Membrane Anchoring of the AgrD N-terminal Amphipathic Region Is Required for Its Processing to Produce a Quorum-sensing Pheromone in Staphylococcus aureus

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Quorum-sensing pheromones are signal molecules that are secreted from Gram-positive bacteria and utilized by these bacteria to communicate among individual cells to regulate their activities as a group through a cell density-sensing mechanism. Typically, these pheromones are processed from precursor polypeptides. The mechanisms of trafficking, processing, and modification of the precursor to generate a mature pheromone are unclear. In Staphylococcus aureus, AgrD is the propeptide for an autoinducing peptide (AIP) pheromone that triggers the Agr cell density-sensing system upon reaching a threshold and subsequently regulates expression of virulence factor genes. The transmembrane protein AgrB, encoded in the agr locus, is necessary for the processing of AgrD to produce mature AIP; however, it is not clear how AgrD interacts with AgrB and how this interaction results in the generation of mature AIP. In this study, we found that the AgrD propeptide was integrated into the cytoplasmic membrane by a conserved α-helical amphipathic motif in its N-terminal region. We demonstrated that membrane targeting of AgrD by this motif was required for the stabilization of AgrD and the production of mature AIP, although this region was not specifically involved in the interaction with AgrB. An artificial amphipathic peptide replacing the N-terminal amphipathic motif of AgrD directed the protein to the cytoplasmic membrane and enabled the production of AIP. Analysis of Bacillus ComX precursor protein sequences suggested that the amphipathic membrane-targeting motif might also exist in pheromone precursors of other Gram-positive bacteria.

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† The abbreviations used are: AIP, autoinducing peptide; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]-ethylglycine.
mature AIP by AgrB remain unclear. Based on the DNA sequences of agr loci and the specific interactions between AIP and AgrC and between AgrD and AgrB, four *S. aureus* groups have been defined (13, 17). AIP from an *S. aureus* strain activates the Agr response in itself and in the same group members, but inhibits the Agr response in heterologous group members. The four *S. aureus* AgrD groups have 46 or 47 residues, and the AIPs have different amino acid sequences and lengths. An alignment of these AgrD groups (group I AgrD, NCBI accession number CAA36782; group II AgrD, accession number AAB63265; group III AgrD, accession number AAB63268; and group IV AgrD, accession number AAG03056) is shown, with the AIP sequences in boldface.

The specific interaction between AgrB and AgrD is not so strict. AgrD of *S. aureus* group II is processed only in the presence of group II AgrB, whereas group I AgrD and group III AgrD are processed by group I AgrB and vice versa (7). In this study, we found that the AgrD propeptide was anchored in the cytoplasmic membrane by an N-terminal amphipathic region. We also demonstrate that the membrane targeting of AgrD by the amphipathic region was required for its normal processing to produce mature AIP, but was not involved in the specific interaction with AgrB. This amphipathic region is present in all staphylococcal AgrD sequences available in the Gen-Bank™/EBI Data Bank as revealed by sequence analysis. Furthermore, analysis of *B. subtilis* ComX sequences revealed the existence of similar amphipathic regions in their N-terminal segments, suggesting that the membrane targeting sequence might play an important role in the processing of the propeptides to generate active mature pheromones in other Gram-positive bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—The *S. aureus* plasmids and strains used in this study are listed in Table I. *S. aureus* cells were grown in CY-GP broth (18), supplemented with antibiotics (5 μg/ml chloramphenicol and 5 μg/ml erythromycin) when necessary. Bacteria grown overnight at 37 °C on GL plates (18) were routinely used to inoculate liquid cultures. Cell growth was monitored with either a Klett-Summerson colorimeter with a green (540 nm) filter (Klett, Long Island City, NY) or a VERSAmax microplate reader (Molecular Devices) at A590 nm. *Escherichia coli* strain MC1061-5 (19) was grown in LB broth, supplemented with tetracycline (20 μg/ml) when necessary.

**Construction of *S. aureus Plasmids**—The group I AgrD-His expression plasmid pLZ4009 was constructed as follows. A PCR product was generated using primers JG56 (5'-GGCTCTAGAAGCTATTACATTATT-3') and JG57 (5'-TCTCTATACGATTGCTTTAGCTAATG-3'), with the His6 coding region in pRSET-A (Invitrogen) ligated to an NcoI site (underlined) and the XbaI site (underlined). The PCR product was then digested with XbaI and cloned into the XbaI and BamHI sites of pRN6913. The PCR products were then digested with EcoRI and BspHI and ligated to the EcoRI and BspHI digested 3.1-kb fragment of pLZ4012.

Plasmids pLIND5, pLIND10, and pLIND12 were constructed as follows. PCR products were amplified from plLZ4012 with primers JG111 (5'-CATGGAATTCTGAACATTATTTTTGTTATATAC-3'), agrD sequence plus an EcoRI site, with the EcoRI site underlined) and LIN25 (5'-AAACATGTTAAAACATTGG-3'), agrB sequence and the AIPs have different amino acid sequences and lengths.

**Electrophoretic Mobility Shift Assays**—Plasmid pLZ4011 was constructed by ligating two T4 polynucleotide kinase-phosphorylated and NcoI-digested PCR products amplified from pRN6958 using primers LZ46 (5'-AACATCGCAGCTTATAGT-3') and tmR (5'-ACCAATGTTTTTTAAAATCCCAG-3'). The plasmid pLZ4009 was constructed by ligating two T4 polynucleotide kinase-phosphorylated and NcoI-digested PCR products amplified from pRN6913: one with primers JG56 and JG57 (5'-CTTATAAATAATTCTGATTGGTAACATCGCAGCTTATAG-3'), with the His6 coding region in pRSET-A (Invitrogen) ligated to an NcoI site (underlined) and the XbaI site (underlined). The PCR products were then digested with EcoRI and BspHI and ligated to the EcoRI and BspHI digested 3.1-kb fragment of pLZ4012.

Plasmids pLIND12 and pLIND14 were constructed as follows. PCR products were amplified from plLZ4010 using primers JG56 and JG57 (5'-AACATCGCAGCTTATAGT-3'), agrB sequence and the AIPs have different amino acid sequences and lengths.

**Ultrafiltration**—The specific interaction between AgrB and AgrD is not so strict. AgrD of *S. aureus* group II is processed only in the presence of group II AgrB, whereas group I AgrD and group III AgrD are processed by group I AgrB and vice versa (7). In this study, we found that the AgrD propeptide was anchored in the cytoplasmic membrane by an N-terminal amphipathic region. We also demonstrate that the membrane targeting of AgrD by the amphipathic region was required for its normal processing to produce mature AIP, but was not involved in the specific interaction with AgrB. This amphipathic region is present in all staphylococcal AgrD sequences available in the Gen-Bank™/EBI Data Bank as revealed by sequence analysis. Furthermore, analysis of *B. subtilis* ComX sequences revealed the existence of similar amphipathic regions in their N-terminal segments, suggesting that the membrane targeting sequence might play an important role in the processing of the propeptides to generate active mature pheromones in other Gram-positive bacteria.
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CAGAATCCAGGGCACAATTTGCAATTCATGTTACCCAGC-3', with the E. coli leader peptidase I gene sequence underlined and the pRSET-A multicloning site sequence italicized), digested with BspHI, and dephosphorylated with shrimp alkaline phosphatase (MBI Fermentas). This fragment was ligated to a BspHI-digested and 5'-phosphorylated PCR fragment amplified from pLZ4011 with primers GJ45 and H11032. This fragment was ligated to a BspHI-digested and 5'-phosphorylated fragment amplified from pLZ4017 with primers GJ94 and H11032. The resulting plasmid contained the coding sequence of the agrD gene and inserted downstream of the P73 promoter (pLZ4010). This plasmid was used as a template to produce PCR products with primer pairs GJ44/LZ17 and H11032. The correct PCR fragment amplified from pGJ4002 (16) was digested with BglII and BamHI. This product was then inserted into pLZ4009 with the BglII site underlined) and GJ94 (5'-CTGAGGGTGCTAATTTAAAAGTTTTAATTTTTTTG-3') and LZ15 (5'-GAAGATCTTTTTTGCAGCTCAG-3') with the BglII and BamHI sites underlined) and GJ94 (5'-CTGAGGGTGCTAATTTAAAAGTTTTAATTTTTTTG-3') and LZ15 (5'-GAAGATCTTTTTTGCAGCTCAG-3') with the BglII and BamHI sites underlined. The resulting plasmid contained the coding sequence of the agrD gene and inserted downstream of the agrD promoter 23.

Membrane Fractions were prepared by ultracentrifugation. The pellets were dissolved in SDS-PAGE and Western Blotting—Protein samples dissolved in SDS sample buffer were incubated at 70 °C for 10 min before being separated by either Tris/glycine/SDS-PAGE (19) or Tris/Tris/SDS-PAGE (22). The separated proteins were then electrochemically transferred to polyvinylidene difluoride membranes (Millipore). After blocking overnight at 4 °C in Tris-buffered saline (19) containing 0.1% Tween 20 and 0.05% bovine albumin (Sigma), the polyvinylidene difluoride membranes were incubated in blocking buffer with a primary antibody (1:2000 dilution of mouse anti-tetrahistidine monoclonal antibody (Qiagen Inc.) or 1:5000 dilution of anti-T7 tag monoclonal antibody (Novagen)) for 1 h at room temperature. The membranes were washed extensively with Tris-buffered saline plus Tween 20 and then probed with horse-radish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences). The immunoblots were detected with an ECL Plus Western blot detection system followed by exposure to Hyperfilm™ ECL™ (Amersham Biosciences).
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promoter of the pRN5548 expression vector (23). Plasmids pLZ4009 and pLZ4010 were transformed into agr-null S. aureus strain GJ2035, creating strains LZ4009 and LZ4010, respectively. Western blot hybridization analysis with the anti-tetrahistidine monoclonal antibody used as a probe revealed a band with an estimated size of 6 kDa from whole cell lysate of LZ4009 or LZ4010 expressing AgrD-His6 (Fig. 1A, lanes 1, 2, 4, and 6), indicating that AgrD is a membrane-associated protein.

To determine whether AgrD is an integral or peripheral membrane protein, we prepared protoplasts from S. aureus LZ4009 cells and lysed the protoplasts in PBS containing 1 M sodium chloride, in 0.2 M sodium carbonate, or in PBS containing 1% sarcosyl. After incubation at 4°C for 2 h, the whole cell lysates were fractionated by ultracentrifugation. Fig. 1B showed the results of Western blot hybridization with the anti-tetrahistidine monoclonal antibody as a probe. AgrD-His6 was not extracted from the membrane fraction by 1 M sodium chloride (Fig. 1B, lane 3) and was only partially extracted from the membrane fraction by 0.2 M sodium carbonate (lane 5). We note that, under both conditions, only peripheral membrane proteins are dissolvable (24, 25). However, this His6-tagged protein was dissolved in 1% sarcosyl (Fig. 1B, lane 7), an anionic detergent proven to preferentially dissolve integral membrane proteins (26). Similar results were obtained from Western blot analysis of LZ4010 expressing group II AgrD-His6 (data not shown). Because the His6 tag is highly hydrophilic and unlikely to be associated with the lipid bilayer, these results strongly suggest that AgrD is an integral membrane protein.

The N-terminal Region of AgrD Is Anchored in the Cytoplasmic Membrane—The full-length RN6390B agrD gene or the 5′-region of the gene coding the N-terminal 23 residues was prepared by PCR and then inserted into the E. coli phoA fusion plasmid pAWLP-2, creating two translational in-frame phoA fusion plasmids, pLZ5001 and pLZ5002, respectively. Expression of the fusion proteins in E. coli MC1061-5 was evaluated by Western blot hybridization of the whole cell lysates with a rabbit anti-E. coli PhoA polyclonal antibody (kindly provided by Dr. Andrew Wright, Tufts University School of Medicine). Both AgrD-PhoA and AgrD-PhoA fusion proteins with correct molecular masses were detected (Fig. 2, lanes 1 and 7). A nonspecific or endogenous PhoA protein band with approximate molecular mass of 55 kDa appeared in samples including the control (Fig. 2, lane 6), as we previously found in strains expressing AgrB-PhoA fusions (16). We noted that, in the pAWLP-2 plasmid, due to addition of the artificial cloning site, the phoA gene was not expressed, so the weaker anti-PhoA response band from cells with this plasmid alone might be due to the unspecific interaction between the anti-PhoA polyclonal antibody and other E. coli proteins and/or the endogenous PhoA protein since the E. coli strain we used is PhoA+. The reason we saw strong bands in cells expressing AgrD-PhoA fusion proteins at the same position as in the control might be due to the degradation of AgrD-PhoA fusion proteins, and these degraded products might be recognized by the polyclonal antibody. The anti-PhoA interactive proteins appeared predominately in the membrane fraction and were completely dissolvable in 1% sarcosyl (Fig. 2, lanes 4 and 10), but were not extracted from the membrane fractions by either 1 M sodium chloride or 0.2 M sodium carbonate (data not shown). Because AgrD-PhoA was anchored in the membrane, these results suggest that the N-terminal half of AgrD is the region that is integrated into the lipid bilayer. The alkaline phosphatase (PhoA) activity of the E. coli strain expressing either the AgrD-PhoA or AgrD-PhoA fusion protein was measured. The PhoA activities of these two strains were no more than that of the strain carrying the vector pAWLP-2 (data not shown), indicating that the C-terminal part of AgrD is located in the cytoplasm. These results suggest that the AgrD propeptide could be either a transmembrane protein with the N terminus outside and the C terminus inside of the membrane or a membrane-anchoring protein with both the N and C termini in the cytoplasm.

An N-terminal Amphipathic Motif Is Conserved in Staphylococcal AgrD Proteins—As our results indicated that AgrD was...
anchored in the cytoplasmic membrane, we asked how AgrD is associated with the membrane lipid bilayer. Hydrophobicity analysis of 24 AgrD sequences from various staphylococcal species retrieved from the GenBankTM/EBI Data Bank failed to find a conserved transmembrane helix among those AgrD sequences (data not shown). A hydrophobic moment plot (27) of each of the 24 AgrD sequences predicted that the N-terminal region of AgrD could form an amphipathic α-helix of various lengths. Helical wheel presentations of the first 18 residues of both group I and II AgrD proteins are shown in Fig. 3. It is clear that all the hydrophilic residues are located on one side of the helix. This feature is conserved in all AgrD proteins but two. These two Staphylococcus aureus AgrD proteins contain one threonine located in the middle of the hydrophobic side that may not interfere with the overall hydrophobic moment. The frequencies of hydrophilic residues are different among AgrD proteins; this may explain why some AgrD sequences were predicted to have transmembrane region and others were not. These results imply that a common α-helical amphipathic motif exists in the N-terminal region of staphylococcal AgrD proteins and that this motif is likely to be anchored in the cytoplasmic membrane.

The N-terminal α-Helical Amphipathic Motif of AgrD Is Required for Its Membrane Anchoring and Processing—To investigate the function of the N-terminal α-helical amphipathic motif of AgrD, we made several AgrD mutants with various residues deleted from the N terminus (Fig. 4). Total cell lysates were prepared from S. aureus cells expressing double-tagged wild-type group I AgrD (TLDH) or the N-terminal deletion mutants and then analyzed by Western blot hybridization using the anti-T7 monoclonal antibody as a probe. As shown in Fig. 5A, protein bands with approximately the same sizes of the predicted molecular masses of TLDH and the AgrD mutants were seen; however, the amounts of the proteins detected were significantly different. S. aureus cells produced similar amounts of TLDH-dN10 (Fig. 5A, lane 4) compared with TLDH-dN5 (lane 3), but much less than TLDH (lane 1). When 12 or more residues were deleted from the N terminus of AgrD in TLDH, the mutant protein levels were significantly lower than those of TLDH, TLDH-dN5, and TLDH-dN10. These results suggest that the N-terminal region of AgrD plays an important role in the stability of the AgrD protein.

To determine the role of the N-terminal α-helical amphipathic motif of AgrD in its subcellular localization, we prepared cytoplasmic and membrane fractions from S. aureus cells expressing TLDH-dN10, TLDH-dN12, or TLDH-dN14. These fractions were analyzed by Western blot hybridization using the anti-T7 monoclonal antibody as a probe. As shown in Fig. 5B (lanes 1, 4, and 7), all three mutant proteins were expressed. TLDH-dN14 was found only in the cytoplasmic fraction (Fig. 5B, lane 8), whereas TLDH-dN12 was detected predominately in the cytoplasmic fraction (lane 5). However, TLDH-dN10 was detected predominately in the membrane fraction (Fig. 5B, lane 3), indicating that the deleted N-terminal region is crucial for membrane anchoring of AgrD.

To establish the relationship between membrane anchoring of AgrD and its processing to generate mature AIP, we cotransformed plasmids encoding TLDH or AgrD N-terminal deletion mutants with plasmid carrying group I wild-type AgrB. The supernatants were then prepared from these strains, and the AIP activities were measured (Fig. 5C). S. aureus cells expressing wild-type AgrB and TLDH-dN10 produced ~50% AIP activities compared with cells expressing AgrB and TLDH, which was likely due to either the lower expression level of TLDH-
Involved in the Specific Interaction with AgrB—The interaction between AgrB and AgrD is group-specific (13), e.g. S. aureus group I AgrD can be processed only by its cognate AgrB, but not by group II AgrB and vice versa. Since AgrB is a multipass transmembrane protein (16), we attempted to address the question of whether the membrane-anchoring amphipathic motif of AgrD is involved in the group-specific interaction with AgrB. Two chimeric C-terminal His6-tagged AgrD proteins (DH-ISII and DH-IISI) (Fig. 4) were constructed by swapping group I and II AgrD proteins, as confirmed by Western blot hybridization with the anti-tetrahistidine monoclonal antibody as a probe (data not shown). The AIPs produced by S. aureus cells coexpressing chimeric AgrD with either group I or II AgrB were then measured. As shown in Fig. 6, AgrD with the C-terminal region from one group was processed by its cognate AgrB no matter whether the N-terminal region was from group I or II AgrD. This result strongly suggests that the N-terminal α-helical amphipathic motif of AgrD is not involved in the group-specific interaction with AgrB.

An AgrD Mutant with Its N-terminal Region Replaced with an Artificial Amphipathic 11-Amino Acid Peptide Is Functional—An artificial 11-amino acid peptide that would form an amphipathic α-helix as predicted by the hydrophobic moment analysis (27) was fused in-frame with the C-terminal region of group I AgrD-His6 (from the 15th residue (isoleucine) to the C terminus). The helical wheel analysis results of the N-terminal region of this AgrD mutant (AmphiNDH) are showed in Fig. 7A. The AmphiNDH protein was expressed in S. aureus (Fig. 7B, lane 1) and was found predominately in the membrane fraction (lane 3) and could not be extracted from the cell membrane by 1 M NaCl (lane 5), confirming that the amphipathic α-helix is anchored in the membrane. When the AmphiNDH protein was coexpressed with group I AgrB, cells produced comparable levels of AIP compared with cells expressing wild-type AgrD-His6 and AgrB (Fig. 7C). These results indicate that the artificial amphipathic α-helix serves as a targeting motif that anchors AgrD in the membrane for its processing.

To test whether replacement of the N-terminal amphipathic region of AgrD with a hydrophobic transmembrane α-helix enables the AgrD C-terminal region to be processed properly, we made an AgrD mutant (tmDH) in which the N-terminal transmembrane α-helix (21 amino acids) of E. coli leader peptidase I (28) followed by two arginine residues was fused with the C-terminal 32 amino acids of AgrD. E. coli signal peptidase I has been widely used in transmembrane topology studies in both prokaryotic and eukaryotic cells (29, 30). This membrane protein has two transmembrane α-helices, and the first α-helix has an N terminus outside and C terminus inside of the membrane topology. We chose the first α-helix of this protein and added two arginine residues at its C terminus according to the so-called “positive inside rule” (31) to increase the possibility that the protein fused at the C terminus of the α-helix would be inside the cytoplasmic membrane. The helical wheel presentation of the first transmembrane α-helix of E. coli signal peptidase I is shown in Fig. 7A. In S. aureus cells, the AgrD tmDH mutant was expressed at comparable levels compared with AgrD-His6 (data not shown). As expected, the mutant protein was found only in the membrane fractions as shown by Western blot hybridization analysis using the anti-tetrahistidine antibody as a probe (Fig. 7C). However, unlike AmphiNDH, no AIPs were produced by cells expressing both tmDH and group I AgrB as determined by AIP activity assays (Fig. 7D). These
results indicate that an amphipathic α-helix is crucial as a targeting motif that anchors AgrD in the membrane for its proper processing. However, we could not rule out the possibility that the transmembrane α-helix from E. coli signal peptidase I used in our studies is not translocated properly on the membrane in S. aureus.

**DISCUSSION**

In this study, we found that AgrD (the propeptide for the Agr quorum-sensing signal AIP) in S. aureus is an integral membrane protein, with its N-terminal region integrated into the cytoplasmic membrane. We have also demonstrated that integration of AgrD into the membrane is required for its stability and processing to generate mature AIP.

Membrane proteins integrate into lipid bilayer in several ways. The majority of them are transmembrane proteins with either α-helical transmembrane segment(s) or β-stranded transmembrane segments. Others are monotypic membrane proteins that are commonly anchored in the membrane by an amphipathic region (32). Thermodynamics studies of amphipathic peptides show that the amphipathic region is usually folded into an α-helix upon interaction with the membrane bilayer (33, 34). Analysis of the predicted staphylococcal AgrD sequences revealed that amphipathic motifs exist in the N-terminal region of AgrD proteins, although the sequences are highly diversified, and the average hydrophobicity values of this region vary. We propose that AgrD is anchored in the cytoplasmic membrane by an N-terminal amphipathic α-helix either on the inner leaflet of the membrane or as a transmembrane segment (Fig. 8, A and B). We found that replacing the N-terminal 14 amino acids of AgrD with an artificial amphipathic 11-amino acid peptide enabled the protein to be targeted correctly to the membrane and processed by AgrB to produce mature AIP. However, replacement of the same AgrD N-terminal region with a well defined E. coli signal peptidase I transmembrane α-helix totally blocked AgrD from being processed by AgrB, even though this AgrD mutant could still be targeted to the cytoplasmic membrane. Assuming that the E. coli signal peptidase I transmembrane α-helix is translocated properly in S. aureus, these results favor the inner leaflet-anchoring model of AgrD as shown in Fig. 8A. Furthermore, the N-terminal T7 tag and the nine-amino acid linker region in TLDH-dN14 are hydrophilic, so it could be considered that the N-terminal 14 amino acids of AgrD were replaced with a 28-amino acid hydrophilic peptide. The fact that this mutant could not anchor in the membrane and could not be processed to generate mature AIP suggests that the amphipathic motif does play a key role in AgrD protein trafficking.

Our previous study shows that AgrB is a multipass transmembrane protein involved in the proteolytic processing of AgrD (16); therefore, membrane localization may be a prerequisite for AgrD to work together with the AgrB protein. Considering that the two proteins may interact around or inside the cytoplasmic membrane, it is of interest to know whether the membrane-anchoring region of AgrD is involved in the interaction. However, our studies on the chimeric AgrD proteins revealed that group-specific processing of AgrD was determined only by its C-terminal region. More important, the AgrD mutant with an artificial N-terminal region containing an amphipathic motif was processed as efficiently as wild-type AgrD, suggesting that the N-terminal amphipathic region of AgrD serves only as a membrane-targeting motif, directing it to the same compartment with AgrB for further processing. Furthermore, significantly lower amounts of AgrD N-terminal deletion mutants that were defective in their abilities to anchor in the membrane were detected compared with the wild-type AgrD sequence, suggesting that anchoring of the AgrD propeptide in the membrane by its N-terminal amphipathic region prevents the nonspecific degradation of the proteins. We hypothesized that the initial interaction between AgrD and AgrB might involve the C-terminal to AIP region of AgrD. The AgrD mutant with four amino acids deleted from its C terminus had no effect on its processing by AgrB to generate mature AIP. However, deletion of six amino acids from the C terminus of
AgrD totally eliminated its ability to be processed. The direct interaction of this region of AgrD with AgrB has yet to be confirmed experimentally.

Examples of proteins that anchor in the membrane by an amphipathic α-helix include plasma lipoproteins (35), ion channels (32), membrane-anchoring enzymes (36), and proteins involved in signal transduction pathways that relay signals from transmembrane receptors to downstream cytoplasmic proteins.
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(37). It is known that bacteriocin-inducing peptides and bacteriocins from lactic acid bacteria are partly amphipathic. They form helical structures upon entering the lipid bilayer, and the helical amphipathic structure is critical for their membrane targeting and normal function (38–40). It has also been proposed that the amphipathic region plays an important role in the formation of ion channels in which the hydrophobic sides of the helices are outside and the hydrophilic sides are inside, forming the channel like a barrel (41). However, the amphipathic motifs of proteins such as T. cruzi protein GRA2 (42), RGS (regulators of G protein signaling) proteins (37), and the bacterial division-related protein MinD (43, 44) are usually anchored on one side of the lipid bilayer and serve as membrane trafficking signals.

Other Gram-positive bacteria may use a mechanism similar to that of AgrD processing to produce mature peptide pheromones from precursor proteins. The B. subtilis ComX pheromone is produced by the C-terminal region of the ComX peptide; however, it is unknown whether a membrane-targeting motif exists in its N-terminal region. We analyzed eight ComX sequences from several Bacillus strains retrieved from the GenBankTM/EBI Data Bank (NCBI accession numbers P45453, AAL59648, AAL67540, AAL67731, AAL67737, AAL67740, AAF82181, and AAF82182) and found that a conserved putative amphipathic motif exists in all. It is possible that an amphipathic membrane-targeting motif may be a common feature of certain quorum-sensing pheromone precursors in Gram-positive bacteria.

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