Identification of the Putative Staphylococcal AgrB Catalytic Residues Involving the Proteolytic Cleavage of AgrD to Generate Autoinducing Peptide*

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The P2 operon of the staphylococcal accessory gene regulator (agr) encodes four genes (agrA, -B, -C, and -D) whose products constitute a quorum sensing system: AgrA and AgrC resemble a two-component signal transduction system of which AgrC is a sensor kinase and AgrA is a response regulator; AgrD, a polypeptide that is integrated into the cytoplasmic membrane via an amphipathic α-helical motif in its N-terminal region, is the peptidase for an autoinducing peptide that is the ligand for AgrC; and AgrB is a novel membrane protein that involves in the processing of AgrD and possibly the secretion of the mature autoinducing peptide. In this study, we demonstrated that AgrB had endopeptidase activity, and identified 2 amino acid residues in AgrB (cysteine 84 and histidine 77) that might form a putative cysteine endopeptidase catalytic center in the proteolytic cleavage of AgrD at its C-terminal processing site. Computer analysis revealed that the cysteine and histidine residues were conserved among the potential AgrB homologous proteins, suggesting that the Agr quorum sensing system homologues might also exist in other Gram-positive bacteria.

Agr two-component signal transduction system, respectively (3, 4); AgrD is the propeptide of the autoinducing peptide (AIP) that is secreted from the bacteria (5) and functions as the ligand for AgrC (6); and AgrB is a membrane protein that is involved in the processing of AgrD (7).

AgrC is a membrane protein with its N-terminal half integrated into the cytoplasmic membrane that contains the AIP binding site (6, 8), and with its C-terminal half located in the cytoplasm that possesses histidine kinase activity (6). Among the identified AgrCs so far from various species of staphylococci, the N-terminal halves are divergent and the C-terminal halves are highly conserved (9, 10). This reflects the fact that the AgrCs are activated only by their cognate AIPs but are inhibited by heterologous AIPs (9, 11–15). Based on the AIP cross-activation and cross-inhibition activities, four specificity groups of Staphylococcus aureus (9, 14) and three groups of Staphylococcus epidermidis (10, 16, 17) have been identified. Upon the binding of AIP, AgrC is autophosphorylated (6). It has been proposed that the phosphoryl group of the phosphorylated AgrC is transferred to AgrA, and the phosphorylated AgrA then interacts with the P2 and P3 promoters to activate the transcription of both RNAII and RNAIII (3, 4, 6). RNAIII is the actual regulator that activates the expression of genes encoding secreted virulence factors and represses those encoding cell surface-associated proteins (1, 18).

AgrD is a membrane protein anchored in the inner leaflet of the cytoplasmic membrane via an amphipathic α-helix formed by its N-terminal region (19). AgrD sequences from various staphylococcal species are remarkably divergent with only 4 identical amino acids (1). The mature AIPs isolated so far from a number of staphylococcal species are 7 to 9 amino acids in length, and all are thiolactone molecules containing a 5-amino acid ring linked by a thioester bond formed between the sulfhydryl group of a conserved cysteine residue and the carboxyl group of the C-terminal amino acid, except the Staphylococcus intermedius AIP, a lactone molecule that contains a ester bond formed between the hydroxyl group of a serine residue (in place of the cysteine residue that is absolutely conserved among other AIPs) and the carboxyl group of the C-terminal residue (11, 12, 15, 20, 21). The AIP sequence is in the middle of the AgrD sequence that is preceded by the N-terminal amphipathic α-helix and followed by a highly hydrophilic C-terminal region. The processing of AgrD to generate mature AIP involves the proteolytic cleavages at two processing sites, the thioester (or ester) bond formation, and the secretion of the mature AIP.

AgrB is a membrane protein with six transmembrane segments including four transmembrane α-helices and two highly hydrophilic regions (22). Like AgrD, the AgrBs sequenced from various staphylococcal species are also divergent, except the N-terminal region located in the cytoplasm and the two highly

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1 The abbreviations used are: agr, accessory gene regulator; AIP, autoinducing peptide; BlaZ, β-lactamase; P_bla, bla promoter; SMM, sucrose-sodium maleate-MgCl2; GDP, green fluorescent protein; AEBSF, 4-[2-aminoethyl]benzenesulfonyl fluoride; TPCK, N-α-tosyl-l-phenylalanine chloromethyl ketone; VPK-CMK, N-Val-Phe-Lys chloromethyl ketone; E-64, 1-trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane; DSP, 3′,3′-dithiodiobi(succinimidyl propionate); nt, nucleotide(s); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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### EXPERIMENTAL PROCEDURES

#### Bacterial Strains and Growth Conditions

S. aureus RN6390B is a derivative of NCTC8325 and is our standard laboratory group I strain. RN6911 is a derivative of RN6390B in which the agr locus is replaced by the tetM gene (18). SA02A is our standard S. aureus group I strain (9) and GJ2035 contains plasmid pLZ412 carrying an inducible plasmid pLZ412, or LZ number 23 promoter 18.

P. aeruginosa PAO1 was used for protein expression. The plasmids used in this study are listed in Table I. S. aureus cells were grown in CYG broth (24), supplemented with either 5 μg/ml chloramphenicol or 5 μg/ml erythromycin or both when necessary. E. coli cells were grown in LB medium (25). Cell growth was monitored with a Klett-Summerson colorimeter with a green (540 nm) filter (Klett, Long Island City, NY). S. aureus cells expressing AgrB or AgrD or both under the control of the staphylococcal P_{bla} promoter were induced with 0.5 μg/ml methicillin. E. coli cells expressing the Agr protein(s) under the T7lac promoter control were induced with 1 μM isopropyl-1-thio-

#### Plasmid Constructions—Plasmid pLZ5001 was constructed by cloning a ClaI DNA fragment containing the S. aureus group I agr-B-His_n gene under the control of the P_{bla} promoter from pLZ2004 (22) into the ClaI site of pRN5541. pLZ5002 was made by ligating two Ncol-digested PCR products amplified from pLZ2004 using T4 polynucleotide kinase-phosphorylated primers: GJ number 50, 5'-GTTCTAGAATCTATTATGAGTGAGCAGCTACTGTA-3' (in agrB, nt 1878–1897 of the S. aureus group I agr, agr-1; GenBank™ accession number X52543) and GJ number 45, 5'-GTA-AATGAGTCCATGGAATATAGCTCTGCAG-3' (around the Ncol site of pRN5548), and primers GJ number 51, 5'-CAATTTTACACCACTCTCAGGCACAGCTACTGTA-3' (in agrB, nt 1758–1778 in agr-1) and GJ number 44, 5'-GTTCTAGAATCTATTATGAGTGAGCAGCTACTGTA-3' (complementary to GJ number 45), respectively. pLZ5003 was constructed in the same way as pLZ5002 except the primer pairs used were GJ number 58 5'-AAAGTCAGCATCACCCTTCTTCTTTTGGT-3' (in P_{bla}) and GJ number 44 (for pLZ5005), or LZ number 23 promoter 18.

#### Strains and plasmids used in this study

| Plasmid       | Genotype and description | Ref.     |
|---------------|--------------------------|---------|
| pET11a        | E. coli T7 tag vector with T7lac promoter | Novagen |
| pGEM-T        | E. coli PCR product cloning vector | Promega |
| pRN5543       | Staphylococcus clonal vector | 18      |
| pRN5544       | Staphylococcal expression vector carrying a staphylococcal inducible P_{bla} promoter | 18      |
| pRN6683       | S. aureus group I agr-B-His_n fusion | 3       |
| pRN6913       | S. aureus group I agrD in pRN5548 | 5       |
| pLZ2003       | S. aureus RN6390B agr-B in pRN5548 | 22      |
| pLZ2004       | S. aureus RN6390B agr-D-His_n in pRN5548 | 22      |
| pLZ4012       | S. aureus RN6390B agrB-D(H) in pRN5548 | 19      |
| pLZ5001       | S. aureus RN6390B agrB-His_n in pRN5541 | This study |
| pLZ5002       | agrB(ΔN), deletion of the N-terminal 34 amino acids in pLZ2004 | This study |
| pLZ5003       | agrB(ΔS81), residues 73–78 ARGHA changed to HHVHHH in pRN6912 | This study |
| pLZ5004       | agrB(ΔP34), residues 123–125 YAP changed to AAA in pLZ2004 | This study |
| pLZ5005       | agrB(ΔPst1), Y123A in pLZ2004 | This study |
| pLZ5006       | agrB(ΔPst2), K129A/K130A in pLZ2004 | This study |
| pLZ5007       | agrB(ΔPst3), Y123A/K129A/K130A in pLZ2004 | This study |
| pLZ5008       | agrB(ΔS80A) in pRN6441 | This study |
| pLZ5009       | agrB(ΔH77A) in pRN6441 | This study |
| pLZ5010       | agrB(ΔH74A) in pRN6441 | This study |
| pLZ5011       | agrB(ΔS81A) in pRN6441 | This study |
| pWP1002       | S. intermedius promoter P_{gfp} fusion in pRN5543 | This study |
| pWP1003       | gfp gene in pRN5543 | This study |
| pWP1004       | S. intermedius promoter P3-blaZ fusion in pRN5543 | 20      |
| pWP1013       | T7 epitope-agrD-Si-His_n (TD-SiH) in pRN5548 | 20      |
| pWP1014       | agr-B-Si and TD-SiH in pRN5548 | 20      |
| pWP1015       | agrB-Si(H77A) in pWP1102 | This study |
| pWP1110       | agrB(ΔS81A) in pWP1104 | This study |
| pWP1111       | agrB-Si(D5A) in pWP1104 | This study |
| pWP1112       | agrB-Si(N20A) in pWP1104 | This study |
| pWP1113       | agrB-Si(H23A) in pWP1104 | This study |
| pWP1114       | agrB-Si(K30A) in pWP1104 | This study |
| pWP1115       | agrB-Si(Q34A) in pWP1104 | This study |
| pWP1116       | agrB-Si(N38A) in pWP1104 | This study |
| pWP1117       | agrB-Si(K42A) in pWP1104 | This study |
| pWP1118       | agrB-Si(H77A) in pWP1104 | This study |
| pWP1119       | agrB-Si(S81A) in pWP1104 | This study |
| pWP1120       | agrB-Si(C84A) in pWP1104 | This study |
| pWP1201       | T7 epitope-agrD-Si-His_n (TD-SiH) in pET11a | This study |
| pWP1202       | agrB-Si-TD-SiH in pET11a | This study |
2172 in agr-1; changed nt, underlined; PsfI site, italic) and GJ number 45 (for pLZ2004). pLZ2004 was constructed the same way as plLZ2002 except the primer pairs used were Lz number 22 and GJ number 44, and Lz number 23 and GJ number 45, and the PCR products were digested with both PstI and NcoI. pLZ2008 was constructed by ligating two NcoI-digested PCR products amplified from pLZ2004 using primers Lz number 24 5'-TTGGTGTATATGGAATGATTACTACT-3' (in agrB, nt 2021–2047 in agr-1; changed nt, underlined) and GJ number 45, and Lz number 25 5'-AAAGAAAGAAGTTCGAGTGACGAC-3' (in agrB, nt 1999–2026 in agr-1) and GJ number 44, followed by cloning a ClaI fragment of the resulting plasmid into the ClaI site of pRN6441. pLZ2009 was made by ligating a NcoI-digested PCR product generated by using oligonucleotides Lz number 21 5'-TGCAAGCACACCTTCTTCCTTCTTTGGTGT-3' (in agrB, nt 2000–2025 in agr-1; changed nt, underlined) and GJ number 45 as primers and pLZ2004 as a template, and an NcoI/BsiHKAI (the BsiHKAI site was blunted with T4 DNA polymerase) DNA fragment of pLZ2004, followed by cloning a ClaI fragment of the resulting plasmid into the ClaI site of pRN6441. To make pLZ5010 to pLZ5012, a HgaII/NcoI-digested PCR product amplified from pLZ2004 was ligated with an appropriate HgaII/NcoI fragment of plLZ2004, and a ClaI fragment carrying the mutated agrB was then cloned into the ClaI site of pRN6441. The primer pairs used were H74A, 5'-CCAGTCACCGCCACTTCCCTTC-3' (P3 promoter region), and SINT11, 5'-CGCCGCTCCCTCTCTTTGTTT-3' (P2 promoter region), as primers, and chromosomal DNA as a template. The PCR product was cloned into an E. coli cloning vector pGEM-T (Promega). The resulting plasmid pGEM-T-P2P3 was digested with SpeI and PsfI, and ligated to a XbaI/PstI DNA fragment prepared from pRN6441-mlp, which contained the genes for methicillin resistance from S. aureus cells expressing AgrB or AgrD were mixed, and the mixtures were snap-frozen in liquid nitrogen followed by thawing at room temperature or at 4 °C three times to fuse the membranes. After incubation at 37 °C, the mixtures were centrifuged at 100,000 × g for 60 min at 4 °C. The supernatants were used for AIP activity assays, and the membrane pellets were suspended in 1× T7 bind/wash buffer (Novagen) plus 2% sodium dodecyl sulfate (SDS), and sonicated (1×) with a sonicator (11). After centrifugation at 12,000 rpm for 20 min, the supernatants were used for the AIP activity assays. Heat and SDS-PAGE sample buffer (25).

**Endopeptidase Activity of AgrB**

**Identification of AgrD Processing Intermediates**—In a previous report (22), we showed that the S. aureus AgrB was involved in the proteolytic cleavage of AgrD to generate mature AIP. However, the identity of the potential AgrD processing intermediate(s) could not be determined because the AgrD used in that study was His6 double-tagged at both the N and C termini, so the processing products containing either the N-terminal or the C-terminal portion of AgrD were not distinguishable. Subsequently, we made a new S. aureus AgrD in which a T7 tag was fused at its N terminus and a His6 tag at its C terminus (TLDH) (19). This double-tagged AgrD was used in this study to identify the possible AgrD processing intermediate(s) by AgrB in S. aureus. In the absence of AgrB, no mature site-directed mutagenesis method according to the manufacturer’s instructions (Stratagene), resulting in plasmids pWP111 to pWP1120 (Table). Plasmid pWP1105 was constructed by cloning an XbaI DNA fragment containing the H77A mutation in agrB-Si prepared from pWP1118 into the XbaI site of pWP1102. The nucleotide sequences of the cloned wild type and mutated genes in the constructed plasmids were confirmed by DNA sequencing.

**Membrane Vesicle Preparation**—S. aureus cells were grown in CYGP broth to 70 Klett units, and induced with 0.5 μg/ml methicillin at 37 °C for 3–5 h. Cells were harvested by centrifugation, washed with 1× succrose-sodium-maleate-MgCl2 (1 × SMM) (24), and suspended in 1× SMM containing 10 μg/ml lysostaphin. After 60 min incubation at 37 °C, protoplasts were prepared and washed with 1× SMM plus 5 mM EDTA. The protoplasts were then lysed by the addition of ice-cold buffer A (20 mM HEPES, pH 7.2, 5% glucose, 5 mM EDTA) followed by brief sonication. After centrifugation at 12,000 × g for 10 min at 4 °C to remove unlysed cells, the total cell lysates were centrifuged at 100,000 × g for 90 min at 4 °C to separate the cell membrane vesicles and the cytoplasmic fractions. The membrane vesicles were suspended in ice-cold buffer A for immediate use or snap-frozen in liquid nitrogen and stored at −80 °C until use.

**In Vitro Processing of AgrD**—Membrane fusion experiments were performed using the freeze-thaw method as described (28). Equal volumes of membrane fractions from S. aureus cells expressing AgrB or AgrD were mixed, and the mixtures were snap-frozen in liquid nitrogen followed by thawing at room temperature or at 4 °C three times to fuse the membranes. After incubation at 37 °C, the mixtures were centrifuged at 100,000 × g for 60 min at 4 °C. The supernatants were used for AIP activity assays, and the membrane pellets were suspended in 1× T7 bind/wash buffer (Novagen) plus 2% sodium dodecyl sulfate (SDS), and sonicated (1×) with a sonicator (11). After centrifugation at 12,000 rpm for 20 min, the supernatants were used for the AIP activity assays with S. intermedius reporter cells harboring plasmid pWP1004 (S. intermedia P3-bloZ) (20).

**Bacterial Strains and Plasmids**—S. aureus strains were grown in CYDP broth to 70 Klett units at 37 °C. Various concentrations of protease inhibitors were then added to the culture. After incubation at 37 °C overnight, cells were washed and suspended in 1× phosphate-buffered saline (25). Cell suspensions (100 μl) were transferred to wells of a 96-well plate (Corning Inc.). GFP was measured using the MFIX Monocline Plate Fluorometer (Dynex Technologies).

**RESULTS**

**Identification of AgrD Processing Intermediates**—In a previous report (22), we showed that the S. aureus AgrB was involved in the proteolytic cleavage of AgrD to generate mature AIP. However, the identity of the potential AgrD processing intermediate(s) could not be determined because the AgrD used in that study was His6 double-tagged at both the N and C termini, so the processing products containing either the N-terminal or the C-terminal portion of AgrD were not distinguishable. Subsequently, we made a new S. aureus AgrD in which a T7 tag was fused at its N terminus and a His6 tag at its C terminus (TLDH) (19). This double-tagged AgrD was used in this study to identify the possible AgrD processing intermediate(s) by AgrB in S. aureus. In the absence of AgrB, no mature site-directed mutagenesis method according to the manufacturer’s instructions (Stratagene), resulting in plasmids pWP111 to pWP1120 (Table). Plasmid pWP1105 was constructed by cloning an XbaI DNA fragment containing the H77A mutation in agrB-Si prepared from pWP1118 into the XbaI site of pWP1102. The nucleotide sequences of the cloned wild type and mutated genes in the constructed plasmids were confirmed by DNA sequencing.
AIP was detected from *S. aureus* cells expressing TLDH alone as determined by AIP activity assay using reporting cells (Fig. 1B), and TLDH was expressed as a single band with an apparent molecular mass of ~9 kDa corresponding approximately to the calculated molecular mass of TLDH (9,182 Da) as detected by Western blot analysis with an anti-T7 tag monoclonal antibody (data not shown). In the presence of AgrB, AIP activity was detected (Fig. 1B), and two additional bands with apparent molecular masses of ~6.5 and 5.5 kDa, respectively, were detected by Western blot analysis with anti-T7 tag antibody (Fig. 1A), but not with anti-penta-His antibody (data not shown). These two additional bands were also detected by Western blot analysis of the purified samples from *S. aureus* cells expressing both TLDH and AgrB using T7 tag antibody-agarose affinity chromatography but not nickel-nitrilotriacetic acid-agarose beads (Fig. 1C). These results indicated that the two additional bands were AgrD processing intermediate peptides B and C (Fig. 1D). Attempts to confirm the identities of these two peptides by mass spectrometry have not been successful due to the failure of obtaining enough purified materials because both peptides were membrane bound (data not shown). We have also not been able to detect peptide D both in the cytosol and the culture supernatant (data not shown), suggesting that peptide D might not be stable.

**Accessory Protein(s) for AgrB**—We clearly demonstrated that AgrB was involved in the processing of AgrD to generate mature AIP. We then asked whether AgrB alone or together with other protein(s) carried out these processes because the processing of AgrD involved two proteolytic cleavages, the thiostear (or ester) bond formation and the secretion of the mature AIP. To address this question, we first used 3,3′-dithiobis(succinimidyl propionate) as a cross-linking reagent in an attempt to identify protein(s) that might be associated with AgrB. However, no AgrB accessory proteins were found using such an approach. We reasoned that if there were additional proteins that might be involved in the processing of AgrD and the secretion of the mature AIP, it was unlikely that these proteins would exist in both *S. aureus* and *E. coli* because no AgrD homologues were identified from *E. coli* by computer analysis. Subsequently, we made two constructs in which the tagged *agrD* or *agrB* genes were cloned into an *E. coli* expression vector. One construct carried DNA sequence encoding the T7 epitope fused at the 5′ end of *agrD* and sequence encoding 6 histidine residues and a stop codon fused at the 3′ end of *agrB*. The other contained both the double-tagged *agrD* and wild type *agrB* genes. In both constructs, the genes were under the control of the inducible T7lac promoter. Whole cell lysates were prepared from *E. coli* cells expressing the double-tagged AgrD with or without AgrB, and the proteins were separated onto SDS-PAGE and detected by Western blot using an anti-T7 monoclonal antibody as a probe. As shown in Fig. 2A, a band with an apparent molecular mass of approximately 8 kDa corresponding to the calculated mass of the double-tagged AgrD (8,223 Da; peptide A* in Fig. 2C) was detected in lanes containing lysates from induced *E. coli* cells expressing the double-tagged AgrD alone, but not in lanes containing lysates from uninduced cells or control cells (cells carrying the cloning vector alone), indicating that the double-tagged AgrD were well expressed in *E. coli*. In the presence of AgrB, an additional anti-T7 antibody reactive band with an apparent molecular mass of 5.5 kDa was detected (Fig. 2A), and the size of this band (Fig. 2B, lane 1) suggested that it likely corresponds to the AgrD processing intermediate peptide B* (Fig. 2C). We note that the sizes of the double-tagged AgrD in *S. aureus* and *E. coli* were different because the length of the amino acid residues preceded the T7 epitope sequence, so the tagged AgrD and its potential processing products in *E. coli* were labeled as A*, B*, and C*, to reflect their size differences from those in *S. aureus*. Interestingly, an additional band with an apparent molecular mass of about 25 kDa was also detected. This band was only seen in lanes containing lysates from *E. coli* cells expressing both the double-tagged AgrD and AgrB (Fig. 2A). The true identity of this band remains unknown. However, we suspected that this band might represent an AgrD processing intermediate covalently bound to AgrB because AgrD was T7 tagged but not AgrB. Repeated attempts to detect the AgrD processing intermediate peptide C* and AIP activity failed. It is possible that in *E. coli* the peptide C* generated is degraded rapidly and the mature AIP that is presumably secreted into the periplasm is either degraded or inactivated by the destruction of the thiostear bond because the thiostear bond is absolutely required for its *agr* activating activity (5, 12). It is equally possible that in *E. coli* AgrB can only proteolytically cleave AgrD at its C-terminal processing site. Taken together, although we could not rule out the possibility that other pro-

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**Fig. 1.** The AgrD processing intermediates in *S. aureus*. *S. aureus* cells were grown, induced with methicillin, and samples were taken at various induction times as indicated. After centrifugation, cell pellets were used to prepare total cell lysates followed by Western blot analysis and the culture supernatants were subjected to AIP activity assays. Cells carrying the cloning vector were used as controls. A, Western blot hybridization of total cell lysates separated by 16% polyacrylamide Tris/Tricine SDS-PAGE and detected by Western blot using an anti-T7 tag monoclonal antibody. B, AIP activities produced from cells expressing TLDH in the presence or absence of AgrB. C, Western blot analyses of purified TLDH and its processing products. *S. aureus* cells expressing AgrB or TLDH, or both were grown and induced for 5 h. Total cell lysates were prepared and proteins were then purified either by anti-T7 monoclonal antibody-agarose beads or by nickel-nitrilotriacetic acid (Ni-NTA) bead chromatography. The purified proteins were separated by 21% polyacrylamide Tris/Tricine SDS-PAGE followed by Western blot analysis using an anti-T7 monoclonal antibody as a probe. D, a schematic diagram of TLDH and its possible processing intermediates. The tags and the AIP region are indicated. The intact TLDH and its processing intermediates and their predicted molecular weights are shown (A–D).

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teins were involved in the processing of AgrD to generate mature AIP, we confidently concluded that the cleavage of AgrD at its C-terminal processing site was carried out by AgrB alone.

**AgrB Is a Putative Endopeptidase**—We used several protease inhibitors in an attempt to block the mature AIP production to determine whether AgrB had peptidase activity. *S. aureus* cells expressing either double-tagged AgrD (TLDH) or TLDH plus AgrB were grown in CYGP broth to ~70 Klett units at 37 °C. Methylcellulose (0.5 µg/ml) together with 10 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64, cysteine protease inhibitor), 50 µM [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-Leu (Bestatin, aminopeptidase B, and leucine aminopeptidase inhibitor), or 5 µM isovaleryl-Val-Val-4-amino-3-hydroxy-6-methylheptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid (peptatin A, aspartic protease inhibitor) were then added. After incubation at 37 °C for 4 h, the cultures were centrifuged. The supernatants (conditioned media) were used for AIP activity assays and the cell pellets were subjected to Western blot analysis using an anti-T7 monoclonal antibody as a probe. AEBSF slightly inhibited cell growth and significantly inhibited the production of AIP (data not shown). In contrast, other inhibitors tested had no effects on either cell growth or AIP production (data not shown). However, AEBSF also inhibited the β-lactamase activities (data not shown) that were used as an indicator for AIP activity (5, 9). Subsequently, we made two new constructs: pWP1003 contained a promoterless green fluorescent protein (gfp) gene, and pWP1002 had the P3′ promoter fused with the gfp gene. These plasmids were then transformed into the wild type *S. aureus* RN6390B. Cells were grown in CYGP media to ~40 Klett units, various concentrations of AEBSF (1.25, 2.5, 5, 10 mM) or E-64 (15, 30, 66, or 100 µM) were then added. After 15 h incubation at 37 °C, the cultures were subjected to GFP assays. E-64 had no effect on the production of GFP (data not shown). However, AEBSF (10 µM) significantly inhibited GFP production in cells carrying the P3′-gfp construct, whereas cells containing the promoterless gfp had no detectable GFP produced (data not shown). These results suggested that AgrB might be a putative protease.

To clearly demonstrate that the processing of AgrD by AgrB can indeed be inhibited by protease inhibitor(s), an in vitro AgrD processing system will be needed. Attempts to use purified double-tagged AgrD (TLDH) and purified membrane vesicles from cells expressing AgrB to develop such a system failed because of the fact that both AgrB and AgrD were membrane proteins (19, 22) and the purified AgrD might not be able to integrate into the membrane properly (data not shown). So the membrane fusion method using purified membrane vesicles from *S. aureus* cells expressing either TLDH or AgrB-His₆ was employed. Equal volumes of the purified membrane vesicles were mixed and the mixtures were then frozen and thawed three times in liquid nitrogen and at room temperature according to the method described (28, 30). We note that the majority of the fused membrane vesicles by the freeze-thaw method are sealed (30). After incubation, the mixtures were separated onto SDS-PAGE and detected by Western blot hybridization with an anti-T7 monoclonal antibody as a probe. Only TLDH proteins were detected in lanes with the mixture of TLDH containing membrane vesicles plus control membrane vesicles prepared from *S. aureus* agr-null mutant GJ2035 carrying the cloning vector pRN5548. In the presence of AgrB containing membrane vesicles, an additional band was detected (Fig. 3A). This band had an apparent molecular mass of 6.5 kDa corresponding to AgrD processing intermediate peptide B (Fig. 1D). We have not been able to detect either the AgrD processing intermediate peptide C or AIP activity from this reaction mixture (data not shown). It is possible that further processing of peptide B to generate peptide C is significantly less efficient in vitro so that peptide C and the AIP generated are not detectable by our assay methods, or that the processing of peptide B, the thioester bond formation, and the secretion of the mature AIP are coupled so that the secretion of the mature AIP cannot be accomplished by our current method. It is also possible that further processing of peptide B requires additional factor(s) even though the identification of AgrB accessory protein(s) have not be successful and the addition of cell extracts from...
S. aureus agr-null mutant RN6691 to our in vitro system had no effects (data not shown).

The newly developed in vitro method was used to determine the effects of protease inhibitors on AgrD processing by AgrB. AEBSF (10 mM) totally inhibited the generation of AgrD processing intermediate fragment B in lanes containing a mixture of TDLDH plus AgrB membranes (Fig. 3B). Two additional protease inhibitors, TPCK (1.2 mM) and n-Val-Phe-Lys chloromethyl ketone (VFK-CMK) (1.2 mM), had the same effects as AEBSF (Fig. 3B). We note that AEBSF, TPCK, and VFK-CMK are serine protease inhibitors. However, both AEBSF and TPCK are also reported to inhibit certain cysteine proteases (31, 32) (Calbiochem’s instructions), and both VFK-CMK and TPCK have the same active group, chloromethyl ketone (CMK). In contrast, bestatin (0.1 mM) and pepstatin A were tested in parallel with CMK (10 mM) and TPCK (1.2 mM) and AEBSF (10 mM) (Fig. 3A). The effects of these inhibitors on AgrD processing are shown in Fig. 3. These results indicated that the N-terminal region of AgrD-Si was replaced by that of S. aureus group I AgrB. The chimeric AgrB could process AgrD-Si as determined by AIP-Si activation assays using S. intermedius reporter cells (Fig. 5A), AIP-Si inhibition assays using S. aureus group II reporter cells (Fig. 5B), and Western blot hybridization analysis using an anti-T7 monoclonal antibody as a probe (Fig. 5C). We note that AgrD-Si used in these experiments was double-tagged with a T7 tag at the N terminus and a His6 tag at the C terminus, which would facilitate the detection of AgrD-Si as well as its potential processing intermediate(s) by Western blot analyses using commercially available antibodies, and the addition of these tags had no effects on AgrD-Si processing and AIP-Si secretion (data not shown). As shown in Fig. 5C, a band with a molecular mass of ~9 kDa corresponding to the calculated molecular mass of the double-tagged AgrD-Si (9,205 daltons) was detected in the lane containing a lysate of cells lacking agrB-Si and producing the tagged AgrD-Si. In the presence of either the wild type AgrB-Si or the chimeric AgrB, an anti-T7 antibody responding band with a molecular mass of ~6 kDa was detected. Whether this band was an AgrD-Si processing intermediate or a final product has not yet been determined. These results suggested that the conserved residues in the N-terminal region of AgrB might be critical for its activity. However, subsequent site-directed mutagenesis analyses of these conserved residues revealed that these mutants (D5A, N20A, H23A, R30A, Q34A, N39A, and K42A) had no significant effects on the AgrB-Si activity (Fig. 5). These results indicated that the N-terminal region of AgrB was not involved in the proteolytic cleavage of AgrD. It is possible that this region is required for the initial binding of AgrD to AgrB that has not yet been determined experimentally.

From the AgrB alignment analysis shown in Fig. 4A, we noticed that three residues (histidine 77, serine 81, and cysteine 84) were identical among AgrBs. It is possible that these amino acids are putative catalytic residues that are involved in the proteolytic processing of AgrD. Accordingly, we replaced these residues with alanines in S. aureus group I AgrB. Interestingly, changing the histidine residue at position 77 (H77) or
The cysteine residue at position 84 (C84) totally eliminated its ability to process AgrD as shown by AIP activity assays (Fig. 6, A and B), Western blot hybridization analysis of total cell lysates (Fig. 6C), and in vitro AgrD processing by membrane fusions (Fig. 6D), whereas mutation of serine 81 (S81) had little effect on the function of AgrB (Fig. 6). Additionally, changing histidine 74 or serine 80 to an alanine residue had no significant effect on AgrB activity. Of note, the total cell lysates and
the in vitro membrane fusion mixtures were also subjected to
Western blot analyses using an anti-penta-His monoclonal an-
tibody as a probe. As shown in Fig. 6, the AgrB-His6 or mutant
proteins were expressed approximately equally from cells used
for cell lysate preparations (Fig. 6E), and membrane vesicles
containing equal amounts of AgrB-His6 proteins were used in
the in vitro experiments (Fig. 6F). These results indicated that
both His77 and Cys84 were required for proteolytic processing of
AgrD. Similar results were obtained using AgrB-Si H77A,
S81A, and C84A mutants (data not shown).

To eliminate the possibility that cysteine84 was involved in
the polymerization of AgrB, we made a new construct
(pWP1105) that contained a copy of the wild type agrD-Si gene,
a copy of a mutated gene (agrD-Si(H77A)) and the agrD-Si
gene. As shown in Fig. 7, S. aureus cells expressing AgrD-Si
and both wild type and mutant AgrB-Si produced mature
AIP-Si in an amount comparable with that of cells expressing
AgrD-Si and the wild type AgrB-Si only. Similarly, no signifi-
cantly different AIPs were produced from S. aureus cells har-
boring plasmid carrying the S. aureus group I wild type of agrB
and agrD genes with or without a copy of the agrB(C84S) gene
(data not shown).

**DISCUSSION**

Quorum sensing systems in Gram-positive bacteria pre-
dominately use peptides as the signals that are the ligands of
membrane receptors (histidine kinases) or that interact with
other protein(s) which in turn regulates the activity of the
response regulator of the signal transduction pathways. The
peptide signal molecules identified so far are processed from
Competence (35, 36)). Another one, which has only been de-
tected (33, 34)), or by a presumed peptidase domain of an ABC
transporter (e.g. the Lactococcus lactis Nis system that involves the production of lantibiotic nisin (32)), or by a presumed peptidase domain of an ABC transporter (e.g. the Streptococcus pneumoniae Com system that regulates the competence (35, 36)). Another one, which has only been described for the Phr system of Bacillus subtilis that is required for competence and sporulation (37–40), involves the cleavage of a leader peptide during secretion via the SecA-dependent transport system. The secreted peptide, a processing intermediate, is subjected to further proteolytic cleavage extracellularly by a peptidase that has not yet been identified and the mature active peptide is then transported into the cytoplasm by the oligopeptide-permease and negatively regulates the Rap-phosphatases activities.

The staphylococcal Agr quorum sensing system has unique characteristics among the peptide-mediated quorum sensing systems studied so far: first, its signal molecule is a thiolactone or lactone molecule; second, the processing of the AgrD precursor involves two proteolytic cleavages and the AIP sequence is in the middle, which is different from other systems in that the precursors are subjected to only one cleavage and the C-terminal portions are served as the signal molecules; and third, the AgrB protein, which has been proposed as transporter for the secretion of the mature AIP (22), does not have the characteristics of an ABC transporter.

Previously, we showed that AgrD was proteolytically cleaved in the presence of the AgrB protein, however, it was not clear whether the reaction was carried out by AgrB or AgrB-associated accessory protein(s) (22). In this study, we showed that AgrB was a putative endopeptidase whose activity could be inhibited by protease inhibitors. We clearly demonstrated that AgrB was involved in the cleavage of AgrD at the C-terminal processing site and identified two amino acid residues (histidine 77 and cysteine 84) in both S. aureus group I AgrB and S. intermedium AgrB that were required for their endopeptidase activities. It is possible that these two amino acids are the putative catalytic residues that form a catalytic center. We note that these two amino acids are at the same positions and conserved among all AgrBs identified so far, and are located juxtapositionally on the inner surface of the cytoplasmic membrane (Fig. 4A) (22), suggesting that the putative catalytic activities are conserved among these AgrBs. Cysteine and histidine are the two most common conserved residues that form the catalytic centers of cysteine peptidases (41), it is possible that AgrB is a putative cysteine endopeptidase although it has no sequence homologies with any known cysteine peptidases. However, why AgrB activity was not sensitive to cysteine protease inhibitors we tested is unknown. One possibility is that the cysteine protease inhibitors cannot reach the AgrB catalytic center because of their membrane permeability, or other amino acid residues surround the AgrB catalytic center and prevent these inhibitors from interacting with their targeted amino acids. Another is that those protease inhibitors cannot reach their inhibitory concentrations in the cytosol. The reason that certain serine protease inhibitors could inhibit AgrB peptidase activity might be because of their inhibitory specificities. We note that there is only one conserved serine residue in AgrBs and replacement of that serine to an alanine residue had no effect on AgrB activity, a finding strongly suggesting that AgrB was not a serine peptidase.

Other Gram-positive bacteria may use a similar mechanism with that of AgrD processing by AgrB to produce mature auto-inducing peptide from precursor proteins. It is interesting to note that the recently identified Fsr quorum sensing system in Enterococcus faecalis (42) is remarkably similar to that of the Agr system. The FsrB (AgrB)s, an AgrB homologue, has been proposed to be the protein responsible for the production of an 11-amino acid residue lactone molecule (gelatinase biosynthesis-activating pheromone) that is likely to be the ligand for FsrC of the FsrC/FsrA signal transduction system (42–44). However, no agrD homologous gene is found in the fsr operon, instead, the gelatinase biosynthesis-activating pheromone sequence is in the C-terminal region of FsrB. It has been proposed that the possible mechanisms of processing and secretion of gelatinase biosynthesis-activating pheromone are similar to those of the AgrB/AgrD (45).

A data base search using S. aureus AgrB as a query sequence revealed the existence of AgrB homologues in other bacteria, including Bacillus cereus G9241 (YP_00237848) (46), Bacillus halodurans (Q9K794) (47), Clostridium acetobutylicum (NCBI accession number Q9TMW3) (48), Clostridium beijerincki (QTWU3), Clostridium perfringens (Q8XM19, Q8XK42, and BAB8057) (49), Lactobacillus plantarum (Q8SS59 and CAD65661) (50), Listeria innocua (Q92FR2, AB1438 and CAC95274) (51), and Listeria monocytesgenes (Q5YAR6, EAL10116, EAL07382, AI1080, and CAC98263) (51, 52). Comparison of all these sequences and FsrB (Q8RQG4) (44) with the AgrBs showed that histidine and cysteine were absolutely conserved (alignment not shown), suggesting that all these proteins might be putative endopeptidases, and the Agr quorum sensing system homologues might exist in these bacteria.

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