Mechanisms and Significance of Bacterial Resistance to Human Cationic Antimicrobial Peptides

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Abstract Cationic antimicrobial peptides (CAMPs) are essential compounds of the innate immunity system possessed by humans. CAMPs protect the host by exerting bactericidal activity, molecular signaling, modulating the immune response, and facilitating the communication between innate and acquired immunity. Over the millennia, bacteria have developed mechanisms to circumvent the antimicrobial activity of CAMPs, thereby promoting their survival during infection. In this chapter, we focus on the mechanisms used by various bacterial pathogens to resist the antibiotic-like action of CAMPs and the consequences of such resistance.

1 Introduction

Regardless of the host, signs of infection caused by a bacterial pathogen are typically noticed after damage to the host has occurred and symptoms are manifested. These symptoms of infection can arise from toxins produced by the pathogen or host inflammatory processes triggered when the host recognizes pathogenic bacteria or their associated virulence factors. Early during infection, mediators of innate immunity are brought to the front line of defense to combat the invader and protect the host. The efficacy of this response can determine the duration, spread, and severity of

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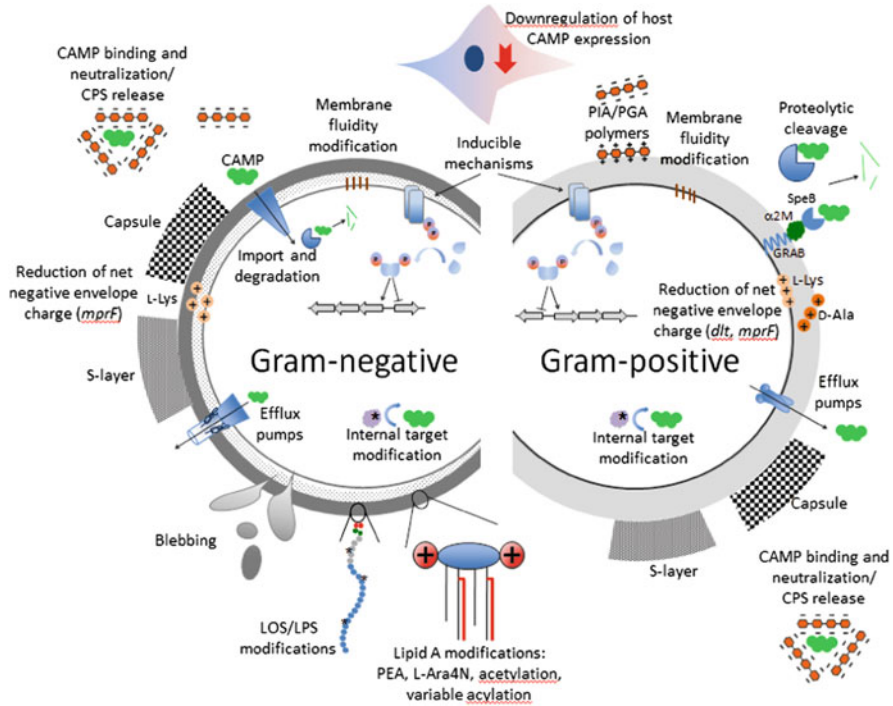
Fig. 1 Schematic representation of the mechanisms of resistance to cationic antimicrobial peptides (CAMP) used by Gram-negative and Gram-positive bacteria. Key: *CAMP* cationic antimicrobial peptide, *CPS* capsule polysaccharide, *D-Ala* D-alanine, *L-Ara4N* 4-amino-4-deoxy-L-arabinose, *L-Lys* L-lysine, *LOS* lipooligosaccharide, *LPS* lipopolysaccharide, *PEA* phosphoethanolamine. These examples are of specific mechanisms of CAMP resistance expressed by Gram-positive and Gram-negative bacteria and should not to be considered exhaustive

disease. Cationic antimicrobial peptides (CAMPs), also appropriately called "host defense peptides" (Brown and Hancock 2006), are important in this response as they can directly or indirectly exert antibacterial activity. Any successful pathogen must find ways to evade the direct action of CAMPs or risk having their numbers severely reduced or even eliminated.

Although thousands of CAMPs exist in nature, humans confront bacteria with three main classes: the α- and β-defensins, the sole human cathelicidin termed LL-37, and peptides derived from protease digestion of proteins that perform important roles in other host response processes (e.g., cathepsin G). Unless otherwise stated, we focus this review on the mechanisms employed by bacterial pathogens to escape the action of gene-encoded CAMPs. It is important to note that CAMPs are, in essence, antibiotics. It is therefore not surprising that many of the general mechanisms developed by bacteria to resist classical antibiotics are in concept also used by bacteria to resist CAMPs. These mechanisms are summarized in Fig. 1.

Studies on mechanisms of bacterial resistance to CAMPs have been facilitated by the ability to construct and use isogenic strains that differ by a single, defined mutation or the presence of a gene that impacts levels of bacterial susceptibility to CAMPs. To assess the significance of such differences, it is first important to have reproducible antibacterial assays, which are discussed below. It is difficult to use purified human CAMPs or synthetic versions, due to availability of materials and cost constraints, to select genetic variants, but these problems can be circumvented by the use of recombinantly produced CAMPs or commercially available compounds that mimic the bactericidal action of CAMPs; the cationic antimicrobial peptide polymyxin B has been a reliable CAMP substitute used by many investigators.

Understanding how bacteria can develop CAMP resistance requires the use of in vitro antimicrobial assays that are reproducible and relatively easy to use. Typically, these assays involve an assessment of direct colony forming unit reduction when bacteria are incubated in liquid media with purified CAMPs. Other standardized assays for determining minimal inhibitory concentrations (MIC) or minimal bactericidal concentrations (MBC) of CAMPs are also routinely employed (Institute Clinical and Laboratory Standard 2009). Typically, actively growing bacteria are diluted and incubated with CAMPs in broth or phosphate buffer-based solutions. These liquid media can be altered in pH and ionic strength to assess the impact of changes in these conditions on the killing efficacy of CAMPs. The radial diffusion agar overlay/underlay assay developed in the Lehrer laboratory (Qu et al. 1996) is also particularly useful and can provide quantitative results as well as allowing one to test how changes in ionic strength, presence of divalent cations, and pH impact CAMP activity. While these assays can collectively provide important information, laboratory growth media have little resemblance to the natural environments in which CAMPs must function in vivo. For instance, it is rare that the presence of other host compounds is taken into account, but these can either have positive or negative actions on the susceptibility to CAMPs. The presence of lysozyme or phospholipase A2 can enhance bacterial killing in CAMP assays. In our own studies with *Neisseria gonorrhoeae*, which typically (and often) infects the human genital tract, it was found that physiologically relevant levels of polyamines (e.g., spermine and spermidine) can decrease bacterial susceptibility to LL-37 (Goytia and Shafer 2010). Additionally, Dorschner and colleagues deduced that physiologically relevant levels of carbonate (CO_3^{2-}) in laboratory media greatly increase the killing potential of numerous and varied CAMPs, but not of the anionic skin-derived antimicrobial peptide (AMP) termed dermcidin (Dorschner et al. 2006). They further found that *Staphylococcus aureus* responds to CO_3^{2-} through a dynamic transcriptional response, thinning its peptidoglycan via repression of the alternative sigma factor, *sigB*, which may explain the increased susceptibility of this pathogen to the tested peptides. Interestingly, others have shown that LL-37 adopts an α-helical (active) conformation in the presence of carbonate (Johansson et al. 1998), which may have played some part in the enhanced killing activity seen in the Dorschner study. Thus, CAMP behavior in the lab setting may not always reflect CAMP-bacteria interactions in vivo.

Environmental conditions (e.g., limitation of iron, anaerobiosis, local pH, ionic strength, and presence of divalent cations) can also significantly impact AMP activity against bacteria. The recent report (Schroeder et al. 2011) that anaerobic conditions can potentiate the antibacterial action of human β-defensin 1 (hBD-1) against commensal gut bacteria nicely illustrates this point. In this instance, reduction of the intramolecular disulfide bonds by the thioredoxin system significantly enhanced HBD-1 activity against anaerobic commensal bacteria, and this was proposed by the authors as a means used by the host to prevent their overgrowth. Interestingly, Nuding et al. found that similar anaerobic conditions can actually weaken the antibacterial action of the related peptide human β-defensin 3 (HBD-3) (Nuding et al. 2009). Such diversity of CAMP function may be a way for the body to fine-tune its gut microbiota population. Alternatively, conditions of hypoxia may induce, in the absence of a surrogate electron acceptor, a state of bacteriostasis for some bacteria. This has been observed in *N. gonorrhoeae*, resulting in gonococcal resistance to antimicrobial proteins and CAMPs (Casey et al. 1985). Bacteria also face iron-starvation conditions imposed by host iron-binding proteins and have a variety of response mechanisms to acquire iron (Ganz 2009). As an example, this environmental stress can influence the level of susceptibility of *Streptococcus pyogenes* to LL-37 (Froehlich et al. 2009).

Ex vivo and in vivo infection models have also been employed to gain insights regarding the significance of mechanisms of bacterial resistance to CAMPs. Perhaps the most widely used ex vivo model is that of polymorphonuclear leukocytes (PMNs). These professional phagocytic cells are used in monolayers or in suspension to evaluate changes in the intraleukocytic microbicidal activity that may be due to AMP resistance mechanisms. CAMPs (e.g., neutrophil defensins termed HNP 1–4 and LL-37) are, along with antimicrobial proteins such as the *b*actericidal/*p*ermeability-*i*ncreasing (BPI) protein (Marra et al. 1990), lactoferrin, cathepsin G, CAP37, and lysozyme, important mediators of nonoxidative killing by PMNs (Sorrell et al. 1978; Spitznagel and Shafer 1985). These antimicrobial compounds are stored within the cytoplasmic specific and azurophilic granules and are delivered into the phagocytic vacuole after granule fusion and degranulation. Bacteria that inhibit phagosome-lysosome fusion can resist CAMPs and antimicrobial proteins (Scott et al. 2003). For instance, *Salmonella* presents a well-studied example where a protein (SipC) belonging to the *Salmonella p*athogenicity *i*sland-2 (SPI-2) is essential to prevent the fusion of *Salmonella*-containing vacuoles with host cell lysosomes (Uchiya et al. 1999). A recent study suggests that *Salmonella* recruits host proteins to the vacuole to inhibit the fusion (Madan et al. 2012). However, once CAMPs are delivered into the developing phagolysosome, they rapidly coat the bacterial surface and can achieve mg/mL concentrations, making it remarkable that any bacteria can survive inside the phagolysosome at all (Lehrer et al. 1988). With PMN models, it is possible to test inferences made regarding the significance of bacterial mutations that alter the susceptibility to isolated, PMN-derived CAMPs; specific examples are described below.

To test the idea that CAMP resistance mechanisms can enhance bacterial survival during infection, numerous studies have employed whole animal models. Mouse models of infection have been particularly useful in this respect, allowing investigators to readily test inferences they have drawn from in vitro and ex vivo tests. In this regard, mouse strains bearing knockout mutations in genes similar to human genes [e.g., CRAMP encoding the murine version of LL-37 (Nizet and Gallo 2002)] or knocked-in human CAMP genes [e.g., the HBD-5 used by N. Salzman and colleagues in their studies dealing with *Salmonella typhimurium* Salzman et al. (2003), Wehkamp et al. (2005)] have been used to test both the significance of CAMPs in host defense and if bacterial resistance mechanisms are important in promoting microbial survival during infection. Briefly, CRAMP knockout mice were more susceptible to invasive group A streptococcal infection than their CRAMP$^{+/+}$ counterparts, while expression of HBD-5 provided mice with increased resistance to a lethal *S. typhimurium* infection.

To date, only a single report has appeared in the literature describing a human bacterial infection model for the purpose of studying the significance of a CAMP resistance mechanism. In this instance, Bauer et al. (Bauer et al. 2006; Bauer and Spinola 2000) utilized the forearm skin puncture model (Spinola et al. 2003) to study host responses to infection by *Haemophilus ducreyi*, the causative agent of the sexually transmitted infection chancroid. *H. ducreyi* is intrinsically resistant to CAMPs and uses two transport systems, the Mtr efflux and Sap importer systems, for this purpose; these systems are described in greater detail below. Loss of these systems significantly decreased the survival of *H. ducreyi* and lesion pathology in this model (Rinker et al. 2011; Mount et al. 2010), indicating that CAMP resistance is important for its ability to cause disease.

2 Mechanisms of Bacterial Resistance to CAMPs

How do we define CAMP resistance? This is not an easy question to answer, as "breakpoints" typically used to differentiate antibiotic-sensitive from antibiotic-resistant strains are seldom considered for CAMP studies undertaken by research laboratories. This matter is complicated by a number of issues: CAMPs can achieve very different concentrations depending on their location; local environmental conditions can be antagonistic or agonistic; inducible resistance can be displayed in the presence of sublethal levels of CAMPs, yet lost under normal conditions; and CAMPs can exert multiple mechanisms of killing which, for a given peptide, might differ depending on the target bacteria. The precise mechanism by which CAMPs kill bacteria is a matter of some controversy, and no unifying mechanism has been readily accepted by the CAMP research community. Certainly, CAMPs must first bind to the microbial surface and traverse the cell envelope. The events occurring post binding that result in bacterial death are where controversy exists, and it has not always been easy to separate direct killing from postmortem events. For instance, changes in membrane integrity and potential, inhibition of cell wall

biosynthesis, and interaction of CAMPs with nucleic acids have been invoked as bactericidal events for certain CAMPs acting on a given bacterial target. Our purpose below is not to review how CAMPs kill bacteria, but rather describe how bacteria use constitutive and inducible mechanisms to circumvent their action.

In order for CAMPs to efficiently kill bacteria, they must reach their target in extracellular fluids or within intracellular compartments avoiding the action of peptidases/proteases, navigate past hydrophilic surface structures such as capsules and *O*-antigen chains of lipopolysaccharide (LPS), interact with negatively charged surface structures, insert into the cell envelope, reach the cytoplasmic membrane, and, in some instances, enter the cytosol. All of these steps provide opportunities for bacterial interference, which can decrease the susceptibility of the target microbe to CAMPs. Briefly, pathogens have evolved several strategies to circumvent the attack by CAMPs: (1) modulate CAMP gene expression, (2) degrade CAMPs by extracellular or intracellular peptidases/proteases, (3) trap CAMPs, (4) reduce binding of CAMPs to the cell surface, (5) export CAMPs by efflux pumps, and (6) alter intracellular targets. CAMP resistance mechanisms are typically expressed constitutively, but many are also under control of regulators of gene expression that respond to environmental cues. Below, we review examples of these strategies from several medically relevant pathogens. Table 1 summarizes specific examples from various pathogens, while Fig. 1 summarizes the different strategies described in this section. We review this subject by beginning with examples of downregulation of CAMP production and then follow CAMPs as they bind and enter target bacteria, providing descriptions of the various systems employed by different bacteria to avoid the killing action of these important peptides.

2.1 Bacterial Modulation of CAMP Gene Expression

Bacteria have developed novel and diverse strategies to modulate the availability of CAMPs in extracellular fluids and within phagolysosomes of phagocytes; the latter subject has been extensively reviewed elsewhere (Ray et al. 2009; Flannagan et al. 2009), and we will concentrate on studies dealing with bacterial modulation of CAMP production. It is important to note that modulation of CAMP production can have profound downstream effects on the overall host immune system, which can facilitate bacterial growth and dissemination during infection. Bacterial products can directly or indirectly modulate CAMP expression and activation of immune responses. For instance, *Escherichia coli* lipopolysaccharide (LPS) can increase mRNA production of human β-defensin 2 (HBD-2) via CD14-activation of neutrophils (Becker et al. 2000). Tada et al. showed that proteases from *Porphyromonas gingivalis* can cleave the macrophage CD14 outer-membrane receptor. CD14 recognizes pathogen-associated molecular patterns (PAMPs), and cleavage of this protein rendered macrophages unresponsive to the presence of this pathogen and prevented CAMP production (Tada et al. 2002). Additionally, uropathogenic *E. coli* (UPEC), collected from patients with urinary tract infections (UTI), express the so-called "curli" fimbriae

Table 1 Examples of CAMP resistance mechanisms expressed by bacteria

Product name/gene	Organism	References
CAMP binding/inactivation		
Staphylokinase	*S. aureus*	Braff et al. (2007), Jin et al. (2004)
SIC	*S. pyogenes*	Akesson et al. (1996)
M1 surface protein	*S. pyogenes*	Lauth et al. (2009)
CAMP proteolytic cleavage		
V8 protease	*S. aureus*	Sieprawska-Lupa et al. (2004)
Aureolysin	*S. aureus*	Sieprawska-Lupa et al. (2004)
ZapA	*P. mirabilis*	Schmidtchen et al. (2002), Belas et al. (2004)
LasA	*P. aeruginosa*	Park et al. (2001), Schmidtchen et al. (2002)
Elastase LasB	*P. aeruginosa*	Schmidtchen et al. (2002)
Gelatinase GelE	*E. faecalis*	Schmidtchen et al. (2002)
Surface protease PgtE	*S. enterica*	Guina et al. (2000)
Metalloproteinase SepA	*S. epidermidis*	Lai et al. (2007)
Metalloprotease DegP	*E. coli*	Ulvatne et al. (2002)
Cysteine protease SpeB	*S. pyogenes*	Nyberg et al. (2004), Schmidtchen et al. (2002), Johansson et al. (2008)
Production of capsular polysaccharides		
PIA, PGA	*S. epidermidis*	Vuong et al. (2004), Kocianova et al. (2005)
Capsule-synthesis gene cluster		
cps cluster	*N. meningitidis*	Frosch et al. (1989)
	K. pneumoniae	Campos et al. (2004)
Mycolic acid synthesis		
kasB	*M. marinum*	Slayden and Barry (2002), Gao et al. (2003)
D-Ala modification of teichoic acids		
dltABCD	*S. pyogenes*	Kristian et al. (2005)
	L. monocytogenes	Abachin et al. (2002)
	S. aureus	Peschel et al. (1999)
Modification of membrane phospholipids with amino acids		
mprF, lysS	*S. aureus*	Fields et al. (1989), Miller et al. (1990), Peschel et al. (2001)
lysX	*M. tuberculosis*	Maloney et al. (2009)
mprF1 and *mprF2*	*C. perfringens*	Roy and Ibba (2008)
Lipid A modifications		
Aminoarabinose addition: *pmrAB, pmrE, pmrFHIJKL, pmrC*	*S. enterica* ser. Typhimurium	Gunn et al. (1998), Lee et al. (2004), Gunn (2008)
Acetylation: *O*-acetyltransferase	*P. mirabilis*	McCoy et al. (2001)
PEA addition: LptA	*N. gonorrhoeae* *N. meningitidis*	Lewis et al. (2009), Tzeng et al. (2005)
Phosphorylcholine substitution: GlpQ	*H. influenzae*	Fan et al. (2001), Lysenko et al. (2000)
Decreased membrane fluidity		
htrB	*H. influenzae*	Starner et al. (2002)

(continued)

Table 1 (continued)

Product name/gene	Organism	References
Production of carotenoids		
crtOPQMN	S. aureus	Mishra et al. (2011)
Plasmid-encoded efflux pump		
QacA	S. aureus	Kupferwasser et al. (1999)
CAMP expulsion		
MtrCDE	N. gonorrhoeae	Shafer et al. (1998)
	N. meningitidis	Tzeng et al. (2005)
MtrCD-GlmU	H. ducreyi	Rinker et al. (2011)
AcrAB-TolC	K. pneumoniae	Padilla et al. (2010)
EpiFEG	S. aureus	Otto et al. (1998)
MefE/Mel	S. pneumoniae	Zähner et al. (2010)
Controlled import leading to degradation		
Sap	S. enterica	Parra-Lopez et al. (1993)
Sap	H. influenzae	Mason et al. (2006)
SapA	H. ducreyi	Mount et al. (2010)
Induction mechanisms		
phoP/phoQ	S. enterica	Groisman et al. (1992), Groisman (2001)
phoP/phoQ	P. aeruginosa	Macfarlane et al. (1999)
pmrA/pmrB	S. enterica	Roland et al. (1993)
pmrA/pmrB	P. aeruginosa	McPhee et al. (2003)
misR/misS	N. meningitidis	Tzeng et al. (2004), Johnson et al. (2001)
covR/covS	S. pyogenes	Froehlich et al. (2009)
agr, sarA, aps/graRSX	S. aureus, S. epidermidis	Lai et al. (2007), Kraus et al. (2008)
Biofilm formation		
icaB	S. aureus	Vuong et al. (2004)
Bacterial regulation of host AMP expression		
Plasmid DNA-mediated decrease in LL-37 and HBD-1 expression	S. dysenteriae, S. flexneri	Islam et al. (2001)
Decreased LL-37 expression	N. gonorrhoeae	Bergman et al. (2005)
Diminished membrane potential		
Small colony variants	Sn aureus	Vesga et al. (1996), Yeaman and Yount (2003)

that modulate the immune system of the host and provide resistance to LL-37. Curli, an amyloid-like fiber expressed in biofilms, promotes cell adherence, increases induction of IL-8 (a human proinflammatory cytokine), binds to LL-37 inhibiting its killing activity, and increases bacterial virulence in a mouse model (Kai-Larsen et al. 2010). Furthermore, Islam et al. showed that *Shigella flexneri* and *S. dysenteriae* downregulate and prevent expression of CAMPs such as LL-37 and HBD-1 by the host. Even though the molecular mechanism was not completely elucidated, the authors suggested a role for plasmid DNA from the bacteria (Islam et al. 2001). Zughaier et al. reported that Neisseria meningitidis capsular polysaccharide (CPS) can

bind LL-37 and, as a consequence, dampen the host immune response against this strict human pathogen (Zughaier et al. 2010). Finally, *N. gonorrhoeae* can impair expression of LL-37 in cervical epithelial cell line ME180 (Bergman et al. 2005). This effect was observed with live bacteria, but not with dead gonococci nor with live commensal *Neisseria* species considered avirulent in a normal host. They concluded that a specific interaction took place between *N. gonorrhoeae* and the ME180 epithelial cell that suppressed expression of LL-37. The gonococcal structures responsible for this suppression of LL-37 production remain to be discovered.

2.2 Degradation of CAMPs

Bacteria can degrade CAMPs by proteolytic cleavage before they reach or pass the bacterial surface. CAMP degradation can occur extracellularly, by secreted and membrane-associated proteases, or intracellularly; the latter is facilitated by importers that deliver CAMPs to the bacterial cytosol, where they are degraded by peptidases and cytosolic proteases.

Studies with *Salmonella enterica* and *Staphylococcus aureus* have contributed significantly to our understanding of the role of bacterial peptidases/proteases in CAMP resistance. *S. enterica* expresses an outer-membrane protease, PgtE, which cleaves LL-37 and other linear CAMPs, and results in increased resistance to LL-37 in vitro and in vivo (Guina et al. 2000). PgtE is similar to the OmpT protein produced by *E. coli*, which cleaves protamine. *E. coli ompT* mutants are more susceptible to human protamine (Stumpe et al. 1998). *S. aureus* produces many proteases, and evidence has been presented that the action of aureolysin, a metalloprotease, and V8, a serine endopeptidase, can enhance its resistance to CAMPs. V8 cleaves and inactivates LL-37 (Sieprawska-Lupa et al. 2004) as well as complement proteins C3a and C4a, which have antimicrobial action (Zipfel and Reuter 2009). Aureolysin cleaves complement protein C3 at a nonphysiological site, rendering its cleavage products inactive (Laarman et al. 2011). Aureolysin-cleaved C3 protein is further degraded by host mechanisms, thus blocking the complement cascade, inactivating the antimicrobial activity of C3a, and preventing the targeting of *S. aureus* by the host immune response (Laarman et al. 2011). Schmidtchen et al. have published a series of elegant papers that collectively emphasize the role of proteases produced by medically important pathogens (and other relevant microorganisms), highlighting their importance for resistance to CAMPs and antimicrobial proteins (Schmidtchen et al. 2001, 2002). Briefly, these studies showed that elastase and alkaline protease from *Pseudomonas aeruginosa*, gelatinase from *Enterococcus faecalis*, a secreted cysteine proteinase (SpeB) from *Streptococcus pyogenes,* and a 50-kDa proteinase from *Proteus mirabilis*, possibly ZapA (Belas et al. 2004), can degrade LL-37 (Schmidtchen et al. 2001). As a secondary effect, these enzymes also degrade proteoglycans from the host's extracellular matrix, releasing negatively charged dermatan and/or heparan sulfate, which significantly inhibits the antimicrobial activity of the α-defensin human neutrophil peptide 1 (HNP-1) (Schmidtchen et al.

2001), and of bactenecin-5 and bactenecin-7 (Park et al. 2001). An additional mechanism developed by *S. pyogenes* involves the bacterial membrane-associated protein GRAB, which binds α-2-macroglobulin (α2M), a host-derived protease inhibitor. α2M, in turn, binds the secreted bacterial protease SpeB, which maintains its proteolytic activity against CAMPs, preventing the action of CAMPs at their target site on the bacterial surface (Nyberg et al. 2004). Thus, *S. pyogenes* binding of host α2M appears to serve two purposes: (1) facilitate cleavage of CAMPs before they reach their target and (2) promote immunological mimicry by presenting self-antigens at the bacterial surface.

Bacteria can also degrade CAMPs intracellularly through the combined action of an importer, which delivers CAMPs to the cytosol, and intracellular peptidases normally used to cleave bacterial peptides and increase the available pool of amino acids. For example, non-typeable *H. influenzae* (NTHi), a commensal Gram-negative bacterium that can cause conjunctivitis, sinusitis, acute and chronic otitis media, and bronchitis (Erwin and Smith 2007), expresses the Sap (*s*ensitivity to *a*ntimicrobial *p*eptides) ABC transporter. This transporter, first identified and characterized in *Salmonella* (Groisman et al. 1992; Parra-Lopez et al. 1993), can increase bacterial resistance to CAMPs by 8-fold and is required for virulence of NTHi in a chinchilla model of otitis media (Mason et al. 2005). SapA binds chinchilla BD-1 as well as human CAMPs (e.g., LL-37, HBD-2 and HBD-3, and HNP-1) (Mason et al. 2011). The binding of CAMPs to SapA upregulates expression of the *sap* operon (*sapABCDFZ*) (Mason et al. 2005), promoting transfer of the CAMPs into the cytosol where they are degraded (Mason et al. 2006). Further research by this group proposed a mechanism where CAMPs are taken up by the periplasmic binding protein SapA then transferred to the cytoplasm through the SapBCDF transporter and SapZ accessory protein (Shelton et al. 2011). Interestingly, this mechanism might increase the intracellular levels of nutrients, since the amino acids from the degraded CAMPs could be recycled. SapA of *H. ducreyi* also increases bacterial resistance to LL-37, but not to α- and β-defensins. However, the most important effect of SapA in *H. ducreyi* was observed in the human forearm model of chancroid in that SapA production was found to increase virulence, probably by promoting resistance to the higher concentrations of LL-37 that are secreted at infection sites in the dermis (Mount et al. 2010).

2.3 Hindering CAMP Localization to the Bacterial Surface

When bacteria find themselves in environments rich in CAMPs, they have strategies other than proteolysis to neutralize or repulse CAMPs, thereby reducing their susceptibility to these antimicrobials. Non-proteolytic mechanisms of CAMP resistance include (1) the presence of CAMP-binding agents, (2) expression of hydrophilic bacterial biopolymers to retard the passage of amphipathic CAMPs in

the electronegative bacterial surface, and (3) architectural constraints imposed by biofilms.

Binding or repulsion of CAMPs by extracellular compounds reduces their capacity to interact with negatively charged target sites on the bacterial surface. Several CAMP-binding compounds have been described. For example, staphylokinase (Sak) and the *s*treptococcal *i*nhibitor of *c*omplement (SIC) bind to and neutralize CAMPs such as HNP 1–3 and LL-37 in the extracellular milieu, effectively decreasing the local CAMP activity as much as 80 % (Jin et al. 2004; Pence et al. 2010). The production of extracellular polymers shields bacteria with an extra layer of protection against CAMPs. Polysaccharide intercellular adhesin (PIA) and poly-γ-glutamic acid (PGA) polymers, produced by staphylococci, inhibit HBD-3 and LL-37 activity (Kocianova et al. 2005; Vuong et al. 2004). It is thought that the charges present on these polymers are a triple threat as they are able to repulse similarly charged antimicrobials, neutralize and sequester oppositely charged antimicrobials, and behave as a mechanical barrier to their entry. Alginic acid produced by *P. aeruginosa* inhibits CAMPs in a similar fashion (Friedrich et al. 1999).

The production of capsule, or glycocalyx (literally, "sugar coat"), is a common defense mechanism also utilized by other human bacterial pathogens, including *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *S. aureus*, and *Bacillus anthracis*. It is notable that many of these pathogens are the cause of mucosal and respiratory tract infections that may progress to the bloodstream, an environment where encapsulated organisms are at a distinct advantage over other bacteria (Yeaman and Yount 2003). However, not all prokaryotic glycocalyces are produced by the pathogen itself and can sometimes be stolen from the host (see pathogenic *Neisseria* below). In the environment, several species of both *Eubacteria* and *Archaea* produce S-layers, which is a glycoprotein shroud that completely surrounds the prokaryotic cell. Interestingly, some eubacterial S-layers can be glycosylated with up to 150 carbohydrate moieties per protein unit (Messner et al. 2008). S-layers may be another protective mechanism against CAMPs in the highly competitive soil and water microbiome.

As with proteolysis, biopolymer sequestration of CAMPs can still function even when physically separated from the cell. Certain Gram-negative bacteria can release membrane vesicles rich in CAMP-binding sites called "blebs," and the act of blebbing could provide an extracellular sink for CAMPs. Some bacteria may also release negatively charged *c*apsular *p*oly*s*accharides (CPS) that titrate CAMPs by electrostatic interactions. Llobet et al. have described this mechanism of resistance in several clinically relevant pathogens, such as *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *S. pneumoniae* (Llobet et al. 2008). They show that CPS from different bacteria at concentrations as low as 1 μg/mL (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) can increase the MIC of HNP-1 between 5- and 30-fold, regardless of the CPS source. However, this mechanism can be rendered inadequate by the presence of polycations that preferentially bind to CPS, freeing CAMPs to react with the bacterial cell wall and kill the targeted cell (Llobet et al. 2008). Additionally, Campos et al. showed that a *K. pneumoniae* mutant lacking CPS is more sensitive to CAMPs such as HNP-1, HBD-1, protamine sulfate, and polymyxin B, compared to the wild-type strain

expressing CPS (Campos et al. 2004). They also showed that the CPS mutant binds more polymyxin B than the wild-type strain, suggesting that CPS protects the bacteria, either by mechanically shielding the bacteria or by titrating the CAMP. Moranta et al. injected wild-type or CPS mutant *K. pneumoniae* into mice and showed decreased levels of β-defensins produced in response to the wild-type strain, as compared to higher levels produced for the CPS mutant isogenic strain (Moranta et al. 2010). This suggested that CPS not only prevented the action of CAMPs at the surface of the bacteria, but also prevented signaling to the host immune system that would normally increase levels of β-defensins. In *N. meningitidis*, Jones et al. observed that expression of LOS and capsule is directly linked to increased resistance to LL-37 compared to the LOS-deficient and capsule-deficient mutants (Jones et al. 2009). Furthermore, they show that incubation of the wild-type bacteria with sublethal concentrations of LL-37 induced the expression of the capsule-associated genes *siaC* and *siaD*, which results in upregulation of capsule biosynthesis (Jones et al. 2009).

CAMPs are typically amphipathic molecules whose hydrophobic domain allows membrane insertion, an event required for killing the target microbe. Accordingly, the presence of bulky hydrophilic structures on the bacterial surface may hinder the migration of CAMPs from the extracellular milieu to the negatively charged bacterial surface structures that form the gateway to hydrophobic lipid bilayers. This may partly explain why the presence of the hydrophilic *O*-antigen in the LPS of Gram-negative enterics endows them with greater CAMP resistance than rough mutants lacking this hydrophilic glycopolymer. Indeed, early work with *S. typhimurium* (Rest et al. 1977) showed that when the *O*-antigen is lost and the inner core sugar chain is progressively truncated, bacterial susceptibility to granule extracts from human neutrophils increases proportionally. These granules are rich in defensins and cationic antimicrobial proteins (Spitznagel 1990). Although these "deep-rough" mutants have increased exposure of their negatively charged lipid A phosphate groups, which are important for CAMP binding, their surface is generally more hydrophobic. This characteristic is conducive for CAMP/membrane interactions and enhances the likelihood of membrane insertion.

The above mechanisms have been defined with planktonic bacteria. However, it is now recognized that many bacterial species form a specialized, highly organized community termed a biofilm. Due to their ultrastructural organization, bacteria within biofilms can exhibit increased resistance to antimicrobials, including CAMPs, compared to their planktonic counterparts (Anderl et al. 2000). Biofilms were correlated with persistent bacterial infections, such as chronic lung infection in cystic fibrosis (Singh et al. 2000). Antimicrobial resistance displayed by bacteria within biofilms appears to be due to mechanisms different from those typically observed in free-floating cells. Since biofilms are multicellular communities of bacteria encased in a hydrated matrix of polysaccharide, proteins, and/or nucleic acids, the capacity of antimicrobials to interact with all members of the community is reduced. Biofilms can also trap and inactivate CAMPs in the complex matrix imposed by its structure. Leid et al. demonstrated that biofilms from *S. aureus* can be penetrated by leukocytes that are active and secrete antimicrobial compounds (including CAMPs). However,

though these leukocytes were able to phagocytose planktonic *S. aureus*, they could not engulf sessile cells (Leid et al. 2002). The authors suggested that the structure of the biofilm is more of a porous hydrogel than a fixed impenetrable structure. Three main hypotheses have been advanced to explain the increased antimicrobial resistance displayed by bacteria in biofilms (Stewart and Costerton 2001). One hypothesis invokes a less permeable and less diffusible environment created by the biofilm with negatively charged compounds (i.e., nucleic acid, polysaccharide) in the matrix that could retard CAMP diffusion. The second hypothesis emphasizes the ultrastructural architecture of the biofilm, where microenvironments might present unfavorable conditions of pH, salt, and anaerobiosis that render CAMPs inactive or inefficient. The third hypothesis speculates that bacteria go through a cell-differentiation process and reach a spore-like metabolic state while in the biofilm, which allows some of them to be resilient to higher concentrations of antibiotics. These hypotheses are not mutually exclusive, and other defense mechanisms described throughout this chapter could contribute to biofilm-mediated CAMP resistance. Another possible mechanism of resistance that needs to be considered is that biofilm formation may trigger an alternate gene expression profile that modulates resistance phenotypes.

Biofilm formation is a dynamic process and can be influenced by environmental conditions, including the presence of certain CAMPs, other host compounds, and bacterial gene products. In the first instance, LL-37 and lactoferrin can prevent biofilm formation by *P. aeruginosa* by promoting bacterial motility (Dean et al. 2011; Overhage et al. 2008). Due to its structural organization, the availability of molecular oxygen may differ at sites within the biofilm complex, and this could influence the antimicrobial action of CAMPs. Accordingly, Schroeder et al. found that hBD-1 was highly efficient against various human flora and select pathogens in its reduced form but lacked efficient antimicrobial activity in its oxidized form (Schroeder et al. 2011). This suggests that an oxidative environment (perhaps present at different degrees within biofilms) could preclude some CAMPs from their killing activity. Finally, a number of bacterial products directly enhance survival of CAMP attack in sessile cells. For example, inducible resistance can be controlled by two-component regulatory (TCR) systems (Mulcahy et al. 2008; Amer et al. 2010) and stand-alone regulators (Warner et al. 2007, 2008; Shafer et al. 2010); the hairlike surface appendage termed curli promotes formation of biofilm structures in UPEC strains of *E. coli* (Kai-Larsen et al. 2010); and periplasmic glucans may bind to CAMPs on their way to the cytoplasmic membrane and sequester them (Mah et al. 2003).

2.4 Envelope Modifications That Decrease CAMP Binding and Permeability

Nearly 50 years ago, Spitznagel and coworkers found that the antimicrobial, arginine-rich, cationic peptides present in neutrophil granules (now known as

defensins) rapidly coat the surface of ingested bacteria (Spitznagel 1961; Zeya and Spitznagel 1966a, b). This electrostatic interaction between CAMPs and bacterial surface structures, and how bacteria modify these structures to inhibit said interaction, is perhaps the most studied mechanism of CAMP resistance (the reader is also directed to several excellent reviews especially those by Peschel 2002; Brogden 2005). In general, eukaryotic membranes are zwitterionic and have low affinity for CAMPs, which may provide them with some immunity to the lytic activity of these peptides. Prokaryotic cell surfaces, on the other hand, are typically negatively charged and so have a higher affinity for CAMPs. In order to prevent the deadly consequences of this attraction, many bacteria have evolved ways to decrease the net negative charge of their exteriors and modify the permeability of their membrane(s). Importantly, these mechanisms are not always specific to CAMPs and may provide protection against a broad spectrum of host and pharmacological cationic antimicrobials, including myeloperoxidase, phospholipase A2, lysozyme, vancomycin, moenomycin, and daptomycin (Peschel 2002).

Though both groups are negatively charged on their exteriors, Gram-negative bacteria are generally more resistant to CAMPs than Gram-positive bacteria, due to the presence of an outer membrane that can retard the passage of CAMPs to the inner membrane and cytoplasm. This is perhaps facilitated by LPS molecules that are held tightly together by (1) van der Waals interactions that exist between acyl chains and (2) salt bridges formed by divalent cations between neighboring carbohydrate chains and between lipid A phosphates. Early studies with deep-rough LPS mutants of *S. typhimurium* (Rest et al. 1977), and the *pmrA* mutants tested by Vaara and coworkers (Vaara et al. 1981; Helander et al. 1994) and Shafer et al. (1984), as well as Farley et al. (1987, 1988), support the concept that the availability of exposed, unsubstituted lipid A phosphate groups are critical to the ionic (and hydrophobic) interactions between CAMPs and the bacterial surface. More recent studies also support this model. For instance, a knockout insertion of a putative LPS synthesis gene *galU* in *Campylobacter jejuni*, a leading food-borne pathogen, decreased the length of LPS and reduced bacterial resistance to polymyxin B (Lin et al. 2009). Similarly, *Bordetella bronchiseptica*, an upper respiratory tract pathogen and close relative of *B. pertussis* (the etiologic agent of whooping cough), appears to require the addition of a negatively charged trisaccharide to LPS by the *wlbA* and *wlbL* genes for full resistance to several phylogenetically diverse CAMPs. It is thought that the uronic acid sugar moieties present in this trisaccharide shield the membrane from antimicrobial attack, perhaps by sequestering the peptides or providing a bulky barrier to entry (Banemann et al. 1998). Phosphorylcholine is produced by *H. influenzae* (Lysenko et al. 2000) and can increase the membrane fraction of zwitterionic phospholipids in the bacterial inner membrane. This would decrease the net negative charge normally present at the exoplasmic leaflet of the cytoplasmic membrane and slow the rate of CAMP self-promoted uptake (see below). Importantly, the investigators also observed modification of *H. influenzae* lipooligosaccharide (LOS) with phosphorylcholine. Such a modification is hypothesized to mimic host membranes (which contain phosphatidylcholine) and further reduce LL-37 binding. The viscosity of the periplasm may also contribute, since this space is densely packed with hydrophilic proteins that may nonspecifically hinder

CAMPs on their way to the inner membrane and cytoplasm, similar to the nonspecific binding of drugs by plasma proteins in the human body (Silhavy et al. 2010).

Gram-negative bacteria can also decrease the net negative charge of their exterior by decorating LPS/LOS lipid A with positively charged small molecules. In *N. meningitidis*, *N. gonorrhoeae*, *Helicobacter pylori*, *E. coli*, and *S. enterica* serovar Typhimurium, the addition of phosphoethanolamine (PEA) not only removes the negative charge once provided by free lipid A phosphate, but also adds a positive charge, thereby decreasing the net negative charge of the outer membrane and perhaps membrane permeability as well (Lewis et al. 2009; Lee et al. 2004; Beceiro et al. 2011). In *Campylobacter jejuni*, PEA can be added to lipid A and to a flagellar protein serving two purposes: (1) increase CAMP resistance and (2) promote bacterial motility (Cullen et al. 2012). Alternatively, 4-amino-4-deoxy-L-arabinose (L-Ara4N) may be added to the same phosphates in some Gram-negative bacteria and provides resistance to CAMPs in a similar manner to phosphoethanolamine (Trent 2004). Bacteria may also use the LpxE lipid A phosphatase to simply remove phosphate from lipid A and reduce negative charge, a phenomenon seen in the plant symbiont *Rhizobium leguminosarum*, as well as the human pathogen *H. pylori*. LpxE orthologues are present in *Francisella tularensis*, *Brucella melitensis*, and *Legionella pneumophila* (Karbarz et al. 2003; Trent 2004). These modifications are not only important for bacterial survival, but also impact the immune response to LOS/LPS or "endotoxin," one of the most potent inducers of septic shock (Peschel 2002). The regulation of these modifications has been very thoroughly studied in *S. enterica* and is controlled by combined efforts of the PhoP/PhoQ and PmrA/PmrB TCR systems (Lee et al. 2004; Gunn et al. 1998); these regulatory systems are discussed in more detail below.

In Gram-positive organisms, polyalditol, polyglycerol, or polyribitol phosphate polymers [teichoic acids (TA)] create a "continuum of negative charge" (Neuhaus and Baddiley 2003) with deprotonated phosphate residues present along each chain. The cationic antimicrobial lysosomal protein cathepsin G appears to use TA as a binding site on *S. aureus* (Shafer and Onunka 1989). Seminal work by Andreas Peschel and coworkers first characterized the CAMP repulsive effect caused by the D-alanylation of TA in *S. aureus*, an ability that endows this pathogen (and others) with decreased susceptibility to diverse CAMPs from different sources (Peschel et al. 1999). The enzymatic pathway encoded by the *dltABCD* operon processes the esterification of D-alanine to TA alditol residues and transforms TA into partly zwitterionic polymers, reducing the net negative charge at Gram-positive surfaces. The expression of the *dlt* operon in *S. aureus* is under the control of the Aps/GraRSX regulatory system (Li et al. 2007a, b). This appears to be a widespread defense mechanism present in other Firmicutes such as *Bacillus, Enterococcus*, and *Streptococcus*. It is also important to mention that *S. aureus* mutants lacking a functional *dlt* operon are more susceptible to the glycopeptide antibiotic vancomycin (Peschel et al. 2000), and in the future it may be possible to counter vancomycin-resistant *S. aureus* (VRSA) with drugs that block D-alanylation of TA.

Changes in membrane rigidity can also influence levels of CAMP resistance. PhoP/PhoQ control of *pagP*, which encodes a palmitoyltransferase that hepta-

acylates *S. enterica* lipid A in response to stresses typically found in a phagosomal environment, can influence membrane rigidity and the capacity of CAMPs to productively insert into bacterial membranes. These stresses include low pH, varying Ca^{2+} and Mg^{2+} ionic strength, and high concentrations of CAMPs (Guo et al. 1998; Prost and Miller 2008). The MsbB protein in *Vibrio cholerae* plays an analogous role by adding an acyl chain to the same position as PagP. This modification was found to greatly enhance resistance to polymyxin B, LL-37 (and its mouse homolog CRAMP), and magainin 2 (Matson et al. 2010). Interestingly, *msbB* deletion mutants were unable to induce a TLR4 response in human embryonic kidney cells, which suggests that efficient recognition and binding of bacterial endotoxin is largely due to lipid A structure. This finding also supports the notion that CAMP resistance mechanisms may protect not only directly, but indirectly through modulating the host immune response. The actions of both PagP and MsbB ultimately increase the stability and hydrophobicity of the outer membrane and decrease its permeability, thus enhancing the fortitude of an already formidable barrier to CAMP entry (Peschel 2002). In *S. aureus*, pigment production through the *crtOPQMN* operon performs a similar function in CAMP resistance by increasing membrane rigidity (Mishra et al. 2011). The cold shock system of *S. aureus* may also be important in resistance as mutants lacking CspA (Katzif et al. 2003) and CspB (Duval et al. 2010) have reduced pigment levels and altered susceptibilities to certain antimicrobials, including cathepsin G-derived CAMPs.

Conversely, decreased membrane rigidity may also provide CAMP resistance. *S. aureus* tPMP (*t*hrombin-induced *p*latelet *m*icrobicidal *p*rotein)-resistant strains were consistently found to have greater membrane fluidity than their tPMP susceptible counterparts, caused by a preponderance of longer chain, unsaturated fatty acids (Bayer et al. 2000). It has also long been known that *S. aureus* membranes contain unsaturated sexa-, hepta-, and octa-isoprenoid menaquinones (Nahaie et al. 1984). Apart from their function as redox molecules in the electron transport chain, they also increase membrane fluidity. Thus, it has been hypothesized that large fluctuations in membrane fluidity to either extreme may distance membrane order from the "sweet spot" required for optimum CAMP bactericidal activity (Mishra et al. 2011; Yeaman and Yount 2003).

One of the most nonspecific and ubiquitous mechanisms of CAMP defense in bacteria is mediated by the MprF (*m*ultiple *p*eptide *r*esistance *f*actor) protein (Peschel et al. 2001). Originally described by Peschel and colleagues (2001), it soon became clear that MprF provides significantly enhanced bacterial resistance to neutrophils and several evolutionarily distinct CAMPs. MprF is present in a wide variety of Gram-positive, Gram-negative, and acid-fast bacteria and is also present in the *Archaea*. This CAMP resistance mechanism is remarkable in that it only requires substrates that are abundant in the bacterial cell—charged tRNAs and membrane phospholipids—and is somewhat indiscriminant when recognizing tRNA donor and phospholipid acceptor molecules. This is thought to be why the MprF mechanism is so widespread (Roy and Ibba 2008). MprF is an integral cytoplasmic membrane protein and may add the positively charged L-lysine, L-alanine, and perhaps, in *Mycobacterium tuberculosis*, L-ornithine (Ernst and Peschel 2011; Khuller and

Subrahmanyam 1970) amino acids to phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). *S. aureus* MprF consists of (1) a transesterase domain which adds the amino acid residue to phosphatidylglycerol (PG) on the cytoplasmic leaflet of the membrane and (2) a "flippase" domain that flips the nascent lysyl-PG to the exoplasmic leaflet of the membrane where it can serve to repulse CAMPs by reducing the net negative charge of the membrane's outer surface. It is noteworthy that MprF is the first flippase to be discovered in prokaryotes (Ernst and Peschel 2011). In *M. tuberculosis*, the *lysX* gene is actually a fusion of *mprF* and *lysU*, a lysyl-tRNA synthase. Here, lysyl-tRNA can be made at the cytoplasmic membrane level by the LysU domain and then shuttled into the MprF reactions described above (Maloney et al. 2009). *Clostridium perfringens* produces two MprF proteins, 1 and 2, which produce alanyl-PG and lysyl-PG, respectively (Roy and Ibba 2008). It is probable that, like other CAMP resistance mechanisms, the activities of MprF are under the control of two- or three-component regulatory systems; indeed, MprF appears to be under the control of the VirR protein of the VirRS TCR in *Listeria monocytogenes* (Mandin et al. 2005) and the Aps/GraRSX TCR in *S. aureus* (Li et al. 2007a, b; Otto 2009).

Intriguingly, it seems that CAMPs themselves are not the only stimuli that can induce CAMP resistance mechanisms in bacteria. A brief but elegant study by Dorrer and Teuber in the 1970s (Dorrer and Teuber 1977) demonstrated that phosphate starvation induced polymyxin B resistance in *Pseudomonas fluorescens* by increasing the membrane fraction of ornithylated lipids, which decreases the net negative charge of the bacterial envelope. Notably, it was later discovered that survival inside of macrophages induced the expression of phosphate importers 9.4-fold in *Salmonella typhimurium* (Valdivia and Falkow 1997). This might indicate that (1) low phosphate levels preclude the use of phosphate on membrane lipids and require that other groups (e.g., ornithine) provide the hydrophilic portion of the membrane lipid to maintain a stable bilayer structure and (2) the host environment may unwittingly hinder its own efforts to kill with CAMPs by inducing the production of these cationic lipid species.

Though the mechanisms described above allow the microorganism to change the envelope structure without dire consequences for growth, there are other CAMP resistance strategies that come at great fitness cost to the bacterium. In *S. aureus*, small colony variants (SCVs) are typically deficient in electron transport and have a diminished membrane potential ($\Delta\psi$) (Yeaman and Yount 2003). They also arise much more readily (10,000-fold) in the host than in laboratory culture (Vesga et al. 1996). This suggests that slowing cell growth may represent a "niche-specific" defense mechanism, triggered by growth in a host, that allows bacteria to depolarize their membrane and decrease the rate of "self-promoted uptake" by host CAMPs (Peschel 2002) (see (Hancock 1997) for a description of this phenomenon). Interestingly, the *S. aureus cspB* mutant studied by Duval et al. described above, exhibited many of the characteristics of SCVs (Duval et al. 2010). In Gram-negative bacteria, reduced growth rates may also help stave off death by CAMP by reducing the occurrence of nascent septa that are a critical component during bacterial binary fission. Sochacki and colleagues used real-time fluorescence microvideography to

show that rhodamine-labeled LL-37 consistently binds *E. coli* cells at their nascent septa first, then proceeds outward in a continuous "circumferential band" towards the distal poles of each developing daughter cell (Sochacki et al. 2011), though LL-37 was still able to bind to nonseptated cells.

2.5 *Export of CAMPs*

Even if CAMPs successfully traverse the formidable barriers described above, bacteria can still circumvent their action by the use of drug efflux pumps that capture and export structurally diverse antimicrobials after they breach the cell envelope. Drug efflux pumps are grouped into superfamilies based on their component stoichiometry, number of transmembrane regions in the transporter, energy source, and type of substrates recognized. Five superfamilies of efflux pumps are known: the *r*esistance-*n*odulation-*d*ivision (RND) superfamily, the *m*ajor *f*acilitator (MFS) *s*uperfamily, the *m*ultidrug *a*nd *t*oxin *e*xtrusion (MATE) superfamily, the *A*TP-*b*inding *c*assette (ABC) transporter superfamily, and the *s*mall *m*ultidrug *r*esistance (SMR) superfamily (see (Piddock 2006) for an excellent review of bacterial efflux pumps). The MtrCDE efflux pump of *N. gonorrhoeae* is a member of the RND superfamily and was the first efflux pump shown to export CAMPs to the extracellular milieu (Shafer et al. 1998). This pump has been studied in detail and will be described later (see below); the analogous pump in *N. meningitidis* also can export CAMPs (Tzeng et al. 2005). Other Gram-negative pathogens have been found to use efflux pumps to resist CAMPs. *Yersinia enterocolitica* can protect itself from CAMP activity by expressing the RosAB efflux pump, which is induced upon growth at 37 °C. The RosA pump activity is powered by the potassium antiporter RosB and is thought to provide resistance through (1) efflux of CAMPs and (2) acidification of the cytoplasm (Bengoechea and Skurnik 2000). *K. pneumoniae* expresses the AcrAB-TolC efflux pump that mediates resistance to human antimicrobial peptides, as an AcrB mutant was more sensitive to HBD-1 and HBD-2 (Padilla et al. 2010). The homologous efflux pump in *E. coli*, AcrAB-TolC, is arguably the most structurally characterized efflux pump to date (Murakami et al. 2006; Husain and Nikaido 2010; Symmons et al. 2009) and will likely become an invaluable tool for the design and testing of an emerging class of antibiotics, the efflux pump inhibitors (EPIs) (Lomovskaya and Bostian 2006).

Efflux pumps also function in Gram-positive bacteria for CAMP resistance. For instance, the EpiFEG efflux pump of *S. epidermidis* exports and increases staphylococcal resistance to various CAMPs. EpiFEG is an ABC transporter that is known to export bacterial-derived CAMPs, such as gallidermin, nisin, and epidermin (Otto et al. 1998). The MefE/Mel efflux pump possessed by certain strains of *S. pneumoniae* is a mechanism used by this pathogen to develop resistance to macrolides. Expression of this pump was found (Zähner et al. 2010) to be inducible by 14- and 15-membered macrolides as well as LL-37/CRAMP and that such induction enhanced pneumococcal resistance to macrolides and LL-37. Maximal

constitutive and inducible cathelicidin resistance expressed by pneumococci required a functional MefE/Mel pump system, although it is yet to be determined if these CAMPs are actual pump substrates. It also appears that some efflux pumps might actually enhance CAMP resistance independently of their efflux capacity. In *S. aureus*, QacA (a plasmid-encoded multidrug MFS efflux pump) mediates resistance to tPMP, but does not affect levels of resistance to HNP-1 or protegrin-1 (Kupferwasser et al. 1999). Curiously, later studies found that QacA-mediated resistance to tPMP was not due to efflux activity and that membrane fluidity seemed to diminish slightly in strains bearing the *qacA* gene (Bayer et al. 2006), but the exact mechanism of how QacA expression protects against tPMP remains to be determined.

Of note is a very intriguing pattern that has emerged among the Gram-positive bacteria that teams ABC-transporter efflux pumps and TCR systems into very close functional associations called "resistance modules." Each component of the module is dependent on the other for resistance against antimicrobial peptides. Extensive phylogenetic analysis suggests a coevolution of efflux pumps and TCR systems in the phylum *Firmicutes* (over 250 resistance modules are estimated). In this partnership, sensor domain-deficient inner membrane histidine kinases (IMHKs) still relay signals through their cognate response regulators, but recognition of the environmental stimulus is carried out by a neighboring permease/transporter protein in the membrane (Dintner et al. 2011). Well-characterized examples of such TCR/efflux pump couplings include the BceRS TCR and BceAB pump in *Bacillus subtilis* and *Streptococcus mutans* (Bernard et al. 2007; Ouyang et al. 2010) and the BraRS TCR and BraED pump in *S. aureus* (Hiron et al. 2011). The VraED pump also appears to play a role in *S. aureus* resistance, but is required only for efflux and not for sensing.

2.6 Modification of Internal Targets

Since the antimicrobial action of most CAMPs is independent of stereochemistry, it has generally been thought that they do not recognize targets with a chiral center (Bessalle et al. 1990; Wade et al. 1990). Furthermore, their killing activity has been linked to processes such as loss of membrane integrity and depolarization. Due to these broad-spectrum killing mechanisms, CAMPs have been likened to "dirty bombs" in contrast to the "smart bomb"-like action of many antibiotic drugs (Peschel and Sahl 2006). This analogy may not be completely correct as it is now clear that CAMPs may also kill bacteria by interfering with internal cellular functions like DNA/RNA/protein synthesis, protein folding, peptidoglycan polymerization, and septum formation (Hale and Hancock 2007; Cudic and Otvos 2002). Just as the bacterial envelope can accumulate changes to hinder CAMP attack, antimicrobial stress selects for mutants containing modified and thus less CAMP-accessible cytoplasmic targets. One example of this is a mutation in the *gyrB* gene in *E. coli*. *gyrB* encodes DNA gyrase, a type II topoisomerase that maintains a level of DNA supercoiling necessary for replication, transcription,

and recombination. GyrB is a target of the well-known class of antibiotics called quinolones, and also the bacteriocin microcin B17, which is produced by *E. coli*. del Castillo and colleagues (2001) found that mutation of residue W751 to hydrophilic amino acids like lysine or arginine imparts a great deal of resistance to microcin B17. The authors hypothesize that this residue may be located on the entry gate through which the intact DNA can be transported (T-segment) and that microcin B17 normally binds and inhibits GyrB activity, leading to cell death (del Castillo et al. 2001).

2.7 Inducible Mechanisms of CAMP Resistance

Inducible mechanisms of CAMP resistance allow bacteria to promptly respond to stressful changes in their environments. TCR systems sense potentially harmful changes, orchestrate a response to the imposed stress, and adapt gene expression to the new context. TCR systems consist of a sensor histidine kinase on the inner membrane and a cytoplasmic regulatory protein. Typically, the sensor kinase detects a signal in the environment, becomes autophosphorylated, and in turn phosphorylates the cognate intracellular regulator, activating it. The activated regulator binds to DNA and alters the expression of different genes. Numerous and functionally distinct TCR systems are found in bacteria. The PhoP/PhoQ TCR system was initially studied in *S. typhimurium* (Fields et al. 1989; Groisman et al. 1989; Miller et al. 1989), and homologs were subsequently found and studied in *P. aeruginosa* (Macfarlane et al. 1999), in various *Enterobacteriaceae*, and in *Neisseria*, where it is named MisR/MisS (Tzeng et al. 2004).

The PhoP/PhoQ and the PmrA/PmrB are well-studied examples of TCRs that have important roles in CAMP resistance. Under favorable conditions, PhoQ is bound by divalent cations such as Mg^{2+} and Ca^{2+} in the environment and is not active. At low concentrations of divalent cations, PhoQ phosphorylates PhoP, which in turn regulates many genes involved in AMP resistance, such as *pagP*, *pgtE*, *slyA,* and *pmrD* (Roland et al. 1994). PmrD is required for activation of the PmrA/PmrB TCR system (Otto 2009; Guina et al. 2000; Macfarlane et al. 2000, 1999; Navarre et al. 2005). When CAMPs are present in the media, it is thought that they displace divalent cations bound to an acidic patch of PhoQ and induce activation of the TCR system (Prost and Miller 2008). In *S. enterica*, PhoP/PhoQ is activated by CAMPs and upregulates genes related to CAMP resistance such as *pagP*, *pagL*, and *lpxO* (Bader et al. 2005; Hancock and McPhee 2005). Using a mutant strain of *S. enterica* showing increased resistance to polymyxin B, azurocidin, and CAP57 (Shafer et al. 1984) due to a *pmrA* mutation, Roland et al. identified the TCR system PmrA/PmrB (Roland et al. 1993). This TCR regulates expression of genes involved in CAMP resistance such as *pmrHFIJKLM* (or *pbg* operon), *cld,* and *cptA*, which are responsible for LPS modifications (Gunn 2008). In *P. aeruginosa*, PmrA/PmrB is induced by low concentrations of Mg^{2+} and by LL-37, which promotes expression of genes, such as *pbgP*, *pbgE,* and *ugd,* involved

in LPS modification and resistance to polymyxin B (Gunn and Miller 1996; McPhee et al. 2003; Moskowitz et al. 2004). It was shown that mutating *pmrAB* in *P. aeruginosa* rendered the bacteria hypersusceptible to killing by LL-37 or by other CAMPs such as polymyxin B (Lewenza et al. 2005).

Li et al. uncovered a novel regulatory system in *S. epidermidis* that they named *aps* for *a*ntimicrobial *p*eptide *s*ensor (Li et al. 2007a, b), also observed in *S. aureus* and named *gra* for *g*lycopeptide *r*esistance *a*ssociated genes (Kuroda et al. 2000; Cui et al. 2005). The system consists of a TCR system with a sensor kinase (*apsS*) and a regulator (*apsR*) and a third protein with unknown yet essential function (*apsX*) (Li et al. 2007a, b). Their research showed that deletion of any or all of these components led to downregulation of the *dlt* and *mprF* genes, which modify cell surface structures and enhance resistance to CAMPs (Li et al. 2007b). Furthermore, Lai et al. have described *agr* and *sarA* in *S. aureus* and *S. epidermidis* as major regulators that are induced in the presence of the anionic AMP dermcidin. These gene regulators increase expression and proteolytic activity of the SepA metalloprotease in presence of dermcidin (Lai et al. 2007).

3 CAMP Resistance in Clinically Relevant Pathogens

In the above sections, we reviewed major mechanisms that bacteria have evolved to resist CAMPs. In order to highlight how such CAMP resistance systems can influence the efficacy of host resistance to infection and bacterial pathogenesis, we discuss them in the context of three major clinically relevant pathogens of public health concern. In this respect, we focus on the obligate human Gram-negative pathogens *N. gonorrhoeae* and *N. meningitidis* and on the Gram-positive bacteria *S. aureus*, particularly the methicillin-resistant strains (MRSA).

3.1 N. gonorrhoeae *and* N. meningitidis

N. gonorrhoeae and *N. meningitidis* are Gram-negative diplococci and strict human pathogens (Shafer et al. 2010). Gonococci (GC) cause the sexually transmitted infection gonorrhea. In contrast, meningococci (MC) are present as commensals in 8–25 % of the human population, but can cause bacterial meningitis and fulminant septicemia (Stephens 2009). Gonorrhea is the second most reported infection in the United States, though many cases are asymptomatic, and can enhance HIV transmission (Klotman et al. 2008; McNabb et al. 2008). Furthermore, although there are vaccines available for many MC serogroups that cause disease, a protective vaccine for serogroup B MC is still under development; no vaccine has been developed that blocks GC infection. Both of these pathogens are also becoming increasingly resistant to antibiotics (Shafer et al. 2010). Worryingly, a recent report described a strain of gonorrhea that is resistant to the last remaining first-line antibiotic used

in empirical treatment, ceftriaxone (Ohnishi et al. 2011). Thus, the pathogenic *Neisseria* represent a significant threat to global health.

As strictly human pathogens, GC and MC have evolved remarkable and redundant mechanisms to defend themselves against host CAMPs. These include capsule production by MC (Jones et al. 2009; Spinosa et al. 2007); host-molecule "cloaking" using the highly anionic polymers heparin/heparan sulfate and short, cationic polyamines (Goytia and Shafer 2010; Jones et al. 2009; Seib et al. 2009); MC sequestration of LL-37 in the bacterial cytosol (Frigimelica et al. 2008); downregulation of host LL-37 production (Bergman et al. 2005); export of CAMPs by the MtrCDE efflux pump (Shafer et al. 1998); decoration of lipid A with PEA (Cox et al. 2003; Tzeng et al. 2005; Lewis et al. 2009); and hexa-acylation of lipid A (Tzeng et al. 2005). Some of these mechanisms have been shown to be under the control of the MisR/MisS TCR system, named for its regulation of *m*eningococcal LOS *i*nner core *s*tructure, which itself is necessary for resistance to CAMPs (Johnson et al. 2001; Newcombe et al. 2004; Tzeng et al. 2004). Other mechanisms may be induced by different inputs, e.g., upregulation of *mtrCDE* expression in GC upon exposure to hydrophobic pump substrates typically present at infection sites (Rouquette et al. 1999).

Is there evidence that any of these resistance mechanisms influence bacterial survival during infection? Briefly, yes: in support of this idea, elegant experiments performed in the laboratory of A. Jerse have shown that loss of the MtrC–MtrD–MtrE efflux pump due to its genetic inactivation decreased the ability of GC to survive in an experimental model of lower genital tract infection in female mice (Jerse et al. 2003). Further work by her group (Warner et al. 2007, 2008) showed that overexpression of MtrC–MtrD–MtrE increases fitness of GC during infection by nearly three orders of magnitude. In contrast, loss of the ability to activate transcription of *mtrCDE* decreased fitness in vivo by 500-fold. Finally, *lptA* mutants of GC that are unable to decorate their lipid A with PEA are more susceptible to CAMPs and less fit in vivo than the parental wild-type strain (Jerse, personal communication, 2011).

3.2 Staphylococcus aureus

S. aureus is a Gram-positive bacterium that has evolved to survive in a commensal capacity on the human host. It can be found on the skin and in the nares in 20 % of the population, but when staphylococci breach host defenses, they can cause many different illnesses, including skin infections, abscesses, and life-threatening diseases such as endocarditis, pneumonia, meningitis, toxic shock syndrome, and sepsis. Importantly, *S. aureus* is one of the most frequent causes of hospital- and community-acquired infections. The incidence of multiple antibiotic-resistant strains of *S. aureus* continues to increase, restricting the options for treatment. *S. aureus* has become one of the most difficult bacterial infections to treat as multidrug-resistant strains have emerged; a typical example is *m*ethicillin-*r*esistant *S. aureus*, or MRSA. MRSA colonizes 2 % of the population, many of whom are immunocompromised due to age (e.g., the elderly and young children) or medical

condition (e.g., pregnant women, HIV-positive, and cancer patients) (Kowalski et al. 2005). MRSA can cause life-threatening infections such as pneumonia, septicemia, and infections following surgery. MRSA resist most β-lactam antibiotics (penicillins and cephalosporins) including penicillin, methicillin, and amoxicillin. Furthermore, it is quite common to see resistance develop when MRSA infection is treated with macrolides and/or fluoroquinolones. Importantly, as of 2007, MRSA infections caused more deaths (>17,000) in the United States than HIV/AIDS (Klevens et al. 2007).

S. aureus has found strategies to impair all of the events associated with CAMP killing activity and sometimes in more than one way. For instance, *S. aureus* secretes proteolytic enzymes, V8 and aureolysin, which are able to degrade and inactivate CAMPs such as LL-37 (Sieprawska-Lupa et al. 2004). It was suggested that loss of these enzymes by molecular modification could render *S. aureus* more susceptible to CAMPs in vitro, ex vivo, and in vivo (Sieprawska-Lupa et al. 2004). Staphylokinase (Sak) is a secreted protein that sequesters LL-37 and increases virulence in vivo (Braff et al. 2007). Burlak et al. demonstrated that *S. aureus* express Sak in vivo, since injection of *S. aureus* in mouse elicited the production of specific antibodies against Sak (Burlak et al. 2007). *S. aureus* produces positively charged polysaccharide intercellular adhesin (PIA) and negatively charged poly-γ-glutamic acid (PGA) at its surface, which increases the net positive charge of the cell surface and, as it was described for *S. epidermidis*, could impair binding of positively charged CAMPs by electrostatic repulsion; however, other mechanisms might be involved as well, since PIA also protected *S. aureus* from the negatively charged AMP dermcidin (Vuong et al. 2004; Kocianova et al. 2005). Moreover, PIA, which is produced by the intercellular adhesion *ica* locus, has been involved in biofilm formation in *S. aureus* (Cramton et al. 1999). *S. aureus* can also express D-Ala and L-Lys at its surface, modifying the net charge, through the *dlt* operon and *mprF* gene, respectively (Peschel et al. 1999, 2001; Staubitz et al. 2004; Collins et al. 2002; Nishi et al. 2004). These mechanisms are also efficient against other CAMPs, such as those derived from lactoferrin and phospholipase A2 (Koprivnjak et al. 2002).

S. aureus regulates these genes and many others involved in CAMP resistance with Aps/GraRSX, an inducible system that is activated in presence of CAMPs (Li et al. 2007a; Kraus et al. 2008). Li et al. showed that a mutant with a deletion of *aspS* was less virulent in an intraperitoneal mouse infection model than the wild-type strain (Li et al. 2007a). Other inducible mechanisms described in *S. aureus* and implicated in CAMP resistance involve Agr and SarA (Huang 2006). Modifications of the membrane involve carotenoid production by the *crtOPQMN* operon, which can suppress nonoxidative host defenses mediated by CAMPs (Mishra et al. 2011). *S. aureus* is also able to prevent CAMP activity by expressing efflux pumps such as the plasmid-encoded QacA and the ABC transporter EpiFEG (though QacA-mediated resistance is independent of efflux activity). Finally, *S. aureus* is able to form biofilms, which are ultrastructures that promote bacterial resistance to AMPs and other killing agents. Internal targets are probable in *S. aureus*, since CAMPs

are able to kill *S. aureus* without significant depolarization or disruption of the membrane (Koo et al. 2001).

4   Conclusions and Perspectives

Bacteria have constantly evolved novel mechanisms to overcome attacks by CAMPs. It seems that for every way CAMPs kill, bacteria have developed a resistance mechanism(s) in response. As mentioned above, the mechanisms of CAMP resistance are for all purposes similar to those developed by bacteria to resist classical antibiotics. At first glance, mechanisms of bacterial resistance to CAMPs would seem to favor the microbe and not the host. However, this view may be overly simplistic; most of the bacteria we interact with on a daily basis are not normally pathogenic, and many are associated with good health. If such commensally carried, helpful bacteria were to be reduced or eliminated in the presence of CAMPs, how would this impact our health? Perhaps CAMP resistance mechanisms evolved not as a way for pathogens to avoid elimination, but rather as a way for the helpful commensals to survive.

CAMPs have been promoted as a new class of therapeutic antimicrobials for treating multi-antibiotic-resistant pathogens, some of which cause infections that are becoming untreatable. Studies exploring various characteristics of CAMPs (charge, amphipathicity, hydrophobicity, etc.) (see Shprung et al. 2012) will help gain insight in the design of ever more efficient synthetic CAMPs. Alternatively, further research focusing on specific bacterial metabolic states could prevent formation of structures such as biofilms that are extremely hard to destroy and that increase the risk of chronic infections and antibiotic resistance development. In this respect, work in the Hancock laboratory on the ability of CAMPs to prevent formation of biofilms is especially important and could be exploited by attaching these peptides to medical devices (de la Fuente-Núñez et al. 2012). The continued advancement of these peptides as therapeutics will require additional studies to further analyze their potential short- and long-term toxic effects, their specificity, their pharmacokinetics, the appearance of resistance patterns, and immunomodula-tory/immunostimulatory secondary effects.

Continued studies on mechanisms of CAMP resistance are also warranted. As therapeutic antimicrobial peptides pass through clinical trials, we can use the knowledge gained from such experiments to predict how bacteria will respond to their presence during treatment (which will likely be at higher levels than what occurs naturally) and if resistance (especially broad spectrum) will develop. We must, however, be cognizant of the possibility that resistance to administered CAMPs may negatively impact innate host defenses mediated by the natural CAMPs that function at different sites in the human body. How this might influence decisions to move forward with the therapeutic application of CAMPs is a matter for future consideration.

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