Accessory Gene Regulator Locus of *Staphylococcus intermedius*

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The accessory gene regulator (*agr*) locus, a candidate system for the regulation of the production of virulence factors in *Staphylococcus intermedius*, has been characterized. Using PCR-based genome walking, we have obtained the first complete sequence (3,436 bp) of the accessory gene regulator (*agr*) gene in this organism. Sequence analysis of the *agr* gene has identified five open reading frames (ORFs), *agrB*, *agrD*, *agrC*, *agrA*, and *hld*. The translated ORF contained amino acid motifs characteristic of the response regulator and histidine protein kinase signal transducer of the classic two-component regulatory system. Sequencing of the *agrD* PCR products amplified from DNA from 20 different isolates has facilitated detection of genetic variation in the putative autoinducing peptide (AIP) within the *agr* gene of *S. intermedius*, revealing the presence of at least three *agr* specificity groups within this species. Classification of the *agr* gene from *S. intermedius* was supported by phylogenetic analysis. Real-time PCR also revealed that the effector molecule of the *agr* system, RNAIII, was regulated in an autocrine manner in *S. intermedius* and demonstrated positive correlation with the temporal gene expression patterns of *luk* and *entC*. Transcription of RNAIII was also dependent on self secreted cues. Cyclic self and nonself peptides were synthesized on the basis of the novel AIPs produced by *S. intermedius*, which lack the cysteine necessary to form the thiolactone ring in analogous peptides from *Staphylococcus aureus* and *Staphylococcus epidermidis*. Experiments with these synthetic cyclic peptides indicated that self peptides led to up-regulation of RNAIII—findings in support of the assumption that activation of the *agr* gene is initiated by growth- and species-specific factors generated during bacterial growth.

Staphylococcus intermedius, first described in 1976 (10), is the most common cause of skin infection in dogs, with canine pyoderma being caused almost exclusively by *S. intermedius* (31). *S. intermedius* is also frequently isolated from dogs with otitis media and externa (3, 4, 28) and is commonly associated with seborrhoea, a cutaneous scaling disorder in dogs. Little is known, however, about the pathogenesis of *S. intermedius*. The results of studies investigating the production of virulence factors are unclear, and no distinct differences in the nature of the toxins produced by isolates from healthy dogs and dogs with pyoderma have been found (1). Thus far, there are differing opinions as to the underlying causes of canine pyoderma. Ihrke suggested factors that predispose dogs to staphylococcal pyoderma infections were due to a wide variety of underlying diseases, such as allergy, metabolic diseases, disruption of normal skin flora, immunodeficiency, and endoparasitic infestation (12), whereas according to Greene and Lammler, host-bacterium interactions and the immune status of the host appeared to be the factors most crucial in determining the outcome of infection (9). Many of the factors believed to be involved in *S. intermedius* virulence have also not been well characterized, and proof of their association with disease pathogenesis is often lacking. It is thus difficult for veterinary clinicians and microbiologists to determine the clinical significance of many *S. intermedius* isolates.

S. intermedius produces a diverse arsenal of virulence factors involving a large number of cell-bound and extracellular proteins (6, 11, 29, 32), which may be likely contributory factors that predispose dogs to staphylococcal pyoderma. The virulent components and products are thought to share overlapping roles, acting either in concert or alone, and may convert host tissue components into nutrients required for bacterial growth. On the basis of studies performed with corresponding virulence factors in *Staphylococcus aureus*, considerable knowledge is available about their contribution to the development of infection in humans (21). Less is known about the virulence factors of *S. intermedius* and their relative importance in canine pyoderma.

In the human pathogen *S. aureus*, the production of virulence factors is known to be controlled, in part, by the accessory gene regulator system. One of a number of two-component regulatory systems identified thus far, the *agr* system has received much attention owing to it being assigned, historically, a central role in the model of *S. aureus* pathogenesis. It is recognized as a quorum-sensing gene cluster that up-regulates production of secreted virulence factors and down-regulates production of cell-associated virulence factors in a cell density-dependent manner (15, 19, 25). When the staphylococci are in lag phase, it is thought that staphylococci initiate infection by synthesizing surface proteins. Once colonization is established, the bacteria multiply and enter exponential phase, activating a density-sensing mechanism that stimulates toxic exoprotein production, thereby enabling them to spread to new sites to prevent overcrowding (30). Synthesis of essential factors only as and when required is important from an economic perspective, as it avoids overwhelming the organism metabolically with unnecessary energy expenditure.

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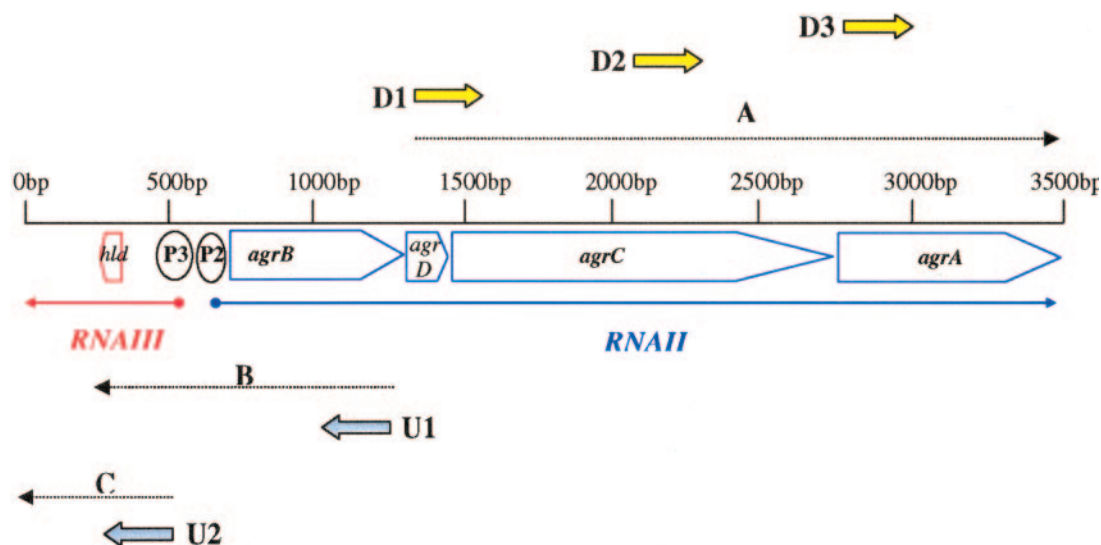
FIG. 1. Schematic representation of the *agr* locus in *S. intermedius*. The extremities of *agr*, which consists of two divergent transcripts, RNAII and RNAIII, were amplified and sequenced using genome walking. For downstream genome walking, the D1 primer was initially designed, allowing amplification of the A fragment. The A fragment was partially sequenced, and from the sequences obtained, the D2 primer was designed and synthesized. Further downstream sequencing of the A fragment was performed using the D2 primer. Sequential primer design (D3) and sequencing were repeated until the whole downstream *agr* sequence in *S. intermedius* was elucidated. For upstream genome walking, PCR amplification with the U1 primer produced the B fragment. From the B fragment, the *S. intermedius*-specific U2 primer was designed, enabling sequencing of the C fragment. The *agrB*, *agrD*, *agrC*, and *agrA* ORFs are transcribed in one direction; while the RNAIII is transcribed in the opposite direction. The individual ORFs are represented by block arrows. The size of each ORF can be seen by the base pair ruler in the middle of the figure.

S. intermedius produces many toxins comparable to those of *S. aureus*, so we hypothesized that it is likely that the production of toxins in the former is under the regulation of a similar global regulator. To determine whether this is so, here we have ascertained the presence of the *agr* locus and analyzed its sequence. Our starting point was the *agrD* gene present in each strain of a collection of 20 *S. intermedius* strains isolated from cases of canine pyoderma. The elucidation of the *agr* locus facilitated assessment of its regulation during the growth of *S. intermedius*. In *S. aureus*, mutation and deletion analyses performed suggested that RNAIII itself, rather than any translation product, is the effector of exoprotein gene regulation (25). We have thereby quantified RNAIII, the effector molecule of *agr*, together with *luk* and *entC*, encoding leukotoxin and enterotoxin C, respectively, using real-time quantitative PCR. We have also synthesized two novel cyclic autoinducing peptides (AIPs), shown to be found in *S. intermedius* (17), but not in *S. aureus* or *Staphylococcus epidermidis*, and used these in functional assays monitoring RNAIII production.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty clinical isolates of *S. intermedius* were collected from the skin of dogs with pyoderma at the Royal Veterinary College. Long-term storage of the isolates was maintained in 20% glycerol in brain heart infusion broth at −70°C, and cultures for routine use were maintained on sheep blood agar plates. Isolates were identified as *S. intermedius* according to their colony morphology, biochemical reactions (production of coagulase and DNase), and on the basis of the analytical profile index (API) (bioMerieux, France).

PCR and sequencing. The collection of 20 *S. intermedius* isolates was screened by direct colony PCR. It was found that the initial hot start of the PCR was sufficient to break down bacterial cell walls from a pinprick colony of *S. intermedius*, allowing these cells to be used as the direct source of DNA. We used the following primers: 5′-TTCCAATCAATCGGTACT-3′ and 5′-TTCCTCCTTCT AACAACTC-3′; their design was based on regions of the partial *agr* sequence derived from a pigeon isolate of *S. intermedius* (GenBank accession number AF346723). PCR was carried out in the GeneAmp PCR system 9700 thermocycler (Perkin-Elmer Biosystems, Foster City, California). Amplification was performed in a final volume of 50 μl containing dATP, dCTP, dGTP, and dTTP (each at a concentration of 200 μM), 10 μM of the corresponding primer, 5 μl of 10× Expand high-fidelity buffer (Roche, Nutley, NJ), and 2.6 U of Expand high-fidelity enzyme mix (Roche). The cycling parameters for PCR were as follows: (i) an initial denaturation step of 2 min at 94°C; (ii) 35 cycles, with 1 cycle consisting of 15 s at 94°C, 30 s at 45°C, and 60 s at 72°C; and (iii) a final extension step of 7 min at 72°C. The amplified fragments were cloned into the pGEM-T Easy vector (Promega, Madison, Wis.). Recombinant DNA molecules were transformed into electrocompetent *Escherichia coli* JM109 cells and sequenced (MWG-Biotech, Ebersberg, Germany).

Genome walking. Genome walking was initiated from the amplified and sequenced *agrD* DNA fragment using the Universal Genome-Walker kit (BD Biosciences Clontech, Palo Alto, CA). Total genomic DNA of *S. intermedius* strain 3 was isolated with Genomic-Tip 100/G (QIAGEN, Crawley, United Kingdom) and digested with DraI, EcoRV, PvuII, and StuI to obtain blunt ends. An aliquot of each DNA sample was run on a 0.7% agarose gel to ascertain quality. After purification, the fragments were ligated to a Genome-Walker adapter provided by the manufacturer (31). The Genome-Walker protocol consists of a series of PCR amplifications, starting initially with the adapter primers (AP1 or AP2) and two nested gene-specific primers designed on the basis of the *agrD* sequence of *S. intermedius* (see above), D1 (5′-CCAATCAATCGGT ACTTTTGCTAGAATCCCTATTAGTACTGGA-3′) and U1 (5′-AGATGAG CCAGAAATCCCTGCCGAGTTGTTAGAAGAGGAC-3′). Sequence extension occurred through further walks using novel gene-specific primers, and the primers were designed on the basis of initial sequence data obtained from previous steps. Care was taken to ensure that the sequences overlapped to some degree. Downstream chromosomal walking was continued with the following primers: D2 (5′-CGTGAAGATGATTTAGAAGGTTTGAAGCAA-3′) and D3 (5′-GCTACCAATGACCCTTATGCTATCTTAGAAACTTCT-3′). The upstream region of *agrD* was analyzed in a similar way using primer U2 (5′-CAA CAACTTACGCACTGGTTAACACAAGGAC-3′). Each amplification was performed in a final volume of 50 μl containing 5 μl 10× genomic PCR buffer, 1.1 mM magnesium acetate, 10 mM 50× deoxynucleoside triphosphate (dNTP)

TABLE 1. Alignment and comparison of the primary sequences of *agrD* from staphylococci

Bacterial source (GenBank no.)	Primary sequence
S. aureus	
Group I (x52543)	MNTLFNLFFD FITGILKN**IG** NIAA<u>YSTCDF IM</u>**DE**VEV**P**K**E L**TQLHE
Group II (AP003135)	MNTLVNMFFD FIIKLAKA**IG** IVGG<u>VNACSS LF</u>**DE**PKV**P**A**E L**TNLYDK
Group III (AF004829)	MKKLLNKVIE LLVDFFNS**IG** YRAA<u>YINCDF LL</u>**DE**AEV**P**K**E L**TQLHE
Group IV (AF288215)	MNTLYKSFFD FITGVLKN**IG** NVAS<u>YSTCYF IM</u>**DE**VEI**P**K**E L**TQL
S. lugdunensis (AF173933).....................................	MNLLSGLFTK GISAIFEF**IG** NFSAQ<u>DICNA YF</u>**DE**PEV**P**Q**E L**IDLQRKQLIESV
S. epidermidis (Z49220)...	MNLLGGLLLK LFSNFMAV**IG** NAAK<u>YNPCAS YL</u>**DE**PQV**P**E**E L**TKLDE
S. intermedius pigeon (AF346723)...............................	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPTSTG FF</u>**DE**PEI**P**A**E L**LEEEK
S. intermedius canine strains	
S3...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEEDK
S11...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**A**E L**LEE
S24...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**LEI**P**A**E L**LEE
S27...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S31...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S33...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S36...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S38...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEE
S40...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEE
S55...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**A**E L**LEE
S64...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEE
S65...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**A**E L**FEE
S69...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEE
S73...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S77...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S81...	MR-ILEVLFN LITNLFQS**IG** TFA<u>RIPISTG FF</u>**DE**PEI**P**A**E L**LEE
S83...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S85...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S88...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE
S95...	MR-ILEVLFN LITNLFQS**IG** TFA<u>KIPTSTG FF</u>**DE**PEI**P**E**E L**LEE

[a] Primary sequences of *agrD* from *S. aureus* group I (GenBank accession number X52543), *S. aureus* group II (AP003135), *S. aureus* group III (AP004829), *S. aureus* group IV (AF288215), *S. lugdunensis* (AF173933), *S. epidermidis* (Z49220), *S. intermedius* of pigeon origin (AF346723), and isolates of *S. intermedius* of canine origin. Conserved amino acid residues in all organisms are in boldface type. Sequences of autoinducing peptides are underlined. Gaps introduced to maximize alignment are indicated by dashes.

mix, 1 μl 50× Advantage genomic polymerase mix (BD Biosciences), and 10 μM of the corresponding primers. A typical PCR cycle consisted of 7 cycles of 94°C for 2 s and 72°C for 3 min, followed by 32 cycles of 94°C for 2 s and 67°C for 3 min, and a final elongation step at 67°C for 4 min. To facilitate PCR amplification of sequences that proved difficult to amplify, we used the Advantage GC genomic polymerase (BD Biosciences).

PCR products from genome walking were purified using the QIAquick gel extraction kit (QIAGEN) and directly sequenced with both primers (adapter and *S. intermedius* specific). For PCR amplicons that were longer than 0.6 kb, sequencing was performed via primer walking, involving repeated cycles of custom oligonucleotide synthesis and direct sequencing. Sequences obtained were confirmed by comparison of independent PCR experiments with different primers and genomic DNA. Alignments of amino acid sequences from the individual protein-coding genes were realized by using the ClustalW algorithm (http://www.ebi.ac.uk/clustalw) and default gap penalties (28). Regions of sequence that were difficult to align were removed from the data file and the sequences realigned. Ambiguous alignment regions, such as the N and C termini, were excluded from the alignments. Overlapping nucleotide sequences of DNA generated were manually aligned to form a contiguous *agr* operon and assessed for complete open reading frames (ORFs). DNA and protein sequence similarity searches were performed by the BLASTN and BLASTP (National Center for Biotechnology Information server). Conserved domains in each ORFs were analyzed using reverse position-specific BLAST (rpsBLAST) (v1.65).

Transmembrane topology prediction. The transmembrane topology of the various ORFs of the *S. intermedius agr* gene was analyzed using ConPredII (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/), a program based on a consensus approach that combines the results of several prediction methods, such as KKD, TMpred, TopPredII, DAS, TMAP, MEMSAT 1.8, SOSUI, TMHMM 2.0, and HMMTOP 2.0 (2).

Assessment of the regulation of leukotoxin, enterotoxin, and RNAIII tran-scription in *S. intermedius*. To examine growth phase-dependent expression, 2-ml cultures of *S. intermedius* (strain 3) were grown overnight with aeration in brain heart infusion broth at 37°C. Cells were then inoculated from the overnight culture to an initial optical density at 600 nm (OD_{600}) of 0.05 and grown with shaking to the desired phase of growth, e.g., early exponential (OD_{600} of 0.5, 1 h), mid-exponential (OD_{600} of 4.5; 4 h), or post-exponential phase (OD_{600} of 8.0; 8 h). The various growth phases were determined in preliminary experiments (not shown). Growth was monitored turbidimetrically with a UV-160A spectrophotometer (Shimadzu) read at 600 nm. The results of growth phase effects were standardized for the number of bacteria, so that the increase in the total amount of protein is not simply a consequence of increasing number of bacterial cells. At indicated times, samples containing equal numbers of cells were immediately stabilized by the addition of 2 volumes of Bacterial Protect reagent (QIAGEN). The bacterial cells were subsequently harvested after 10 min by centrifugation at $13,000 \times g$ for 10 min at 4°C, prior to RNA extraction.

Characterization of the activating factor for *luk*, *entC*, and RNAIII expression in *S. intermedius*. Cultures of *S. intermedius* strains 3 and 27 and *S. aureus* strain J3945 were inoculated from 1-day-old single colonies on sheep blood agar plates into 2 ml brain heart infusion broth. The cultures were grown overnight at 37°C with orbital shaking for 15 h. To synchronize cultures to an active mode of growth and to eliminate potential quorum signals that may have accumulated, 0.5 ml of overnight culture was centrifuged at $3,000 \times g$ for 5 min at 4°C. After the supernatant was discarded and the bacteria were resuspended in fresh prewarmed medium (50 ml), they were grown at 37°C with shaking. Aliquots (10 ml) were harvested at intervals of 1, 4, and 8 h corresponding to the early, mid-, and post-exponential phases of bacterial growth. Bacterial cells were removed by centrifugation at $13,000 \times g$ for 15 min at 4°C, and thereafter, the supernatants were filter sterilized through a 0.22-μm-pore-size filter (Millipore). All the supernatant filtrates obtained were lyophilized and stored at −80°C until use as a source of putative AIP.
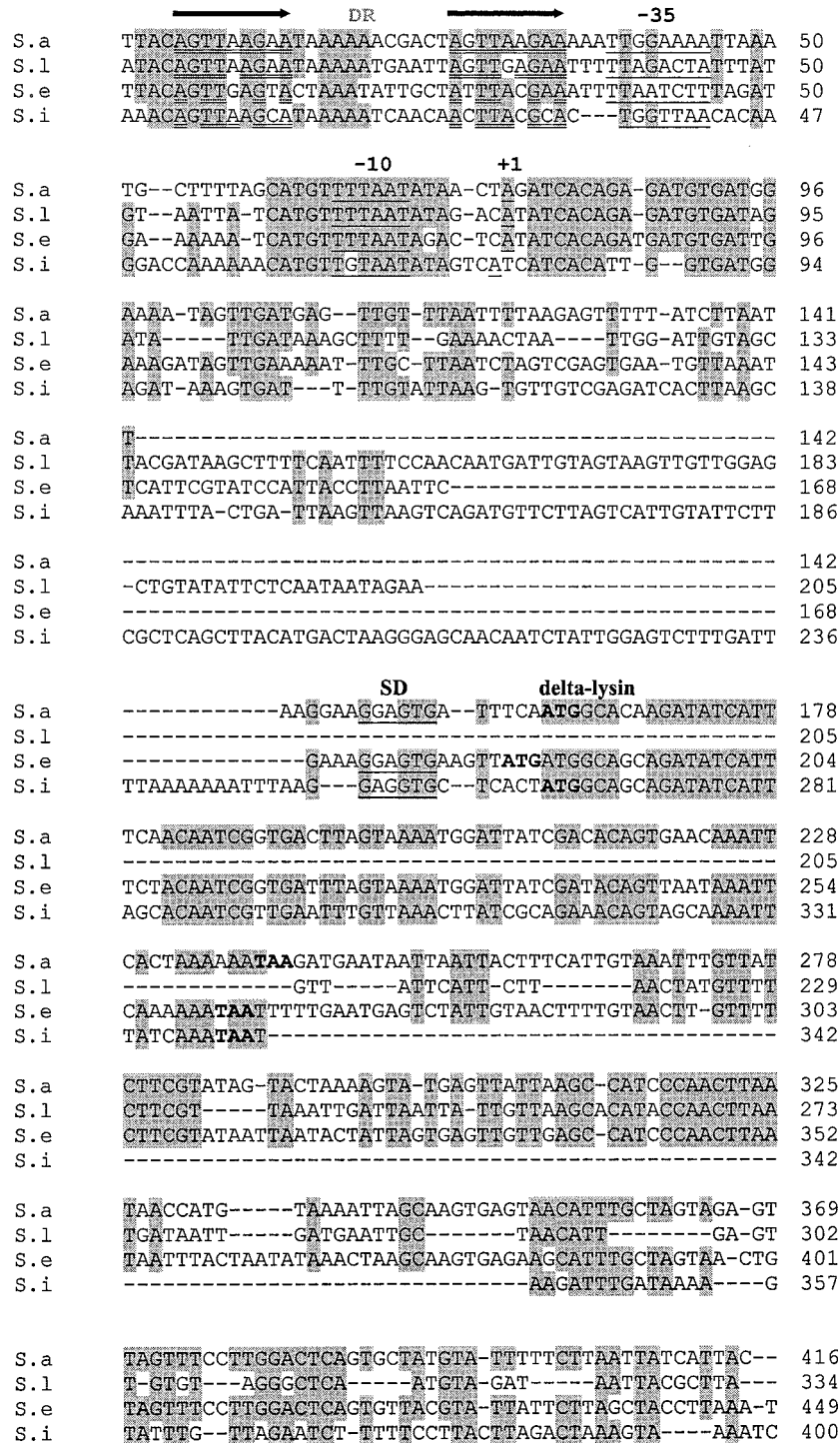
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                                        DR                          −35
        S.a    TTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAATTGGAAAATTAAA      50
        S.l    ATACAGTTAAGAATAAAAATGAATTAGTTGAGAATTTTTAGACTATTTAT      50
        S.e    TTACAGTTGAGTACTAAATATTGCTATTTACGAAATTTTAATCTTTAGAT      50
        S.i    AAACAGTTAAGCATAAAAATCAACAACTTACGCAC---TGGTTAACACAA      47

                              −10             +1
        S.a    TG--CTTTTAGCATGTTTTAATATAA-CTAGATCACAGA-GATGTGATGG      96
        S.l    GT--AATTA-TCATGTTTTAATATAG-ACATATCACAGA-GATGTGATAG      95
        S.e    GA--AAAAA-TCATGTTTTAATAGAC-TCATATCACAGATGATGTGATTG      96
        S.i    GGACCAAAAAACATGTTGTAATATAGTCATCATCACATT-G--GTGATGG      94

        S.a    AAAA-TAGTTGATGAG--TTGT-TTAATTTTAAGAGTTTTT-ATCTTAAT     141
        S.l    ATA-----TTGATAAAGCTTTT--GAAAACTAA----TTGG-ATTGTAGC     133
        S.e    AAAGATAGTTGAAAAAT-TTGC-TTAATCTAGTCGAGTGAA-TGTTAAAT     143
        S.i    AGAT-AAAGTGAT---T-TGTATTAAG-TGTTGTCGAGATCACTTAAGC     138

        S.a    T-------------------------------------------------     142
        S.l    TACGATAAGCTTTTCAATTTTCCAACAATGATTGTAGTAAGTTGTTGGAG     183
        S.e    TCATTCGTATCCATTACCTTAATTC-------------------------     168
        S.i    AAATTTA-CTGA-TTAAGTTAAGTCAGATGTTCTTAGTCATTGTATTCTT     186

        S.a    --------------------------------------------------     142
        S.l    -CTGTATATTCTCAATAATAGAA---------------------------     205
        S.e    --------------------------------------------------     168
        S.i    CGCTCAGCTTACATGACTAAGGGAGCAACAATCTATTGGAGTCTTTGATT     236

                              SD                delta-lysin
        S.a    ------------AAGGAAGGAGTGA--TTTCAATGGCACAAGATATCATT     178
        S.l    --------------------------------------------------     205
        S.e    -------------GAAAGGAGTGAAGTTATGATGGCAGCAGATATCATT     204
        S.i    TTAAAAAAATTTAAG---GAGGTGC--TCACTATGGCAGCAGATATCATT     281

        S.a    TCAACAATCGGTGACTTAGTAAAATGGATTATCGACACAGTGAACAAATT     228
        S.l    --------------------------------------------------     205
        S.e    TCTACAATCGGTGATTTAGTAAAATGGATTATCGATACAGTTAATAAATT     254
        S.i    AGCACAATCGTTGAATTTGTTAAACTTATCGCAGAAACAGTAGCAAAATT     331

        S.a    CACTAAAAAATAAGATGAATAATTAATTACTTTCATTGTAAATTTGTTAT     278
        S.l    -------------GTT-----ATTCATT-CTT-------AACTATGTTTT     229
        S.e    CAAAAAATAATTTTTGAATGAGTCTATTGTAACTTTTGTAACTT-GTTTT     303
        S.i    TATCAAATAAT---------------------------------------     342

        S.a    CTTCGTATAG-TACTAAAAGTA-TGAGTTATTAAGC-CATCCCAACTTAA     325
        S.l    CTTCGT-----TAAATTGATTAATTA-TTGTTAAGCACATACCAACTTAA     273
        S.e    CTTCGTATAATTAATACTATTAGTGAGTTGTTGAGC-CATCCCAACTTAA     352
        S.i    --------------------------------------------------     342

        S.a    TAACCATG-----TAAAATTAGCAAGTGAGTAACATTTGCTAGTAGA-GT     369
        S.l    TGATAATT-----GATGAATTGC-------TAACATT--------GA-GT     302
        S.e    TAATTTACTAATATAAACTAAGCAAGTGAGAAGCATTTGCTAGTAA-CTG     401
        S.i    ----------------------------AAGATTTGATAAAA----G     357

        S.a    TAGTTTCCTTGGACTCAGTGCTATGTA-TTTTTCTTAATTATCATTAC--     416
        S.l    T-GTGT---AGGGCTCA-----ATGTA-GAT-----AATTACGCTTA---     334
        S.e    TAGTTTCCTTGGACTCAGTGTTACGTA-TTATTCTTAGCTACCTTAAA-T     449
        S.i    TATTTG--TTAGAATCT-TTTTCCTTACTTAGACTAAAGTA----AAATC     400
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FIG. 2. Nucleotide sequence alignment of the RNAIII of *S. aureus* (S.a), *S. lugdunensis* (S.l), *S. epidermidis* (S.e), and *S. intermedius* (S.i). Identical nucleotides that are present in at least three of the sequences are indicated by shading. The predicted delta-lysin open reading frames (ATG) and the stop codons (TAA) are in boldface type. The putative ribosome-binding Shine-Dalgarno (SD) sites upstream of the delta-lysin are underlined. Arrowheads and double-underlined sequences indicate direct (DR) and indirect repeat (IR). Sequences are manipulated by introducing gaps (indicated by dashes) to fit transcription start points and the delta-lysin open reading frames. Putative transcriptional start sites (+1) and the −10 and −35 promoter elements are underlined.

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S.a   AGATAATTATTTCTAGCATGTAAGCTATCGTAAACAACAT-CGATTTATC   465
S.l   -GCTACTTAACTGTAGAAGGTAAT-TATTGTA--CTA----GTGTTA-C    374
S.e   AGGTAATTATTTCTAGCATGTAAGCTATCGTAAACAACATTCAATTTATC   499
S.i   AGGT------TTCGAT-ATTGAAGCCATTGTAG-CAATATTTAATA-ATC    441

S.a   ATTATTT---GATAAAT-----AAAATTTTTTTCATAATTAATAACATCC   507
S.l   TCTAGTC----AGAAGA------------TTTTCATAATTAATAACATCC   408
S.e   AT-GTAAATAGATAAATTCACTAAAATTTTTT-CATAATTAATAACATCC   547
S.i   ----------GGTAAATTCACTAAA-TTTTTT-CATAATTAATAACATCC   479

S.a   CCAAAAA--TAGATTGAAAAAATAACTGTAAA---A-CATTCCCTTAATA   551
S.l   CCAAATT--ATTATTGAAAA--TAACTGTAAA---AACATTCCCTTAATA   451
S.s   CCAAAAA--TAGATAGAAAA--TAACTGTAAA---AACATTCCCTTAATA   591
S.i   CCAATTAAATAGA-------A-TAACTGTAAATAAAACATTCCCTTAATA   521
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                        ─────────────────►        IR        ◄────────────
S.a   ATAAGT-AT----GGTCGTGAGCCCCTCCCAAGCTCGCGGCCT        589
S.l   AGCGAA-AA-AA--GTCGTGAGTCACTCCCAAACTCACGACTT        490
S.e   ATAAGTTATCAA--GCCGTGAGTCTCTCCCAAGCTCACGGCT--       631
S.i   TGAAG-----AACAGGCG---AGTCCTCCCAAGCTCGTCTGTTC       558
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FIG. 2—*Continued*.

Synthesis of the AgrD cyclic peptides. The cyclic peptides RIPISTGFF and KIPTSTGFF were custom synthesized by Peptide Protein Research Company Ltd. (Eastleigh, United Kingdom). Both peptides were cyclized by a lactone ring between the serine side chain and the C terminus and purified in acetonitrile and water containing 0.1% trifluoroacetic acid. The purified peptides were 95% homogenous as indicated by high-performance liquid chromatography and mass spectrometry.

In vitro biological activity assay. The spent culture supernatants or synthetic peptides (50 μg or 100 μg) were added separately to early exponentially growing *S. intermedius* strain 3 cells and incubated with shaking for 30 min, prior to stabilization with Bacterial Protect reagent (QIAGEN) and harvesting, in accordance with the manufacturer's recommendation. Following RNA isolation, the expression levels of *luk*, *entC*, RNAII,I of the internal control 16S rRNA were analyzed by real-time reverse transcription-PCR.

RNA preparation and cDNA synthesis. Total bacterial RNA was extracted from *S. intermedius* and *S. aureus* using the RNeasy Mini kit (QIAGEN). DNase digestion of 7 μl of total RNA was performed with 2 U RNase-free DNase I (Promega) and 1 μl of the supplied buffer in a total volume of 10 μl for 30 min at 37°C. RNA concentrations were determined using the RiboGreen RNA quantification reagent (Molecular Probes, Leiden, The Netherlands). To assess RNA integrity, 2 μg of each RNA sample was electrophoresed in a 1.2% agarose–0.66 M formaldehyde gel. For cDNA synthesis, 5 μl of total RNA was mixed with 0.8 μl (400 ng) of random hexamer (Promega), 2 μl of 10× first-strand buffer, 2 μl of 5 mM dNTP, 10 U of RNasin (Promega), 1 μl of Sensiscript reverse transcriptase (QIAGEN), and distilled water to a total volume of 20 μl. Samples were incubated for 30 min at 37°C, followed by heat inactivation at 65°C for 10 min. Random priming was used to generate cDNA so as to reduce the number of reverse transcriptase reactions required.

Real-time quantitative PCR of RNAIII, leukotoxin, and enterotoxin C transcription in *S. intermedius*. Amplicon standards were generated for each primer pair by amplifying 100 to 500 ng of genomic *S. intermedius* DNA in a thermocycler. The following primers were used: 5′-GCCTAATACATGCAAGTC-3′ and 5′-CATGTTATCCGGCATTAG-3′ for 16S rRNA, 5′-CAGTTCACCATATGAAACAG-3′ and 5′-TCCATTCTTTGTTGTAAGGTG-3′ for enterotoxin C, 5′-GCAAGAGGTAACTTTAATCCTG-3′ and 5′ATCAGAATGTGTTTTGATTAG-3′ for leukotoxin, and 5′-GCAGCAGATATCATTAGC-3′ and 5′-TGCTACAATGGCTTCA-3′for RNAIII. The design of the forward and reverse primers for RNAIII was based on the sequences obtained from genome walking. Amplicons were gel purified using QIAquick gel extraction kit (QIAGEN) before the copy number was calculated. The real-time PCR templates for each gene included fresh dilutions of the standards, cDNA samples, and distilled water for a negative control. A control for each primer pair and RNA sample also included sham cDNA synthesis reactions that lack reverse transcriptase, followed by PCR amplification to identify RNA preparations contaminated by residual genomic DNA. All reactions were performed in triplicate in a DNA Engine Opticon 2 system (MJ Research, Waltham, MA). Each reaction tube contained 10 μl of 2× Quantitect SYBR green PCR master mix (HotStarTaq DNA polymerase, Quan-

titect SYBR green PCR buffer, dNTP mix, SYBR green I, and 5 mM MgCl$_2$), 0.3 μM of gene-specific forward and reverse primers, and 2 μl template, made up to a final volume of 20 μl with distilled water. Cycling parameters were as follows: initial activation step at 95°C for 15 min, denaturation at 94°C for 15 s, annealing at 55°C for 30s, extension at 72°C for 30 s, and fluorescence data acquisition at 72°C for 15 s. Melting curve analysis was performed at 50°C to 95°C with stepwise fluorescence acquisition at every 1°C s^{-1}. Melting curves observed for each gene were confirmed to correspond to the correct amplicon size by agarose gel electrophoresis of PCR products (not shown). Sequence-specific standard curves were generated using 10-fold serial dilutions (10^3 to 10^7 copies μl^{-1}) of the specific RNA standards. On the basis of the given copy number values for amplicon standards, the Opticon Monitor analysis software (version 2.01) generated a standard curve that was used to quantitate representative gene cDNA copy numbers in each sample. The mean cDNA copy number values obtained for each gene were divided by the corresponding mean 16S rRNA values to standardize for the number of bacterial cells present in each sample. Relative comparisons between corrected values were performed using the analysis of variance test in GraphPad Prism software package (version 4.0) applying the Bonferroni posthoc test to allow multiple comparisons for significance.

Nucleotide sequence accession number. The DNA sequence of the 3.436-kb fragment has been deposited in GenBank under the accession number AY965912.

RESULTS AND DISCUSSION

Cloning and sequencing of the *agrD* gene revealed that the 20 canine *S. intermedius* strains tested could be divided into three *agr* specificity groups based on the putative autoinducing peptide produced by each strain. Elucidation of the complete 3′ *agr* (RNAIII) and the upstream *agr* flanking sequences was achieved using primers U1 and AP2 and primers U2 and AP2. For the downstream region of *agrD*, nested PCR gave a 5.5-kb amplified fragment. By repeated sequential PCR and primer walking, the entire *agr* determinant and further downstream flanking sequences were obtained (GenBank accession number AY965912) (Fig. 1).

Through genome walking, the *agr* locus in *S. intermedius* was found to span a total of 3,436 bp, with five ORFs, *agrB*, *agrD*, *agrC*, *agrA*, and *hld*, in two divergently transcribed transcripts, RNAII and RNAIII. The start codons of *agrB*, *agrD*, and *agrC* of *S. intermedius* are preceded by the sequence 5′-AGGAGGA-3′, which is similar to the typical consensus ribosome-bind-

TABLE 2. Comparison of the predicted amino acid sequences of delta-lysin of staphylococci[a]

Staphylococcus species	Predicted amino acid sequence of delta-lysin	% Identity with *S. aureus* (human) delta-lysin
S. aureus (human)	MAQ**D**IIST**IG** **DL**V**K**W**II****DTV** **NKF****T****KK**	100.0
S. aureus (canine)	MAA**D**IIST**IV** **EF**V**K**L**IAETV** **EKF****IKK**	65.4
S. epidermidis	MAA**D**IIST**IG** **DL**V**K**W**II****DTV** **NKF****KK**	92.0
S. simulans	MAG**D**IVGT**IG** **EF**V**K**L**IIETV** **QKF****TQK**	65.4
S. warneri (I)	MAA**D**IIST**IG** **DL**V**K**L**IINTV** **KKF****QK**	80.0
S. warneri (II)	MTA**D**IIST**IG** **DF**V**K**W**IL****DTV** **KKF****TK**	80.0
S. intermedius	MAA**D**IIST**IV** **EF**V**K**L**IAETV** **AKF****IK**	64.0

[a] The predicted amino acid sequences of delta-lysins of *S. aureus* (GenBank accession number AP003364), *S. epidermidis* (GenBank accession number Z49220), *S. simulans* (GenBank accession number AJ223775), *S. warneri* (I and II, two nonidentical copies of delta-lysine) (GenBank accession number AJ223776), and *S. intermedius* (canine S3 isolate, GenBank accession number AY965912) are compared. A BLASTP search was conducted using the translated amino acids to assemble homologous delta-lysin sequences. Charged residues are indicated by bold type. Conserved amino acids are underlined.

ing site 5′-AGGAGGT-3′ in gram-negative bacteria. For *agrA*, the ribosome-binding site is 5′AGGATGT-3′.

The amino acid sequences of AgrB, AgrD, AgrC, and AgrA of *S. intermedius* were compared to those of known Agr proteins from other staphylococci, and analyzed by manual examination and publicly available computer programs. Only the *agr* loci from *S. aureus* group I (GenBank accession number X52543), *S. aureus* group II (GenBank accession number AP003135), *S. aureus* group III (GenBank accession number AP004829), *S. epidermidis* (GenBank accession number Z49220), *Staphylococcus lugdunensis* (GenBank accession number AF173933), and *S. intermedius* (GenBank accession number AY965912, this study) were incorporated in the multiple-alignment analysis, since sequence data for other staphylococci are incomplete. The identified *agrB*, *agrD*, and *agrC* regions of RNAII of *S. intermedius* share some similarity with the corresponding sequences of other staphylococci. However, regions of difference are found, and those regions of RNAII that show little similarity among the staphylococcal species are thought to be important for species-specific peptide processing and recognition involved in the *agr* response.

AgrA of *S. intermedius*. In the 3′ region of RNAII, high sequence conservation throughout the *agrA* genes of *S. intermedius* and *S. aureus* is evident, with 83.6% identity. Evidence for the role of AgrA as a response regulator can also be inferred from analyses of its sequences using reverse position-specific BLAST (rpsBLAST) and the Superfamily 1.63 HMM library and genome assignments server. Analysis using ConPredII found no likely transmembrane helices in the sequence of AgrA of *S. intermedius*, in agreement with the expected cytoplasmic localization of response regulators (not shown). Being a putative response regulator, AgrA of *S. intermedius* is likely to have little direct involvement with the specificity of processing and of the ligand-receptor interactions that constitute the putative quorum-sensing circuits, which may account for the low sequence divergence among the AgrA proteins of various staphylococci.

AgrB of *S. intermedius*. Analysis of the amino acid sequence (188 residues) of the AgrB protein of *S. intermedius* reveals that it shares 41.5% identity to the AgrB proteins of *S. aureus*, *S. lugdunensis*, and *S. epidermidis*. Transmembrane topology analysis predicted the AgrB of *S. intermedius* to be a membrane-spanning protein with five potential helices, consistent with the proposed function of AgrB as seen in *S. aureus*, where it is responsible for processing of the AIP expressed by AgrD. Since AIP is derived from an internal fragment of the *agrD*-coding sequence and its synthesis involves the product of the *agrB* gene (15), it would be expected that polymorphism in the *agrD* gene would require *agrB* to vary accordingly, so as to retain the specificity of peptide processing. This may account for the high sequence variation of AgrB in the various staphylococcal species.

AgrC of *S. intermedius*. The *S. intermedius* AgrC amino acid sequence (433 residues) shows 15.7% identity with analogous products from *S. aureus*. Transmembrane topology analysis of AgrC of *S. intermedius* suggested the presence of a N-terminal hydrophobic domain. A search of conserved domains (rpsBLAST) revealed that the C terminus of AgrC is categorized as a member of the histidine protein kinase family. As *agrC* presumably encodes a receptor responsible for binding to the autoinducing peptide derived from *agrD*, divergence in the N terminus of the *agrC* of staphylococci would allow binding to its corresponding polymorphic AIP, thus retaining the specificity of the receptor-ligand interaction. This divergence may be responsible for speciation within staphylococci, similar to divergence within the structurally related *comAP* locus required for competence in *Bacillus* (33).

AgrD of *S. intermedius*. The short *S. intermedius* AgrD amino acid sequence (45 residues) shows only 24.4% similarity with the equivalent prepheromone of *S. aureus*. Characterization of the AIP of *S. aureus* by Ji and colleagues revealed that it is a modified peptide whose sequence is contained within an internal fragment of the *agrD*-coding sequence (15). On the basis of the primary sequence of the pheromone peptides determined from PCR analysis, four different *agr* subgroups have been classified in *S. aureus* (15, 26), and one group has been detected in 15 investigated strains of *S. epidermidis* (27). Our similar PCR sequence analyses of the *agrD* genes among 20 strains of *S. intermedius* isolated from clinical cases of canine pyoderma revealed the distribution of three AIP allelic variants. Among the 20 strains, 5% (1 strain) produce the AIP RIPTSTGFF, while 35% (7 strains) and 60% (12 strains) produce the RIPISTGFF (this study) and KIPTSTGFF peptides, respectively (Table 1). The AIP gene in the three different groups of *S. intermedius* was highly conserved and shows nucleotide polymorphisms that led to conservative amino acid changes only.

With *S. aureus*, the highly divergent AIP sequences in the different groups vary in length from seven to nine amino acyl residues. Through extensive structure-activity relationship studies, the AIP is found to be a thiolactone molecule containing a

ring of five 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 two to four amino acid residues, depending on the staphylococcal species and groups within the same species (13, 15, 22, 27). Prediction of the *S. intermedius* AIPs from the corresponding nucleotide sequences of *agrD* revealed that in contrast to the AIP sequences in the four groups of *S. aureus*, there are no conserved sequences of five cysteine residues from the COOH terminus in the putative AIPs of all three specificity groups in *S. intermedius*. This is consistent with the findings by Kalkum and colleagues, who reported that instead of a thiolactone structure, a lactone structure was discovered in a pigeon isolate of *S. intermedius* (17). Despite the high divergence of the *agrD* sequences among the different staphylococcal species, there is nonetheless a more or less conserved negatively charged C terminus. This negatively charged terminus has been proposed to be involved in the processing of AgrD (35), and it contributes to the translocation of the AIP (15).

RNAIII of *S. intermedius*. Upstream of the transcription start site, putative −10 and −35 promoter elements, similar to those in other staphylococci, were found in *S. intermedius* (Fig. 2). A direct repeat immediately upstream of the −35 promoter element was found in all species of staphylococci. Sequence conservation among these elements suggests their functionally important roles in regulating RNAIII gene expression (24), with similar control mechanisms likely to exist in all staphylococcal species. The 3′ regions of the RNAIII sequences of the different staphylococcal species also showed a considerable degree of sequence conservation (Fig. 2). In *S. aureus*, the 3′ region was found to be essential for the production of exoproteins, such as α and β toxin (18). Similarity in this region among the different staphylococci suggests that they may play roles to that of *S. aureus*.

The RNAIII gene also contains an open reading frame encoding the delta-lysin homologue. Delta-lysin is encoded by the *hld* gene, which gives rise to a transcript of 75 nucleotides in *S. intermedius*. The predicted delta-lysin genes of the various staphylococcal species are found at different distances from the transcription start point, with most being localized in the 5′ half of RNAIII (Fig. 2). In *S. intermedius*, there is an additional insertion upstream of the structural gene for the delta-lysin open reading frame (Fig. 2). The open reading frame of the delta-lysin in *S. intermedius* possesses a reasonably high degree of sequence identity to those of *S. aureus* and other coagulase-negative staphylococci (Table 2). In *S. intermedius*, 16 of the 25 amino acids of delta-lysin are identical to those of *S. aureus*. From the alignment of the primary sequence of delta-lysin in Table 2, it can be seen that the distribution of the charged residues was conserved between all predicted molecules. Consequently, it is likely that the delta-lysin of *S. intermedius* may function as a pore-forming toxin, as has been reported for *S. aureus* delta-lysin (8).

Fitton et al. and Turner et al. determined the amino acid sequence of delta-lysin of *S. aureus* from human and canine isolates and found that the toxin obtained from *S. aureus* of the human strain differs from that made by the canine strain by nine amino acids (Table 2) (7, 34). Interestingly, the delta-lysin from a canine isolate of *S. intermedius* (this study) compared with that from a canine isolate of *S. aureus* (34) differs only by a single amino acid (Table 2). Given the higher similarity
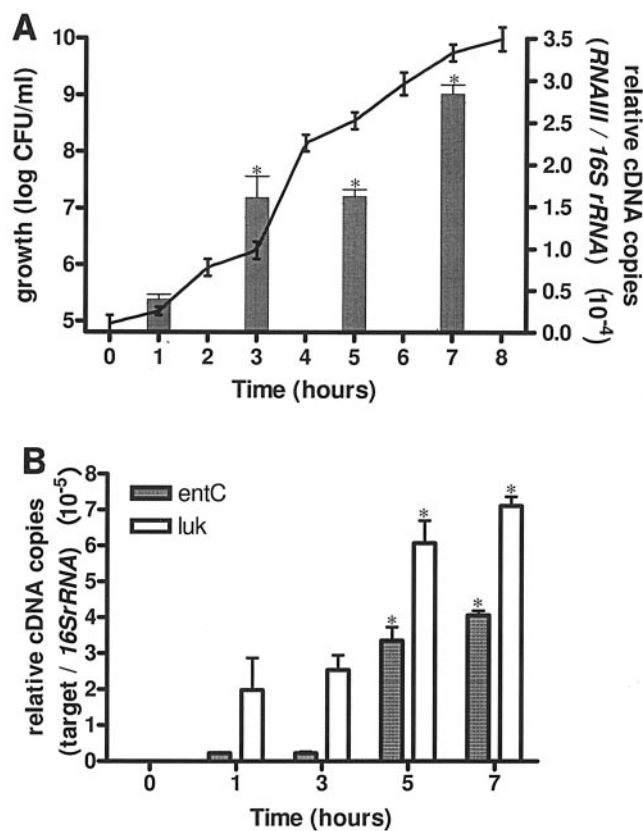

FIG. 3. (A) Growth curve (line graph) and transcription profile (bar graph) of RNAIII and (B) temporal regulation of *luk* and *entC* in *S. intermedius* (strain 3) from early exponential to post-exponential phase of growth. The expression level of each sample was normalized to that of *S. intermedius* 16S rRNA signal. Values that were significantly different compared to the respective control value at 0 h ($P < 0.05$) are indicated by asterisks. Values are means ± standard errors of the means (error bars) ($n = 3$).

between *S. aureus* and *S. intermedius* of canine origin compared to that between *S. aureus* of human and canine origin, one might question the true identity of the suggested *S. aureus* of canine origin.

Temporal regulation of RNAIII, leukotoxin, and enterotoxin C expression. Despite differences in the primary nucleotide sequences of RNAIII from *S. aureus* and *S. intermedius*, the kinetics of transcription in both are comparable—notably, the expression of RNAIII reaches its maximum in late logarithmic phase of growth (Fig. 3A). The temporal regulation of RNAIII suggests that activation of the P3 promoter, which drives the expression of RNAIII, is growth phase dependent. Regulation of RNAIII in an autocrine manner is consistent with its hypothesized function as the effector of the *agr* system. Expression of enterotoxin C and leukotoxin were similarly temporally regulated, displaying a significant increase in the amount of transcripts in the post-exponential phase (Fig. 3B). Thus far, there is very little data available concerning the relationship between *S. intermedius* cutaneous infection and the production of leukotoxin and enterotoxin, respectively, because little is known about the local and general effects of the toxin products in vivo. The *luk* gene was chosen for this study, as all 20 clinical
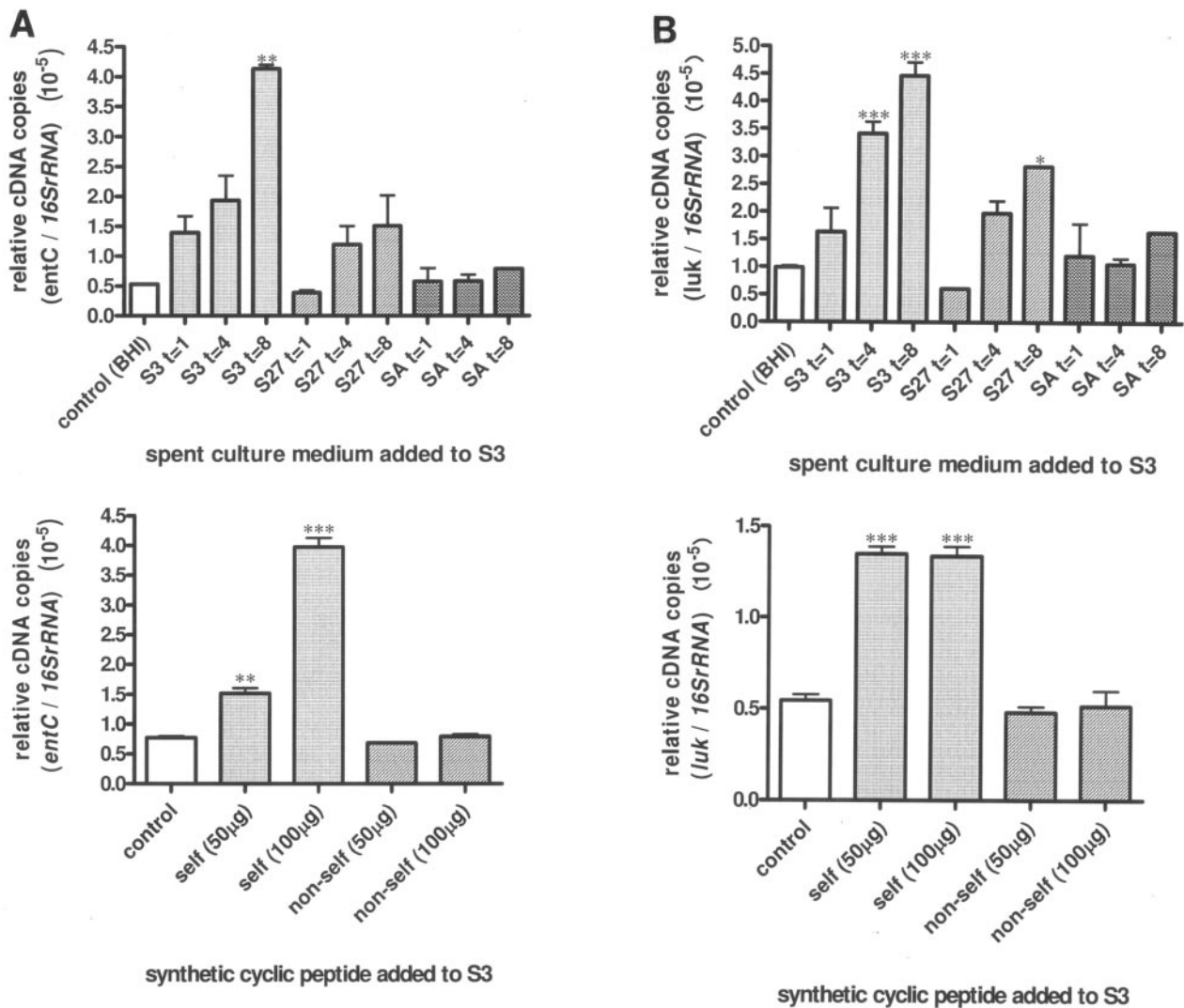
FIG. 4. Supernatants from 1-, 4-, and 8-h cultures of *S. intermedius* strain 3 (S3), *S. intermedius* S27 (S27), and *S. aureus* (SA) (top graphs) or synthetic self and nonself cyclic peptides (50 μg or 100 μg) (bottom graphs) were added to early exponential *S. intermedius* S3 cells. The expression of *entC* (A), *luk* (B), and RNAIII (C) were monitored during subsequent growth of bacteria by real-time quantitative PCR. Data are means and standard errors of the means (error bars) of three determinants performed in duplicate. Values that are significantly different from the control (brain heart infusion [BHI]) values are indicated with asterisks ($P < 0.05$ *, $P < 0.001$ **, and $P < 0.001$ ***).

isolates of *S. intermedius* isolated from cases of canine pyoderma were found to harbor the two cotranscribed genes of leukotoxin, *lukF* and *lukS* (not shown), suggesting the likely role and importance of this toxin in the virulence of *S. intermedius*, at least in cutaneous infection. Staphylococcal enterotoxins, on the other hand, are known to have potent effects on cells of the immune system in general, functioning as superantigens to cause excessive nonspecific T-cell proliferation. In *S. intermedius*, the expression of both virulence factors followed a trend similar to that of RNAIII and was positively correlated (RNAIII versus *entC*, $r = 0.8191$ and $P = 0.0898$; RNAIII versus *luk*, $r = 0.9001$ and $P = 0.0373$), suggesting that changes in the regulation of both toxins studied may be attributed to the differential transcription of RNAIII.

Characterization of the activating factor for *luk*, *entC*, and RNAIII expression in *S. intermedius*. Given that the AgrC

protein of *S. intermedius* resembles the classical sensory transducing proteins of bacteria, it seemed likely that *agr*, and hence RNAIII activation, would be initiated by an environmental signal generated during bacterial growth. To investigate whether quorum sensing via the *agr* gene was responsible for the exponential-phase induction of toxin transcription, the effects of cell-free spent culture supernatants obtained from different phases of growth from *S. intermedius* strains 3 and 27 and *S. aureus* strain J3945 on gene expression in *S. intermedius* strain 3 were assessed. The spent media collected were individually added to early exponential cultures of *S. intermedius* strain 3 at a time when expression of each gene studied was minimally activated, as determined in preliminary studies (not shown). The results obtained demonstrate that post-exponential-phase culture supernatants of *S. intermedius* contain a substance that, when added to early exponential self cultures,
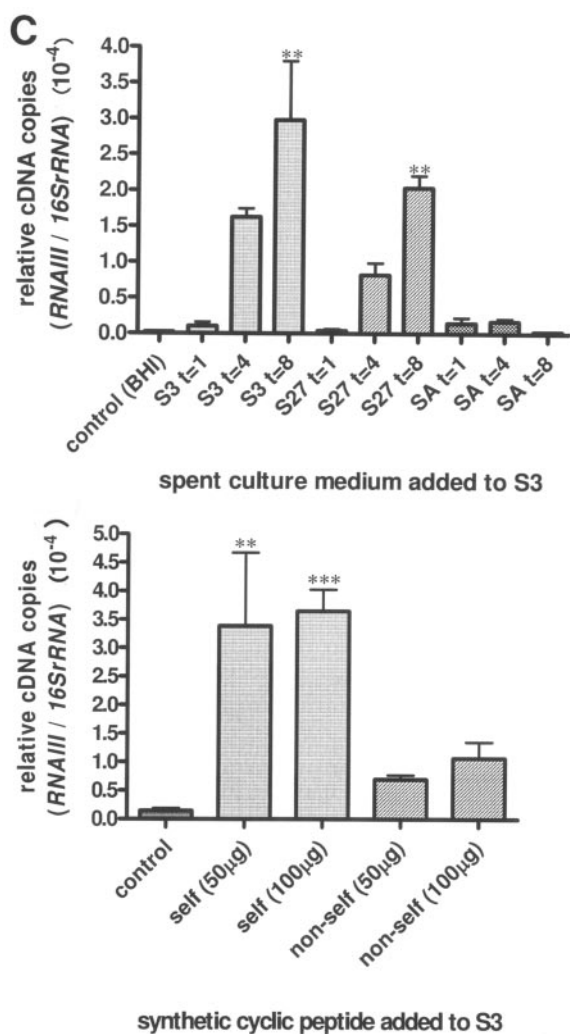
spent culture medium added to S3

synthetic cyclic peptide added to S3

FIG. 4—*Continued*.

ferent strain, *S. intermedius* strain 27 (S27), was added to *S. intermedius* strain 3 cells, there was higher expression of RNAIII ($P < 0.01$) and *luk* ($P < 0.05$) than when the medium-only control was added (Fig. 4). To verify that activation of the above-mentioned transcripts following the addition of post-exponential-phase spent medium is due to specific signals elaborated by the bacterium itself, cyclic peptides were synthesized, according to the sequenced putative AIPs of strain 3 (self) and S27 (nonself) in order to test their efficacy to induce gene expression in *S. intermedius* strain 3. Up-regulation of RNAIII ($P < 0.001$), *luk* ($P < 0.001$), and *entC* ($P < 0.001$) expression was observed following the addition of "self" peptide (RIPIS TGFF) to *S. intermedius* strain 3. With respect to the "nonself" peptide (KIPTSTGFF), there was no convincing activation of the *agr* response, in contrast to that observed when nonself spent culture medium (S27) was added to *S. intermedius* strain 3. The difference could be attributable to changes in environmental factors as *S. intermedius* enters the post-exponential phase, such as alterations in the levels of acidity, or to the presence of nonspecific endogenous substances in the spent culture medium.

With *S. aureus* and *S. epidermidis*, although the various peptide groups differ in their amino acid sequences, each has been shown to possess a cyclic thiolactone moiety (15, 22, 27). Previous studies have reported the essentiality of the high-energy thioester linkage between the central cysteine and the C-terminal carboxyl group in the thiolactone ring of these AIPs for full *agr* activation activity (20, 22, 23, 27). Despite being potent intergroup inhibitors, the corresponding lactone and lactam analogues of the group II AIP of *S. aureus* were also found to be inactive, as were linear version of this and other AIPs (14, 22). In our study, the addition of "self" synthetic peptide (RI PISTGFF) with a lactone cyclic structure to early exponentially growing *S. intermedius* activated transcription of RNAIII. The putative nonapeptide, RIPISTGFF, is thus the activating factor that turns on the *agr* response in *S. intermedius* strain 3. Results obtained from this study also suggest that the thiolactone ring is not an absolute requirement for *agr* activation in *S. intermedius*, as was proposed for other staphylococci.

Taken together, the response defined on the basis of increases in transcript levels of quorum-sensing-controlled genes, such as RNAIII of *agr*, indicates that *S. intermedius* is an active participant in activating its own virulence regulatory system. This type of regulation is a form of quorum sensing, whereby a population of bacteria responds in concert when a critical cell density is reached. The identification of the *agr* gene in *S. intermedius* is an important step towards understanding its pathogenesis. Given the similarities in the orientation, organization, and conservation of certain residues and domains of the *agr* gene of *S. intermedius* to those of *S. aureus*, it is likely that the *agr* system may play a comparable role in the pathology of *S. intermedius* canine pyoderma.

caused immediate activation of RNAIII, enterotoxin C, and leukotoxin transcription, with expression of RNAIII and both virulence factors being positively correlated (RNAIII versus *entC*, $r^2 = 0.9705$ and $P = 0.0295$; RNAIII versus *luk*, $r^2 = 0.9821$ and $P = 0.0179$) (Fig. 4). Exhibition of a concentration-dependent activation suggests that the production of virulence factors in *S. intermedius* is initiated by a growth-specific cue generated during bacterial growth. The activating substance appears to accumulate in the supernatant during the growth of *S. intermedius*, and upon reaching a threshold level, it regulates virulence factor gene expression. This type of kinetics represents a cell density-sensing mechanism similar to that observed with *Pseudomonas*, *Vibrio*, *Agrobacterium*, and other bacterial species that use homoserine lactones as autoinducers (5, 16, 36).

The abundance of mRNA from *S. intermedius* RNAIII, leukotoxin, and enterotoxin genes demonstrated dependency on secreted biological cues. With self biological cues, activation of the transcripts encoding three genes studied occurred when supernatants obtained from late-exponential growth of *S. intermedius* strain 3 ($t = 8$ h) were added to the same growing strain (Fig. 4). When post-exponential supernatant from a dif-

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