PFGE and southern hybridyzation

Of the 5 *P. rettgeri* isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity (Figure 1). Three of these isolates had a plasmid harboring bla_{NDM-1} and one had a plasmid harboring bla_{OXA-72}, with plasmid sizes ranging from 9.42 to 23.1 kbp (data not shown).

Genomic structures surrounding bla_{NDM-1} and bla_{OXA-72}

The genetic environments surrounding bla_{NDM-1} (Accession no. AB828598) was bla_{NDM-1}-ble_{MBL}-*trpF-dsbC-cutA1*. All 3 isolates harboring bla_{NDM-1} (IOMTU1, 91 and 99) had the same genetic environments. The bla_{OXA-72} gene was flanked by conserved inverted repeats at the XerC/XerD binding sites [21], indicating mobilization by site-specific recombination mechanisms. The *rep1* gene was located downstream of bla_{OXA-72} (Accession no. AB857844).

Discussion

The relatively high MICs to piperacillin/tazobactam and carbapenems of the five *P. rettgeri* isolates were likely due to the presence of bla_{NDM-1} or bla_{OXA-72}. The enzymatic activities of metallo-β-lactamases, including NDM-1, were not inhibited by tazobactam [22], a β-lactamase inhibitor, in agreement with the MIC profiles of these isolates to piperacillin/tazobactam. The high MICs of all 5 isolates to ceftazidime, cefepime and aztreonam were likely due to the presence of bla_{VEB-1} [23], and the presence of *armA* in these isolates was likely associated with their extremely high resistance to all aminoglycosides tested [11]. Point mutations in the quinolone-resistance-determining regions of *gyrA* and *parC* have been associated with high resistance to quinolones [24]. Point mutations in *pmrCAB* operon have been associated with the resistance of *Acinetobacter* spp. [25] and *Pseudomonas aeruginosa* [26] to polymxyin and colistin; and the presence of *fos* genes, including *fosA*, *fosA2*, *fosA3*, *fosC* and *fosC2*, has been associated with resistance to fosfomycin in Gram-negative bacteria [27-29].

Plasmids containing bla_{NDM-1} or bla_{OXA-72} may be disseminated among Gram-negative pathogens in Nepal. The genetic environments surrounding bla_{NDM-1} in our *P. rettgeri* strains (bla_{NDM-1}-ble_{MBL}-*trpF-dsbC-cutA1*) were also observed in other plasmids, including *A. baumannii* plasmid pAbNDM-1 from China (Accession no. JN377410), *Citrobacter freundii* plasmid pYE315203 from China (Accession no. JX254913), *E. coli* plasmid pNDM102337 from Canada (Accession no. JF714412), *K. pneumoniae* plasmid pKP-NCGM18-1 from Nepal (Accession no. AB824738) [30], *K. pneumoniae* plasmids pKPX-1, pKPN5047 and pNDM-HN380 from China (Accession nos. AP012055, KC311431 and JX104760, respectively), and *P. rettgeri* plasmid pFR90 (Accession no. JQ362415) from China. In addition, the genetic structures of OXA-72 producing *Acinetobacter* spp [31-34] and *K. pneumoniae* (Accession no. JX268653 and AB825955 deposited in 2012 and 2013, respectively) had the same genetic structure (bla_{OXA-72}-*rep1*) as our strain of *P. rettgeri*.

Conclusions

To our knowledge, this is the first report describing *P. rettgeri* strains harboring bla_{NDM-1} or bla_{OXA-72} and *armA* isolated from patients in Nepal. These 5 strains were highly resistant to both β-lactams and aminoglycosides and expanded in a clonal manner in the hospital.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

TT: Performed PCR and sequencing, analyzed data and drafted the manuscript. TMA: Performed entire genome sequencing. RKD and MKS: Performed drug susceptibility tests. HO: Supervised this study. KS: Performed pulsed-field gel electrophoresis and its pattern analysis. TK and BMP: Designed protocols and supervised this study. All authors read and approved the final manuscript.

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References

1. Stock I, Wiedemann B: **Natural antibiotic susceptibility of *Providencia stuartii*, *P. rettgeri*, *P. alcalifaciens* and *P. rustigianii* strains.** *J Med Microbiol* 1998, **47**:629–642.
2. Yoh M, Matsuyama J, Ohnishi M, Takagi K, Miyagi H, Mori K, Park KS, Ono T, Honda T: **Importance of *Providencia* species as a major cause of travellers' diarrhoea.** *J Med Microbiol* 2005, **54**:1077–1082.
3. Shiroto K, Ishii Y, Kimura S, Alba J, Watanabe K, Matsushima Y, Yamaguchi K: **Metallo-beta-lactamase IMP-1 in *Providencia rettgeri* from two different hospitals in Japan.** *J Med Microbiol* 2005, **54**:1065–1070.
4. Nishio H, Komatsu M, Shibata N, Shimakawa K, Sueyoshi N, Ura T, Satoh K, Toyokawa M, Nakamura T, Wada Y, Orita T, Kofuku T, Yamasaki K, Sakamoto M, Kinoshita S, Aihara M, Arakawa Y: **Metallo-beta-lactamase-producing Gram-negative bacilli: laboratory-based surveillance in cooperation with 13 clinical laboratories in the Kinki region of Japan.** *J Clin Microbiol* 2004, **42**:5256–5263.
5. Lee HW, Kang HY, Shin KS, Kim J: **Multidrug-resistant *Providencia* isolates carrying bla_{PER-1}, bla_{VIM-2}, and *armA*.** *J Microbiol* 2007, **45**:272–274.
6. Gefen-Halevi S, Hindiyeh MY, Ben-David D, Smollan G, Gal-Mor O, Azar R, Castanheira M, Belausov N, Rahav G, Tal I, Mendelson E, Keller N: **Isolation of genetically not related bla_{NDM-1} positive *Providencia rettgeri* in Israel.** *J Clin Microbiol* 2013, **51**:1642–1643.
7. Carvalho-Assef AP, Pereira PS, Albano RM, Beriao GC, Chagas TP, Timm LN, Da Silva RC, Falci DR, Asensi MD: **Isolation of NDM-producing *Providencia rettgeri* in Brazil.** *J Antimicrob Chemother.* in press.

8. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR: **Characterization of a new metallo-beta-lactamase gene, bla_{NDM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India.** *Antimicrob Agents Chemother* 2009, **53**:5046–5054.
9. Cornaglia G, Giamarellou H, Rossolini GM: **Metallo-beta-lactamases: a last frontier for beta-lactams?** *Lancet Infect Dis* 2011, **11**:381–393.
10. Pillai DR, McGeer A, Low DE: **New Delhi metallo-beta-lactamase-1 in Enterobacteriaceae: emerging resistance.** *CMAJ* 2011, **183**:59–64.
11. Wachino J, Arakawa Y: **Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update.** *Drug Resist Updat* 2012, **15**:133–148.
12. Sheng WH, Badal RE, Hsueh PR, SMART Program: **Distribution of extended-spectrum beta-Lactamases, AmpC beta-Lactamases, and carbapenemases among enterobacteriaceae isolates causing intra-abdominal infections in the Asia-Pacific region: results of the Study for Monitoring Antimicrobial Resistance Trends (SMART).** *Antimicrob Agents Chemother* 2013, **57**:2981–2988.
13. Bueno MF, Francisco GR, O'Hara JA, de Oliveira Garcia D, Doi Y: **Co-production of 16S Ribosomal RNA Methyltransferase RmtD and RmtG with KPC-2 and CTX-M-group ESBLs in *Klebsiella pneumoniae*.** *Antimicrob Agents Chemother* 2013, **57**:2397–2400.
14. Galani I, Souli M, Panagea T, Poulakou G, Kanellakopoulou K, Giamarellou H: **Prevalence of 16S rRNA methylase genes in Enterobacteriaceae isolates from a Greek university hospital.** *Clin Microbiol Infect* 2012, **18**:E52–E54.
15. Zacharczuk K, Piekarska K, Szych J, Zawidzka E, Sulikowska A, Wardak S, Jagielski M, Gierczynski R: **Emergence of *Klebsiella pneumoniae* coproducing KPC-2 and 16S rRNA methylase ArmA in Poland.** *Antimicrob Agents Chemother* 2011, **55**:443–446.
16. Wu Q, Liu Q, Han L, Sun J, Ni Y: **Plasmid-mediated carbapenem-hydrolyzing enzyme KPC-2 and ArmA 16S rRNA methylase conferring high-level aminoglycoside resistance in carbapenem-resistant *Enterobacter cloacae* in China.** *Diagn Microbiol Infect Dis* 2010, **66**:326–328.
17. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH: **Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli.** *J Clin Microbiol* 1998, **36**:3674–3679.
18. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG: **Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA.** *Appl Environ Microbiol* 1998, **64**:795–799.
19. Simmon KE, Croft AC, Petti CA: **Application of SmartGene IDNS software to partial 16S rRNA gene sequences for a diverse group of bacteria in a clinical laboratory.** *J Clin Microbiol* 2006, **44**:4400–4406.
20. National Committee for Clinical Laboratory Standards: **Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 9th ed.** In *Approved standard M07-A9*. 8th edition. Wayne, Pa: Clinical and Laboratory Standards Institute; 2012.
21. D'Andrea MM, Giani T, D'Arezzo S, Capone A, Petrosillo N, Visca P, Luzzaro F, Rossolini GM: **Characterization of pABVA01, a plasmid encoding the OXA-24 carbapenemase from Italian isolates of *Acinetobacter baumannii*.** *Antimicrob Agents Chemother* 2009, **53**:3528–3533.
22. Bush K, Jacoby GA: **Updated functional classification of beta-lactamases.** *Antimicrob Agents Chemother* 2010, **54**:969–976.
23. Poirel L, Naas T, Guibert M, Chaibi EB, Labia R, Nordmann P: **Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum beta-lactamase encoded by an *Escherichia coli* integron gene.** *Antimicrob Agents Chemother* 1999, **43**:573–581.
24. Jacoby GA: **Mechanisms of resistance to quinolones.** *Clin Infect Dis* 2005, **41**(Suppl 2):S120–S126.
25. Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA: **Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system.** *Antimicrob Agents Chemother* 2009, **53**:3628–3634.
26. Moskowitz SM, Ernst RK, Miller SI: **PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A.** *J Bacteriol* 2004, **186**:575–579.
27. Beharry Z, Palzkill T: **Functional analysis of active site residues of the fosfomycin resistance enzyme FosA from *Pseudomonas aeruginosa*.** *J Biol Chem* 2005, **280**:17786–17791.
28. Xu H, Miao V, Kwong W, Xia R, Davies J: **Identification of a novel fosfomycin resistance gene (fosA2) in *Enterobacter cloacae* from the Salmon River, Canada.** *Lett Appl Microbiol* 2011, **52**:427–429.
29. Wachino J, Yamane K, Suzuki S, Kimura K, Arakawa Y: **Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes.** *Antimicrob Agents Chemother* 2010, **54**:3061–3064.
30. Tada T, Miyoshi-Akiyama T, Dahal RK, Mishra SK, Ohara H, Shimada K, Kirikae T, Pokhrel BM: **Dissemination of multidrug-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal.** *Int J Antimicrob Agents* 2013, **42**:372–374.
31. Werneck JS, Picao RC, Carvalhaes CG, Cardoso JP, Gales AC: **OXA-72-producing *Acinetobacter baumannii* in Brazil: a case report.** *J Antimicrob Chemother* 2011, **66**:452–454.
32. Wang H, Guo P, Sun H, Wang H, Yang Q, Chen M, Xu Y, Zhu Y: **Molecular epidemiology of clinical isolates of carbapenem-resistant *Acinetobacter* spp. from Chinese hospitals.** *Antimicrob Agents Chemother* 2007, **51**:4022–4028.
33. Montealegre MC, Maya JJ, Correa A, Espinal P, Mojica MF, Ruiz SJ, Rosso F, Vila J, Quinn JP, Villegas MV: **First identification of OXA-72 carbapenemase from *Acinetobacter pittii* in Colombia.** *Antimicrob Agents Chemother* 2012, **56**:3996–3998.
34. Goic-Barisic I, Towner KJ, Kovacic A, Sisko-Kraljevic K, Tonkic M, Novak A, Punda-Polic V: **Outbreak in Croatia caused by a new carbapenem-resistant clone of *Acinetobacter baumannii* producing OXA-72 carbapenemase.** *J Hosp Infect* 2011, **77**:368–369.

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Bacteriocin Protein $BacL_1$ of *Enterococcus faecalis* Is a Peptidoglycan D-Isoglutamyl-L-lysine Endopeptidase*

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Background: Enterococcal bacteriocin $BacL_1$ shows bactericidal activity in the co-presence of BacA.
Results: Recombinant $BacL_1$ alone acts as a D-isoglutamyl-L-lysine endopeptidase against *E. faecalis* peptidoglycan independently of BacA.
Conclusion: $BacL_1$ is a peptidoglycan hydrolase and potentially lyses viable bacterial cells in the presence of BacA.
Significance: This study of bacterial fratricide mediated by bacteriocin provides new insight into the ecological physiology of bacteria.

Enterococcus faecalis **strains are commensal bacteria in humans and other animals, and they are also the causative agent of opportunistic infectious diseases. Bacteriocin 41 (Bac41) is produced by certain *E. faecalis* clinical isolates, and it is active against other *E. faecalis* strains. Our genetic analyses demonstrated that the extracellular products of the *$bacL_1$* and *bacA* genes, which are encoded in the Bac41 operon, coordinately express the bacteriocin activity against *E. faecalis*. In this study, we investigated the molecular functions of the $BacL_1$ and BacA proteins. Immunoblotting and N-terminal amino acid sequence analysis revealed that $BacL_1$ and BacA are secreted without any processing. The coincidental treatment with the recombinant $BacL_1$ and BacA showed complete bacteriocin activity against *E. faecalis*, but neither $BacL_1$ nor BacA protein alone showed the bacteriocin activity. Interestingly, $BacL_1$ alone demonstrated substantial degrading activity against the cell wall fraction of *E. faecalis* in the absence of BacA. Furthermore, MALDI-TOF MS analysis revealed that $BacL_1$ has a peptidoglycan D-isoglutamyl-L-lysine endopeptidase activity via a NlpC/P60 homology domain. These results collectively suggest that $BacL_1$ serves as a peptidoglycan hydrolase and, when BacA is present, results in the lysis of viable *E. faecalis* cells.**

Bacteriocins are antimicrobial proteins or peptides produced by a wide variety of bacteria. Bacteriocins usually show a narrow spectrum of antimicrobial activity that is specifically active against species that are identical or closely related to the strain that is producing the bacteriocin (1). Meanwhile, bacteriocin-producing bacteria also have specific immunity factors that protect the producer strain from being killed by the cognate bacteriocin. The production of bacteriocin is thought to provide a competitive advantage to the producer strain in an ecological niche that has closely related strains present.

Many clinical isolates of *Enterococcus faecalis* have been reported to produce various bacteriocins (2, 3). These enterococcal bacteriocins are often encoded on a pheromone-responsive conjugative plasmid (4–7). They have been classified into five groups based on the bactericidal spectrum identified in our previous study (6). Class 1 is active against a wide variety of Gram-positive bacteria (6, 8). The β-hemolysin/bacteriocin (cytolysin), which belongs to class 1-type bacteriocins, shows not only bactericidal activity but also hemolytic activity against mammalian cells, and it is associated with virulence in an animal model (9–11). Class 2 is active against a broad spectrum of bacteria, including *E. faecalis*, the other *Streptococcus* spp., and *Staphylococcus aureus*. The class 2 bacteriocins contain the peptide antibiotics AS-48 (12) and bacteriocin 21 (13). Class 3 shows activity against *E. faecalis*, *Enterococcus hirae*, and *Listeria monocytogenes* and includes bacteriocin 31 (6). Class 4 and class 5 show activity only against *E. faecalis* and *E. hirae*, respectively (4, 14–16).

Bacteriocin 41 (Bac41)[2] is a class 4 type bacteriocin found in the pheromone-responsive plasmid pYI14 of the clinically isolated strain *E. faecalis* YI14 (4). Our subsequent epidemiologic study showed that more than 50% of *E. faecalis* clinical strains, but not laboratory strains, produce Bac41 (17). Our previous genetic analysis revealed several features of Bac41. Bac41 is specifically active only against *E. faecalis* and has no activity against *Enterococcus faecium*. The determinant of Bac41 is encoded in the EcoRI fragments A (12.6 kbp) and H (3.5 kbp) of pYI14 and consists of six genes, including *$bacL_1$*, *$bacL_2$*, *bacA*, and *bacI*.

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[2] The abbreviations used are: Bac41, bacteriocin 41; SH3, Src homology 3; THB, Todd-Hewitt broth; CBB, Coomassie Brilliant Blue; Ni-NTA, nickel-nitrilotriacetic acid; PSD, postsource decay.

TABLE 1
Bacterial strains and plasmids used in this study

	Description	Source/Reference
Strains		
E. faecalis OG1S	*str*; derivative of OG1	Ref. 26
E. coli DH5α	Host for DNA cloning	Bethesda Research Laboratories
E. coli BL21 Rosetta	Host for protein expression	Novagen
Plasmids		
pAM401	*E. coli-E. faecalis* shuttle plasmid; *cat, tet*	Ref. 38
pHT1100	pAM401 containing wild-type Bac41	Ref. 4
pHT1101	pAM401 containing Bac41 without *bacA*	Ref. 4
pMG1106	pAM401 containing Bac41 without *bacL$_1$*	Ref. 4
pAM401::*bacL$_1$/bacL$_2$*	pAM401 containing *bacL$_1$* and *bacL$_2$*	Ref. 4
pAM401::*bacL$_1$-his/bacL$_2$*	pAM401 containing *bacL$_1$-his* and *bacL$_2$*	This study
pAM401::*flag-bacL$_1$-his/bacL$_2$*	pAM401 containing *flag-bacL$_1$-his* and *bacL$_2$*	This study
pMGS100	*E. coli-E. faecalis* shuttle expression plasmid; *cat, tet*	Ref. 39
pMGS100::*bacA*	pMGS100 containing *bacA*	This study
pMGS100::*bacA-his*	pMGS100 containing *bacA-his*	This study
pMGS100::*flag-bacA-his*	pMGS100 containing *flag-bacA-his*	This study
pET22b(+)	Expression vector of His-tagged protein in *E. coli*	Novagen
pET::*bacA*	pET22b (+) containing *bacA*	This study
pET::*bacL$_1$*	pET22b (+) containing *bacL$_1$*	This study
pET::*bacL$_1$Δ1*	pET22b (+) containing *bacL$_1$* truncate Δ1	This study
pET::*bacL$_1$Δ2*	pET22b (+) containing *bacL$_1$* truncate Δ2	This study
pET::*bacL$_1$Δ1Δ2*	pET22b (+) containing *bacL$_1$* truncate Δ1Δ2	This study
pET::*bacL$_1$Δ3*	pET22b (+) containing *bacL$_1$* truncate Δ3	This study

The bacteriocin activity of Bac41 is complementarily expressed by two extracellular components: the *bacL$_1$*- and *bacA*-encoded proteins, BacL$_1$ and BacA. ORF *bacL$_2$* is required for the expression of *bacL$_1$* and *bacL$_2$* itself (18). ORF *bacI* is an immunity factor protecting the Bac41-harboring strain from being killed by BacL$_1$ and BacA (4).

BacL$_1$ is a 595-amino acid protein (64.5 kDa) consisting of two domains located in the 3–140 and 163–315 amino acid regions of the amino acid sequence. The domains show homology to the bacteriophage-type peptidoglycan hydrolase and the NlpC/P60 family peptidoglycan hydrolase, respectively (19, 20). A C-terminal, three-repeat structure located in the 329–590 amino acid region of BacL$_1$ shows homology with the bacterial Src homology 3 (SH3) domain that is reported to bind to the bacterial cell wall (21, 22). BacA is a 726-amino acid protein (79.1 kDa) showing a significant degree of homology to YbfG and YkuF of *Bacillus subtilis* (23). The functions of YbfG and YkuF are unknown, but a putative peptidoglycan-binding domain and a domain similar to the GH25 family peptidoglycan hydrolase are detected in the 81–140 and 208–491 amino acid regions of BacA, respectively (4, 24).

The N termini of BacL$_1$ and BacA are predicted to have a signal peptide, presumed to be secreted in a *sec*-dependent manner (4). These investigations suggest that BacL$_1$ and BacA are cell wall lytic enzymes able to induce the bacteriolytic killing of target cells. However, the precise molecular functions of BacL$_1$ and BacA remain elusive. In this report, we demonstrate the biochemical analysis of BacL$_1$ and BacA and reveal that BacL$_1$ has the enzymatic activity of a peptidoglycan hydrolase.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, oligonucleotides, Media, Antimicrobial Reagents, and Antibodies—The bacterial strains and plasmids used in this study are shown in Table 1. The oligonucleotides used in this study are listed in Table 2. A standard plasmid DNA methodology was used (25). *E. faecalis* and *Escherichia coli* strains were grown in Todd-Hewitt broth (THB; Difco) and Luria-Bertani medium (LB; Difco) at 37 °C, respectively (26), unless otherwise noted. *E. coli* strains were grown in Luria-Bertani medium at 37 °C. The antibiotic concentrations for the selection of *Escherichia coli* were as follows: ampicillin, 100 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}. The concentration of chloramphenicol for the selection of *E. faecalis* was 20 μg ml^{-1}. All antibiotics were obtained from Sigma. Anti-BacL$_1$ and -BacA sera were prepared by immunization of rabbits with recombinant BacL$_1$-His and BacA-His proteins, respectively (Operon Technologies, Alameda, CA). Anti-FLAG antibodies were purchased from Invitrogen.

Construction of Expression Plasmids—The amplification of the respective genes for the plasmid construction was performed by the PCR method using the corresponding primers as indicated in Table 2. The constructed plasmids were sequenced to confirm that the desired sequence had been inserted.

Preparation of Whole Cell and Culture Supernatant Proteins from E. faecalis—Overnight cultures of *E. faecalis* strains were inoculated into fresh THB broth and incubated at 37 °C for the indicated time. The bacterial pellet was resuspended with distilled water, and the culture supernatant was filtered (0.22 μm; Millipore (Billerica, MA)). Trichloroacetic acid was then added to each sample at a final concentration of 10%. After incubation on ice for 15 min, the supernatants were centrifuged at 10,000 rpm for 10 min. The precipitated protein samples were neutralized with 2 M Tris-base and dissolved in sample buffer. The resulting protein samples were separated with SDS-PAGE and then subjected to CBB staining or immunoblot analysis.

Isolation of Recombinant His$_6$-tagged Proteins—An overnight culture of *E. faecalis* expressing the recombinant protein was inoculated into 500 ml of fresh THB and incubated at 37 °C for 18 h. The *E. coli* BL21 Rosetta strains expressing recombinant protein were inoculated into 500 ml of fresh LB and cultured at 37 °C with shaking until an optical density of 0.5–0.7 at 600 nm was obtained. Then isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM for induction, follow-

TABLE 2
Oligonucleotides used in this study
The underlines indicate the following endonuclease recognition sequences: GAATTC, EcoRI, GCCGGC, EagI; TCGCGA, NruI; CATATG, NdeI; CTCGAG, XhoIV.

Oligonucleotides	Sequence	Generated plasmid	Source/ Reference
B9P2842F	CCGGAATTCTAGCAACCGAAAACCACGTTGG	pAM401::*bacL$_1$/bacL$_2$* pAM401::*bacL$_1$-his/bacL$_2$* pAM401::*flag-bacL$_1$-his/bacL$_2$*	Ref. 4
B9P5773R	GCGGAATTCATTGCGCAGCAAATCATTGC	pAM401::*bacL1/bacL2* pAM401::*bacL$_1$-his/bacL$_2$* pAM401::*flag-bacL$_1$-his/bacL$_2$*	Ref. 4
F-His-L1_stop	CACCACCATCACCATCATTAGTACAAATTATATTGCTT	pAM401::*bacL$_1$-his/bacL$_2$* pAM401::*flag-bacL$_1$-his/bacL$_2$*	This study
R-His-L1_stop	ATGATGGTGATGGTGGTGATTAAAGAATCCTTTGCCCC	pAM401::*bacL$_1$-his/bacL$_2$* pAM401::*flag-bacL$_1$-his/bacL$_2$*	This study
F-FLAG-L1_start	GATTATAAAGATGACGATGACAAAAATTACAGTCAAAAAGCAAT	pAM401::*flag-bacL$_1$-his/bacL$_2$*	This study
R-FLAG-L1_start	TTTGTCATCGTCATCTTTATAATCCATAAACTTCACCTCATATT	pAM401::*flag-bacL$_1$-his/bacL$_2$*	This study
F-EagI-bacA	TTTTTCGGCCGGCATGGATGAAATGGTTTTA	pMGS100::*bacA*, pMGS100::*bacA-his*	This study
R-NruI-bacA	ATTTTTTCGCGATTAAGCTAATGCAGCAAAAA	pMGS100::*bacA*	This study
F-FLAG-bacA	AAGATGACGATGACAAAGATGAAATGGTTTTAGGTA	pMGS100::*flag-bacA-his*	This study
F-EagI-FLAG	TTTTTCGGCCGGCATGGATTATAAAGATGACGATGACAAA	pMGS100::*flag-bacA-his*	This study
R-His-bacA	ATGATGGTGATGGTGGTGAGCTAATGCAGCAAAAAATG	pMGS100::*bacA-his* pMGS100::*flag-bacA-his*	This study
R-NruI-His	ATTTTTTCGCGATTAATGATGGTGATGGTGGTG	pMGS100::*bacA-his* and pMGS100::*flag-bacA-his*	This study
F-NdeI-bacA	CGCCATATGGATGAAATGGTTTTAGG	pET::*bacA*	This study
R-XhoI-bacA	CCGCTCGAGAGCTAATGCAGCAAAAAATG	pET::*bacA*	This study
F-NdeI-bacL1	CGCCATATGATGAATTACAGTCAAAAAGC	pET::*bacL$_1$*	This study
R-XhoI-bacL1	CCGCTCGAGATTAAAGAATCCTTTGCCCC	pET::*bacL$_1$*	This study
F-del-L1_Lys1	AAGTTTATGAATACAGCCCTTTATCTTGAAGG	pET::*bacL$_1$*Δ1, pET::*bacL$_1$*Δ1Δ2	This study
R-del-L1_Lys1	ATAAAGGGCTGTATTCATAAACTTCACCTCAT	pET::*bacL$_1$*Δ1, pET::*bacL$_1$*Δ1Δ2	This study
F-del-L1_Lys2	CGTATTGGTTTTTATCCTGGAGATTCTTCTGG	pET::*bacL$_1$*Δ2, pET::*bacL$_1$*Δ1Δ2	This study
R-del-L1_Lys2	ATCTCCAGGATAAAAACCAATACGTGCGTGAT	pET::*bacL$_1$*Δ2, pET::*bacL$_1$*Δ1Δ2	This study
F-del-L1-His_SH3	GATTCAGTGAATAAAGGATTCTTTAATCACCA	pET::*bacL$_1$*Δ3	This study
R-del-L1_SH3	AAAGAATCCTTTATTCACTGAATCTCCTTTTG	pET::*bacL$_1$*Δ3	This study

ing an additional incubation at 30 °C with shaking for 3 h. The bacterial cells were collected by centrifugation and resuspended in 10 ml of lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 10 mg ml^{-1} lysozyme, pH 8.0) with EDTA-free protease inhibitor mixture (Complete MINI EDTA-free, Roche Applied Science) to be enzymatically lysed at 37 °C for 30 min. The bacterial suspension was further lysed by sonication on ice using a sonicator (Ultrasonic Disruptor UD-201; TOMY, Tokyo, Japan) set at power level 6, at 40% duty, for 20 min and then clarified by centrifugation at 15,000 rpm for 10 min. The resulting soluble lysate was added to 1 ml of 50% Ni-NTA nickel chromatography resin (Ni-NTA purification system; Invitrogen) pre-equilibrated with lysis buffer, and the column was washed with 40 ml of wash buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, pH 8.0). The His_6-tagged protein was eluted with elution buffer (25 mM Tris-HCl, 150 mM NaCl, 200 mM imidazole, pH 8.0). The eluent containing the His_6-tagged protein was subjected to ultracentrifugation using Amicon Ultra (Millipore, Billerica, MA) and resuspended in PBS. The protein concentration was determined by the Bradford method (Bio-Rad protein assay kit) using BSA (Sigma) as the standard.

N-terminal Amino Acid Sequence Analysis—An overnight culture of *E. faecalis* expressing the recombinant protein was inoculated to 500 ml of fresh THB and incubated at 37 °C for 18 h. The culture supernatant was prepared by centrifugation at 1,300 × *g* for 10 min at 4 °C and filtered (0.22 μm; Millipore). The supernatant sample was added to 1 ml of 50% Ni-NTA nickel chromatography resin (Ni-NTA purification system; Invitrogen), and the His_6-tagged protein was eluted as described above. The His_6-tagged protein prepared from the supernatant was separated by SDS-PAGE and transferred to a 0.2-μm pore nitrocellulose membrane (Immobilon-PSQ, Millipore) and stained with CBB. The target band was excised and subjected to Edman degradation amino acid sequence analysis to determine its N-terminal structure (GENOSTAFF, Tokyo, Japan).

Soft Agar Assay and Liquid Phase Bactericidal Assay—The soft agar assay for bacteriocin activity was performed as described previously (27). Briefly, 1 μl of the bacterial culture supernatant or recombinant protein sample was spotted onto THB soft agar (0.75%) containing the indicator strain and was then incubated at 37 °C for 24 h. For the liquid phase bactericidal assay, an overnight culture of indicator strain was diluted with fresh THB and adjusted to an optical density at 600 nm of 0.2, and then the recombinant proteins were added, and the sample was incubated at 37 °C. The change in optical density at 600 nm was monitored by a spectrometer (DU730, Beckman Coulter, Fullerton, CA). For the morphological analysis, the bacterial suspension that had been treated with recombinant protein was sampled and subjected to Gram-staining (Faber G, Nissui, Tokyo, Japan) and then analyzed by microscopy (Axiovert 200, Carl Zeiss, Oberkochen, Germany).

Zymograph Analysis—The zymogram gel was prepared by adding a sample of autoclaved indicator strain, *E. faecalis* OG1S, to a final optical density of 0.4 at 600 nm. The protein samples were separated by SDS-PAGE using the zymogram gel prepared above. After electrophoresis, the gel was washed with 20 mM Tris-HCl (pH 8.0), 1% Triton X-100 at room temperature with gentle shaking for 1 h and then incubated in 20 mM Tris-HCl (pH 8.0), 0.1% Triton X-100 at 37 °C for 36 h (28). The plaques that were observed on the gel were analyzed by densitometer (GS-800 calibrated densitometer, Bio-Rad).

Preparation of the Cell Wall Fraction—*E. faecalis* grown in THB at the exponential phase was collected by centrifugation. The bacterial pellet was rinsed with PBS and resuspended in 10 ml of 4% SDS and boiled at 95 °C for 30 min. The pellet was washed with distilled water four times and treated with 0.5 mg ml^{-1} trypsin (0.1 M Tris-HCl (pH 6.8), 20 mM $CaCl_2$) at 37 °C for 16 h. The sample was further washed with distilled water four times and was resuspended in 10% TCA and incubated at 4 °C for 5 h and then given additional washes with distilled

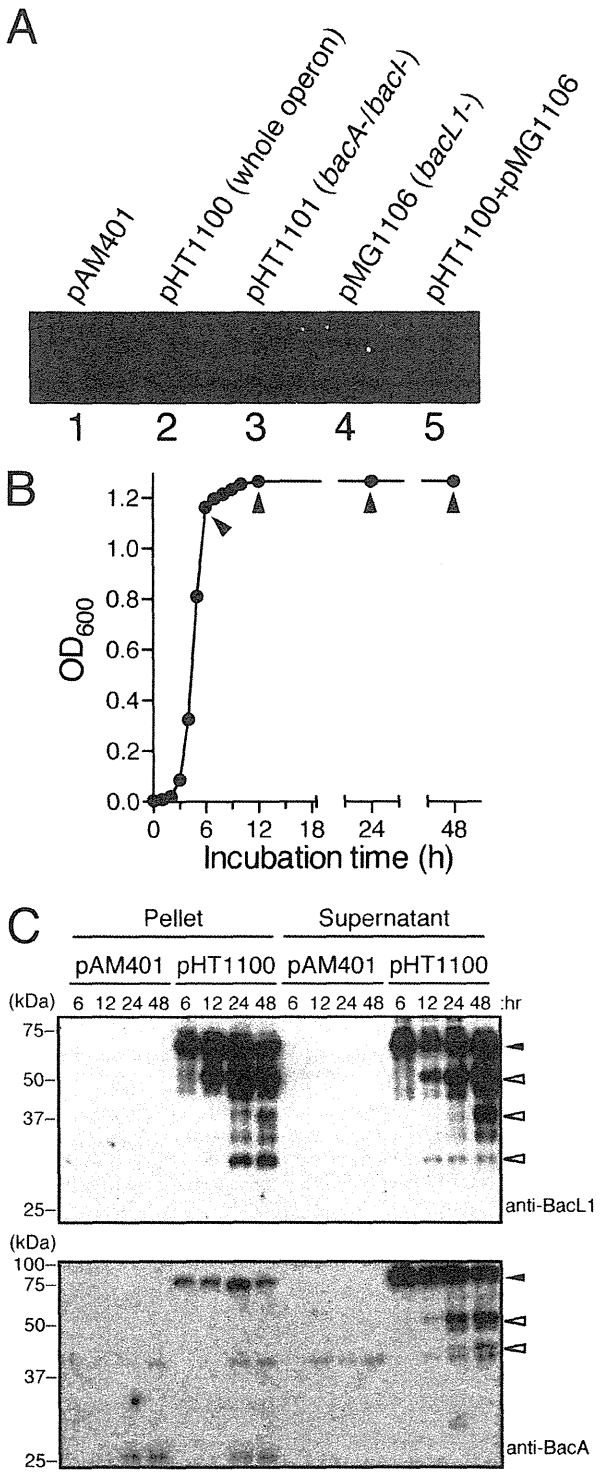

FIGURE 1. **Bacteriocin activity and secreted protein profile in the culture supernatant of *E. faecalis* carrying Bac41 genes.** *A*, culture supernatants of *E. faecalis* carrying pAM401 (*lane 1*), pHT1100 (*lane 2*), pHT1101 (*lane 3*), or pMG1106 (*lane 4*) were spotted onto a THB soft agar plate (0.75%) containing the indicator strain *E. faecalis* OG1S without any plasmid. A 1:1 mixture of the culture supernatants of cells harboring pHT1101- and pMG1106-carrying *E. faecalis* strains was spotted in *lane 5*. The plate was incubated at 37 °C for 24 h, and the formation of halos was evaluated. *B*, a seed bacterial culture of *E. faecalis* was inoculated into fresh THB broth and incubated at 37 °C. The turbidity at an optical density of 600 nm was monitored. *Arrowheads*, time points at which samples of the bacterial culture were collected to prepare protein samples. *C*, the bacterial pellets and the culture supernatant proteins were prepared from cultures of *E. faecalis* carrying pAM401 or pHT1100 at the indicated time point during the incubation period. The resulting protein samples were separated with SDS-PAGE and analyzed by immunoblotting using anti-BacL$_1$ (*top*) and anti-BacA (*bottom*) serum. *Filled arrowheads* or *open arrowheads* indicate the band position of the predicted intact molecule or the degraded product of the respective proteins.

water four times (29). Finally, the cell wall fraction was resuspended with PBS and quantified from the optical density at 600 nm for the cell wall degradation assay. Mutanolysin (Sigma) was used as a positive control for the cell wall degradation enzyme.

Identification of Cleavage Site in Peptidoglycan by Mass Spectrometry—The identification of the enzymatic cleavage site was performed as described previously (30). Briefly, the cell wall fraction prepared as described above was treated with the indicated enzyme. This was followed by the addition of 20% phosphoric acid to adjust the pH to 4.0 and then incubation at 95 °C for 5 min to stop the reaction. The mixture was then added to 1.5 M sodium borate buffer (pH 9.0) and 10 mg solid sodium borohydride and incubated at room temperature for 15 min. The excess borohydride was quenched using 20% phosphoric acid until effervescence disappeared. The muropeptide solution was then adjusted to pH 2.5 using 4 N HCl prior to centrifugation at 10,000 × *g* for 15 min. The supernatant was passed through a membrane filter (0.22 μm) to remove the insoluble contaminants. Separation of the muropeptides was carried out by reverse-phase HPLC using a Hypersil ODS (5 Å, 250 × 4.6 mm) column. A linear gradient was prepared from 5% (v/v) methanol in 50 mM sodium phosphate buffer (pH 2.5) to 30% (v/v) methanol in 50 mM sodium phosphate buffer (pH 2.8) over 210 min. The muropeptides were detected from their UV absorbance at 206 nm. For MALDI-TOF MS analysis, the HPLC fractions containing the muropeptides of interest were desalted with a C-18 ZipTip (Millipore). The samples (1 μl) were co-spotted with an equal volume of the matrix, a saturated solution of α-cyano-4-hydroxy cinnamic acid in CH_3CN/TFA (50:50). Mass spectrometric measurements were performed in a reflextron positive mode on a Biflex iV MALDI-TOF MS instrument (Bruker Daltonics, Billerica, MA). In MALDI post-source decay (PSD) experiments, the timed ion selector was used to select the $[M + Na]^+$ value of the precursor ion. To determine the BacL$_1$ cleavage site of peptidoglycan, each structure of the detected degradation products was compared with the peptidoglycan structure of *E. faecalis* (29–31).

Binding Assay—2 mg (wet weight) of cell wall fraction prepared as above and 10 μg of recombinant protein were mixed in PBS and incubated for 1 h at 4 °C. After incubation, a part of the mixture was kept for the following analysis. The pellet and the supernatant fractions were separated by centrifugation at 13,000 rpm for 1 min, and the pellet was further washed with PBS three times. Each fraction was subjected to SDS-PAGE and CBB staining. The resulting gel was analyzed by densitometer, and the signal intensities of the bands were quantified by ImageJ (National Institutes of Health, Bethesda, MD). The signal of each band intensity in the pellet or the supernatant fraction was normalized by comparison with that of the total fraction.

RESULTS

Secretion Profile of BacL$_1$ and BacA—The culture supernatant of *E. faecalis* carrying pHT1100, which encodes the entire Bac41 operon, including the *bacL$_1$* and *bacA* genes, shows bactericidal activity against *E. faecalis* (Fig. 1*A*). In contrast, this bactericidal activity was no longer detected in the supernatant of *E. faecalis* carrying pHT1101 or pMG1106, which are deficient for either the *bacA* or *bacL$_1$* gene from pHT1100, respectively. The mixture of the culture supernatants of *E. faecalis* (pHT1101 and pMG1106) showed the same bactericidal activity as the culture supernatant of *E. faecalis* (pHT1100). These facts clearly indicate that BacL$_1$ and BacA are secreted into the culture supernatant and are essential for the bactericidal activity of Bac41.

Many bacteriocins in *E. faecalis* have been reported to be secreted in a *sec*-dependent manner. The N-terminal signal sequence of the substrate is cleaved during its secretion process. Previously, we predicted the signal sequences for the *sec*-dependent secretion in the N-terminal amino acid sequence of BacL$_1$ and BacA (4). However, it was not checked whether the N-terminal sequences of these proteins are actually processed during their secretion. Thus, we set out to identify the extracellular forms of BacL$_1$ and BacA to determine whether any processing occurs during their secretion.

We prepared whole intracellular and extracellular proteins from the bacterial pellet and the supernatant fractions, respectively, of *E. faecalis* carrying pAM401 or pHT1100 at several points during the growth phase (Fig. 1*B*). No difference was recognized between the whole-protein profile of *E. faecalis* (pAM401) and *E. faecalis* (pHT1100) (data not shown). The expression and secretion profile of BacL$_1$ and BacA was analyzed by immunoblotting using anti-BacL$_1$ or anti-BacA sera (Fig. 1*C*). The signal of BacL$_1$, in either the pellet or supernatant, gradually increased during the later phase of growth. In contrast, the signal of BacA in the pellet or the supernatant was detected even at a relatively early phase in growth, and the extracellular BacA in the supernatant appeared to be degraded during the later period. Degraded products of BacL$_1$ and BacA were also detected.

Unexpectedly, we did not detect any shift in mobility with either BacL$_1$ or BacA because of processing of their N termini, suggesting that BacL$_1$ and BacA were not processed during their secretion. To confirm this conclusion, we tagged the N termini of BacL$_1$ and BacA with the FLAG peptide and examined whether the N-terminal FLAG tag is still detectable after secretion (Fig. 2*A*). As shown in Fig. 2*A*, the signal of N-terminally tagged FLAG peptide was detectable. The addition of the FLAG tag at the N terminus of BacL$_1$ drastically interfered with its secretion efficiency.

The extracellular or intracellular C-terminal His$_6$-tagged BacL$_1$ (BacL$_1$-His) was purified from the culture supernatant of *E. faecalis* (pAM401::*bacL$_1$-his*) using an Ni-NTA-agarose column. The electrophoretic mobility of the purified extracellular BacL$_1$-His was apparently the same as that of intracellular BacL$_1$-His (Fig. 2*B*). We directly confirmed this finding by sequencing the N-terminal amino acid sequence of BacL$_1$. The band corresponding to the extracellular BacL$_1$-His was excised

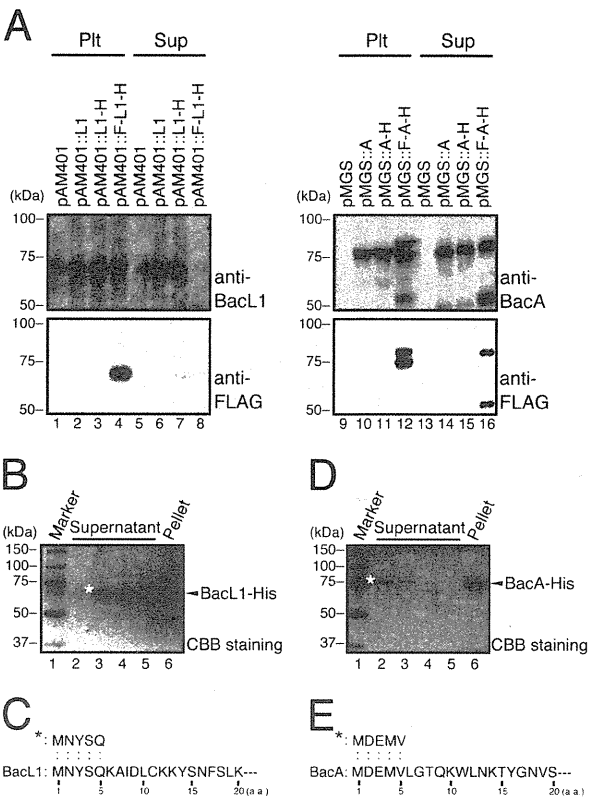

FIGURE 2. **Identification of the secreted forms of BacL$_1$ and BacA in the culture supernatant of *E. faecalis*.** *A*, the bacterial cell pellets (*Plt*; *lanes 1–4* and *9–12*) and the culture supernatant proteins (*Sup*; *lanes 5–8* and *13–16*) prepared from the bacterial culture of *E. faecalis* carrying pAM401 (*lanes 1* and *5*), pAM401::*bacL$_1$/bacL$_2$* (*pAM401::L$_1$*; *lanes 2* and *6*), *pAM401::bacL$_1$-his/bacL$_2$* (*pAM401::L$_1$-H*; *lanes 3* and *7*), pAM401::*flag-bacL$_1$-his/bacL$_2$* (*pAM401::F-L$_1$-H*; *lanes 4* and *8*), pMGS100 (*pMGS*; *lanes 9* and *13*), pMGS100::*bacA* (*pMGS::A*; *lanes 10* and *14*), pMGS100::*bacA-his* (*pMGS::A-H*; *lanes 11* and *15*), or pMGS100::*flag-bacA-his* (*pMGS::F-A-H*; *lanes 12* and *16*) were subjected to immunoblotting analysis using anti-BacL$_1$ serum, anti-BacA serum, or anti-FLAG antibody. *B*, secreted C-terminal hexahistidine-tagged BacL$_1$ (*BacL$_1$-His*; *lanes 2–6*) was purified from the filtered (0.22-μm pore size) culture supernatant of *E. faecalis* OG1S carrying pAM401::*bacL$_1$-his/bacL$_2$*, as described under "Experimental Procedures." Extracellular BacL$_1$-His bound to the immobilized nickel-NTA column was sequentially eluted with 200 mM (*lanes 2–4*) and 500 mM imidazole (*lane 5*). Intracellular BacL$_1$-His was purified from the bacterial cell pellet (*lane 6*). Each sample was separated by SDS-PAGE and stained with CBB. A molecular marker was applied to *lane 1*. The *asterisk* indicates the band in *lane 3* that was subjected to N-terminal sequence analysis. *C*, the N-terminal 5 amino acids of secreted BacL$_1$-His were determined by Edman sequencing (*upper sequence*). The *lower sequence* shows the predicted amino acid sequence of BacL$_1$, including the N-terminal 20 amino acids from the start methionine that was obtained from DNA sequence data (4). *D*, secreted C-terminal hexahistidine-tagged BacA (*BacA-His*; *lanes 2–6*) was purified from the filtered (0.22-μm) culture supernatant of *E. faecalis* OG1S carrying pMGS::*bacA-his*, as described under "Experimental Procedures." Extracellular BacA-His on the immobilized nickel-NTA column was sequentially eluted with 200 mM (*lanes 2–4*) and 500 mM imidazole (*lane 5*). Intracellular BacA-His was purified from the bacterial cell pellet (*lane 6*). Each sample was separated by SDS-PAGE and stained with CBB. A molecular marker was applied in *lane 1*. The *asterisk* indicates the band in *lane 2* that was subjected to the N-terminal sequence analysis shown in *E*. *E*, the N-terminal 5 amino acids of the secreted BacA-His were revealed by Edman sequencing and are shown in the *upper sequence*. The *lower sequence* shows the predicted amino acid sequence of BacA, including the N-terminal 20 amino acids from the start methionine that were obtained from the DNA sequence data (4).

and subjected to N-terminal amino acid sequencing by the Edman method. The N-terminal amino acid sequence of extracellular BacL$_1$-His was MNYSQ and identical to the 5-amino acid sequence from the start methionine of the BacL$_1$ ORF (Fig. 2*C*). Similarly, the extracellular BacA-His was also purified

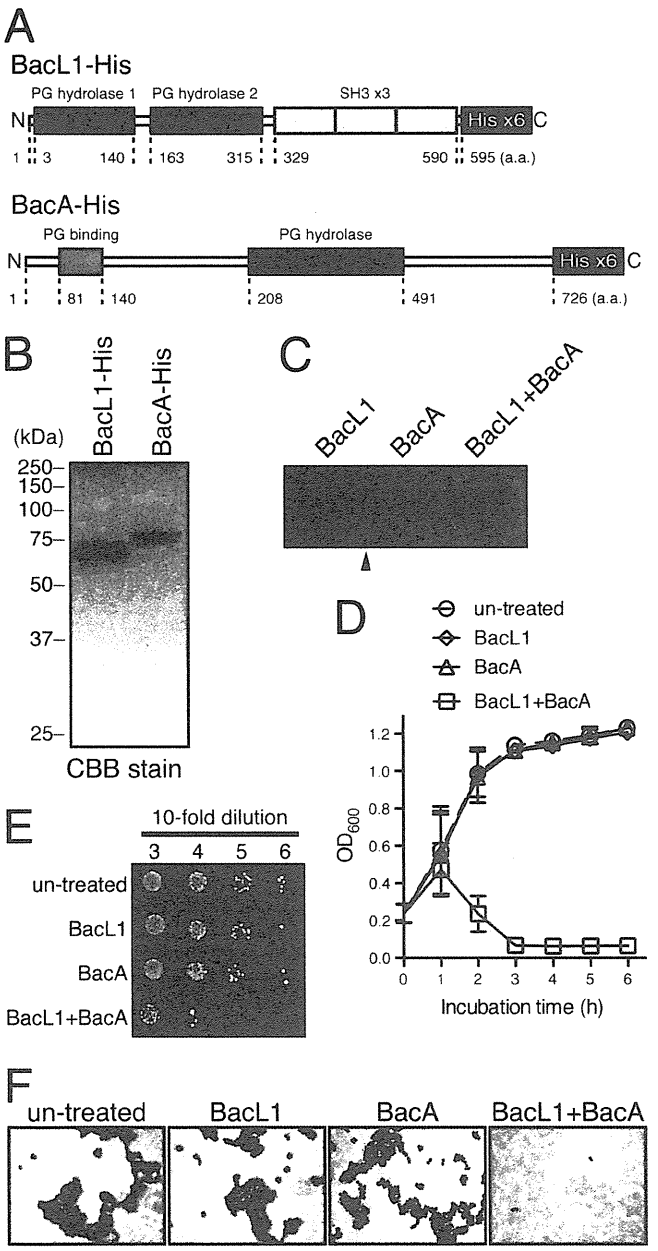

FIGURE 3. **Bacteriocin activity of recombinant BacL$_1$ and BacA proteins.** *A*, conserved domain structures of the recombinant BacL$_1$ and BacA are shown. The hexahistidine tag was added to the C terminus of each wild-type protein for purification with the Ni-NTA system. *B*, recombinant BacL$_1$-His and BacA-His proteins prepared from *E. faecalis* OG1S carrying pAM401::*bacL$_1$-his/bacL$_2$* and *E. coli* BL21 Rosetta carrying pET22-*bacA-his*, respectively, were separated by SDS-PAGE and stained with CBB. *C*, recombinant BacL$_1$-His (25 ng), BacA-His (25 ng), or a mixture of both proteins (25 ng each) was spotted on the THB soft agar (0.75%) containing the indicator strain *E. faecalis* OG1S. The plate was incubated at 37 °C for 24 h, and the formation of halos was evaluated. The *arrowhead* indicates the area in which the bactericidal activity of BacL$_1$ and BacA complemented each other. *D*, a culture of *E. faecalis* was diluted with fresh THB broth to adjust the optical density at 600 nm to 0.2. Recombinant BacL$_1$-His (5 μg/ml), BacA-His (5 μg/ml), or a mixture of both proteins (5 μg/ml each) was added into the bacterial suspension and incubated at 37 °C. The turbidity was monitored during the incubation period. The data for each case are presented as the means ± S.E. (*error bars*) of three independent experiments. *E*, *E. faecalis* was treated with BacL$_1$, BacA, or both proteins as in Fig. 3*C*. After incubation for 6 h, the bacterial suspensions were serially diluted 10-fold with fresh THB broth and then spotted onto a THB agar plate. The plate was incubated at 37 °C for 24 h, and colony formation was evaluated as a measure of bacterial viability. The data for each case are presented as the means ± S.E. of three independent experiments. *F*, *E. faecalis* was treated with BacL$_1$, BacA, or both proteins as in *C*. After incubation for 2 h, the bacterial suspensions were subjected to Gram staining and analyzed by microscopy.

using a Ni-NTA-agarose column to sequence its N-terminal amino acids. Its molecular weight was identical to that of intracellular BacA-His in SDS-PAGE (Fig. 2*D*). The N-terminal amino acids of secreted BacA (MDEMV) were also identical to the 5-amino acid sequence from the start methionine of the BacA ORF (Fig. 2*E*). Collectively, these results demonstrate that BacL$_1$ and BacA are secreted without *sec*-dependent signal peptide cleavage or any N-terminal processing.

Bactericidal Activity of Recombinant BacL$_1$ and BacA—To investigate the molecular function of BacL$_1$ and BacA in detail, we prepared the recombinant BacL$_1$-His (65.3 kDa) and BacA-His (79.9 kDa) (Fig. 3*B*). In a soft agar assay, the bactericidal activity against the indicator strain *E. faecalis* was observed when using a mixture of BacL$_1$-His and BacA-His proteins and also between the spotted area of BacL$_1$-His and BacA-His (Fig. 3*C*). However, neither individual protein was sufficient for the bactericidal activity.

To examine bacteriolysis in the aqueous phase, a suspension of *E. faecalis* in fresh THB broth had either BacL$_1$-His, BacA-His, or the mixture of both proteins added, and the turbidity of the suspension was monitored (Fig. 3*D*). In the absence of any treatment, the turbidity of the suspension gradually increased due to bacterial growth. However, when BacL$_1$-His and BacA-His were both added to the bacterial suspension, a remarkable reduction in turbidity was observed after 2 h of incubation, and after 3 h of incubation, the optical density at 600 nm was less than 0.1. Furthermore, the viability of cells co-treated with BacL$_1$-His and BacA-His was greatly reduced compared with untreated cells (Fig. 3*E*), and the lysed cells did not stain well in Gram staining (Fig. 3*F*). In contrast, treatment with either BacL$_1$-His or BacA-His alone did not affect bacterial growth (Fig. 3*D*), and no significant effects on the viability or morphology of the cells were observed when the bacterial suspension was treated with each individual protein (Fig. 3, *E* and *F*).

Peptidoglycan Hydrolase Activity of BacL$_1$ and Its Cleavage Site—To further investigate the enzymatic activity of BacL$_1$ and BacA, the constructs were subjected to zymography analysis using a gel containing autoclaved *E. faecalis* cells as a substrate (Fig. 4*A*). BacL$_1$-His alone was sufficient for bacteriolytic activity in the presence or absence of BacA-His. Furthermore, the purified cell wall fraction was prepared from *E. faecalis* and treated with BacL$_1$-His, BacA-His, a mixture of the two constructs, and mutanolysin (Fig. 4*B*). The cell wall treated with BacL$_1$-His alone or with the mixture of BacL$_1$-His and BacA-His was gradually degraded, as after mutanolysin digestion. In contrast, the activity of BacA-His was completely undetectable. These data clearly show that BacL$_1$ alone was sufficient for the degradation of the cell wall component and that it acts as a peptidoglycan hydrolase, although it has no bactericidal activity against viable cells.

Thus, BacL$_1$ appears to act as a peptidoglycan-degrading enzyme. To determine the BacL$_1$ cleavage site, the soluble product generated from BacL$_1$-treated peptidoglycan was sep-

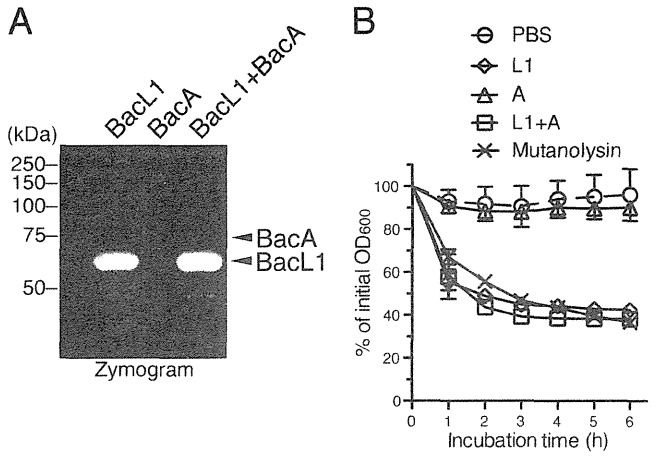

FIGURE 4. **Degrading activity of recombinant BacL$_1$ against the cell wall fraction of *E. faecalis*.** *A*, recombinant BacL$_1$-His (400 ng; *lane 1*), BacA-His (400 ng; *lane 2*), and a mixture of both proteins (400 ng each; *lane 3*) were separated by SDS-PAGE with the zymogram gel containing autoclaved *E. faecalis* cells, following static incubation at 37 °C for 36 h. The resulting gel was analyzed using a densitometer. The *top* and *bottom arrowheads* indicate the band positions of BacA and BacL$_1$, respectively. *B*, a cell wall fraction prepared from *E. faecalis* at exponential phase was diluted with fresh PBS. Recombinant BacL$_1$-His (5 μg/ml), BacA-His (5 μg/ml), and a mixture of both proteins (5 μg/ml each) or mutanolysin (1 μg/ml) was added into the cell wall suspension and incubated at 37 °C. The turbidity at optical density of 600 nm was quantified at the indicated times of incubation. The values presented are the percentages of the initial turbidity of the respective samples. The data for each case are presented as the means ± S.E. (*error bars*) of three independent experiments.

arated by reverse-phase HPLC on an ODS-Hypersil column (Fig. 5*A*). The HPLC fractions were analyzed by MALDI-PSD analysis to determine their molecular structures as described under "Experimental Procedures" (Fig. 5, *B* and *C*). The data indicate that BacL$_1$ has an endopeptidase activity that hydrolyzes the peptide bond between D-isoglutamine and L-lysine in the stem peptide (Fig. 5*D*).

Domain Analysis of BacL$_1$—To determine the molecular function of BacL$_1$, we constructed truncated derivatives of the recombinant BacL$_1$-His proteins. BacL$_1$Δ1 (49.9 kDa), BacL$_1$Δ2 (48.4 kDa), BacL$_1$Δ1Δ2 (33.0 kDa), and BacL$_1$Δ3 (37.7 kDa) had a deletion in domain 1 or domain 2, in both domain 1 and 2, or in domain 3, respectively (Fig. 6, *A* and *B*). Unlike the wild-type BacL$_1$-His, each truncated protein no longer showed bactericidal activity against viable *E. faecalis* in a soft agar assay or a liquid bacteriolytic assay, even in the presence of BacA (Fig. 6, *C* and *D*). However, in the zymography and cell wall lysis assays, the protein with the truncated domain 1 (BacL$_1$Δ1) still showed similar cell wall-degrading activity as the wild-type BacL$_1$-His (Fig. 6, *E* and *F*). Furthermore, treatment of the cell wall fraction with the BacL$_1$Δ1 protein generated the soluble fragments L-Lys-L-Ala$_2$-(D-Ala-L-Lys-L-Ala$_2$)$_n$ and L-Lys-(D-Ala$_2$)-L-Ala$_2$-(D-Ala-L-Lys-L-Ala$_2$)$_n$, which were identical to the results obtained with wild-type BacL$_1$ (Fig. 5, *B* and *C*). In contrast, these peptides were not generated from the cell wall treated with BacL$_1$Δ2, BacL$_1$Δ1Δ2, or BacL$_1$Δ3 (data not shown), indicating that the D-isoglutamyl-L-lysine endopeptidase activity is dependent on domains 2 and 3 of BacL$_1$.

Cell Wall Targeting Mediated by Repeated SH3 Domain of BacL$_1$—The data shown in Fig. 6 also indicate that the C-terminal repeated domain showing homology with the SH3

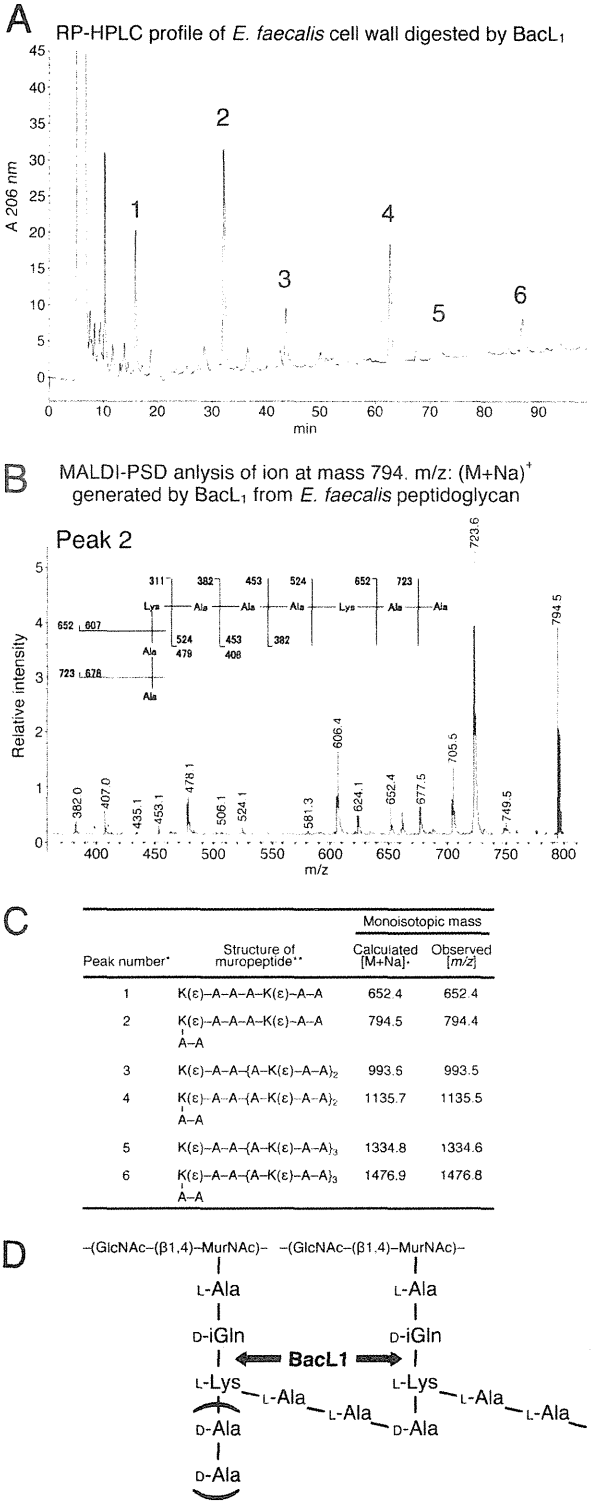

FIGURE 5. **Cleavage site of *E. faecalis* peptidoglycan by BacL$_1$.** *A*, the *E. faecalis* cell wall fraction was incubated with wild-type BacL$_1$-His. The solubilized peptidoglycan component was separated on a C18 reverse-phase HPLC column, as described under "Experimental Procedures." The *numbers above* the *peaks* indicate the fractions isolated for the following MALDI-PSD MS analysis. *B*, the compound corresponding to the purified peak 2 of reverse-phase HPLC in *A* was subjected to MALDI-PSD MS. *C*, possible muropeptide structures and their calculated masses [M + Na]$^+$ are shown as observed mass (*m/z*) in MADLI-PSD analysis (*B*). *, *peak numbers* refer to the HPLC chromatogram (*A*). **, *K*, lysine; *A*, alanine. *D*, predicted cleavage site of BacL$_1$ in *E. faecalis* peptidoglycan (*filled arrows*).

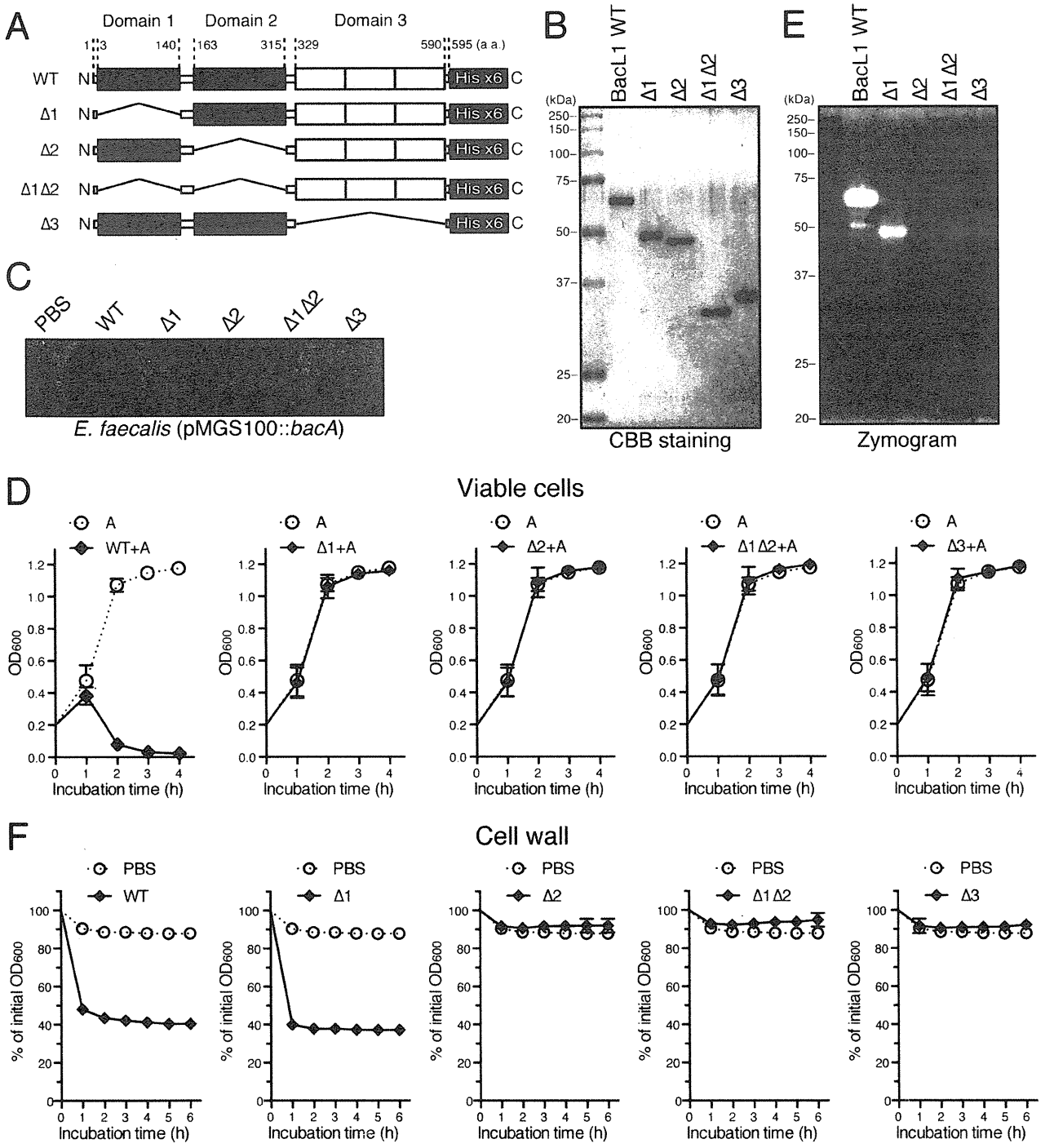

FIGURE 6. **Domain analysis of BacL$_1$.** *A*, diagrammatic representation of the truncated BacL$_1$ constructs. The wild-type BacL$_1$ (WT) consists of 595 amino acids, with three domain structures in the region of 3–140 (domain 1), 163–315 (domain 2), and 329–590 (domain 3) amino acids, respectively. BacL$_1$Δ1, -Δ2, or -Δ3 is the truncated derivative from which domain 1, 2, or 3 from wild-type BacL$_1$ has been deleted, respectively. BacL$_1$Δ1Δ2 is the derivative from which both domain 1 and 2 have been deleted. The hexahistidine tag was added to the C terminus of each truncated protein for purification with the Ni-NTA system. *B*, the recombinant truncated BacL$_1$ derivatives (400 ng) were separated by SDS-PAGE and stained with CBB. *C*, the recombinant truncated BacL$_1$ derivatives (25 ng) were spotted onto the THB soft agar (0.75%) containing the indicator strain *E. faecalis* carrying pMGS100::*bacA*, which produces BacA. The plate was incubated at 37 °C for 24 h, and the formation of halos was evaluated. *D*, a culture of *E. faecalis* was diluted with fresh THB broth to adjust the optical density at 600 nm to 0.2. The mixture of each truncated BacL$_1$ derivative and BacA-His (5 μg/ml each) was added into the bacterial suspension and incubated at 37 °C. The turbidity was monitored during the incubation period. The result of the BacA-His$_6$-treated sample without any BacL$_1$ derivatives is presented in each graph as the negative control. The data are presented as the means ± S.E. (*error bars*) of three independent experiments. *E*, the recombinant truncated BacL$_1$ derivatives (400 ng) were separated by SDS-PAGE on the zymogram gel containing autoclaved *E. faecalis* cells, followed by static incubation at 37 °C for 36 h. The resulting gel was analyzed using a densitometer. *F*, a cell wall fraction prepared from *E. faecalis* at exponential phase was diluted with fresh PBS. The recombinant truncated BacL$_1$ derivatives (5 μg/ml) were added into the cell wall suspension and incubated at 37 °C. The turbidity at 600 nm was quantified at the indicated times after incubation. The values presented are the percentages of the initial turbidity of the respective samples. The PBS-treated sample is presented in each graph as a negative control. The data are presented as the means ± S.E. of three independent experiments.

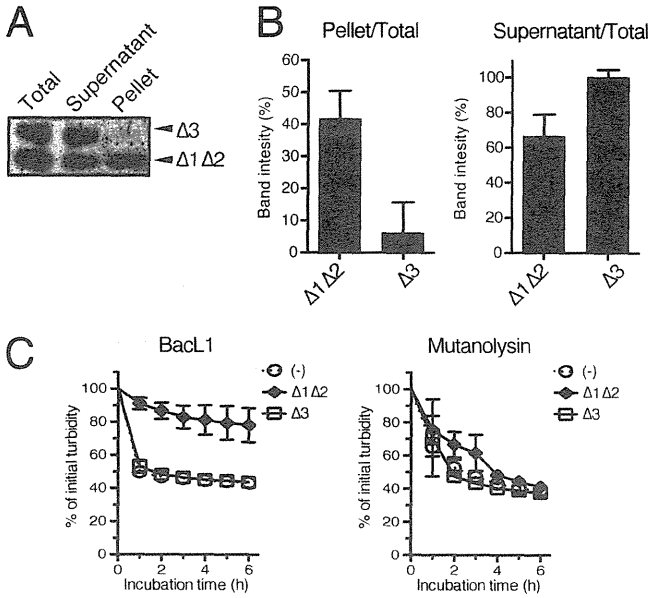

FIGURE 7. **Binding activity of SH3 domain to the cell wall of *E. faecalis*.** *A*, a cell wall fraction prepared from *E. faecalis* at exponential phase (2 mg) was mixed with the recombinant BacL$_1$Δ1Δ2 and BacL$_1$Δ3 (*Total*). After incubation at 4 °C for 1 h, the cell wall-bound (*Pellet*) and unbound (*Supernatant*) fractions were separated by centrifugation and then analyzed by SDS-PAGE followed by CBB staining. The *arrowheads* indicate the specific band positions of the respective proteins. *B*, quantified protein levels (percentages) of the pellet or supernatant fractions were determined by defining the relative band intensity of the total fraction as 100%. The data for each case are presented as the means ± S.E. (*error bars*) of three independent experiments. *C*, a cell wall fraction prepared from *E. faecalis* was preincubated with BacL$_1$Δ1Δ2 or BacL$_1$Δ3 at 4 °C for 1 h. After preincubation, wild-type BacL$_1$ (*left*) or mutanolysin (*right*) was added, and the turbidity at an optical density of 600 nm was monitored. The values presented are the percentages of the initial turbidity of the respective samples. The data for each case are presented as the means ± S.E. of three independent experiments.

domain of ALE-1 of *S. aureus* is required for both bacteriolytic and cell wall-degrading activity. The repeated SH3 domain of ALE-1, a peptidoglycan hydrolase of *S. aureus*, has been reported to be a targeting domain that binds to peptidoglycan (22). The SH3 domain of BacL$_1$ (domain 3) presumably also binds to peptidoglycan. We therefore assessed the binding efficiency of the truncated BacL$_1$ proteins to the cell wall fraction of *E. faecalis* (Fig. 7, *A* and *B*).

Domain 3 of BacL$_1$ (BacL$_1$Δ1Δ2) showed 40% binding efficiency. In contrast, the binding efficiency of BacL1 with a deleted domain 3 (BacL$_1$Δ3) was less than 10%, showing that domain 3 of BacL$_1$ binds to the substrate. Furthermore, preincubation with BacL$_1$Δ1Δ2 interfered with the cell wall-degrading activity of BacL$_1$, but not of mutanolysin. In contrast, the truncated protein BacL$_1$Δ3 did not affect the degradation activity of BacL$_1$. Collectively, these results suggest that domain 3 of BacL$_1$ works as a cell wall-targeting domain of BacL$_1$.

DISCUSSION

In this study, we investigated the biochemical features of two Bac41 components, BacL$_1$ and BacA, using their recombinant proteins. First, we confirmed that the secreted BacL$_1$ and BacA were the enzymatically active forms. Many enterococcal bacteriocins have an N-terminal *sec* signal sequence and are translated as preproteins to be secreted by the *sec* pathway (6, 13–16). The N-terminal *sec* signal peptide, which consists of N-terminal positively charged residues, a hydrophobic core, and a polar C-terminal cleavable site, is recognized by the *sec*-dependent secretion machinery (32–34). Upon the translocation via *sec* machinery, the *sec* signal peptide at the N terminus of preprotein is cleaved. The mature form of the protein is exported, exerting the bactericidal effect against target cells (34).

Our previous sequence analysis predicted the existence of a signal sequence in the N terminus of BacL$_1$ and BacA (4) (Fig. 8). However, we observed no trace of the cleavage of an N-terminal peptide from BacL$_1$ or BacA (Fig. 1C) and confirmed that the N termini of postsecretion BacL$_1$ and BacA were completely intact (Fig. 2). Thus, the full-length recombinant proteins of BacL$_1$ and BacA were sufficient for the bacteriocin activity in the culture supernatant of *E. faecalis* (pHT1100), which carries wild-type Bac41-related genes, including *bacL$_1$* and *bacA* (Fig. 3). Based on these observations, we concluded that BacL$_1$ and BacA are secreted without N-terminal processing and that the proteins as they translated are already in an active form.

However, we also noted that the N-terminal regions of BacL$_1$ and BacA appear to be involved in the efficiency of their secretion or the cytosolic state, respectively (Fig. 2). The secretion efficiency of BacL$_1$ was strikingly reduced by tagging with the N-terminal FLAG peptide but not by tagging with the C-terminal hexahistidine. In contrast, an N-terminal FLAG tag did not affect the intracellular amount of BacL$_1$ (Fig. 2*A*), suggesting that the intact N terminus is required for the efficient secretion of BacL$_1$. The N-terminal peptide fusion to BacA also resulted in an unexpected artificial effect (Fig. 2*A*). The band expected for the intact secreted FLAG-BacA-His in the culture supernatant was observed at the predicted position and behaved similarly to BacA-His or non-tagged BacA (Fig. 2*A*). However, the cytosolic FLAG-BacA-His was shifted to larger and smaller sizes than predicted from the molecular mass (Fig. 2*A*). Although we cannot explain this phenomenon, the artificial N-terminal tagging probably affected the cytosolic state of BacA. Hence, the N-terminal regions of BacL$_1$ and BacA appear to play a role in their secretion and in their cytosolic form.

As described above, it does not seem that BacL$_1$ and BacA are secreted in a typical *sec*-dependent manner. Indeed, the SignalP 4.1 program server does not predict an obvious *sec*-dependent signal in the BacL$_1$ or BacA sequence. Despite that, the N-terminal amino acid sequence of BacL$_1$ or BacA contains multiple lysine residues and a potential hydrophobic core. Taken together with the result shown in Fig. 2, BacL$_1$ and BacA might be secreted via a *sec*-dependent pathway but not cleaved by signal peptidase during translocation.

Another possible secretion mechanism for BacL$_1$ and BacA is leakage due to cell disruption. The *Streptococcus pneumoniae* cytolysin, pneumolysin, is a secreted protein that shows cytotoxicity to mammalian cells. Nevertheless, pneumolysin lacks a signal peptide sequence at its N terminus. The extracellular secretion of pneumolysin is thought to depend on the disruption of cells upon the characteristic autolysis that is induced during stationary or death phase (35). Further investigation is needed to elucidate this issue.

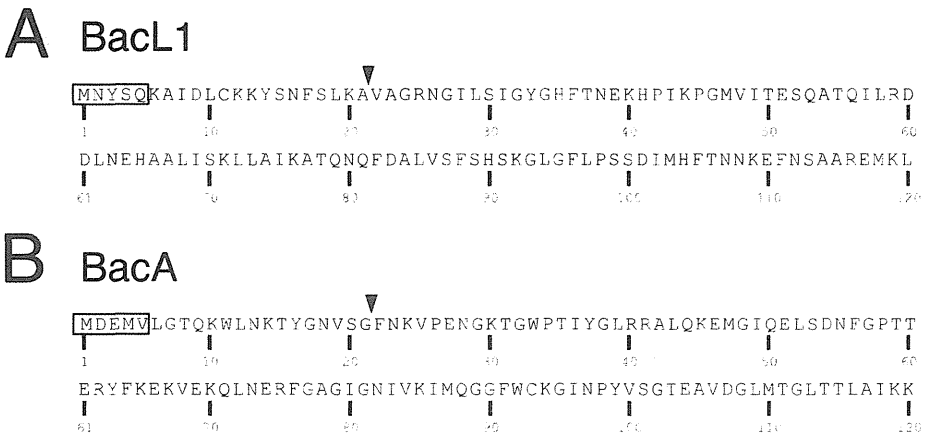

FIGURE 8. **N-terminal amino acid sequences of BacL$_1$ and BacA.** Amino acid sequences of 1–120 residues in BacL$_1$ (*A*; accession number BAG12399) and BacA (*B*; accession number BAG12403) are presented (4). Residues in the *frame* are the resulting sequences from the N-terminal amino acids sequence analysis as in Fig. 2. *Arrows* indicate the processing site predicted in the previous study (4).

BacL$_1$ contains two distinct peptidoglycan hydrolase domains, referred to as domains 1 and 2 in this paper. Domain analysis showed that the BacL$_1$Δ1 protein, but not BacL$_1$Δ2, was able to degrade peptidoglycan (Fig. 6, *E* and *F*) and showed D-isoglutamyl-L-lysine endopeptidase activity (Fig. 5) similar to wild-type BacL$_1$. The data suggested that domain 2 of BacL$_1$, rather than domain 1, is responsible for the D-isoglutamyl-L-lysine endopeptidase activity. When the structural model of BacL$_1$ was analyzed using the Phyre program, domain 2 was predicted to be similar to the NlpC/P60 family peptidoglycan hydrolases. The NlpC/P60 family peptidoglycan hydrolases have been found in various Gram-positive or -negative bacteria (36).

Most Nlp/P60 family proteins are reported to be endopeptidases that cleave the peptide bond between D-isoglutamine and mesodiaminopimelic acid or L-lysine in the stem peptide of peptidoglycan. Indeed, mass spectrometry analysis revealed that BacL$_1$ is an endopeptidase that digests the linkage between D-isoglutamine and L-lysine in the stem peptide of *E. faecalis* peptidoglycan (Fig. 5). Thus, the endopeptidase activity of domain 2 is likely to play a critical role in the lytic activity against *E. faecalis* cells.

By contrast, because the truncated BacL1Δ2 with an intact domain 1 did not degrade the *E. faecalis* cell wall (Fig. 6, *E* and *F*), the activity of domain 1 was not detected, despite its significant homology with the bacteriophage-type lysozyme with peptidoglycan hydrolase activity (4, 37). However, domain 1 was still required for bactericidal action against viable cells (Fig. 6, *C* and *D*) but not for degradation of the purified cell wall fraction (Fig. 6, *C* and *F*). Probably, domain 1 does not have cell wall-degrading activity itself but has an accessory function for the efficient endopeptidase activity of domain 2. Another possibility is that the deletion of domain 2 resulted in a conformational change that interferes with the function of domain 1. Further study is required to understand the role of domain 1 of BacL$_1$ in bactericidal activity against viable cells.

The data in Fig. 3 clearly demonstrate that BacL$_1$ and BacA are both required for bacteriolysis against *E. faecalis*. Nevertheless, BacL$_1$ still showed cell wall degradation activity, even in the absence of BacA (Fig. 4), indicating that the peptidoglycan hydrolase activity of BacL1 does not immediately result in the bacteriolysis of viable *E. faecalis*. In addition, BacA has a putative peptidoglycan binding domain (4) and a GH25 family peptidoglycan hydrolase-like domain (24), suggesting that BacA also binds to the target cell wall rather than just being an activator of BacL$_1$. Nevertheless, the binding activity of full-length recombinant BacA-His to peptidoglycan was poorly detected (data not shown). Thus, we need to address the role of BacA in a future study. The molecular mechanism of BacL$_1$-induced bacteriolysis is clearly complex and is associated with additional factors, the state of the target cells, and the unknown function of BacA.

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REFERENCES

1. Jack, R. W., Tagg, J. R., and Ray, B. (1995) Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* **59,** 171–200
2. Clewell, D. B. (1981) Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. *Microbiol. Rev.* **45,** 409–436
3. Ike, Y., Hashimoto, H., and Clewell, D. B. (1987) High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. *J. Clin. Microbiol.* **25,** 1524–1528
4. Tomita, H., Kamei, E., and Ike, Y. (2008) Cloning and genetic analyses of the bacteriocin 41 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI14. A novel bacteriocin complemented by two extracellular components (lysin and activator). *J. Bacteriol.* **190,** 2075–2085
5. Ike, Y., and Clewell, D. B. (1992) Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. zymogenes can be determined by plasmids in different incompatibility groups as well as by the chromosome. *J. Bacteriol.* **174,** 8172–8177
6. Tomita, H., Fujimoto, S., Tanimoto, K., and Ike, Y. (1996) Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. *J. Bacteriol.* **178,** 3585–3593
7. Fujimoto, S., Tomita, H., Wakamatsu, E., Tanimoto, K., and Ike, Y. (1995) Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. *J. Bacteriol.* **177,** 5574–5581
8. Franz, C. M., van Belkum, M. J., Holzapfel, W. H., Abriouel, H., and Gálvez, A. (2007) Diversity of enterococcal bacteriocins and their group-

ing in a new classification scheme. *FEMS Microbiol. Rev.* **31,** 293–310
9. Ike, Y., Hashimoto, H., and Clewell, D. B. (1984) Hemolysin of *Streptococcus faecalis* subspecies zymogenes contributes to virulence in mice. *Infect. Immun.* **45,** 528–530
10. Chow, J. W., Thal, L. A., Perri, M. B., Vazquez, J. A., Donabedian, S. M., Clewell, D. B., and Zervos, M. J. (1993) Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob. Agents Chemother.* **37,** 2474–2477
11. Jett, B. D., Jensen, H. G., Nordquist, R. E., and Gilmore, M. S. (1992) Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infect. Immun.* **60,** 2445–2452
12. Martínez-Bueno, M., Maqueda, M., Gálvez, A., Samyn, B., Van Beeumen, J., Coyette, J., and Valdivia, E. (1994) Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. *J. Bacteriol.* **176,** 6334–6339
13. Tomita, H., Fujimoto, S., Tanimoto, K., and Ike, Y. (1997) Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. *J. Bacteriol.* **179,** 7843–7855
14. Inoue, T., Tomita, H., and Ike, Y. (2006) Bac 32, a novel bacteriocin widely disseminated among clinical isolates of *Enterococcus faecium*. *Antimicrob. Agents Chemother.* **50,** 1202–1212
15. Todokoro, D., Tomita, H., Inoue, T., and Ike, Y. (2006) Genetic analysis of bacteriocin 43 of vancomycin-resistant *Enterococcus faecium*. *Appl. Environ. Microbiol.* **72,** 6955–6964
16. Yamashita, H., Tomita, H., Inoue, T., and Ike, Y. (2011) Genetic organization and mode of action of a novel bacteriocin, bacteriocin 51. Determinant of VanA-type vancomycin-resistant *Enterococcus faecium*. *Antimicrob. Agents Chemother.* **55,** 4352–4360
17. Zheng, B., Tomita, H., Inoue, T., and Ike, Y. (2009) Isolation of VanB-type *Enterococcus faecalis* strains from nosocomial infections. First report of the isolation and identification of the pheromone-responsive plasmids pMG2200, encoding VanB-type vancomycin resistance and a Bac41-type bacteriocin, and pMG2201, encoding erythromycin resistance and cytolysin (Hly/Bac). *Antimicrob. Agents Chemother.* **53,** 735–747
18. Tomita, H., Todokoro, D., and Ike, Y. (2010) Genetic analysis of the bacteriocin 41 encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI14. *3rd International ASM Conference on Enterococci*, Abstr. 30B, pp. 37–38, American Society for Microbiology, Washington, D. C.
19. Pecenková, T., Benes, V., Paces, J., Vlcek, C., and Paces, V. (1997) Bacteriophage B103. Complete DNA sequence of its genome and relationship to other *Bacillus* phages. *Gene* **199,** 157–163
20. Sheehan, M. M., García, J. L., López, R., and García, P. (1997) The lytic enzyme of the pneumococcal phage Dp-1. A chimeric lysin of intergeneric origin. *Mol. Microbiol.* **25,** 717–725
21. Tettelin, H., Masignani, V., Cieslewicz, M. J., Eisen, J. A., Peterson, S., Wessels, M. R., Paulsen, I. T., Nelson, K. E., Margarit, I., Read, T. D., Madoff, L. C., Wolf, A. M., Beanan, M. J., Brinkac, L. M., Daugherty, S. C., DeBoy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Lewis, M. R., Radune, D., Fedorova, N. B., Scanlan, D., Khouri, H., Mulligan, S., Carty, H. A., Cline, R. T., Van Aken, S. E., Gill, J., Scarselli, M., Mora, M., Iacobini, E. T., Brettoni, C., Galli, G., Mariani, M., Vegni, F., Maione, D., Rinaudo, D., Rappuoli, R., Telford, J. L., Kasper, D. L., Grandi, G., and Fraser, C. M. (2002) Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 12391–12396
22. Lu, J. Z., Fujiwara, T., Komatsuzawa, H., Sugai, M., and Sakon, J. (2006) Cell wall-targeting domain of glycylglycine endopeptidase distinguishes among peptidoglycan cross-bridges. *J. Biol. Chem.* **281,** 549–558
23. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., and Danchin, A. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390,** 249–256
24. Martinez-Fleites, C., Korczynska, J. E., Davies, G. J., Cope, M. J., Turkenburg, J. P., and Taylor, E. J. (2009) The crystal structure of a family GH25 lysozyme from *Bacillus anthracis* implies a neighboring-group catalytic mechanism with retention of anomeric configuration. *Carbohydr. Res.* **344,** 1753–1757
25. Sambrook, J. J., and Russell, D. D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Dunny, G. M., Craig, R. A., Carron, R. L., and Clewell, D. B. (1979) Plasmid transfer in *Streptococcus faecalis*. Production of multiple sex pheromones by recipients. *Plasmid* **2,** 454–465
27. Ike, Y., Clewell, D. B., Segarra, R. A., and Gilmore, M. S. (1990) Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*. Tn917 insertional mutagenesis and cloning. *J. Bacteriol.* **172,** 155–163
28. Sugai, M., Komatsuzawa, H., Miyake, Y., and Suginaka, H. (1991) Visualization of endo-β-*N*-acetylglucosaminidase, lysozyme, and lysostaphin after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. *Zentralbl Bakteriol.* **275,** 156–161
29. Eckert, C., Lecerf, M., Dubost, L., Arthur, M., and Mesnage, S. (2006) Functional analysis of AtlA, the major N-acetylglucosaminidase of *Enterococcus faecalis*. *J. Bacteriol.* **188,** 8513–8519
30. Kajimura, J., Fujiwara, T., Yamada, S., Suzawa, Y., Nishida, T., Oyamada, Y., Hayashi, I., Yamagishi, J., Komatsuzawa, H., and Sugai, M. (2005) Identification and molecular characterization of an *N*-acetylmuramyl-L-alanine amidase Sle1 involved in cell separation of *Staphylococcus aureus*. *Mol. Microbiol.* **58,** 1087–1101
31. Uchiyama, J., Takemura, I., Hayashi, I., Matsuzaki, S., Satoh, M., Ujihara, T., Murakami, M., Imajoh, M., Sugai, M., and Daibata, M. (2011) Characterization of lytic enzyme open reading frame 9 (ORF9) derived from *Enterococcus faecalis* bacteriophage phiEF24C. *Appl. Environ. Microbiol.* **77,** 580–585
32. Schneewind, O., and Missiakas, D. M. (2012) Protein secretion and surface display in Gram-positive bacteria. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **367,** 1123–1139
33. Thanassi, D. G., Bliska, J. B., and Christie, P. J. (2012) Surface organelles assembled by secretion systems of Gram-negative bacteria. Diversity in structure and function. *FEMS Microbiol. Rev.* **36,** 1046–1082
34. Feltcher, M. E., and Braunstein, M. (2012) Emerging themes in SecA2-mediated protein export. *Nat. Rev. Microbiol.* **10,** 779–789
35. Martner, A., Dahlgren, C., Paton, J. C., and Wold, A. E. (2008) Pneumolysin released during *Streptococcus pneumoniae* autolysis is a potent activator of intracellular oxygen radical production in neutrophils. *Infect. Immun.* **76,** 4079–4087
36. Arai, R., Fukui, S., Kobayashi, N., and Sekiguchi, J. (2012) Solution structure of IseA, an inhibitor protein of DL-endopeptidases from *Bacillus subtilis*, reveals a novel fold with a characteristic inhibitory loop. *J. Biol. Chem.* **287,** 44736–44748
37. Pritchard, D. G., Dong, S., Kirk, M. C., Cartee, R. T., and Baker, J. R. (2007) LambdaSa1 and LambdaSa2 prophage lysins of *Streptococcus agalactiae*. *Appl. Environ Microbiol.* **73,** 7150–7154
38. Wirth, R., An, F. Y., and Clewell, D. B. (1986) Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. *J. Bacteriol.* **165,** 831–836
39. Fujimoto, S., and Ike, Y. (2001) pAM401-based shuttle vectors that enable overexpression of promoterless genes and one-step purification of tag fusion proteins directly from *Enterococcus faecalis*. *Appl. Environ. Microbiol.* **67,** 1262–1267

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Interference of bacterial cell-to-cell communication: a new concept of antimicrobial chemotherapy breaks antibiotic resistance

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Bacteria use a cell-to-cell communication activity termed "quorum sensing" to coordinate group behaviors in a cell density dependent manner. Quorum sensing influences the expression profile of diverse genes, including antibiotic tolerance and virulence determinants, via specific chemical compounds called "autoinducers". During quorum sensing, Gram-negative bacteria typically use an acylated homoserine lactone (AHL) called autoinducer 1. Since the first discovery of quorum sensing in a marine bacterium, it has been recognized that more than 100 species possess this mechanism of cell-to-cell communication. In addition to being of interest from a biological standpoint, quorum sensing is a potential target for antimicrobial chemotherapy. This unique concept of antimicrobial control relies on reducing the burden of virulence rather than killing the bacteria. It is believed that this approach will not only suppress the development of antibiotic resistance, but will also improve the treatment of refractory infections triggered by multi-drug resistant pathogens. In this paper, we review and track recent progress in studies on AHL inhibitors/modulators from a biological standpoint. It has been discovered that both natural and synthetic compounds can disrupt quorum sensing by a variety of means, such as jamming signal transduction, inhibition of signal production and break-down and trapping of signal compounds. We also focus on the regulatory elements that attenuate quorum sensing activities and discuss their unique properties. Understanding the biological roles of regulatory elements might be useful in developing inhibitor applications and understanding how quorum sensing is controlled.

Keywords: quorum sensing, antagonist, inhibitor, antibiotic resistance, virulence control, autoinducer, homoserine lactone

OUTLINE OF "QUORUM SENSING" IN GRAM-NEGATIVE BACTERIA

The development of antibiotics originated with penicillin, and this approach to the treatment of bacterial infection has been an enormous success. However, the widespread use of antibiotics has resulted in bacteria acquiring resistance in addition to their innate tolerance derived from mechanisms such as biofilm formation and drug efflux. Since the discovery of bacterial quorum sensing in a marine bacterium 40 years-ago, similar systems have been discovered in many organisms, including animal and plant pathogens, and these systems have been characterized along with virulence and drug tolerance determinants. Thus, quorum sensing is now regarded as a potential target for the development of antibacterial agents. In the last 20 years, various quorum sensing inhibitors have been isolated and characterized from natural and chemically synthesized libraries. Some animal and plant infection models have demonstrated the antibacterial efficacy of these agents against quorum sensing pathogens. In this paper, we focus on quorum sensing inhibitors as a novel type of antibacterial agent and also provide an update on recent progress in quorum sensing studies. In the first section, we will review the background and literature relating to bacterial cell-to-cell communication, which is currently termed "quorum sensing."

DISCOVERY AND HISTORY

Bacteria are single cell organisms, however, they conduct a bacterial "cell-to-cell" communication activity with the same and/or different species via diffusible chemical compounds, and exhibit group behaviors similar to eukaryotic cells. This concept of social activity between bacteria has been termed "sociomicrobiology" (Parsek and Greenberg, 2005).

Sociomicrobiology was first described in a study carried out in the early 1970s on the bioluminescence phenomenon found in *Vibrio fischeri*, a marine bacterium associated with Hawaiian squid, (Nealson et al., 1970). When the bacteria were grown in shake flasks, expression of the luminescence gene (*lux*) was shown to be relatively low during early exponential growth, but was then followed by a rapid increase in expression during the late exponential and early stationary phases. The luminescence gene in exponential phase cultures can be activated by the addition of cell-free fluid extracts from stationary phase cultures. These observations implied that *Vibrio fischeri* has an environmental sensing system

to monitor its own population density, and a signaling substance termed "autoinducer," which was later shown to be 3-oxohexanoyl-homoserine lactone, activates *lux* expression in high cell density cultures (Eberhard et al., 1981). Currently, over 100 species of bacteria are known to produce autoinducer molecules in a cell density dependent manner similar to *Vibrio fischeri* and this signaling mechanism is now termed "quorum sensing" (Fuqua et al., 1994). Bacteria use three classes of autoinducer for quorum sensing. Acyl-homoserine lactone (AHL) is the most common class of autoinducer used by Gram-negative bacteria, whereas oligopeptide is the major class of autoinducer in Gram-positive bacteria (Dunny and Leonard, 1997). Most of these signals are highly specific and are produced and recognized by a single species. The other class of autoinducer is a 4,5-dihydroxy-2,3-pentanedione (DPD) derivative termed autoinducer-2 (AI-2; Bassler, 2002). It has been suggested that AI-2 is a non-species specific signal which mediates interspecies communication among Gram-negative and Gram-positive bacteria. Although the activity of AI-2 signals has been demonstrated in over 100 species of bacteria, their structures remain largely unknown. Only a few structures of the AI-2 ligand-receptor complex (from *Vibrio harveyi*, *Salmonella* Typhimurium, *Sinorhizobium meliloti*, and *Yersinia pestis*) have been described (Chen et al., 2002; Miller et al., 2004; Pereira et al., 2008; Kavanaugh et al., 2011). In this review, we will focus on the AHL quorum sensing mechanism, as that is the most well defined system of the three quorum sensing families. We will also review progress in the study of effectors which disrupt or attenuate the AHLs-mediated quorum sensing as potential antimicrobial targets.

ELEMENTS AND REGULATORY SYSTEM OF AHLs QUORUM SENSING

The first AHL molecule to be described was 3-oxohexanoyl-homoserine lactone (typically abbreviated 3-oxo-C_6-HSL) from *Vibrio fischeri* (Eberhard et al., 1981). Two elements, a signal generator LuxI and the cognate receptor LuxR, regulate the quorum sensing mediated by 3-oxo-C_6-HSL in this bacterium. The 3-oxo-C_6-HSL is biosynthesized in a catalytic reaction mediated by LuxI (Engebrecht and Silverman, 1984). The molecule can diffuse into and out of cells, and once a threshold concentration is reached, the 3-oxo-C_6-HSL binds the cognate receptor, LuxR (Kaplan and Greenberg, 1985; Hanzelka and Greenberg, 1995). This results in a conformational change in LuxR which leads to the activation of the luciferase genes (*lux*) by binding to a specific DNA sequence on the gene promoter.

Since the discovery of the *luxIR* genes, homologous genes have been identified in more than 100 species of Gram-negative bacteria and in some species their quorum sensing activities have also been demonstrated. AHL molecules contain a common homoserine lactone moiety from *S*-adenosyl-methionine (abbreviated SAM) and a specific fatty acid side chain from the bacterial cellular pool. The side chain varies within different species. Therefore, the specificity for AHL signals is conferred by the length and modifications to the fatty acyl groups. Fatty acyl groups are usually 4–18 carbons in length, and some are modified by a 3-oxo or 3-hydroxy substituent, a terminal methyl branch or various degrees of unsaturation (**Figure 1.**). Recently, a new category of homoserine lactone signals that have non-fatty acid side chain substrates has been reported. They utilize phenyl-carbonic acids derived from plant metabolites, or a branched amino acid generated in the process of bacterial amino acid biosynthesis (Schaefer et al., 2008; Ahlgren et al., 2011; Lindemann et al., 2011).

The signal generator LuxI family protein is an enzyme catalyzing the generation of homoserine lactone molecules from SAM and a specific acyl group. However, this enzyme uses the acyl-carrier protein (ACP)-modified thioester form of carbonic acids as a side chain substrate rather than their free form (Schaefer et al., 1996; Parsek et al., 1999). They also have a low affinity for Coenzyme A (CoA)-modified carbonyl substrates.

The LuxR family protein has dual roles as an AHL receptor and a transcriptional regulator. The protein interacts with specific AHLs at an N-terminal signal receiver domain and forms hydrogen bonds between amino acids in the protein and the AHL molecule, which is then subjected to a conformational change in the C-terminal helix-turn-helix (HTH) domain, which enables it to bind to a conserved inverted repeat DNA sequence termed the "Lux-box" located upstream of the target gene's promoter. LuxR family proteins usually respond to AHLs produced by the corresponding synthase, however, there are some proteins that have a wide range of AHL-binding specificity. For example, CviR, a LuxR homolog from *Chromobacterium violaceum* is able to respond to AHLs with side chains that are C_4 to C_8 in length (Mcclean et al., 1997).

Quorum sensing is known to control a variety of bacterial genes involved in bioluminescence, plasmid transfer, virulence, the biosynthesis of secondary metabolites and antibiotics, and biofilm formation. Comprehensive transcriptome and computational promoter analyses have revealed quorum sensing-controlled genes in several organisms. For example, in *Vibrio fischeri*, only (0.6% of total) genes are controlled by LuxR-3-oxo-C_6-HSL (Antunes et al., 2007), whereas more than 300 genes (6% of total) are regulated during quorum sensing in the opportunistic pathogen *Pseudomonas aeruginosa* (Schuster et al., 2003).

QUORUM SENSING AND BACTERIAL INFECTIOUS DISEASES

Although quorum sensing was originally discovered in a bioluminescence study using a marine organism, extensive studies in this area have been performed with pathogenic bacteria. There is increasing evidence that bacteria use the quorum sensing mechanism to regulate their own virulence genes. Quorum sensing is considered to be a strategic tool enabling bacteria to accomplish their infection processes and survive in the host. The physiological benefit allows the bacterial cells to multiply without displaying overt virulent behavior until a certain threshold population density is reached. As a consequence, a coordinated immunological response by the host is only made when the bacterial population is high, which increases the likelihood that any defenses will be successfully overwhelmed, thereby enhancing the survival prospects of the bacteria. In this section, we will summarize the studies that have investigated the contribution of quorum sensing to bacterial virulence and infectious disease in *Pseudomonas aeruginosa*, which is the quorum sensing pathogen studied in the most detail.

Pseudomonas aeruginosa is an opportunistic pathogen, is commonly associated with nosocomial infections and is infectious in immune-compromised patients. This organism is also known

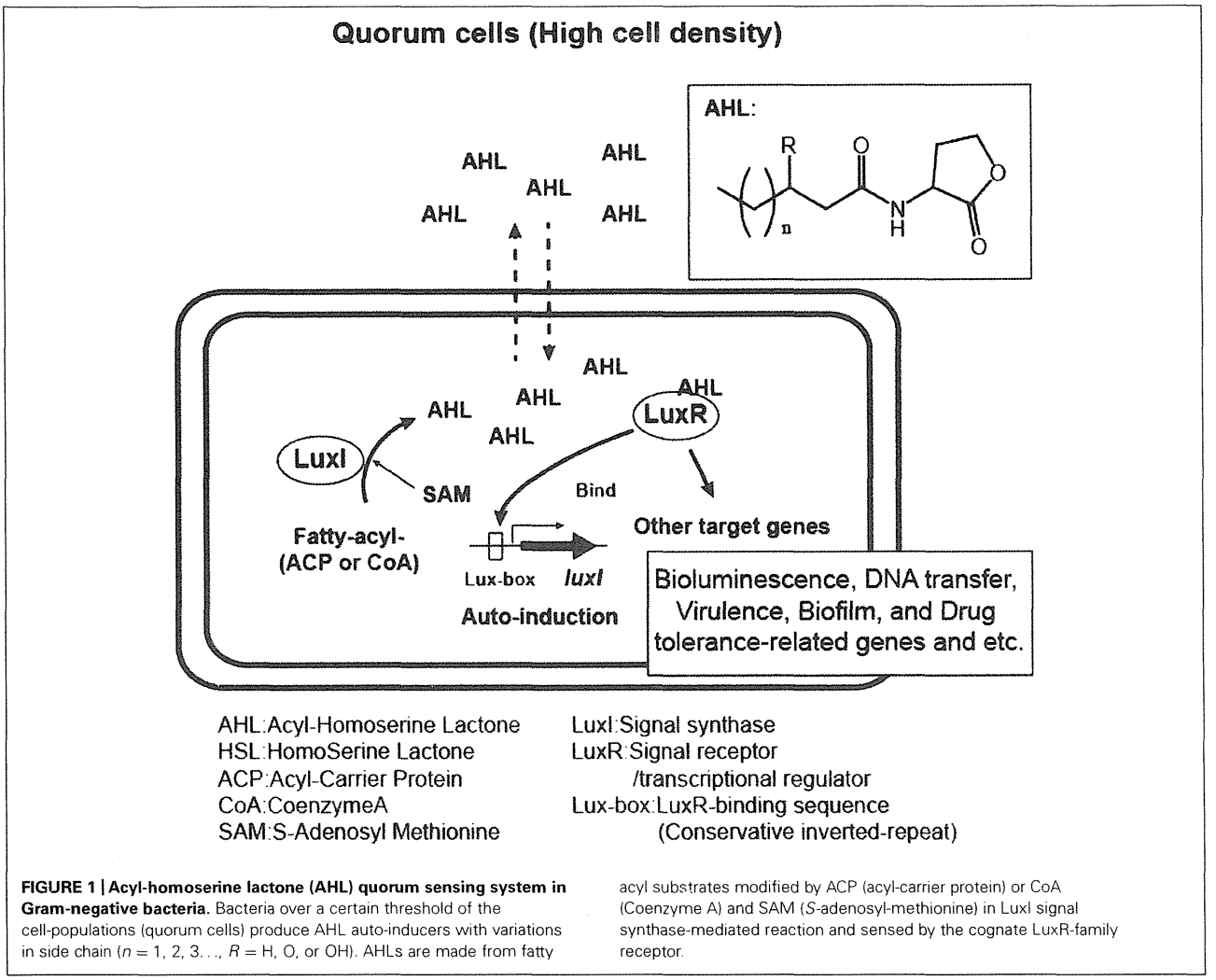

FIGURE 1 | Acyl-homoserine lactone (AHL) quorum sensing system in Gram-negative bacteria. Bacteria over a certain threshold of the cell-populations (quorum cells) produce AHL auto-inducers with variations in side chain ($n = 1, 2, 3\ldots$, R = H, O, or OH). AHLs are made from fatty acyl substrates modified by ACP (acyl-carrier protein) or CoA (Coenzyme A) and SAM (*S*-adenosyl-methionine) in LuxI signal synthase-mediated reaction and sensed by the cognate LuxR-family receptor.

for the chronic infection it causes in individuals with the genetic disease cystic fibrosis (CF) which can result in respiratory failure. In addition, this bacterium is regarded as a "model organism" in the quorum sensing field. The AHL signals produced by *Pseudomonas aeruginosa* are 3-oxododecanoyl-homoserine lactone (3-oxo-C_{12}-HSL; Pearson et al., 1994) and butanoyl-homoserine lactone (C_4-HSL) (Pearson et al., 1995). They are generated by AHL synthases called LasI and RhlI and subsequently bind to the cognate receptors called LasR and RhlR, respectively. This dual quorum sensing system is hierarchical. When the bacterial cell density reaches a particular threshold, the LasIR quorum sensing system is initiated. The 3-oxo-C_{12}-HSL-LasR complex activates *rhlI* expression as well as LasR-controlled genes including *lasI*, the cognate signal synthase, which then leads to activation of the RhlIR system. Either or both the *las* and *rhl* systems activate the production of virulence factors such as elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectines, and superoxide dismutase (Smith and Iglewski, 2003). In addition to LasR and RhlR, there is a third LuxR-family protein named QscR that is a homolog of LasR/RhlR, but does not have the cognate signal synthase (Chugani et al., 2001). QscR can bind with 3-oxo-C_{12}-HSL as well as LasR, and also with heterologous C_{12}, C_{10}, 3-oxo-C_{10}, and 3-oxo-C_6-HSLs (Lee et al., 2006; Ouuma and Greenberg, 2011). Apart from these AHLs, *Pseudomonas aeruginosa* also produces one non-AHL quorum sensing molecule termed "the *Pseudomonas* quinolone signal (PQS)" (Pesci et al., 1999). The PQS is synthesized by PqsABCD and PqsH from anthranilate that is an intermediate in the tryptophan biosynthetic pathway, and responded by PqsR (MvfR), a LysR-like protein. According to some studies, PQS and AHL-quorum sensing (*las* and *rhl*) are interlinked. The production of PQS is activated by *las* system and PQS influences the expression of C_4-HSL-regulated genes in *rhl*-dependent and –independent manners, suggesting that PQS could be also important for virulence of the organism. Highlight of PQS studies is reviewed elsewhere (Diggle et al., 2006; Huse and Whiteley, 2011)

Infection studies with mice have demonstrated the contribution of quorum sensing in the pathogenesis of *Pseudomonas aeruginosa*. *Pseudomonas* strains with mutations in quorum sensing-regulated genes induce less tissue destruction and pneumonia

and result in lower mortality compared with the wild-type (Rahme et al., 1995; Tang et al., 1996; Rumbaugh et al., 1999, 2009). Additional studies using alternative infection models with *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Dictyostelium discoideum* also have illustrated decreases in virulence with quorum sensing mutants (Rahme et al., 1995; Tan et al., 1999; Cosson et al., 2002). These model studies using an acute-infected animal host illustrate the contributions of the quorum sensing system to *Pseudomonas aeruginosa* infections. There are a few reports that *Pseudomonas aeruginosa* quorum sensing is also responsible for chronic lung infections. *Pseudomonas aeruginosa* with mutations of *lasI* and/or *rhlI* showed milder lung infections in mice and rat models (Wu et al., 2001; Imamura et al., 2005). However, the contribution of quorum sensing to chronic infections should be discussed in caution because accumulation of *lasR* mutants has been frequently observed in both many clinical isolates from CF patients and long-term laboratory cultures (Cabrol et al., 2003; Diggle et al., 2007). The *lasR* variants are considered social cheaters (Sandoz et al., 2007; Dandekar et al., 2012). LasR activates genes encoding for extracellular proteases which undertake proteolyses to cooperatively crop common metabolic/energy substrates, reasonably, the LasR-mediated quorum sensing can be promoted under nutrient-limited conditions in the presence of the particular protease substrates. The *lasR* cheaters exploit the social benefit provided by the cooperators (*lasR*-intact parent strain) saving biological costs to conduct their quorum sensing. It might be critical to answer about total quorum sensing activity of the bacterial population in infection sites and frequency of the mutant appearance.

Moreover, quorum sensing has also been shown to influence biofilm development. Biofilm is a biological architecture of aggregated microbes on a surface. It is closely associated with virulence because biofilm cells embedded within an extracellular matrix are less susceptible to antibacterial reagents than free floating cells (Nickel et al., 1985; Mah and O'Toole, 2001; Drenkard, 2003). As a result, biofilm infections tend to be chronic and difficult to eradicate. *Pseudomonas aeruginosa* is the principal pathogen in the lungs of patients with CF. The bacterium is known to exist there as a biofilm and produce significant amounts of quorum sensing molecules (Singh et al., 2000). There is a report that the *Pseudomonas aeruginosa* 3-oxo-C_{12}-HSL signal is involved in the maturation of a biofilm. A *lasI* mutant formed immature biofilms that, unlike wild-type biofilms, were sensitive to sodium dodecyl sulfate (SDS; Davies et al., 1998). Another report predicted that the *rhl* system is also important for biofilm development. The *rhl* defective *Pseudomonas aeruginosa* does not produce the rhamnolipid surfactants that are important for maintaining the biofilm architecture in the later stages (Davey et al., 2003). However, it should be noted that there is some conflict of opinion and confusion regarding the degree of involvement of quorum sensing in biofilm regulation. Biofilm architecture is easily influenced by growth conditions and wild-type, and the quorum sensing mutants might form identical biofilm under certain conditions (See reviews; Parsek and Greenberg, 2005; Parsek and Tolker-Nielsen, 2008). A number of animal infection studies carried out with different objectives have shown that quorum sensing is required for virulence, therefore in the context of virulence, it is generally believed that quorum sensing contributes to the formation of a functional biofilm. Studies of biofilm dynamics to define complexity and trace the development process have being developed over the last 10 years and these will provide insights into the actual role of quorum sensing on biofilm biology. In addition to biofilm development, quorum sensing induces the expression of the drug efflux system MexA-MexB-OprM and confers tolerance to a wide range of antimicrobial agents by extruding them from the cytoplasm (Poole, 2001; Maseda et al., 2004). Thus, quorum sensing is a key factor in determining the success of infection in host animals for *Pseudomonas aeruginosa*

QUORUM SENSING INTERFERENCE

Interfering with quorum sensing is expected to become a powerful strategy to control virulence and antibiotic tolerance in quorum sensing pathogens, and can be applied within antimicrobial chemotherapy to overcome bacterial infections. To date, methods that can be used to disrupt quorum sensing include (1) Antagonizing signal binding to LuxR-family receptor, (2) Inhibition of signal production, (3) Degrading signals, (4) Trapping signals, and (5) Suppression of synthase and receptor activities, stabilities or productions (**Figure 2**). A brief background and recent progress in these studies will be given in the following section.

ANTAGONIST FOR LuxR-FAMILY RECEPTOR

The initial step in quorum sensing is to bind a specific AHL signal to a LuxR protein. Thus, antagonists that interfere with the AHL-the receptor binding are potential quorum sensing inhibitors. Various natural and synthetic compounds have been tested for their antagonistic activity (Chemical structures of representative inhibitors are drawn in **Figure 3**.). In general, analogs are potential antagonists of the native AHL signal. In three early studies, analogs with alternations in the acyl side chain of 3-oxo-C_6-HSL for *Vibrio fischeri*, 3-oxo-C_{12}-HSL for *Pseudomonas aeruginosa* and 3-oxo-C_8-HSL for *Agrobacterium tumefaciens* were demonstrated to inhibit the binding of native AHLs (Passador et al., 1996; Schaefer et al., 1996; Zhu et al., 1998). These studies focused particularly on the length of the acyl side chains. These cognate receptors are able to bind some analogs at a higher affinity than native AHL ligands, but the analogs then inactivate gene expression, thus they are antagonists.

Following earlier studies, a library of synthetic analogs to the *Pseudomonas aeruginosa las* quorum sensing molecule 3-oxo-C_{12}-HSL was constructed by Smith et al. (2003a,b). The homoserine lactone moiety of their compounds was replaced with different alcohols, amines and/or a 5- or 6-membered ring. In their high throughput screening using a *lasI* promoter-fused *gfp* reporter strain, three compounds acted as antagonistics against 3-oxo-C_{12}-HSL-LasR-mediated quorum sensing. 3-Oxo-C_{12}-(2-aminophenol) and 3-oxo-C_{12}-(aminocyclopentanol) are able to inhibit the LasR activation attributed to the 3-oxo-C_{12}-HSL-LasR interaction. On the other hand, 3-oxo-C_{12}-(aminocyclohexanone) appeared to target not only LasR but also RhlR, which is the second quorum sensing receptor in *Pseudomonas aeruginosa*, although this antagonist still has a dodecanoyl (C_{12})-side chain. A synthetic analog of C_4-HSL, *N*-decanoyl cyclopentyl-amide (abbreviated C_{10}-CPA), has also been found to target both LasR and RhlR

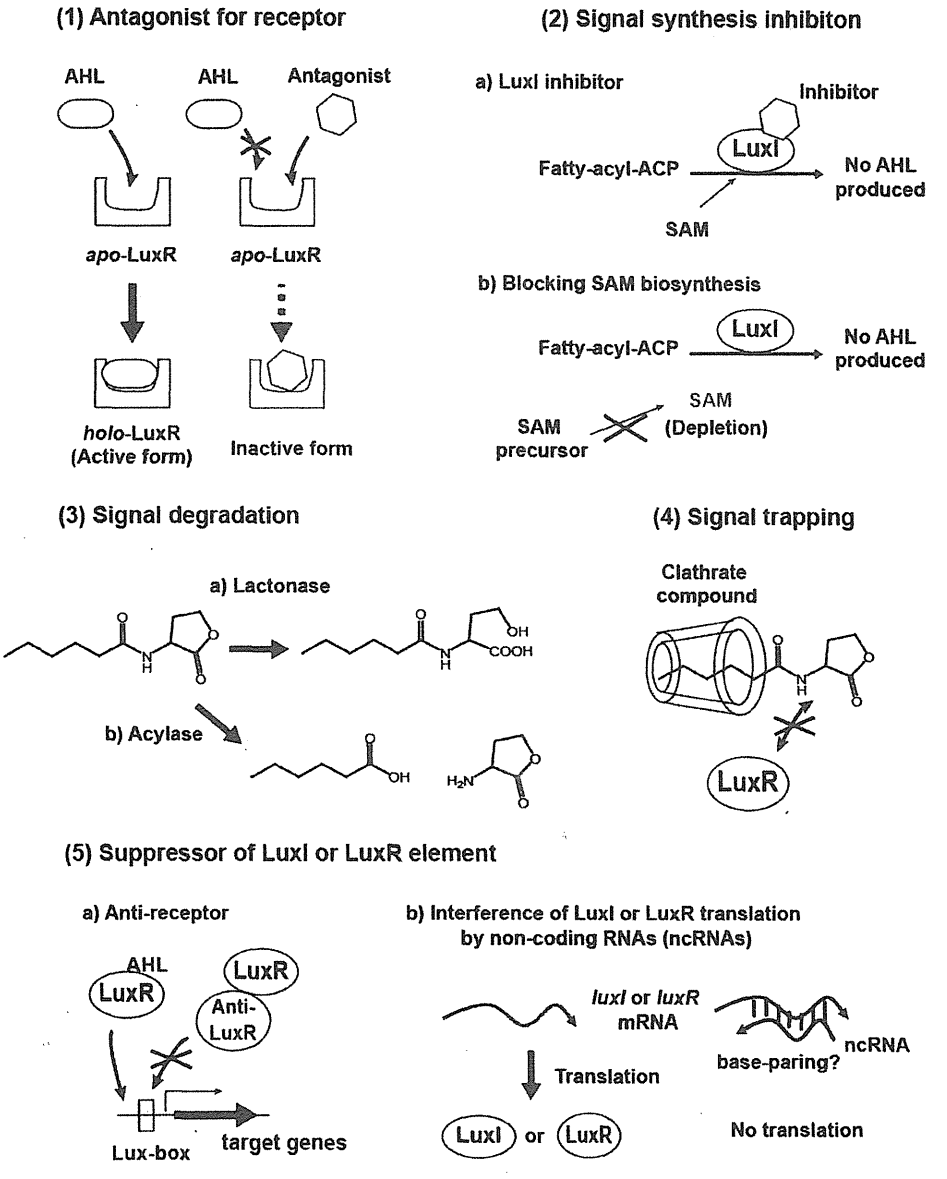

FIGURE 2 | Proposed methods for AHL quorum sensing interference.

proteins (Ishida et al., 2007). C_{10}-CPA inhibits *lasB* and *rhlA* gene activation by 3-oxo-C_{12}-HSL and C_4-HSL with IC50 (half-maximal inhibition) of 80–90 μM, respectively, and inhibition results in reductions in elastase, pyocyanin, and rhamnolipid levels and biofilm formation. Most of these analog compounds are modified with a homoserine lactone moiety and/or a side chain. Changes in the amide function bridging the lactone ring and the fatty acid also influences the AHL binding activity to receptor proteins, since the amide forms hydrogen bonds with a conserved tyrosine and aspartic acid in the AHL binding pocket (Vannini et al., 2002; Zhang et al., 2002). Changing the amide to a sulfonamide and/or a urea has been predicted to result in the formation of an additional hydrogen bond between a tyrosine residue in the ligand pocket and the sulfonamide, and to strengthen the hydrogen bond between the aspartic acid and the external NH of urea (Castang et al., 2004; Frezza et al., 2006, 2008). Compounds with either or both modifications have showed antagonistic behaviors with the *Vibrio fischeri* LuxR receptor. In recent studies, selective and broad-spectrum antagonists active across multiple species have also been developed. C_8-HSL, C_{10}-HSL, 4-bromophenylpropionyl-HSL and 4-iodophenylacetyl-HSL simultaneously antagonize the AHL-bindings to the receptor proteins of TraR in *Agrobacterium tumefaciens*, LuxR in *Vibrio fischeri* and LasR in *Pseudomonas aeruginosa*, while several other analogs work on two of these species (Geske et al., 2007).

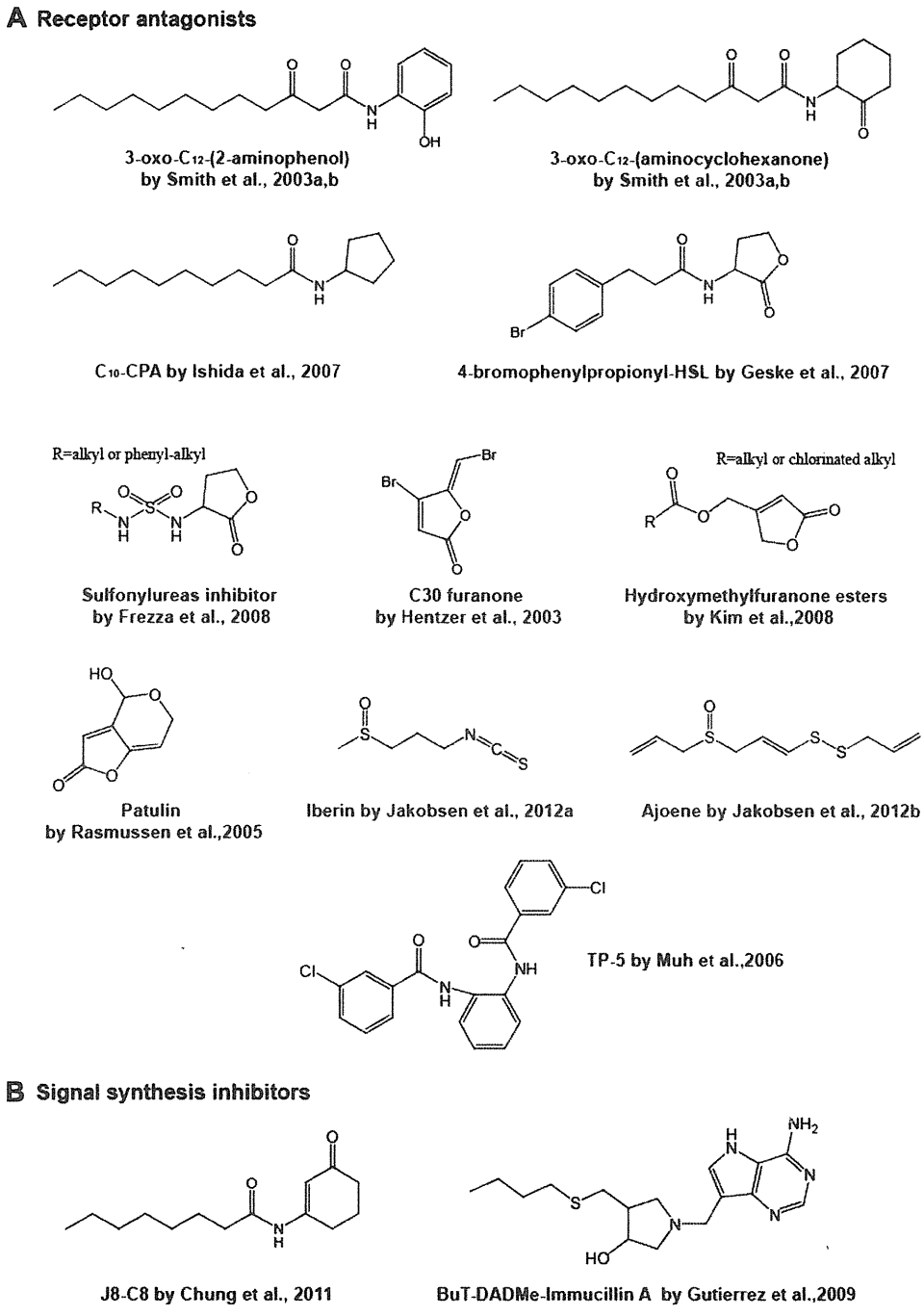

FIGURE 3 | Chemical structures of representative receptor antagonists (A) and signal synthesis inhibitors (B).

Some natural compounds have also been demonstrated to behave as antagonists. Halogenated acyl-furanones, which are structurally similar to AHLs, and are derived from the marine algae *Delisea pulchra* are one of the most-studied antagonist groups (Givskov et al., 1996). These naturally occurring compounds displace the 3-oxo-C_6-HSL signal from its cognate LuxR receptor protein, thus inhibiting the quorum sensing-mediated gene expression (Manefield et al., 1999). The SwaR receptor for C_4-HSL in *Serratia liquefaciens* is also a target for the antagonists, and the failure of C_4-HSL-SwaR interaction results in a reduction in the swarming motility linked to surface colonization and biofilm formation (Rasmussen et al., 2000). Gram et al. (1996) have reported

that a furanone isolated from the furanones pool of secondary metabolites produced by *Delisea pulchra* inhibits swarming motility in *Proteus mirabilis* without affecting cell growth and swimming motility, whereas other furanones have no inhibitory activity. However, the regulatory target in this system is not known. Although natural furanones have a limited inhibitory effect on the quorum sensing of *Pseudomonas aeruginosa*, the synthetic analogs "C30" and "C56," which lack the alkyl side chain, exhibit interference in the LasR-mediated *Pseudomonas aeruginosa lasB* gene expression (encoding the elastase that is associated with the virulence), increase the susceptibility to antimicrobial agents on biofilm cells, promote the bacterial clearance on the lung of infected mice and prolong the survival time of the mice (Hentzer et al., 2002, 2003; Wu et al., 2004; Christensen et al., 2012). Taha et al. (2006) have discovered three LasR antagonists by *in silico* screening with pharmacophore modeling utilizing the authentic furanone inhibitors C-30 and C-56 as leading compounds. These are phenyl compounds incorporating a mercury or lead atom bound by covalent bonds and have been shown to inhibit quorum sensing-driven pyocyanin and pyoverdin production. Subsequent studies have developed other furanone derivatives based on natural furanone core structures. A series of hydroxymethylfuranone esters condensed with fatty acids that have a modified carbon length or are chlorinated at the terminal has been synthesized (Kim et al., 2008). These compounds have been shown to repress the LasR-driven reporter expression in a *lasI-lacZ* fusion by competing with exogenous 3-oxo-C_{12}-HSL binding to a recombinant LasR in *Escherichia coli*, and they also inhibit biofilm formation on the flow cell system in *Pseudomonas aeruginosa*. The authors have built the inhibitor-LasR protein docking models. According to the *in silico* modeling analyses, the inhibitors are predicted to bind preferentially to the receptor rather than the natural ligand, but fail to change the conformation of LasR to the "active" form, which suggests they have antagonistic activities. In the same year, another group reported a series of furanone antagonists whose structures are closer to AHL. They replaced the homoserine lactone moiety of AHL with a furanone. These molecules dock with the LuxR protein at its binding pocket (Estephane et al., 2008). However, we should note a report that halogenated furanones destabilize LuxR receptors rather than antagonizing (Manefield et al., 2002). Therefore, as for exact action mode of this type of inhibitors, there is still some confusion. Remarkably, a very recent study has highlighted the possibility that furanone resistance might arise in *Pseudomonas aeruginosa* (Maeda et al., 2012). Maeda et al. (2012) have observed that a mutation in the *mexR* gene in *Pseudomonas aeruginosa* decreases its susceptibility to the synthetic C-30 furanone. As the *mexR* gene product functions as a repressor for the *mexAB-oprM* operon encoding a multidrug efflux system, the inactivation of *mexR* gene presumably leads to overexpression of MexAB-OprM, thereby enhancing the efflux of the furanone inhibitor.

In addition, other natural AHL inhibitors have been identified, such as patulin and penicillic acid produced by fungi (Rasmussen et al., 2005), iberin from horseradish extracts (Jakobsen et al., 2012a) and ajoene from garlic (Jakobsen et al., 2012b). These natural products inhibit *Pseudomonas aeruginosa* quorum sensing of either or both the *las* and *rhl* systems. Patulin and penicillic acid have structures reminiscent of the furanone compounds originally discovered in *Delisea pulchra*, whereas iberin and ajoene are structurally unrelated linear sulfide compounds, but can compete with *Pseudomonas aeruginosa* AHLs for binding to LasR or RhlR.

Aside from AHL structural analogs, a research group has identified three inhibitors for the LasR protein in combination studies by screening a library of 200,000 compounds and *in silico* structure modeling. A tetrazole and a phenyl ring compound with a common 12-carbon alkyl tail designated "PD12" and "V-06-018", respectively, were isolated from the library (Muh et al., 2006b). Both compounds inhibited LasR-controlled gene expression and expression of the virulence factors elastase and pyocyanin. A triphenyl compound designated "TP-5" was originally predicted to be an antagonist by the LasR-ligand docking model built from the 3D-structure of the ligand-bound TraR protein in *Agrobacterium tumefaciens* (Vannini et al., 2002; Zhang et al., 2002), which is highly homologous to LasR. Interestingly, it is more likely that TP-5 interacts with LasR by forming a hydrogen bond with Asp-73 of the polypeptide and exhibits LasR-inhibitory activity despite being structurally unrelated to the natural ligand. This compound was derived from a parent compound designated "TP-1" from their library of compounds. TP-1 mimics 3-oxo-C_{12}-HSL activity, and therefore acts as an agonist for LasR (Muh et al., 2006a).

Information on the 3D-structures of the LuxR-family receptors has enabled us to discover a variety of antagonists. However, this information is not enough. We need to know how the conformational change in the receptor occurs to distinguish between a "true ligand" (agonist) and "fake ligand" (antagonist). The crystal structure only provides a snapshot of the information required. Although the structures of receptor-AHL binding form are available for a few species, we need to obtain both structures; an antagonist-binding form and a ligand-free form. Recently, the crystal structure of CviR from *Chromobacterium violaceum* with an antagonist has been reported and so far this is the only example (Chen et al., 2011). On the other hand, analysis of the signal-free form is currently a difficult task because LuxR-family proteins are considered to be unstable in the absence of the cognate signal, and are eventually subjected to proteolysis (Zhu and Winans, 2001). However, although a major challenge, it is important to gain information about these structures.

SIGNAL SYNTHESIS INHIBITION

Interfering with AHL synthesis is another straightforward approach to the inhibition of quorum sensing. Simply, if no AHL is produced, no quorum sensing occurs. Early studies on quorum sensing inhibitors have focused on antagonists of AHL receptors. However, there have been few studies on inhibitors of AHL synthesis and data is very limited. In one of these studies, it was found that several analogs of SAM, which is the second substrate for LuxI synthases, inhibit the LuxI reaction (Parsek et al., 1999). However, two breakthrough studies have been recently carried out. The first study identified a C_8-HSL analog that binds to AHL synthase, thereby inhibiting its enzymatic activity (Chung et al., 2011). The second study described compounds targeting the reaction activity of 5′-methylthioadenosine nucleosidase (MTAN, alternatively abbreviated "Pfs") involved in SAM recycling (Gutierrez et al., 2009).