Transmembrane Topology of AgrB, the Protein Involved in the Post-translational Modification of AgrD in *Staphylococcus aureus*#

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The accessory gene regulator (agr) of *Staphylococcus aureus* is the central regulatory system that controls the gene expression for a large set of virulence factors. This global regulatory locus consists of two transcripts: RNAII and RNAIII. RNAII encodes four genes (agrA, B, C, and D) whose gene products assemble a quorum sensing system. RNAIII is the effector of the Agr response. Both the agrB and agrD genes are essential for the production of the autoinducing peptide, which functions as a signal for the quorum sensing system. In this study, we demonstrated the transmembrane nature of AgrB protein in *S. aureus*. A transmembrane topology model of AgrB was proposed based on AgrB-PhoA fusion analyses in *Escherichia coli*. Two hydrophilic regions with several highly conserved positively charged amino acid residues among various AgrBs were found to be located in the cytoplasmic membrane as suggested by PhoA-AgrB fusion studies. However, this finding is inconsistent with the putative transmembrane profile of AgrB by computer analysis. Furthermore, we detected an intermediate peptide of processed AgrD from *S. aureus* cells expressing AgrB and a 6 histidine-tagged AgrD. These results provide direct evidence that AgrB is involved in the proteolytic processing of AgrD. We speculate that AgrB is a novel protein with proteolytic enzyme activity and a transporter facilitating the export of the processed AgrD peptide.

*Staphylococcus aureus* is an important bacterial pathogen that causes a great variety of human diseases. The pathogenicity of *S. aureus* largely depends on a set of virulence factors. These include cell wall-associated proteins involved in attaching the bacteria to host cells or extracellular matrices and protecting the bacteria against host defenses. Other factors are secreted proteins that attack host cells, degrade components of extracellular matrices, and interfere with immune responses (1). The expression of these virulence factor genes is primarily regulated by a quorum sensing system encoded by the global regulatory locus, the accessory gene regulator (agr). At low cell density, the agr genes are continuously expressed at basal levels. A signal molecule, autoinducing peptide (AIP), produced and secreted by the bacteria, accumulates outside of the cells (2, 3). When the cell density increases and the AIP concentration reaches a threshold, it activates the agr response (2), i.e. activation of secreted protein gene expression and subsequent repression of cell wall-associated protein genes (1, 4–6).

The agr locus consists of two operons, P2 and P3 (6–7). The P3 transcript, RNA III, the effector of the agr response, functions as the regulator controlling the expression of virulence factor genes by a yet to be defined mechanism (6, 8–12). The P2 transcript, RNA II, encodes four genes, agrA, B, C, and D (6, 7). AgrC, a transmembrane protein, is a sensor kinase of the classic bacterial two component signal transduction system: the N-terminal half is the input domain that interacts with a signal molecule produced by the bacteria, and the C-terminal half is a transmitter that is autophosphorylated at a conserved histidine upon stimulation by the signal molecule (2, 13, 14). AgrA resembles a response regulator, which is required for the activation of both agr promoters P2 and P3 (2, 7, 15), although it is not clear whether AgrA binds to these two promoters directly (16, 17). It is possible that either phosphorylated AgrA would bind to the agr promoters or AgrA would interact with another global regulator, SarA, to control the agr expression (17–19).

Both agrB and agrD genes are essential for the production of the signal molecule, AIP (2, 3). The AgrD propeptide is ribosomally synthesized and subsequently processed and secreted from the bacteria (2, 3, 20). The AIP is a thiolactone molecule containing a ring of 5 amino acids formed by a thioester linkage between the sulfhydryl group of a cysteine residue and the C-terminal carboxyl group, and a tail ranging from 2 to 4 amino acid residues depending on staphylococcal species and groups within the same species (3, 21–23). This thioester bond is critical for its agr activation activity (14, 21, 22, 24). AgrB is predicted to be a transmembrane protein (7), and the association of *Staphylococcus epidermidis* AgrB with the cell membrane has been demonstrated recently (25). AgrB is absolutely required for the production of AIP (2, 3). Because the production of mature AIP involves several steps, including proteolytic digestion, thioester bond formation, and secretion, we reasoned that AgrB was probably the protein to carry out all these processes. In this study, we confirmed the membrane localization and proposed the transmembrane topology of *S. aureus* AgrB. Further more, we provided direct evidence showing that AgrB was involved in the proteolytic processing of AgrD.

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The amino acid sequences of these proteins can be accessed through NCBI Protein Database under NCBI accession numbers CAA36781, AAC8295, and AAC71976.

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* The abbreviations used are: AIP, autoinducing peptide; agr, accessory gene regulator; BlaZ, β-lactamase; P_{blaZ}, blaZ promoter; PhoA, alkaline phosphatase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; DAS, Dense Alignment Surface.

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Plasmids
pRN5548 Vector carrying a staphylococcal inducible $P_{\text{ lux}}$ promoter 8
pRN6441 Cloning vector 8
pRN6893 agr RNAIII-blaZ fusion 7
pRN6911 $agr$ in pRN5548 2
pRN6912 $agr$ in pRN5548 2
pRN6913 $agr$ in pRN5548 2
pGJ2001 $agr$ in pRN5548 $P_{\text{lux}}$ This study
pGJ4002 $agr$ in pRN6441 This study
pGJ4004 $6x$his-$agr$ in pRN5548 This study
pLZ2003 $agr$ in pRN6441 This study
pLZ2004 $agr$-6xhis in pRN5548 This study
pLZ4005 $agr$-6xhis in pRN5548 This study

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—S. aureus strains and plasmids used in this study are listed in Table I. S. aureus cells were grown in CY-GP broth (26), supplemented with antibiotics (chloramphenicol, 5 µg/ml, erythromycin, 5 µg/ml) when necessary. Bacteria grown overnight at 37 °C on GL plates (26) were routinely used to inoculate liquid cultures. Growth of cells 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 OD$_{600}$. Expression of genes driven by staphylococcal $\beta$-lactamase promoter ($P_{\text{ lux}}$) was induced with 0.5 µg/ml methicillin. *Escherichia coli* strain MC1061 (27) was grown in LB broth and supplemented with tetracycline (20 µg/ml) when necessary.

**S. aureus Plasmid Construction**—All *S. aureus* plasmids used in this study were based on either pRN5548 or pRN6441 (7) (see Table I). pGJ2001 was constructed by digesting pRN6912 (2) with SphI and SallI, blunting with DNA polymerase I large fragment (Klenow), and religating. A *Clai* DNA fragment from pGJ2001 containing agrB gene under the control of the staphylococcal $P_{\text{ lux}}$ was inserted into the *Clai* site of pRN6441 to give rise to pLZ2003. To construct the C-terminal His$_6$-tagged AgrB (AgrB-6xHis) expression plasmid pLZ2004, a PCR product containing oligonucleotides GJ#14 5'-GCTCATAGCTGATAATGAAGCTATTACATTATTACC-3' (in italic, with an added ClaI site, underlined), and GJ#27 5'-TAATGATGATGATGATGTTCGTGTAATTGTGTAATTC-3' (6 histidine codons and a stop codon underlined) as primers, and pGJ2001 as the template was generated. The PCR product was digested with XbaI and cloned into the *XbaI* and *EcoRI* sites of pRN6441. The resulting plasmid was transformed into *E. coli* MC1061 strain MC1061 (27) grown in LB broth.

**Construction of agrB-phoA Fusions and Alkaline Phosphatase Activity Assay**—PCR products using the forward primer GJ#17 5'-GAATTACATTATTATTAGCTGATAATGAAGCTATTACATTATTACC-3' (in italic, with an added EcoRI site) and the reverse primer (with an added BamHI site) within agrB gene, and pGJ2001 plasmid DNA as the template were prepared, digested with BamHI and BamHI, and ligated into the BamHI site of the *E. coli* phoA fusion vector pAWLP-2 or pAWLP-3 (kindly provided by Dr. Andrew Wright, Tufts University School of Medicine). The resulting plasmids were transformed into *E. coli* MC1061. The junction between agrB and phoA and the agrB region of each construct was verified by DNA sequencing. PhoA (alkaline phosphatase) activity was measured using overnight cell cultures in LB broth. The colorimetric reaction product was measured using Sigma 104 phosphatase substrate (Sigma) as described previously (29).

**Cell Fractionation**—*S. aureus* cells were grown in CYP broth and induced with 0.5 µg/ml methicillin at 37 °C for 3 h. After harvesting, the cell pellet was suspended in 1 × sucrose-sodium maleate-MgCl$_2$ buffer containing 10 µg/ml lysostaphin and incubated for 30 min at 37 °C. The protoplasts were then collected and lyed by addition of 1 × phosphate-buffered saline supplemented with 1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in a solution of 0.5% Triton X-100.
nylonmethanesulfonyl fluoride and incubated on ice for 30 min. The cell lysate was briefly sonicated, and the unlysed cells were removed by centrifugation at 7,000 × g for 10 min at 4 °C. The cell lysate was then centrifuged at 200,000 × g for 2 h at 4 °C to separate the cell membrane and cytoplasmic fractions. The membrane pellet was washed three times with ice-cold water, resuspended in water, and stored at −80 °C. Protein concentration was measured with a detergent-compatible protein assay kit (Bio-Rad). E. coli MC1061 cells containing the phoA expression vector or agrB-phoA fusion plasmid were grown in LB broth overnight at 37 °C with shaking. After centrifugation, cell pellets were lysed by brief sonication in buffer consisting of 1% Triton X-100, 50 mM Tris-Cl, 1 mM MgCl₂, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The Triton X-100-insoluble fraction of the whole cell lysate was prepared by ultracentrifugation at 200,000 × g for 2 h at 4 °C. The pellets were dissolved in 1 × SDS sample buffer (27) 5 min, and stored at −20 °C.

**Western Blotting**—Whole cell lysate, membrane, cytoplasmic fraction, or purified His₆-tagged protein samples were boiled for 5 min in 1× SDS sample buffer. Proteins were separated either by Tris-glycine SDS-PAGE (27) (for AgrB-6xHis and AgrB-PhoA fusion proteins) or by Tris-Tricine SDS-PAGE (30) (for AgrB-DDH protein). The separated proteins were then electrophoretically transferred to PROTRAN nitrocellulose membranes (Schleicher & Schuell). After blocking overnight at 4 °C with TBST buffer (27) plus Tween 20 (0.05%) containing 5% nonfat milk, the membranes were incubated in the blocking buffer with the primary antibody (1:5000 dilution of mouse anti-pentaHis monoclonal antibody, Qiagen; 1:1000 dilution of rabbit polyclonal anti-PentaHis antibody, kindly provided by Dr. Andrew Wright, Tufts University School of Medicine; 1:5000 dilution of anti-T7-tag monoclonal antibody, Novagen) for 1 h at room temperature. The blots were washed extensively and probed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Amersham Biosciences). The immuneblots were detected with the SuperSignal West Pico Chemiluminescent detection kit (Pierce) followed by exposure to X-Omat film (Kodak).

**Results**

*S. aureus* AgrB Is Located in the Cytoplasmic Membrane—To directly detect and purify the AgrB protein from *S. aureus*, we made a construct (pLZ2004) in which 6 histidine codons were added to the 3′-end of RN6390B agrB gene. This plasmid, or the cloning vector pRN5548, was transformed into an agr-null *S. aureus* strain GJ2035, creating strains LZ2004 and LZ2001 respectively. Western blot hybridization analysis with an anti-pentaHis monoclonal antibody as a probe revealed a band with an estimated size of about 23 kDa from whole cell lysate of LZ2004 expressing the His₆-tagged AgrB (data not shown). This band was not observed from LZ2001 cell lysate (data not shown). The size of the protein detected was consistent with that of the predicted molecular weight of the His₆-tagged AgrB. This result suggests that the AgrB-6xHis protein was expressed in *S. aureus*. The effect of the 6 histidine residues on the AgrB processing of AgrD was determined as follows. Conditioned media were collected from cells expressing only wild-type AgrD (LZ4200) or both wild-type AgrB and wild-type AgrD (LZ4201). In parallel, conditioned media were prepared from cells expressing the His₆-tagged AgrB and wild-type AgrD (LZ4202). The AIP activities in the conditioned media were measured. LZ4202 produced comparable AIP activity with that of strain LZ4201 (data not shown), indicating that the determinants His₆-tagged AgrB function normally in *S. aureus*.

To determine the cellular localization of the AgrB protein in *S. aureus*, cytoplasmic and membrane fractions were prepared by ultracentrifugation from LZ0001 or LZ2004 strains. Western blot hybridization with anti-pentaHis monoclonal antibody as a probe showed that the 23-kDa band as detected in the whole cell lysate of LZ2004 appeared in the membrane fraction of the same strain (data not shown). To confirm this result, affinity chromatography with nickel-nitrotriacetic acid-agarose was employed to partially purify proteins under denaturing conditions from the cytoplasmic and membrane fractions of LZ0001 or LZ2004 cells. This method provides about five times more concentrated proteins. Again, Western blot hybridization analysis showed the 23-kDa protein presented only in the membrane fraction from cells expressing the His₆-tagged AgrB (data not shown). These results, consistent with a previous report on the *S. epidermidis* AgrB (25), confirmed that AgrB is located in the cytoplasmic membrane.

**Transmembrane Topology of AgrB by PhoA Fusions**—To facilitate our experimental design in constructing AgrB-PhoA fusions, hydropathy profiles of AgrB proteins from the four groups of *S. aureus* (3, 23) as well as the AgrB homologs from *S. epidermidis* (21) and *Staphylococcus lugdunensis* (31) were analyzed. A Kyte-Doolittle (32) hydrophobicity plot for these AgrB proteins is shown below in Fig. 2A. The AgrB proteins were also analyzed with various programs available through the Internet. These programs include TopPred 2 (33) (bioweb.pasteur.fr/seqanal/interfaces/toppred.html), TMHMM (34) (www.cbs.dtu.dk/services/TMHMM-2.0/), and Dense Alignment Surface method (DAS) (35) (www.ncbi.nlm.nih.gov/). Despite the highly variable sequences among the AgrBs, all programs gave similar results in hydrophobicity plots, although the predicted transmembrane α-helices were slightly different for some AgrBs. A total of five possible transmembrane segments were predicted from these analyses.

To confirm the topology predicted from the hydrophobicity analyses, various lengths of the 5′ region or the full-length of the agrB gene from RN6390B were prepared by PCR and then inserted into the *E. coli* phoA fusion plasmid pAWLP-2 or pAWLP-3 creating a series of agrB-phoA fusion plasmids. A total of 23 such fusion plasmids were generated. The expression of the fusion proteins in *E. coli* MC1061 carrying the agrB-phoA fusion plasmid was evaluated by Western blot hybridization of whole cell lysates with a rabbit anti-*E. coli* PhoA polyclonal antibody. Proteins corresponding to the molecular weight of all the fusion proteins were identifiable, however, high numbers of nonspecific bands also appeared due to the cross-reaction of the polyclonal antibody with other proteins (data not shown). To eliminate the background problem, Triton X-100-insoluble fractions prepared from whole cell lysates were used for Western blot analysis. This non-ionic detergent could dissolve most of the membrane proteins but not all of them, especially when the proteins are highly expressed as in these experiments in which the fusion proteins are expressed under the control of a constitutive P₆₃₅ promoter on a high copy number plasmid. A similar method was used by Saenz et al. (25) to detect *S. epidermidis* AgrB. As shown in Fig. 1A, all the AgrB-PhoA fusion proteins with the correct molecular weights were detected. The amounts of the fusion proteins expressed in each strain were comparable, although in a few cases there were different amounts of degradation products. A protein band with an approximate molecular mass of 55 kDa was present in samples, including the controls, suggesting that it might be a nonspecific band or endogenous PhoA protein.

The alkaline phosphatase (PhoA) activity of each strain expressing the AgrB-PhoA fusion protein was measured (Fig. 1B). The location of the fusion point of each fusion protein was then assigned based on the PhoA activity. PhoA-positive suggests that the PhoA portion, thus the fusion point of the protein, is in the periplasm of *E. coli*, whereas PhoA-negative indicates that the fusion point is either in the membrane or in the cytoplasm. The fusions Gln-10, Asn-19, Gln-28, and Ala-37 failed to show any PhoA activity, suggesting that the N-terminal portion of AgrB, a highly hydrophilic region, was in the cytoplasm. We
note that AgrB does not seem to have a signal peptide at its N-terminal portion, because deletion mutation of AgrB from amino acid residues 2 to 34 had no effect on the integration of AgrB into S. aureus membrane. The full-length AgrB-PhoA fusion (Lys-189) had no PhoA activity, indicating that the C terminus of AgrB is also in the cytoplasm. The transmembrane segments I, II, IV, and V predicted from the computer analysis (Fig. 2A) were partially confirmed based on the PhoA activity of Ala-37 and Thr-62, Ser-88 and Pro-96, Arg-140 and Pro-162, and Ala-175 and Lys-189 fusions. However, both Ile-104 and Ser-116 fusions predicted to be in the transmembrane segment III had PhoA activities. Considering that both fusions Pro-96 and Thr-128 were also PhoA active, this flanking region was probably located outside of the cytoplasmic membrane. Fusions Arg-71, Ala-76, Ala-78, Pro-79, and Ser-88 and fusions Ile-133, Arg-136, Leu-137, and Arg-140 all lacked PhoA activity, suggesting these amino acid residues were either in the transmembrane segments or in the cytoplasm. Since both the N terminus and the C terminus were positioned in the cytoplasm, it was reasonable to assign the regions from Thr-65 to Ala-76 and from Ala-126 to Ile-138 as transmembrane segments. Based on these AgrB-PhoA fusion results and computer analyses, a putative AgrB transmembrane topology model was proposed, as shown in Fig. 2B. In this model, the AgrB protein contains six transmembrane segments. Two segments consist of highly hydrophilic amino acid residues and a relatively highly hydrophobic loop positions outside of the membrane.

**AgrB Is Involved in the Proteolytic Processing of AgrD**—We previously showed that the agrB gene is required for the production of the mature AIP in staphylococci (2–3), however, no direct evidence showing the involvement of AgrB in the proteolytic processing of AgrD has been reported. To study the role of AgrB in this process, we made a construct (pLZ4005) in which 6 histidine codons were added to the 3′-end of RN6390B agrD gene and a DNA fragment containing His6 as well as a T7 epitope coding region was added at the 5′-end. A diagram of the double-tagged AgrD (AgrD-HDH) fusion protein is shown in Fig. 3A. S. aureus LZ2403 expressing both RN6390B AgrB and AgrD-HDH produced mature AIP, although the mature AIP activity was lower than that of Gj2404 expressing the wild-type AgrB and AgrD (Fig. 3B), indicating the AgrD-HDH fusion protein was functional. Western blot hybridization with anti-pentaHis monoclonal antibody was performed using whole cell lysates from cells expressing AgrD-HDH with or without AgrB. As seen in Fig. 3C, a specific pentaHis antibody-reactive band of ~12 kDa in size, corresponding to the predicted molecular weight of AgrD-HDH, was present from cells expressing AgrD-HDH (LZ4005, LZ0403, and LZ2403) (Fig. 3C, lanes 2–4) but not from cells not expressing AgrD-HDH (LZ0001) (Fig. 3C, lane 1). An additional protein band with an approximate molecular mass of 9 kDa appeared from LZ2403 cells expressing both AgrB and AgrD-HDH (Fig. 3C, lane 4). This same protein band was also detected in the Western blot with LZ2403 cell lysate and anti-T7 tag monoclonal antibody as a probe (Fig. 3D, lane 4). These results indicate that the 9-kDa protein was an AgrD-HDH-processing product consisting of the His6-T7 tag and the N-terminal part of AgrD and that AgrB was required for the production of this AgrD-processing product. However, it was not clear whether the 9-kDa protein was an AgrD-HDH-processing intermediate (Fig. 3A, fragment A) or a final product (Fig. 3A, fragment C) or whether AgrB alone or AgrB together with other protein(s) carried out this proteolytic reaction. As noted in lane 4 of Fig. 3 (C and D), another weak band of about 7 kDa in size was present in both blots. This protein was larger than the His6-T7 tag (fragment E, 5807 Da) but smaller than fragment C (8477 Da), suggesting that this protein was a degradation product of the 9-kDa protein and the cleavage site was within the N-terminal part of AgrD.

### DISCUSSION

Because the agr system coordinately regulates the expression of a series of virulence factors, cross inhibition of the agr gene expression among different species or groups of the same species of staphylococci by AIP may have great clinical significance in controlling the pathogenicity (3–5, 20). Although significant advances have been made on the structure and activity of the AIPs (14, 22, 24, 36), it remains unclear how AgrD is processed in vivo to form the active mature AIP. Our previous research showed that agrB encodes a protein required for the
processing of the AgrD propeptide, and despite the sequence divergence of AgrB and AgrD, both retain the specific interactions required for maturation of the peptide derivatives of AgrD (2–4).

In this study, we expressed His₆-tagged AgrB in *S. aureus* and confirmed that AgrB is anchored in the cytoplasmic membrane. These results are consistent with a previous report on the AgrB homolog from *S. epidermidis* (25). AgrD is processed in a complicated way; it involves proteolytic digestion of the propeptide at two sites, further modification by the formation of a thioester linkage, and secretion into the surrounding environment (2–3, 22). In addition, the fact that AgrB is required for the production of the mature AIP molecule, which accumulates outside of the cells, makes it plausible to hypothesize that AgrB may be involved in all these processes. Therefore, the understanding of the secondary structure of AgrB is critical to elucidate the mechanism of the AgrD processing by the AgrB protein and to facilitate the identification of domains that play important roles in the AgrD processing.

The AgrB membrane topology we proposed in this report was based on the PhoA fusion analyses in *E. coli* in conjunction with computerized hydrophobicity analysis. Such a PhoA fusion analysis method has been successfully used in many transmembrane topology studies such as the membrane topology of LcnC, a protein involved in both maturation and exporting of lactococcin (37), and various *S. aureus* membrane proteins (13, 38–39). PhoA fusion results confirmed that four of the five hydrophobic domains were transmembrane helices as predicted by computer analysis. The hydrophobic region between residue Ile...
104 and Ala-124 was assigned as an outside loop based on the PhoA activities of Pro-96, Ile-104, Ser-116, and Thr-128. However, Ser-116 had an intermediate level of alkaline phosphatase activity, suggesting that this hydrophobic region of the AgrB protein was not clearly periplasmic but was in some intermediate zone, i.e. a small region around residue 116 could be in a transition state between the outer surface and the membrane. Thus this region of AgrB might play an important role in the secretion of AIP or in the processing of the AgrD propeptide and secretion of AIP. PhoA fusions Arg-71, Ala-76, Ser-88, Ile-133, Arg-136, Leu-137, and Arg-140 all lacked alkaline phosphatase activity, although these fusion points were located in two highly hydrophilic regions. There were three possible ways to position these two regions. First, they are periplasmic. It has been reported that a PhoA fusion to a known periplasmic domain may have low alkaline phosphatase activity because of the presence of a positively charged residue in the membrane-spanning segment to the N-terminal side (40). However, this would not explain our results, because both areas are preceded by fusion points that are PhoA-active. Second, they are in the cytoplasm. But the confirmation of the four computer-predicted transmembrane segments by our PhoA fusion analyses made this possibility less likely. Finally, they are transmembrane segments. The hydrophobicity is not a necessity for the formation of a transmembrane segment. As reported by Ota et al. (41), an internal signal-anchor sequence with N$_{\text{NaxC}}$/C$_{\text{Cyto}}$ topology is able to locate a preceding hydrophilic segment through the membrane when multispanning membrane proteins are integrated into the endoplasmic reticulum. We believe that the positioning of these two regions in the membrane is appropriate and would be consistent with our data when the whole picture of the AgrB membrane topology is considered. Because both regions contain highly conserved positively charged amino acid residues among all the AgrBs identified so far, we hypothesized that the two hydrophilic transmembrane segments are somehow contained in the four hydrophobic transmembrane segments, and this configuration might be crucial for the processing and exporting of AgrD peptide.

We provided direct evidence showing that AgrB is involved in the proteolytic processing of AgrD. We could not exclude the possibility that there may be other protein(s) associated with AgrB with the actual enzymatic activity to carry out this reaction. The 9-kDa fragment generated from AgrD propeptide in the presence of AgrB needs to be further characterized to identify the precise cleavage site within AgrD. The one or more active sites of AgrB involved in the proteolytic processing of AgrD have not been identified. However, our preliminary results showed that, although the mature AIP is secreted, the other possible AgrD-processing products by AgrB and the AgrD propeptide were membrane-bound, and these peptides were not found either in the cytosol or in the culture supernatant. No AgrD-processing intermediates were detected in the absence of AgrB (Fig. 3, C and D). These results suggest that the AgrD proteolytic processing may occur within the membrane. It is also possible that this process may occur either in the cytosol or outside of the cytoplasmic membrane, and the resulting processing products with the exception of the mature AIP remain membrane-bound. It would be ideal to utilize the agrB-phoA fusion constructs we made to identify the possible AgrB active site(s) in E. coli. However, our attempts to generate functional mature AIP from E. coli cells expressing wild-type AgrB and AgrD were not successful so far. The reason for this is not clear.

We note that Gram-positive bacteria that utilize peptide autoinducers to activate the quorum sensing systems, such as in lactobacilli (42), streptococci (43), and Bacillus subtilis (44), the autoinducing peptides are processed and secreted by proteins consisting of two domains, a proteolytic domain carrying out the enzymatic reaction, and an ATP-binding domain forming an ATP-binding cassette transporter that exports the mature autoinducing peptide (45, 46). These proteins are encoded by genes in the same operon as those that encode propeptides and the two component signal transduction systems. These operons have similar gene arrangement as that of the agr P2 operon. We also note that AgrB is not homologous with any of the known proteinases found in bacteria, and it does not have a recognizable ATP binding motif. It is possible that S. aureus utilizes a different mechanism to generate an autoinducer from those found in other Gram-positive bacteria. We proposed the following hypothetical model for the processing of AgrD propeptide and the secretion of the mature AIP in S. aureus: The propeptide is proteolytically cleaved at two sites within AgrD by AgrB. Cleavage of the N-terminal peptide bond could be a simple proteolytic cleavage. Processing at the C terminus could involve cleavage of the C-terminal peptide bond resulting in a peptide-enzyme acyl adduct, which would then undergo trans-esterification with the cytoeine thiol group. No external energy (ATP) would be required for these reactions. It could be rapidly followed by desolation of the peptide concomitantly with its export, and again there would not be any obligatory requirement for ATP. Further study on the confirmation of the AgrB dual functions and the identification of its functional domains will be required to define it as a novel protein.

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