interact with lipid bilayers. And indeed, much of the research into AMP mechanisms has focussed on their interaction with membranes, either as their direct mechanism of killing via membrane permeabilization, or as a means of getting inside the cell to disrupt intracellular targets. Membrane interactions are likewise implicated in the specificity of positively charged AMPs which have stronger interactions with anionic membranes, for example bacterial or cancer cell membranes. The non-

Abbreviations: AFM, atomic force microscopy; AMP, antimicrobial peptides; CD, circular dichroism; CFU, colony forming unit; CL, cardiolipin; d_{31}-PA, deuterated palmitic acid; DHPC, dihexanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPC, dodecylphosphocholine; DSC, differential scanning calorimetry; LPS, lipopolysaccharides; MAS, magic-angle spinning; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGN, peptidoglycan; PE, phosphatidylethanolamine; PI, phosphatidylinositol; POPC, palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); REDOR, rotational-echo double resonance; SS-NMR, solid-state nuclear magnetic resonance

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http://dx.doi.org/10.1016/j.bbapap.2017.07.018
Received 22 April 2017; Received in revised form 13 July 2017; Accepted 25 July 2017
Available online 24 August 2017
1570-9639/

specific nature of AMP pathogen interactions is thought to underlie the relative rarity of resistance development to AMPs as compared to conventional small molecule drugs [3,18–23]. However, there is a growing awareness that it is also critical to consider the interactions between AMPs and non-lipidic components of the target cell, such as the peptidoglycan layer or intracellular AMP targets. Consequently, many biophysicists who study AMPs are starting to include more whole cell experiments [24–27], along with the more traditional model membrane work.

2. AMP mechanisms

Mechanisms of AMP killing and growth inhibition of bacteria include both direct effects on the membrane, i.e. permeabilization, as well as targeting of intracellular components and modulation of the host cell immune system [19,28–30]. Intracellular targets of AMPs are proposed to include DNA, RNA, ribosomes, chaperone proteins, and enzymes [22,31–35].

Studies of AMPs interacting with model lipid bilayers have led researchers to suggest a variety of possible mechanisms for membrane disruption, including toroidal pores, disordered toroidal pores, carpet and barrel-stave models [22,23,36] as illustrated in Fig. 1. In toroidal pores, for example, the polar/positively charged face of the amphipathic AMP structure interacts with the headgroup of the negatively charged lipids, while the hydrophobic AMP face makes contact with the lipid acyl chains, inducing bending of the bilayer, and thus stabilizing lipid pore structures. Such defects may only need to be lined by just one or two AMPs [37,38]. Although the various membrane disruption models are frequently presented as different mechanisms, many of them can be unified by considering a phase diagram of AMP mechanism as a function of peptide concentration and lipid composition [39]. While models of membrane disruption mechanisms provide valuable insight into how AMPs function, it is clear that they are not the whole story, as there is often very poor correlation between membrane permeabilization induced by AMPs and cell growth inhibition or death [40–43]. Such observations have led many researchers to suggest that at least some AMPs kill target pathogens via a multi-hit mechanism that may well include membrane permeabilization, but with other important targets as well (e.g. [22,30,34,41,44]). In addition to intracellular AMP targets, there are a variety of non-lipid components in the cell envelope of pathogens that are likely to affect AMP activity.

3. Bacterial cell envelopes and AMP interactions

Cell envelope components that may complicate the picture of AMP-bacteria interactions derived from model lipid studies include the peptidoglycan (PGN) and lipopolysaccharide (LPS) layers, as well as membrane proteins, membrane domains, bilayer asymmetry, and the specifics of lipid composition [45–47]. Understanding how non-lipidic components affect AMP activity is critical. For instance, some cell wall constituents appear to protect bacteria from certain AMPs, while for other AMPs the opposite is true; the presence of non-lipidic cell envelope components appear to sensitize or attract AMPs to the bacteria [48–50].

The architecture of the bacterial cell envelope is different for Gram-positive and Gram-negative bacteria (Fig. 2) [51,52]. In Gram(+)

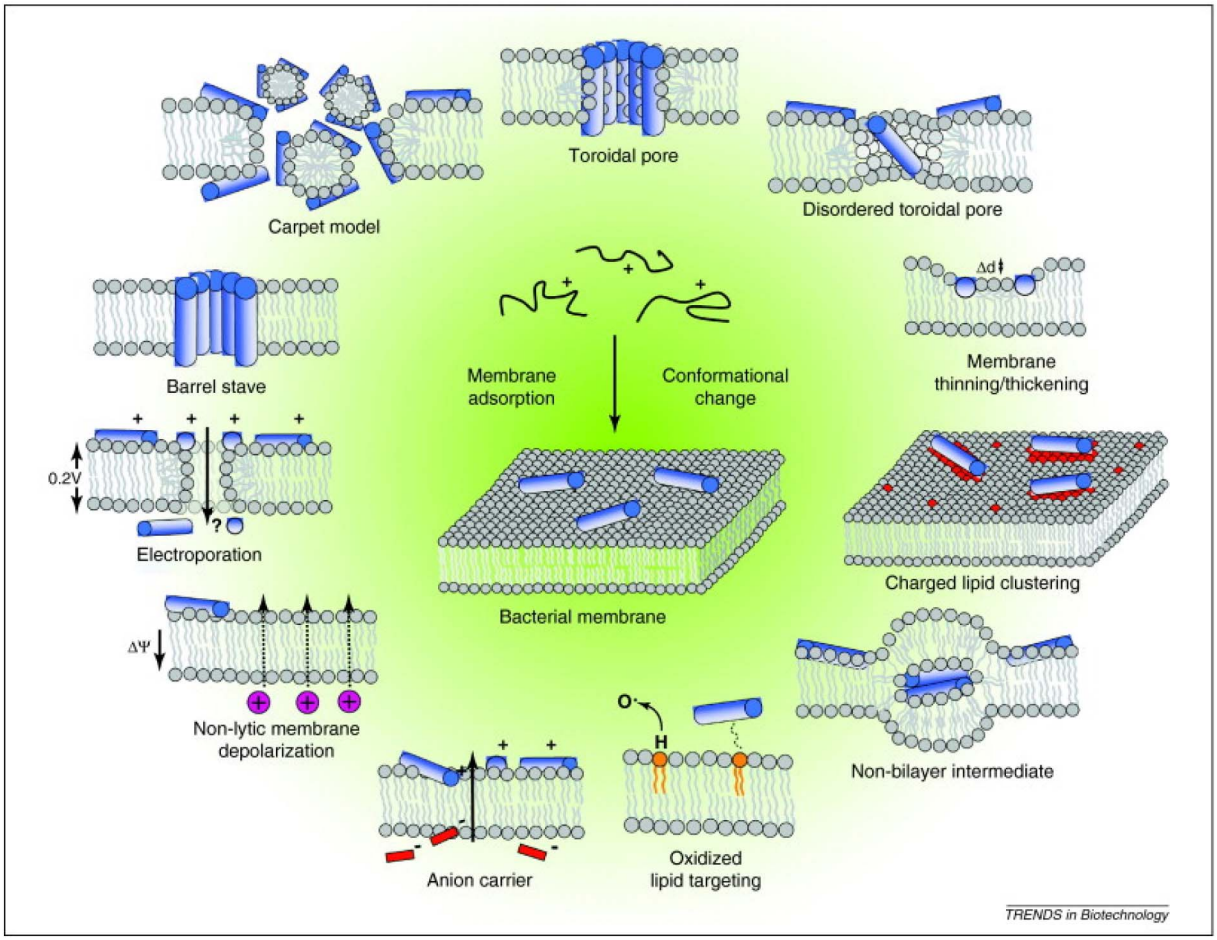

Fig. 1. Schematic of possible membrane disruption mechanisms by antimicrobial peptides.
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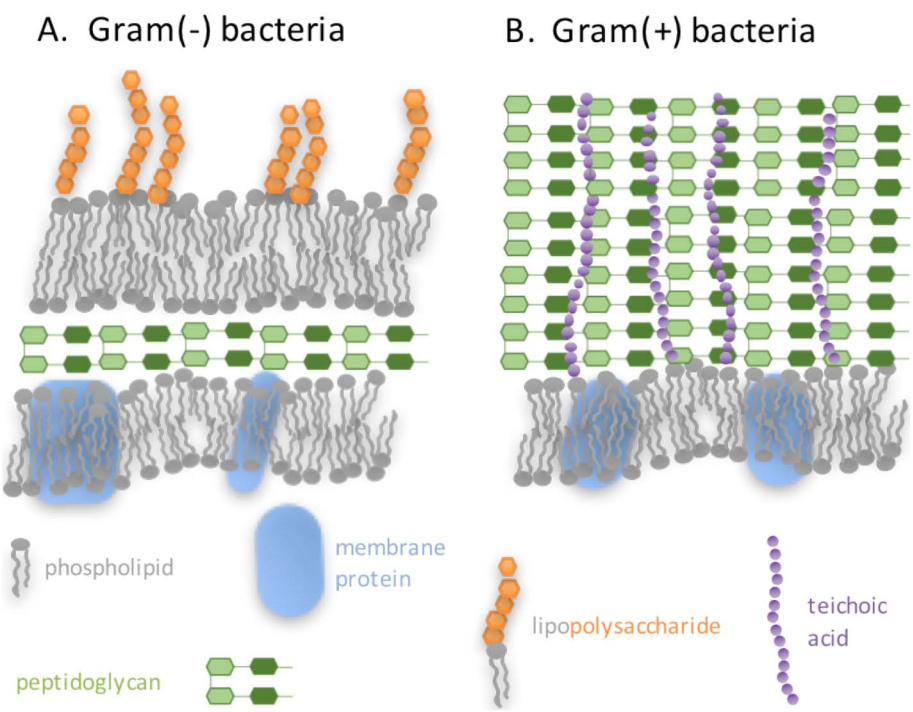

Fig. 2. Schematic of Gram-negative (A) and Gram-positive (B) bacterial cell envelopes.
Adapted from Laadhari et al. [80] with permission.

bacteria, there is a single lipid bilayer and a thick (20–80 nm) PGN layer, while Gram(−) bacteria have a much thinner (6–8 nm) PGN layer but are enveloped by a LPS layer, the outer membrane, that is not present in the Gram(+) bacteria. The PGN layer is comprised of repeating units of disaccharide-peptide building blocks that are cross-linked, via both the peptide and carbohydrate components, to form a continuous network. The length and composition of the peptide stem varies from species to species [53]. Also integral are the teichoic acids that are covalently linked to the PGN. The teichoic acids supply structural integrity to the cell well and also assist infection by contributing to adhesion and host interactions [54]. Because the teichoic acids confer a substantial negative charge to the cell envelope, they are potentially important considerations for interactions with cationic AMPs.

Another negatively charged cell envelope component is LPS, a major constituent of the outer membrane of Gram(−) bacteria. LPS has lipid A, core oligosaccharide, and O-antigen components. Some of the carbohydrate components of lipid A are highly phosphorylated, which is what confers the negative charge to the LPS. Divalent cations (Mg^{2+} and Ca^{2+}) bind the phosphate groups and thus help to link the LPS molecules together. AMPs have been proposed to move across the LPS via a "self-promoted" uptake mechanism, whereby the AMPs displace the LPS stabilizing divalent cations, destabilizing the outer membrane and allowing the AMP to permeate the LPS [55]. As reviewed in Malanovic and Lohner [50], non-lipidic cell wall components may either entrap AMPs and prevent them from reaching the inner lipid bilayer or, alternatively may attract AMPs and cause them to accumulate near the membrane surface. In the Booth lab, preliminary work has found that disrupting the PGN of *Bacillus subtilis* increases the activity of one AMP, MSI-78, but reduces the activity of another AMP, CA(1-8)ME(1-10) [49]. And, for the histidine-rich Gad-1 and Gad-2 AMPs, the effect of PGN disruption on AMP activity depends on the pH [48].

Lipid composition itself, and in particular the anionic content of lipids, is known to be an important factor in AMP selectivity and activity [15,22,56]. More than one group has noted how alterations in lipid composition can affect AMP membrane interactions, even when the overall anionic/zwitterionic composition is conserved [57,58]. There is a substantial variation in the lipid composition of membrane found in different pathogens. For example, while most bacteria contain phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL), their relative proportions vary tremendously. There have also been reports of asymmetry between the lipid composition of the inner and outer leaflets of Gram(−) bacteria, with PG preferentially localizing to the outer leaflet, phosphatidylinositol (PI) and PE to the inner leaflet, while CL is evenly distributed through both leaflets [59]. As illustrated in Table 1, the inner membranes of Gram(−) bacteria such as *Escherichia coli* has a different relative proportion in PG and PE compared to a Gram(+) bacterium such as *B. subtilis*. Even more striking, while both *B. subtilis* and *Staphylococcus aureus* are Gram(+) bacteria, *S. aureus* has no PE, but only PG and CL [60] (Table 1).

4. Traditional NMR approaches to study AMPs' action mechanism

To assess the effect of AMPs on lipid ordering and determine their action mechanism on membrane specifically, model phospholipid membranes have been traditionally used in combination with ^{31}P- and ^{2}H solid-state (SS) NMR. The location of the phosphorus atom in the lipid headgroup provides information on perturbation of the bilayer surface by ^{31}P SS-NMR. In this review, we focus on ^{2}H SS-NMR which, used in combination with acyl-chain deuterated phospholipids, reveals effects in the hydrophobic portion of the bilayer. Generally, the lipid composition used aims at mimicking that of the natural bacterial membrane (Table 1). Mimicking is never perfect when trying to reproduce headgroup and acyl chain diversity, let alone the asymmetry in composition between inner and outer leaflets. The anionic character of

Table 1
Average lipid composition of selected bacteria expressed in average weight % of total lipids. Values from Warschawski et al. [61] and Marcotte and Booth [60], and references therein.

Bacterium	PE	PG	CL	LPS	GL
E. coli					
Inner	60	33	7	0	0
Outer	61	13	1	25	0
B. subtilis	13	77	4	0	6
S. aureus	0	58	42	0	0

PE = phosphatidylethanolamine, PG = phosphatidylglycerol, CL = cardiolipin, LPS = lipopolysaccharides, GL = galactolipids.

the bacterial membrane is therefore usually insured by the use of negatively charged PG, which is mixed with zwitterionic PE or, more often zwitterionic phosphatidylcholine (PC), since perdeuterated PCs are commercially available and more affordable than PE or PG. The PG proportion is quite variable, and in some cases, especially for ^{31}P NMR experiments, pure PG membranes are used, such as dimyristoylPG (DMPG) with thanatin [62]. For example, a PC/PG molar ratio of 4:1 was used to study the interaction of aurein 1.2 with lipids [63], while a PC/PG 3:1 molar ratio was employed for the study of PGLa [64], magainin 2 and aurein 3.3 [65] in model membranes, and a PC/PG 2:1 ratio was used with fallaxidin 4.1a [66]. Saturated lipids are employed, but also unsaturated ones such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG), which with their low gel-to-fluid phase transition temperatures ensure membrane fluidity at room temperature, similar to natural bacterial membranes [67].

Different model membranes can be employed, such as multilamellar vesicles (MLVs), magnetically-oriented bicelles and mechanically-oriented bilayers deposited on glass plates. These membranes have been thoroughly reviewed [61] and show different pros and cons. Briefly, the multilayer nature of MLVs lacks biological relevance, but the local constraints provided by the vesicle size and curvature resemble that of a cell. Also, the high lipid concentration is an asset for NMR analysis of peptide-lipid interactions. The membrane insertion of fallaxidin 4.1a [66], and AMPs from frog skin such as aurein 1.2 [63,68], citropin 1.1 maculatin 1.1 and caerin 1.1 [68], were, for example, studied by ^{2}H SS-NMR with PC/PG MLVs. Given the orientational dependence of the quadrupolar splitting, as will be detailed below, oriented samples provide simplified spectra and facilitate the assessment of the quadrupolar splittings and insertion degree of AMPs. Bicelles are disk-shaped bilayers, typically made of DMPC and dihexanoylPC (DHPC); however up to 25 mol% DMPC can be replaced by DMPG to prepare bacterial-like oriented membranes, as reviewed by Marcotte and Auger [69]. Their planar surface offers morphology close to biological membranes. In some particular conditions (lipid composition, temperature range, hydration level, etc.) bicelles spontaneously orient in the magnetic field (B_0) with the bilayer normal perpendicular to B_0. In other conditions, for example when DMPC/DHPC molar ratio (q) $<$ 2, small size bicelles leads to their fast tumbling in solution [61,70]. This size tunability is advantageous as it enables the use of bicelles in solution NMR as well as other spectroscopic techniques such as circular dichroism (CD). Therefore the molecular structure of AMPs can be studied in a similar membrane environment with a variety of biophysical methods. For example, the structure of mastoparan X from wasp venom [71], and alamethicin from the fungus *Trichoderma viride* [72] were determined by ^{1}H NMR in q = 0.5 bicelles. Bilayers can also be supported on glass plates that can be stacked in an appropriate NMR probe [61]. Their orientation with respect to B_0 direction can be varied, but a 0° orientation of the lipid normal provides maximum quadrupolar splitting and sensitivity to membrane perturbations, as will be shown below. Despite their low hydration level and tedious preparation, as reviewed elsewhere [61], mechanically aligned lipid bilayers have been combined to ^{2}H SS-NMR to study the interaction mechanism of AMPs, for example tachyplesin [73] or dermcidin [74] in POPE/POPG aligned bilayers.

5. Approaches to study AMPs interacting with membranes in whole cells

SS-NMR is a great tool to study molecular interactions, because it can focus on specific parts of a molecule, even within a system as complex as a whole microorganism. NMR offers various ways to focus on a specific chemical environment, for example filtering either rigid or dynamic ^{13}C-labeled molecules [75], or observing ^{31}P nuclei present in the lipid headgroups. But the most common way to do so is to isotopically enrich the molecule of interest [76]. Knowledge of bacterial metabolism is of great help and has allowed us to probe bacterial membrane order, by focusing on lipid acyl chains labeled with ^{2}H isotopes. Since the natural abundance of ^{2}H is 0.01% and that high ^{2}H-labeling of the bacterial lipids is reached (typically ~70%) with the methodologies described below, the ^{2}H NMR spectrum of a complex cell sample will predominantly show the labeled acyl chains.

Unlike many other cells, bacteria provided with free fatty acids will ingest them during growth and incorporate most of them, without modification, to some of their lipid acyl chains. At the same time, the bacterium may also produce additional lipids, trying to maintain the natural diversity in its acyl chain distribution (chain length, number of unsaturations, etc.). In the case of Gram(−) bacteria (*E. coli* and *Vibrio splendidus* for example), we have noticed that when the bacteria are provided with saturated deuterated fatty acids (generally palmitic acid), the final balance is displaced in favor of saturated acyl chains. In other words, the metabolic adaptation, that usually maintains a particular balance in saturation/unsaturation [67], fails to some extent here when bacteria are exposed to a high level of saturated fatty acids. Thus, it is helpful to incorporate non-deuterated fatty acids in the growth media, generally oleic acid, to help compensate for this unbalance. An appropriate balance of saturated and unsaturated fatty acids in the growth media also improves the viability of the cells during the NMR experiment. In some bacteria, such as *E. coli*, a 1:1 ratio of palmitic to oleic acid works well [77–79], whereas for others, such as *B. subtilis*, a lower level of oleic acid, or even none at all, is necessary [78,80] (see protocol section for more details).

Static ^{2}H SS-NMR provides spectra that reflect the membrane lipid order, and how it is affected under external perturbation, such as temperature change or the addition of AMPs. Initial attempts at ^{2}H NMR of bacteria dates back to 1972 with *Acholeplasma laidlawii* [81], but was greatly improved by the development of the solid echo technique [82], allowing to measure order parameters, transition temperatures, spectral moments, relaxation times, the effect of cholesterol, etc. The method was extended to other intact bacteria such as *E. coli* [77,83,84], *Mycoplasma capricolum* [85], *Bacillus megaterium* [86], or even intact sea urchin sperm cells [87]. However it suffers one inconvenience: since the amount of ^{2}H in the sample is small, since ^{2}H SS-NMR is not very sensitive, and since ^{2}H spectra are broad, each experiment lasts at least a couple of hours.

For some fragile samples, this leads to cell degradation over time, and thus we have recently come up with an accelerated version of this approach by performing the same experiment under magic-angle spinning (MAS). Similar information can therefore be obtained in 20 min, as illustrated in Fig. 3 [79,80]. Issues regarding the detrimental aspect of MAS on living cells have been addressed. As seen by our and other groups' [88] viability assays, the centrifugal force under MAS does not seem to affect the integrity of the bacterial envelop, nor induce any leakage.

Deuterium is a quadrupole with a spin 1, resulting in a wide quadrupolar doublet for each nucleus. The splitting of this doublet, $\Delta\nu_Q$, is proportional to the motionless quadrupolar splitting ν_Q, reduced by the order parameter of the CD bond at this deuterium position, S_{CD}, described by Eq. (1). While for simple spectra, the linewidth of each resonance can be related to the dynamics of the corresponding spin, ^{2}H SS-NMR spectra (whether static or MAS) require a mathematical formalism to extract spectral “moments” that provide similar information. Spectral moments are normalized and weighted integrals of the spectrum, and they are also related to the order parameter through a factor a_n (Eq. (2)). Their measurements can be simplified in the case of a spectrum obtained under MAS (Eq. (3), ω_r being the spinning rate and A_N being the area of the Nth sideband). First and second moments, M_1 and M_2, reflect the average membrane order parameter and squared order parameter, respectively. An additional parameter, called Δ_2, characterizes the *distribution* of order parameters (Eq. (4)), and informs on membrane heterogeneity [89]. These parameters can be extracted and compared, at various temperatures, with or without AMP. It should

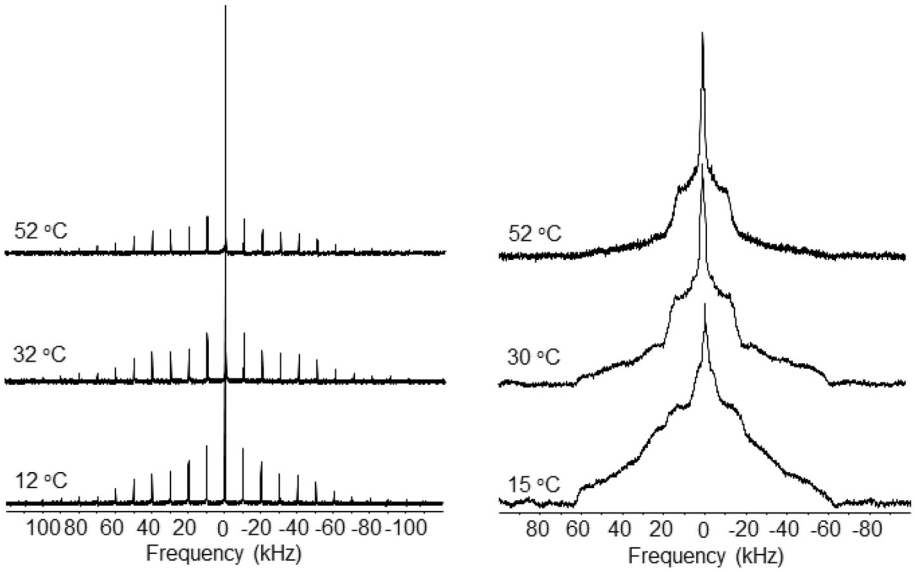

Fig. 3. ^{2}H NMR spectra of intact *B. subtilis* acquired at different temperatures with MAS at 10 kHz using 8k scans (left) compared to static samples using 100k scans. Adapted from Laadhari et al. [80] with permission.

be reminded that in biological samples low sensitivity can translate into high uncertainty on M_1 and M_2 values that propagates into a very high uncertainty on Δ_2 values. Consequently, Δ_2 parameter should be used cautiously as a qualitative indication of heterogeneity increase or decrease rather than a quantitative measure of it.

$$\Delta\nu_Q = \frac{3}{4}\nu_Q S_{CD} \tag{1}$$

$$M_n = \frac{\int_0^\infty \omega^n f(\omega)d\omega}{\int_0^\infty f(\omega)d\omega} = a_n {\nu_Q}^n \langle {S_{CD}}^n \rangle \tag{2}$$

$$M_n = \omega_r^n \frac{\sum_{N=0}^{\infty} N^n A_N}{\sum_{N=0}^{\infty} A_N} \tag{3}$$

$$\Delta_2 = \frac{M_2}{1.35{M_1}^2} - 1 \tag{4}$$

As expected, when large quantities of AMPs are introduced, membrane disruption translates into an important spectral moment reduction, which is what we see [77,80]. More surprising is the effect observed when small amounts of AMPs are added to the cells: depending on the peptide and bacteria, the membrane can either be slightly disordered (in most cases) or, unexpectedly, slightly more ordered (in the case of aurein with *B. subtilis*), indicating a different initial type of interaction between the peptide and the cell wall or membrane, and therefore probably a different mechanism [80].

6. Deuteration and viability assessment protocols

Wild-type and mutated bacterial strains (*E. coli* fatty acid auxotroph strains, such as L51 or LA8) have been used in the past [77,78,83] and in both cases are grown at 37 °C under moderate shaking in LB (Luria broth) medium. Good knowledge of metabolic pathways can help to design precise specific ^{2}H labeling, and this review covers the successful attempts that have so far focussed mainly on simple acyl chain labeling methods. For deuteration of non-mutated strains, the bacterial culture is supplemented with 0.2 mM deuterated palmitic acid (incorporated into 1 mM dodecylphosphocholine (DPC) micelles) and, when necessary, with 0.2 mM *non-deuterated* oleic acid. Cells are harvested in the mid-log growth phase, pelleted, rinsed three times with a final saline solution made of deuterium-depleted water, pelleted again and studied fresh by SS-NMR. Lipid fatty acid chain profile is determined by gas-phase chromatography combined with mass spectrometry, which allows for the quantification of deuterated fatty acids. For non-mutated strains ca. 70% of the saturated fatty acid chains are deuterated [79,80,84].

Cell viability after the NMR experiments can be assessed either by colony forming unit (CFU) assays [77] or by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays [84]. Between 40% and 80% of the bacteria remain viable after several hours in the NMR spectrometer [77], and spinning the sample only lowers this amount by 10% [79,80]. For peptide treatment, the pellet is re-suspended and the appropriate amount of peptide is added and incubated for 5 min. The sample is then pelleted again and studied fresh by SS-NMR. We need to emphasize that our SS-NMR experiments are performed on bacterial pellets, and hence the number and concentration of bacteria (and hence lipids) examined are several orders of magnitude greater than the cell amounts and concentrations employed in MIC assays. Thus, in the Marcotte lab the approach has been to treat a relatively large (compared to MIC assays) number of cells at the MIC concentration of AMP, while the Booth lab has taken the approach of controlling the AMP:bacteria dry weight (and hence AMP:lipid) ratio. To facilitate comparison between the two methods, an estimate of the AMP concentration is provided in Table 4 based on the approximate volume of the cell suspension used in the AMP treatment phase.

7. In vivo NMR study of aurein 1.2 and caerin 1.1

Aurein 1.2 and caerin 1.1 are active peptides against bacteria, fungi and cancer cells [90,91]. They are cationic AMPs produced from the skin secretion of Australian tree frogs from the *Litoria genus* [13,92]. As shown in Table 2 aurein 1.2 and caerin 1.1 are respectively composed of 13 and 25 amino acid residues. The C-terminus of both peptides is amidated – a functional group that adds a positive charge and is important for their antimicrobial activity [93]. They also share a common Gly-Leu motif at the N-terminus. Glycines are known as “helix breakers” and often contribute to the conformational flexibility that is thought to participate to AMP function and selectivity [94]. While random coil in

Table 2
Amino acid sequences of antimicrobial peptides and overall charge.

AMP	Sequence	Charge at pH 7
Aurein 1.2	GLFDIIKKIAESF-NH_2	+ 1
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	+ 1.3
MSI-78 (pexiganan)	GIGKFLKKAKKFGKAFVKILKK-NH_2	+ 10
CA(1-8)M(1-10)	MKWKLFKKIGIGAVLKVLT–NH_2	+ 6

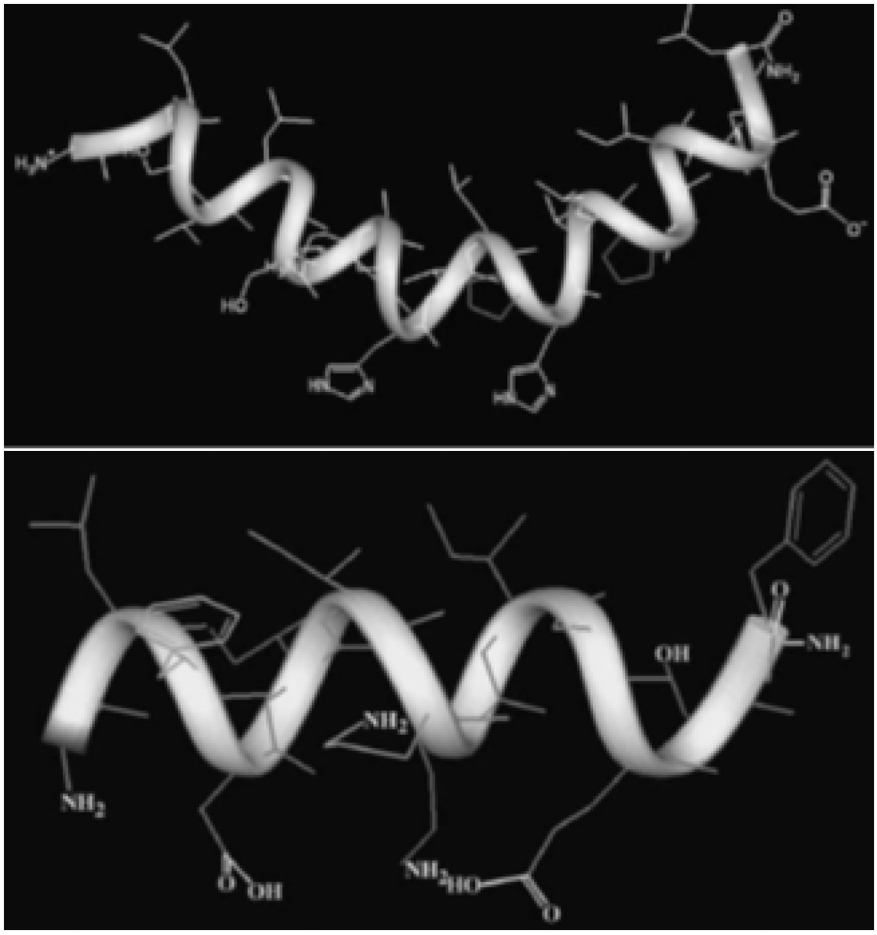

Fig. 4. Structures of caerin 1.1 (top) and aurein 1.2 (bottom) in 2,2,2-trifluoroethanol (TFE)/water solution mimicking the hydrophobic environment of lipid membranes.
Adapted from Apponyi et al. [96] with permission.

solution, these AMPs adopt an α-helical conformation in a membrane environment (Fig. 4), as demonstrated by CD with model membranes and intact bacteria [93,95,96]. Contrarily to aurein 1.2, caerin 1.1 is composed of two helices separated by a flexible region initiated by a proline residue (Pro15) [14,91] which facilitates the orientation of hydrophilic and hydrophobic regions with respect to the membrane, important for the antimicrobial activity [93,97].

Aurein 1.2 and caerin 1.1 are active against several Gram(−) bacteria, and more specifically towards Gram(+) [87]. Extensive work from the Separovic's group on the action mechanism of these AMPs has elucidated their action towards model membranes [13,63,98]. Using several techniques, including atomic force microscopy (AFM) with model DMPC/DMPG membranes, they showed that caerin 1.1 could lyse red blood cells at very high concentrations (> 250 μg/mL), but that aurein 1.2 was unable to make holes in membranes, even at concentrations where cell lysis is observed [63]. ^{31}P and ^{2}H SS-NMR experiments showed that aurein 1.2 had a disordering effect on the top region of the lipid acyl chains, indicating that this peptide remains on the bilayer surface [63]. These studies suggest that aurein 1.2 acts via a carpet mechanism to provoke cell lysis. This group also showed that caerin 1.1 inserts into the lipid membrane via a toroidal pore mechanism. Indeed a combination of ^{31}P- and ^{2}H SS-NMR analyses showed that this AMP induced coexistence of gel, fluid and isotropic lipid phases in DMPC/DMPG bilayers, consistent with the formation of lipid domains of different acyl chain orders observed for highly curved regions of toroidal pores [98].

Recently, we have studied the interaction of aurein 1.2 and caerin 1.1 with intact *E. coli* and *B. subtilis* by ^{2}H SS-NMR, to better take into account the presence of all cell wall constituents, and thus refine the action mechanism of these AMPs [80]. To do so, the lipid acyl chains of non-mutated bacteria were deuterated using PA-d_{31} in the growth medium. Bacteria were first exposed to AMPs at their minimal inhibitory concentration (MIC) of about 100 μg/mL for *E. coli* and 30 μg/mL for *B. subtilis*, respectively, for both peptides. When looking at *E. coli*, the effects observed on intact cells were in agreement with the results published with model membranes. Indeed, spectra shown in Fig. 5 reveal that both peptides have a membrane disordering effect on *E. coli* membranes. This is further appreciable from the spectral moment analysis (Table 3), where the values of M_1 and M_2 decreased in the presence of the AMPs, especially aurein 1.2. Some membrane disruption was revealed by an increase in the isotropic component. These results agreed with the more abrupt destabilization of aurein 1.2 through its carpet mechanism. As for caerin 1.1, its insertion into bilayers by formation of transmembrane pores has a less destructive effect on bacterial membranes.

While membrane dynamics was increased in all cases upon large addition of AMPs, we have observed a difference in bacterial behavior when AMPs were added in smaller quantities, around the MIC. In the case of Gram(+) bacterium *B. subtilis*, reduced membrane dynamics were induced by both AMPs at their MIC (Fig. 5 and Table 3) – an effect that was not observed on *E. coli*, when exposed at, or even below, the MIC. It is only when exposed to caerin 1.1 at higher concentrations, such as 45 μg/mL, that lower M_1 and M_2 values revealed lipid disordering and increased isotropic component indicated some membrane lysis. Our results thus suggest that there must be some different mechanism in *B. subtilis* vs. *E. coli*, for example an interaction with PGN components such as the negatively-charged wall teichoic and lipoteichoic acids, which are absent in *E. coli*, or a different pathway towards a different intracellular target. Without AMP, the isotropic peak comes from natural abundance D_2O (despite the use of ^{2}H-depleted

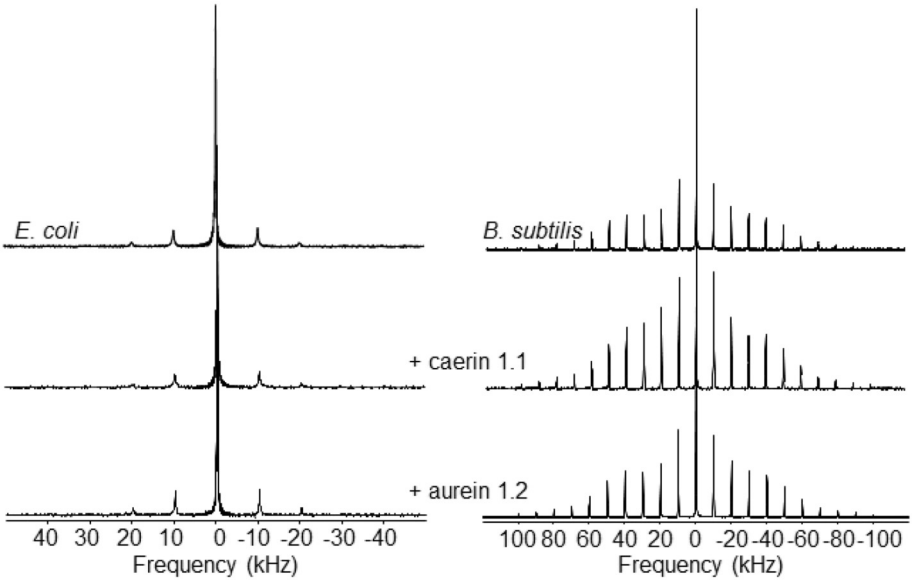

Fig. 5. MAS (10 kHz) ^{2}H NMR spectra of intact *E. coli* and *B. subtilis* acquired at 37 °C and with addition of MIC of caerin 1.1 or aurein 1.2.
Reprinted from Laadhari et al. [80] with permission.

Table 3
Spectral moment analysis of the MAS (10 kHz) ^{2}H NMR spectra of labeled *E. coli* and *B. subtilis*, without and with the presence of AMPs at different concentrations.
Adapted from Laadhari et al. [80] with permission.

Sample	AMP concentration (μg/mL)	M_1 (10^4 s^{-1})	M_2 (10^9 s^{-2})
B. subtilis[a]		9.2 (0.2)[a]	15 (2)
+ caerin 1.1	30 (~MIC)	9.4 (0.6)	15 (1)
	45	6.6 (0.3)	8 (1)
	60	4.0	3.3
+ aurein 1.2	30 (~MIC)	12 (2)	23 (3)
	45	13 (3)	27 (4)
	60	3.8 (0.6)	3 (2)
E. coli[a]		3.2 (0.3)	1.6 (0.7)
+ caerin 1.1	100 (~MIC)	2.8 (0.3)	1.6 (0.6)
+ aurein 1.2	100 (~MIC)	2.5 (0.2)	1.4 (0.2)

[a] Standard deviation based on four measurements.

water) or lipids, but it is small relative to the rest of the signal. With AMP, this isotropic signal is sometimes unaffected, and sometimes largely increased (Fig. 5). Bear in mind that the central component does not contribute much to the spectral moments (Eq. (2)), and the observation of an isotropic phase induced by AMPs will be accompanied by an increase in membrane dynamics and a decrease in moment values.

The membrane disordering and disruption was enhanced at 60 μg/mL of caerin 1.1. As for aurein 1.2, the increased lipid disordering in *B. subtilis* membranes was only observed at 60 μg/mL of peptide (Fig. 6 and Table 3). These results indicated that a higher concentration of aurein 1.2 is actually required to exert its action on the membrane. Aurein 1.2 is shorter and more cationic than caerin 1.1, and most likely to be retained by teichoic and lipoteichoic acids, thus reducing their local concentration on the lipid membrane, and limiting their interaction with negatively-charged lipids.

The similar structure of aurein 1.2 and caerin 1.1 determined in different membrane mimetics such as trifluoroethanol, large unilamellar vesicles or intact bacteria suggest a similar interaction with the membrane environment [93,95,96]. Indeed when studied by ^{2}H SS-NMR in oriented DMPC/DHPC bicelles and in less hydrated DMPC bilayers mechanically-aligned on glass plates, the same trends were observed in terms of lipid perturbation [13]. Therefore we can assume that the nature of the interaction of AMPs with lipids is the same, in models of different morphologies, different hydration and in cells.

As discussed before, and detailed in Wimley et al. [41], it is difficult

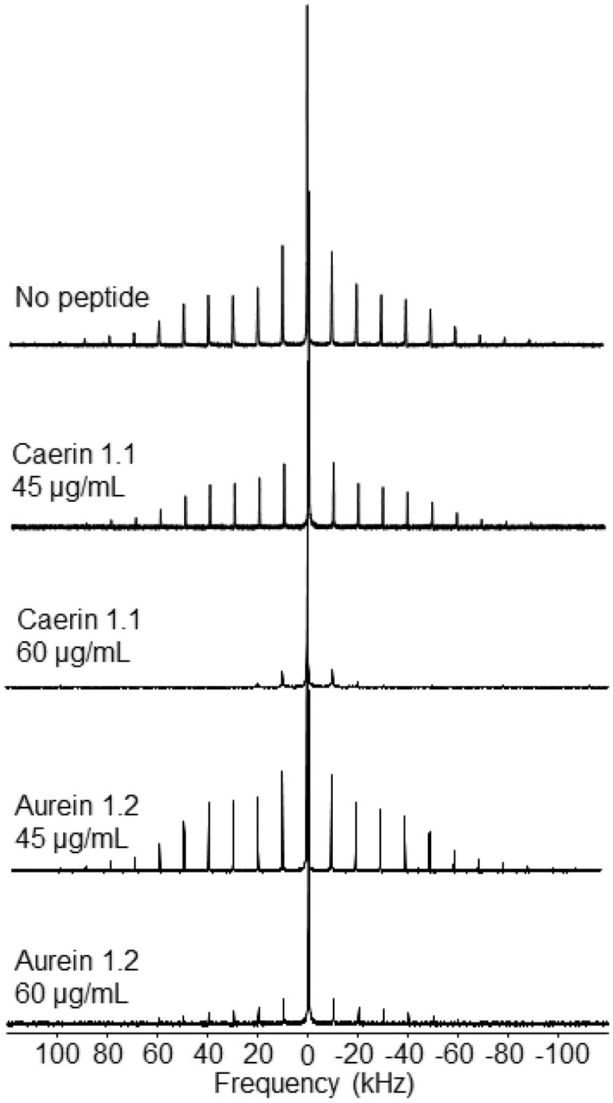

Fig. 6. ^{2}H MAS NMR spectra of intact *B. subtilis* acquired at 37 °C and with the addition of different concentrations of (a) caerin 1.1, and (b) aurein 1.2.
Adapted with permission from Laadhari et al. [80].

to compare NMR and other biophysical experiments with biological assays such as MIC, typically carried out at 10^4–10^6 bacterial cells/mL. In *in vivo* NMR experiments, bacteria are pelleted and thus samples contain more cells than in biological assays, affecting the AMP-to-lipid ratio studied. Wimley et al. [41] estimate a bound peptide-to-lipid ratio of 100:1 for 5 μM peptide in a sample of 10^5 cells/mL, suggesting that AMPs must be interacting with other non-lipid components. Also, it only takes 10–15 min to AMPs to bind bacteria. Even if SS-NMR experiments were done using two orders of magnitude higher in cell concentration, the peptide:lipid ratio will still be elevated. The lipid interaction that is measured is the same, and this is what SS-NMR probes.

8. In vivo NMR study of MSI-78 and CA(1-8)M(1-10)

An alternate approach to employing natural AMP sequences such as aurein 1.2 and caerin 1.1, is to design AMP sequences de novo, or to modify natural sequences with the goal of improving properties such as potency, specificity and stability. Two of the best studied artificial sequences are MSI-78 and CAME (both reviewed, for example, in [16]). MSI-78, also known as pexiganan, is a 22-residue, highly positively charged (Table 2), synthetic analog of magainin 2, an AMP from the skin of *Xenopus laevis* [15]. It has been shown to have a broad spectrum of activity against fungi and both Gram-positive and -negative bacteria [15,99], while maintaining relatively low activity against red blood cells. MSI-78 has been thoroughly studied, in large part due to its initial promise as a topical therapeutic [15]. MSI-78 is unstructured in solution and takes on a helical structure in the presence of POPC lipid vesicles [100]. Solution NMR was used to solve its structure in zwitterionic DPC micelles. The peptide was shown to take on a dimeric antiparallel α-helical coiled structure, where the dimer is stabilized by a phenylalanine zipper [101].

A number of biophysical experiments, including SS-NMR, have been carried out to probe MSI-78's interactions with model lipids. ^{15}N SS-NMR of MSI-78 in mechanically-oriented POPC and 3:1 POPC/POPG bilayers suggested the peptide orients parallel to the bilayer, at least at the particular AMP:lipid molar ratio employed (3 mol%) [100]. Peptide orientation was looked at in more detail in a later study, and it was found that the peptide tilt angle depends on both the peptide-to-lipid ratio and the choice of lipids used in the model membranes [102]. Oriented peptide/lipid samples are generally thought to be less well hydrated than vesicles and so it is also beneficial to consider NMR studies of MSI-78 with vesicles. MSI-78 was shown to increase acyl chain disorder in ^{2}H NMR spectra of POPC-d_{31} vesicles and could permeabilize both 3:1 POPC/POPG vesicle membranes and the outer membrane of *E. coli* [100], in keeping with the suggestion that membrane permeabilization [103] may be the mechanism by which this AMP inhibits pathogens. A clue as to the mechanism for permeabilization comes from dynamic scanning calorimetry (DSC) data with MSI-78-POPE dispersions that show MSI-78 can induce positive curvature strain [100]. On the other hand, DSC studies carried out with intact *E. coli* showed that MSI-78 binds, destabilizes and inhibits *E. coli* ribosomes [32]. This finding brings up the possibility that MSI-78 acts against both membranes and ribosomes, or even that its main target is inside the bacteria and the membrane permeabilization observed is just coincident with MSI-78's penetration into the cells. Another non-lipidic cell component that affects MSI-78's ability to inhibit cell growth is the PGN layer. Preliminary work in our lab has shown that disrupting *E. coli* PGN prior to AMP treatment increases MSI-78's ability to inhibit cell growth – in contrast with CA(1-8)M(1-10),where PGN disruption protects bacteria from the AMP [49].

The CAME series of peptides encompass a number of different sequence variations, but are all based around hybrids of cecropin A from moths, and melittin A from bees [16,17]. Peptides in this group include CA(1-7)M(2-9), composed of an N-terminal region comprised of residues 1–7 from cecropin A and a C-terminal region of melittin A residues 2–9 [104], as well as CA(1-8)M(1-12) [105], CA(1-8)M(1-18) [104], BP100 [56], CAMEL0 [106] and more [107–109]. CAME peptides have shown promise in animal models of human disease, for instance in a sepsis model of resistant *Acinetobacter baumannii* infection [110]. NMR and other biophysical experiments with model membranes have been used to try to pick apart the structure-function relationships of CAME peptides. For example, solution NMR was used to determine the high resolution structure of CA(1-8)M(1-12) in DPC micelles [111] and to examine the role of a central hinge (either proline or Gly-Ile-Gly) known to be important for function [109]. CA(1-7)M(2-9) and CA(1-8)M(1-18) take on α-helical structures in the presence of model membranes and membrane interactions are highly dependent on the balance of zwitterionic and anionic lipids in the model membranes [104].

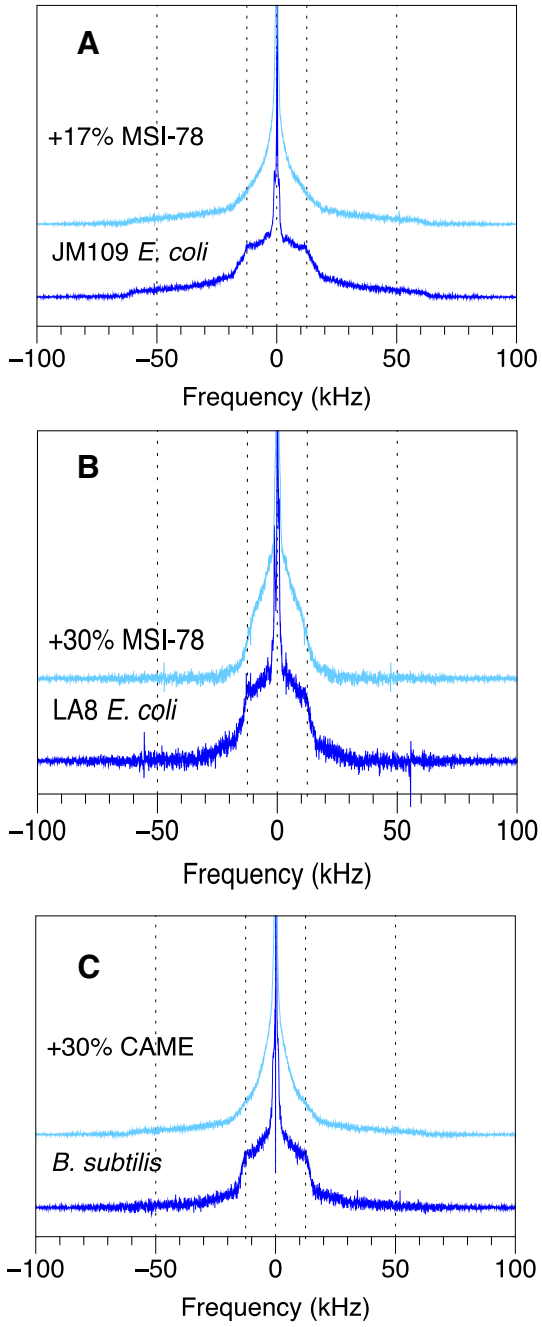

Fig. 7. Selected static solid-state NMR spectra for ^{2}H-membrane-enriched bacteria with and without MSI-78 and CA(1-8)M(1-10).

In Fig. 7A is shown the static spectra of ^{2}H-membrane-enriched *E. coli* with and without the AMP MSI-78. Without MSI-78, there is a well-

defined shoulder at ± 12 kHz arising from deuterons in the motionally-constrained region of the deuterated chains near the headgroup. The presence of 17% (by dry weight of the bacteria) MSI-78 causes a shift in the intensity from larger splittings to smaller splittings. This shift in ^{2}H-splitting intensity indicates decrease in average orientational order, i.e., even in the presence of the entire bacterium we detect an AMP-induced increase in lipid chain disorder. This effect on lipid order is qualitatively similar to what is seen in ^{2}H SS-NMR studies of AMPs interacting with model lipid bilayers, but in the presence of the bacterium, a much greater AMP:lipid ratio, about 100 ×, is require to see an effect on chain order, as compared to the same experiment in a model lipid system [77]. Fig. 7B also shows ^{2}H-membrane-enriched *E. coli*, but the strain of *E. coli* employed, LA8, has been mutated so that it cannot synthesize or metabolize lipid acyl chains and hence may reduce the "background" ^{2}H signal. MSI-78's effect on the two *E. coli* strains is very similar. Unlike *E. coli*, *B. subtilis* is a Gram(+) bacterium and thus has a very different cell envelope. However, in Fig. 7C we see that it gives a very similar static ^{2}H SS-NMR spectrum to *E. coli*. The narrowness of the ^{2}H splittings in Fig. 7C as compared to Fig. 5 most likely derives from the differences in the fatty acids the *B. subtilis* were grown on; in Fig. 5, no unsaturated fatty acids were used [78] and in Fig. 7C a small (compared to palmitic acid) amount of oleic acid was included [80]. This serves to illustrate how sensitive the ^{2}H NMR profile is to differences in growth conditions. When 30% of the AMP CA(1-8)M(1-10) is added to *B. subtilis*, we see a shift in the intensity from wider splittings to smaller splittings, similar to that seen for *E. coli*, indicating a decrease in acyl chain order. It is interesting to note that although CA(1-8)M(1-10) has a substantially smaller positive charge than MSI-78, + 6 as opposed to + 10 (Table 2), there is a qualitatively similar effect on lipid chain order.

The AMP-induced change in the ^{2}H SS-NMR spectra can be quantified by calculating the first and second moments of the spectra (M_1 and M_2) (Table 4). The values are very dependent on where the cursors for the integration are set, and also depend on the details of sample preparation – i.e. depending on how the sample is prepared we often observe a highly ordered component in the spectra which we include in the calculation of M_1 and M_2. Like caerin 1.1, MSI-78 can be seen to reduce both M_1 and M_2 in a concentration dependent manner. On the other hand, the measured M_1 and M_2 actually increase with the addition of CA(1-8)M(1-10), to *B. subtilis*, although the appearance of the spectra indicates a shift to smaller splittings. We believe this incongruence is due to the rigid (wide splitting) component in these *B. subtilis* spectra which contributes substantially to the M_1 and M_2 values and masks the AMP induced changes in the more central part of the spectra.

It is important to note that the AMP:lipid ratios in the MSI-78 and CA(1-8)M(1-10) experiments is much lower than would be present in MIC experiments – i.e. we expect this to be a sublethal dose of AMP. Equally important, we have shown that ~75% or more of the cells do remain viable, even after 6–8 h in the spectrometer (see the protocol section).

Table 4
Spectral moment analysis from static ^{2}H SS-NMR spectra of ^{2}H-membrane-enriched bacteria [77,78] [and unpublished results].

Cells	AMP (% of dry weight of bacteria)	Approximate AMP concentration (μg/mL)	M_1 (10^4 s^{-1})	M_2 (10^9 s^{-2})
E. coli LA8	None	–	4.6[a]	3.7[a]
E. coli LA8	10% MSI-78	~150 (25 × MIC)	4.1	2.8
E. coli LA8	20% MSI-78	~300 (50 × MIC)	3.6	2.4
E. coli LA8	30% MSI-78	~450 (70 × MIC)	3.4[a]	2.2[a]
E. coli LA8	60% MSI-78	~900 (150 × MIC)	2.8	1.5
E. coli JM109	None	–	10.0	1.9
E. coli JM109	17% MSI-78	~250 (10 × MIC)	8.3	1.6
B. subtilis	none	–	6.5[b]	8.4[b]
B. subtilis	30% CA(1-8)M(1-10)		7.3[b]	12.2[b]

[a] Average of two measurements (all others are single experiments).
[b] Values are dominated by a highly ordered component of the spectrum. Although M_1 and M_2 increase with AMP, inspection of the spectra show that, like the rest of the data in the table, there is a shift in intensity towards the centre of the spectrum with AMP.

9. Conclusion

^{2}H SS-NMR is a valuable method to investigate *in vivo* how AMPs - or other antimicrobial agents - act on the actual bacterial membrane. This is advantageous considering that AMP interactions can depend on model membrane composition [104]. Lipid perturbations induced through mechanisms such as the carpet model with aurein 1.2 or the toroidal pore with caerin 1.1 (Fig. 1) can be revealed through spectral changes. Varying the AMP concentration can also allow help elucidate different events in the action of antibacterial agents, including loss of lipid mobility (observed for example with cetyltrimethylammonium bromide detergent [84]) that can occur before an actual increase in membrane fluidity, and of course reveal membrane loss of integrity through micellization. The experimental design is easy to establish. Indeed deuteration of lipids in bacteria membranes can be readily carried out by supplementing the appropriate saturated and unsaturated fatty acids in the growth medium. Moreover, the NMR experiments are simple and robust. This methodology certainly enables ascertaining how much of the mechanism is lipid-related vs. other mechanisms, involving the cell wall or other proteins for example.

How does the effect of AMPs on the ^{2}H NMR spectra of ^{2}H-labeled membranes in intact bacteria compare to the same results for ^{2}H-labeled model membranes? As might be expected, the answer depends on the AMP in question. One challenge in synthesizing the results of different kinds of AMP experiments is in quantifying how much AMP is required to see a particular effect. MIC measurements provide a concentration of AMP needed to see growth inhibition. However, MICs are measured at much more dilute cell densities than NMR experiments employ and so have a difference in molar AMP to lipid ratio of many orders of magnitude [41,77].

While the data presented in this review come from two different research groups and consequently use somewhat different protocols for preparing cells for NMR, the AMP treatments employed are generally in the 10s to 100s of μg/mL, i.e., around the MIC concentration or higher (Tables 3 and 4). One of the most notable results is that, compared to ^{2}H SS-NMR studies with model lipids, the AMP:lipid molar ratio needed to see an effect on the spectra of intact bacteria is on the order of 100 times greater [73]. There are at least two reasons why more peptide might be needed to see comparable effects on the membrane of intact bacteria compared to model lipid bilayers. One possibility is that most of the AMP reaches the lipid bilayer but, for some reason, is less able to disrupt bilayer chain order at low concentrations than is the case in model systems where lower concentrations are sufficient to cause observable effects. Another possibility is that the AMP is only transiently in the membrane on its way to an intracellular target. A more likely possibility, though, is that much of the peptide does not reach the lipid membrane. It may be bound up in other components of the cell envelope, such as the LPS layer of Gram(−) bacteria, or the teichoic acid in Gram(+)'s PGN, as detailed by Hancock et al. [12]. Thus, ^{2}H SS-NMR of the membranes of intact bacteria emphasizes the importance of AMP interactions with non-lipid components of bacteria in the bactericidal activity, considering how little the membrane is perturbed at the MIC for AMPs such as caerin 1.1 and MSI-78. This suggests that optimizing AMPs for clinical use should also take into consideration AMP interactions with non-lipid components.

While this review covers the study of AMPs interaction with living bacteria by ^{2}H SS-NMR, it is also the template of other studies, such as other molecules like detergents and pigments interacting with bacteria [84], other microorganisms such as microalgae for example [75]. Other nuclei such as ^{31}P, ^{15}N, ^{13}C have also been exploited to characterize

intact bacteria and investigate the action of antibiotics. For example, wall teichoic acids have been studied by ^{31}P solid-state NMR, and the PGN explored using through-bond and through-space ^{13}C correlation experiments [112] under hydrated conditions. Interactions with the PGN have been studied using ^{13}C-^{15}N rotational-echo double resonance (REDOR) [113] of lyophilised labeled bacteria, as well as ^{13}C-^{19}F and ^{15}N-^{19}F REDOR to determine the structure of [^{19}F]-antibiotic-PGN complexes [114,115]. The ^{2}H SS-NMR results summarized here show that not only whole-cell but *in vivo* solid-state NMR is a valuable strategy, and one of the rare tools that can scrutinize molecules at an atomic level within a living organism. This is only the beginning of a new chapter in NMR.

Transparency document

The http://dx.doi.org/10.1016/j.bbapap.2017.07.018 associated with this article can be found, in online version.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (grant 326750-2013 to I.M., grant 312676 - 2011 to V.B.) and the Centre National de la Recherche Scientifique (UMR 7099 to D.E.W.). M.L. is grateful to the Groupe de Recherche Axé sur la Structure des Protéines (GRASP), to Pharmaqam and to the Faculté des Sciences of the Université du Québec à Montréal for the award of scholarships.

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