

Isolation and Characterization of an Autoinducer Synthase from *Acinetobacter baumannii*[▽]

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The opportunistic human pathogen *Acinetobacter baumannii* strain M2 was found to produce distinct acyl-homoserine lactone (AHL) signals based on the use of an *Agrobacterium tumefaciens traG-lacZ* biosensor. An *A. baumannii* gene, designated *abaI*, was cloned and directed AHL production in recombinant *Escherichia coli*. The *AbaI* protein was similar to members of the LuxI family of autoinducer synthases and was predicted to be the only autoinducer synthase encoded by *A. baumannii*. The primary AHL signal directed by *AbaI* was identified by mass spectrometry as being *N*-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C₁₂-HSL). Minor amounts of at least five additional AHLs were also identified. The expression of *abaI* at the transcriptional level was activated by ethyl acetate extracts of culture supernatants or by synthetic 3-hydroxy-C₁₂-HSL. An *abaI*::Km mutant failed to produce any detectable AHL signals and was impaired in biofilm development.

Acinetobacter baumannii is a nonmotile, gram-negative member of the class *Pseudomonadales* of the *Gammaproteobacteria*. *A. baumannii* is an important nosocomial pathogen and can cause a variety of infections including wound infections, bloodstream infections, ventilator-acquired pneumonia, and urinary tract infections (2, 5, 6, 11, 13, 14, 21, 23, 31, 41, 43). In addition, *A. baumannii* has been extremely problematic for U.S. military personnel in Iraq and Afghanistan, where this pathogen has been responsible for a large number of infections (1, 3, 8, 19). Of particular concern with *A. baumannii* is the increasing frequency of multidrug-resistant strains that leave few, if any, therapeutic options for treatment (16, 19, 24, 32).

In gram-negative bacteria, cell-to-cell signaling is often mediated by the production of *N*-acyl-homoserine lactone (AHL) signaling molecules (12). To detect these signals, a variety of bacterial biosensor strains have been constructed (27, 35, 44). These strains detect AHL signals by the activation of a reporter gene such as *lacZ* or *lux* or by the production or inhibition of a purple pigment in *Chromobacterium violaceum*. Using these biosensors, previous studies showed AHL production by *Acinetobacter* spp. (15). In a variety of gram-negative bacteria, it has also been demonstrated that biofilm development can be dependent on AHL signaling (7, 18, 26). In *A. baumannii*, very little is known regarding factors required for biofilm formation (25, 39, 40). A putative chaperone that is required for this process has been identified (39). In addition, a homolog of a staphylococcal biofilm-associated protein (Bap) has been characterized in *A. baumannii*, where it appears to act as an extracellular adhesin (25).

In this study, we report the identification and characterization of an autoinducer synthase from *A. baumannii* that was

designated *AbaI*. Mass spectrometry was used to identify AHL signals that were directed by *AbaI*. The *abaI* gene was activated in a positive-feedback loop by an *AbaI*-dependent AHL signal(s). An *abaI* mutant was impaired in the later stages of biofilm development, and this phenotype was rescued by ethyl acetate extracts of cell supernatants from a wild-type strain.

***A. baumannii* produces AHL signals.** An *A. tumefaciens traG::lacZ* fusion, responsive to a variety of 3-oxo, 3-hydroxy, and 3-unsubstituted AHL signals of various acyl chain lengths (35), was strongly activated by a diffusible factor from an *A. baumannii* clinical isolate designated M2 that was obtained from the MetroHealth Medical Center, Cleveland, OH (Fig. 1A). To further characterize the extracellular signal(s) produced by *A. baumannii* M2, cell-free supernatants were prepared from cells grown to an optical density at 600 nm (OD₆₀₀) of at least 1.1 in 500 ml of M9 minimal salts medium containing 1% glucose and 0.3% Casamino Acids (28). Cell-free supernatants were prepared by pelleting cells and filter sterilizing the resulting supernatant with a 0.22-μm filter. Supernatants were then extracted twice with an equal volume of acidified ethyl acetate (0.1 ml acetic acid per liter), and the extracts were dried to a volume of 50 μl.

Concentrated extracts were analyzed on a C₁₈ reverse-phase thin-layer chromatography (TLC) plate (JT Baker) using a method described previously by Shaw et al. (35), with a few modifications. A concentrated extract (1 μl) was applied onto a C₁₈ TLC plate and developed in a glass chamber with methanol-water (60:40). The TLC plate was developed overnight and then overlaid with a soft agar lawn containing the *Agrobacterium tumefaciens traG::lacZ* indicator strain. The plate was sandwiched between two Pyrex glass containers, one containing water at the bottom to maintain humidity, and sealed with plastic wrap. Plates were incubated at 28°C until blue spots appeared, which indicated the presence of putative AHL signals.

As shown Fig. 1B, lane 3, there are three putative AHL signals (S1, S2, and S3) produced by *A. baumannii* that activate

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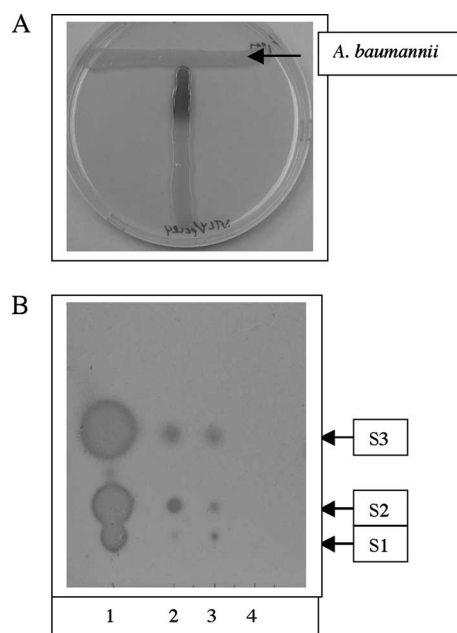


FIG. 1. AHL signal production by *A. baumannii*. (A) A line of *A. baumannii* cells (horizontal top line) was placed perpendicular to a line of *A. tumefaciens* cells containing an AHL-responsive *traG::lacZ* fusion. Plates were incubated at 28°C for 12 h. (B) For each condition, ethyl acetate extracts were prepared from 500-ml cultures, dried by rotary evaporation, and resuspended to a final concentration of 8,000 $\times$. For all samples, 1 μ l of concentrated extract was applied to a C_{18} TLC plate and developed with methanol-water (60:40). Plates were dried at room temperature for 2 h and overlaid with a soft agar suspension containing the *A. tumefaciens* *traG-lacZ* biosensor as described previously (35). Plates were then incubated overnight at 28°C until blue spots appeared (typically 24 h). Samples are as follows: *E. coli* MG1655/pSK.abaI (lane 1), a 1/1,000 dilution of *E. coli* MG1655/pSK.abaI extract (lane 2), *A. baumannii* M2 wild-type extract (lane 3), and *A. baumannii* M2 *abaI::Km* (lane 4).

the *traG-lacZ* biosensor. All three *A. baumannii* signals lacked the trailing, comet tail pattern characteristic of 3-oxo derivatives (35), suggesting that they were 3-hydroxy or 3-unsubstituted AHLs.

Cloning of an *A. baumannii* gene that directs AHL signal production. To identify a gene that directed the production of the AHL signal(s), we employed a screening procedure that allowed us to directly identify *Escherichia coli* transformants containing an autoinducer synthase gene. For this screen, LB agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) were overlaid with approximately 10^5 cells of the *A. tumefaciens* *traG-lacZ* biosensor. Next, a pooled recombinant library of *A. baumannii* Sau3AI DNA fragments in pACYC184 (4) transformed into *E. coli* MC4100 cells was directly plated onto the *A. tumefaciens* lawn. After overnight growth at 28°C, *E. coli* transformants were visible within the *A. tumefaciens* lawn. Colonies containing recombinant plasmids with a putative autoinducer synthase were identified by a blue halo that resulted from the activation of the *A. tumefaciens* *traG-lacZ* fusion in the indicator lawn.

Using the above-described screen, we isolated a pACYC184 derivative that encoded a putative autoinducer synthase. Re-transformation of the plasmid from positive colonies verified

that the recombinant plasmid directed the production of the activating signal. A recombinant plasmid with an approximately 5-kb insert was subjected to transposon mutagenesis using the GPS Tn7 in vitro mutagenesis system (New England BioLabs) to identify the putative autoinducer synthase encoded within the inserted DNA fragment. Insertions that inactivated the gene responsible for signal production were identified by cross-streaking colonies from an insertional library against the *A. tumefaciens* indicator strain. Tn7Km insertions that abolished signal production were then sequenced using the N and S primers that read outward from the ends of Tn7Km. All insertions that prevented signal production mapped to the same open reading frame encoding a 129-amino-acid protein with extensive homology to autoinducer synthases of the LuxI family, which was therefore designated *abaI* (*Acinetobacter baumannii* LuxI).

The *abaI* gene was recloned from the chromosome of *A. baumannii* M2 as a 2.0-kb chromosomal EcoRI fragment. This was necessary because the original 5-kb Sau3AI fragment was found to contain a scrambled insert. However, this did not alter the interpretation of the DNA sequence. A pBluescript SK derivative containing this 2-kb insert (pSK.abaI) was used for all further experiments. *E. coli* MG1655 containing pSK.abaI produced AHL signal profiles in the TLC overlay assay that were essentially identical to those of native *A. baumannii* M2, with three areas of signal activity (Fig. 1B, lanes 1 and 2). However, the relative amounts of each signal varied between *E. coli*/pSK.abaI and *A. baumannii* M2. The amount of S2 was greater in *E. coli*/pSK.abaI (Fig. 1B, lane 2) than in *A. baumannii* M2 (Fig. 1B, lane 3), and the amount of S1 was greater in *A. baumannii* M2. In addition, quantitative analysis of signal production revealed that *E. coli*/pSK.abaI produced approximately 1,000-fold more signal than did *A. baumannii* M2 (Fig. 1B, compare lanes 1 and 3).

Organization of the *abaI* region in *A. baumannii*. Compared to the recently published *A. baumannii* ATCC 17978 genome (36), the *AbaI* protein was 96% identical to the corresponding protein (GenBank accession number NC_009085.1). However, the *AbaI* protein identified from strain M2 was predicted to be 56 amino acids shorter at the amino terminus. Using the BLAST analysis program, the *AbaI* protein exhibited 54% identity and 69% similarity to the AfeI protein of *Acidithiobacillus ferrooxidans* and to LuxI proteins from *Burkholderia cepacia* (46% identity and 64% similarity) and *Ralstonia solanacearum* (41% identity and 65% similarity). Sequence analysis of the M2 genome revealed that immediately upstream from the *abaI* gene, an open reading frame (A1S_0110) encoding a protein of unknown function was present and was also seen in the 17978 genome (Fig. 2). Interestingly, an open reading frame similar to A1S_0110 was present immediately upstream of putative autoinducer synthases from *A. ferrooxidans* (37% identity and 48% similarity) and *Burkholderia pseudomallei* (38% identity and 62% similarity) (9, 33). Based on the 17989 genome, an open reading frame divergently transcribed from the A1S_0110 and *abaI* open reading frames was predicted to encode a LuxR protein designated *AbaR* (Fig. 2). The *AbaR* protein was 198 amino acids long and exhibited the best match to AfeR from *A. ferrooxidans* (46% identity) and to a LuxR-type protein from *Burkholderia pseudomallei* (38% identity). The similarity of gene organizations in *A. baumannii*,

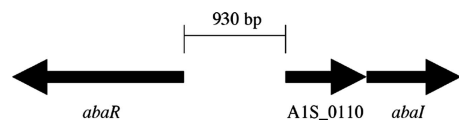


FIG. 2. Organization of the *abaI* region of *A. baumannii*. The genomic region surrounding *abaI* in both *A. baumannii* M2 and ATCC 17978 is shown. The arrows above each gene indicate the orientation of each gene. The coding regions of *abaR* and the open reading frame of unknown function (A1S_0110) are separated by 930 bp. The *abaR* location is based on the published sequence of the ATCC 17978 genome (36).

A. ferrooxidans, and *B. pseudomallei* suggests a common origin for these genes.

Structural identification of the AHL signals produced by *A. baumannii*. To characterize the AHL signals produced by *A. baumannii*, concentrated ethyl acetate extracts of spent culture supernatants were analyzed by liquid chromatography (LC)/mass spectrometry (MS) and LC/tandem MS (MS/MS) (Fig. 3). Initially, each parent ion was screened by MS/MS fragmentation for an ion with an m/z of 102, characteristic of the homoserine moiety (17, 29, 35). Putative AHLs with a breakdown ion with an m/z of 102 were observed and centered at 27, 42, 44, 48, and 50.5 min of elution from the reversed-phase C_{18} column (Fig. 3A). These areas were designated areas 1 to 5, respectively. The peaks at 33.93 and after 50.87 did not contain any ions that were identifiable as being HSL derivatives and were not examined further. For each peak, a full MS profiling of the parent ion was performed (Fig. 3B), and the profile was compared to those of known 3-oxo-, hydroxy-, and unsubstituted AHL signals (17, 29, 35). Based on this analysis, the primary AHL signal produced was 3-hydroxy- C_{12} -HSL (m/z of 300.2) (Fig. 3B1) and was present in the material that eluted from 26 to 28 min (Fig. 3A). Synthetic 3-hydroxy- C_{12} -HSL migrated in the same position in the reversed-phase column and had the same m/z of 300.2. In addition, at this time, a peak with an m/z of 282.2 was present but in significantly smaller amounts (Fig. 3B1). This species could correspond to the unsaturated form of C_{12} -HSL or unsaturated 3-oxo- C_{11} -HSL. At position 2 in Fig. 1A, two ions were identified, with m/z values of 256.2 and 282.2, that matched the values for unsubstituted C_{10} -HSL (m/z of 256.2) and unsaturated C_{12} -HSL or unsaturated 3-oxo- C_{11} -HSL (m/z of 282.2) (Fig. 3B2). The ion at m/z 256.2 fragmented in MS/MS to m/z 102 (Fig. 3B2). However, MS/MS analysis of the m/z 282.2 ion did not give an obvious fragment with an m/z of 102. Additional AHL signals that were identified at other elution times in significantly smaller amounts than 3-hydroxy- C_{12} -HSL included unsaturated C_{14} -HSL or unsaturated 3-oxo- C_{13} -HSL (m/z 310.3; 44 min) (Fig. 3B3), unsubstituted C_{12} -HSL (m/z 284.3; 48 min) (Fig. 3B4), and unsaturated C_{16} -HSL or unsaturated 3-oxo- C_{15} -HSL (m/z 338.3; 50.1 min) (Fig. 3B5). Subsequent MS/MS analyses of all the above-described ions indicated the presence of the m/z 102 ion, verifying the presence of the HSL moiety (Fig. 3B).

To further verify the role of *AbaI* in AHL production, concentrated ethyl acetate extracts were prepared from supernatants of *E. coli* MG1655 containing the cloned *abaI* gene on a high-copy plasmid (pSK.*abaI*). The prominent AHL in *E. coli*/pSK.*abaI* had an m/z of 300.2, consistent with 3-hydroxy- C_{12} -HSL. Ions of m/z 256.2 and m/z 284.2 were also present in

minor amounts, corresponding to unsubstituted C_{10} -HSL and C_{12} -HSL, respectively (data not shown). *E. coli*/pSK.*abaI* also produced a AHL with an m/z of 272.2 that was not observed in *A. baumannii*. This corresponded to 3-hydroxy- C_{10} -HSL.

We attempted to assign each signal to the corresponding spots (S1 to S3) observed in the TLC plates (Fig. 1B). Each spot was individually isolated from the C_{18} TLC matrix. The spot corresponding to S1 contained three AHL signals, and the primary product exhibited an m/z of 300.2, consistent with 3-hydroxy- C_{12} -HSL. Moreover, synthetic 3-hydroxy- C_{12} -HSL exhibited an R_f value identical to that of the material in S1 (data not shown). Minor peaks at m/z 284 and m/z 312 that were consistent with unsubstituted C_{12} -HSL and unsubstituted C_{14} -HSL, respectively, were also present in S1. The S2 spot contained a signal with an m/z of 284, corresponding to unsubstituted C_{12} -HSL. In addition, the material eluted from the S2 spot exhibited an R_f value on TLC plates identical to that of a synthetic C_{12} standard (data not shown). Lastly, the material in S3 exhibited an m/z of 256, consistent with unsubstituted C_{10} -HSL. However, the material purified from this spot did not comigrate with a C_{10} -HSL standard. One explanation for this result is that a second AHL that accounts for the activity in the S3 spot is present but that the concentration of this AHL was too low to detect by mass spectrometry.

Role of the *abaI* gene in AHL signal production. To inactivate the chromosomal copy of *abaI*, plasmid pSK.*abaI* was mutagenized with Tn7Km, and insertions that prevented the production of the AHL signal were identified using the *A. tumefaciens* biosensor containing *traG-lacZ*. An insertion that disrupted *abaI* in the middle of the coding region was cloned along with flanking chromosomal DNA as a *SalI*-*XbaI* fragment into suicide vector pKNG101 (22). This plasmid, pKNG101.*abaI*::Km, was introduced into the *A. baumannii* chromosome by filter mating with *E. coli* SM10/pKNG101.*abaI*::Km, followed by selection for kanamycin- and streptomycin-resistant exconjugants. These exconjugants contained pKNG101.*abaI*::Km integrated at the wild-type *abaI* gene, an event that results in a wild-type copy of *abaI* and a second copy containing the *abaI*::Km' disruption. To excise the integrated plasmid, cells were grown for 5 to 10 generations without antibiotic selection, and dilutions were plated onto LB plates without sodium chloride and containing 10% sucrose, followed by incubation at room temperature. All sucrose-resistant colonies were streptomycin sensitive, indicating that they had lost the integrated pKNG101 plasmid. These colonies were then tested for kanamycin resistance, and all colonies with this phenotype contained the *abaI*::Km disruption as determined by Southern blot analysis (37) using a probe internal to *abaI* generated by PCR using primers 5'-GTACAGTCGACGTATTTGTTGAATATTTGGG-3' and 5'-CGTACGTCTAGAGTAATGAGTTGTTTGCGCC-3'.

The *abaI*::Km mutant failed to produce any detectable signals in the TLC overlay using the *A. tumefaciens* biosensor (Fig. 1B, lane 4). In addition, there were no detectable ions present at an m/z of 256, 284, or 300 in ethyl acetate extracts from the *abaI*::Km mutant (data not shown).

***abaI* is transcriptionally activated by AHL signals in a positive-feedback loop.** Transcription of an autoinducer synthase is often positively regulated by the accumulation of the cognate AHL signal (12), although exceptions to this can exist (38). To

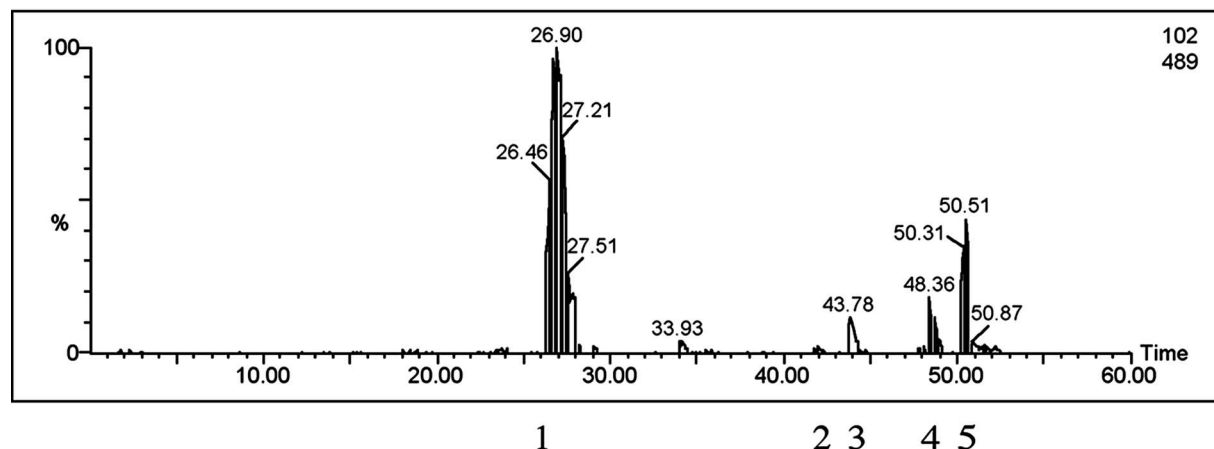
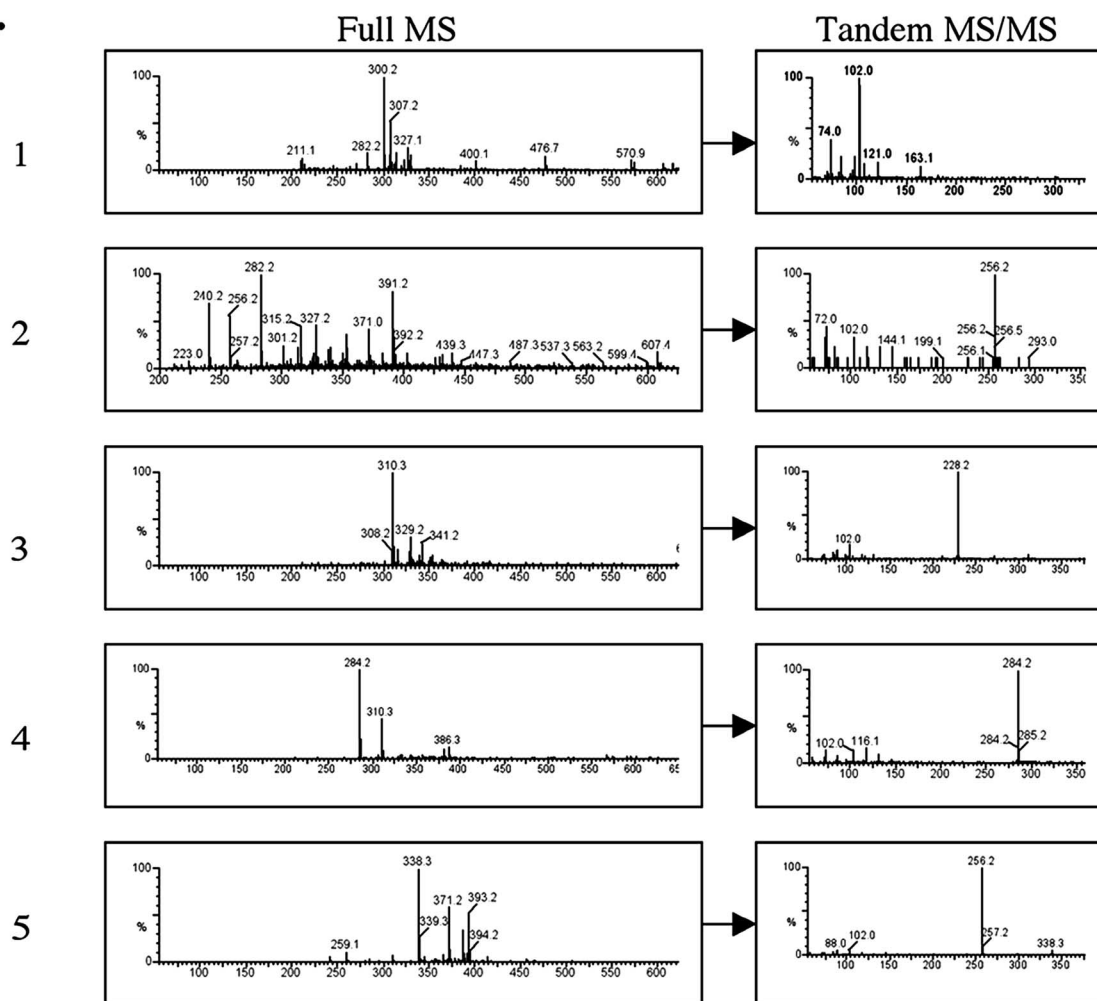
A.**B.**

FIG. 3. MS profiles of AHL signals. A 10,000-fold-concentrated ethyl acetate extract of *A. baumannii* M2 supernatant was diluted 1:3 in 30 μ l of methanol for LC/MS and LC/MS/MS analysis. Ten microliters was injected onto a 3- μ m, 100- by 2.1-mm, C_{18} reverse-phase column (Restek) installed on a Waters Alliance 2695 high-performance liquid chromatograph (Waters Corporate, Milford, MA) operated at a flow rate of 200 μ l/min with the effluent flowing directly into the mass spectrometer. Solvent A consisted of water with 0.1% formic acid, and solvent B consisted of acetonitrile with 0.1% formic acid. A gradient elution method was utilized, which started at 5% solvent B for 5 min, went to 95% solvent B over 30 min, and remained isocratic at 95% solvent B for 15 min. Electrospray ionization-MS was performed by using a Waters Q-TOF micro-MSD instrument (Waters Corporate, Milford, MA). The instrument parameters were as follows: capillary voltage of 2,800 V, cone voltage of 25 V, and collision energy of 25 V. Nitrogen was used as the collision gas. All signal peaks containing fragments with an m/z of 102 were analyzed using MassLynx 4.0 software. The elution times (min) off the C_{18} column of species with an MS/MS breakdown ion at m/z 102 are shown (A). Each of the five peaks was then analyzed by MS to identify the parent ion, and the full MS profile for each ion is shown. The MS/MS profile of each parent ion is shown on the right.

TABLE 1. Activation of *abaI-lacZ* by AHL signals

Growth condition ^a	Relative level of expression of <i>abaI-lacZ</i> (Miller units) $\pm$ SD ^b
OD ₆₀₀ of 0.5	
LB + EtAc control	1.0 $\pm$ 0
LB + 1 μ M 3OH-C ₁₂ -HSL	2.1 $\pm$ 0.2
LB + 10 μ M 3OH-C ₁₂ -HSL	4.9 $\pm$ 0.7
LB + wild-type EtAc extract	4.0 $\pm$ 1.1
LB + <i>abaI::Km</i> EtAc extract	1.1 $\pm$ 0.005
OD ₆₀₀ of 1.2	
LB + EtAc control	1.0 $\pm$ 0.02
LB + 1 μ M 3OH-C ₁₂ -HSL	5.8 $\pm$ 0.55
LB + 10 μ M 3OH-C ₁₂ -HSL	13.7 $\pm$ 1.45
LB + wild-type EtAc extract	8.9 $\pm$ 0.1
LB + <i>abaI::Km</i> EtAc extract	1.1 $\pm$ 0.005

^a Cells were grown in LB broth, and ethyl acetate (EtAc) extracts of culture supernatants were added at a 10 $\times$ concentration.

^b Relative expression values are reported as Miller units of β -galactosidase activity in cells grown under the indicated growth conditions divided by the values in cells grown in LB broth only.

determine if *abaI* expression was altered by AHL signals, a promoterless *lacZ* cassette obtained from pQF50 (10) as an SmaI-ScaI fragment was inserted into the *abaI* coding region by ligation with pKNG101.*abaI::Km* digested with PmeI, which excises out the kanamycin resistance cassette within *abaI*. The introduction of the pKNG101.*abaI-lacZ* fusion into the M2 chromosome was done by a conjugal mating with *E. coli* SM10 containing this plasmid. *A. baumannii* exconjugants were then selected for sucrose resistance to excise the integrated plasmid by a second crossover. The presence of the correct single-copy *abaI-lacZ* fusion in *A. baumannii* M2 was verified by PCR and DNA sequence analysis of the PCR product. It is important that this *lacZ* cassette creates a null allele in *abaI*, a phenotype that was verified by the lack of AHL production in cross-streak assays with the *A. tumefaciens traG-lacZ* biosensor (data not shown). When cells were harvested at an OD₆₀₀ of 0.5, the expression levels of *abaI-lacZ* in the presence of 1 μ M and 10 μ M 3-hydroxy-C₁₂-HSL were increased 2.1- and 4.9-fold, respectively, above the levels in cells grown in LB only (Table 1). The addition of an ethyl acetate extract of a culture supernatant from wild-type M2 activated the *abaI-lacZ* fusion fourfold. However, an ethyl acetate extract from the *abaI::Km* mutant failed to activate the *abaI-lacZ* fusion. When cells were harvested at a higher density (OD₆₀₀ of 1.2), the *abaI-lacZ* fusion was activated to a greater extent by AHL signals, 5.8- and 13.7-fold in the presence of 1 μ M and 10 μ M, respectively, of 3-hydroxy-C₁₂-HSL. In addition, ethyl acetate extracts of wild-type M2 activated the *abaI-lacZ* fusion 8.9-fold. These results indicate that the transcription of the *abaI* gene is positively activated by the AHL signals directly resulting from *AbaI* enzymatic activity.

AHL-dependent quorum sensing is required for biofilm development in *A. baumannii*. The ability of the wild type and the isogenic *abaI::Km* mutant to form biofilms was examined in cells grown for 8, 16, and 24 h in wells of a microtiter plate. The growth rates of the wild type and the *abaI::Km* mutant were identical (31-min generation time) and were at similar optical densities after each of the indicated times. For cells grown for

8 h, the biofilm-forming abilities were similar for the wild type and the *abaI::Km* mutant as measured by crystal violet staining of adherent cells (*A*₅₈₀ values of 0.52 and 0.55, respectively) (Fig. 4A). However, the biofilm-forming ability of the *abaI::Km* mutant was markedly lower at 16 and 24 h, at 40% and 41%, respectively, than that of the wild type (Fig. 4A). To

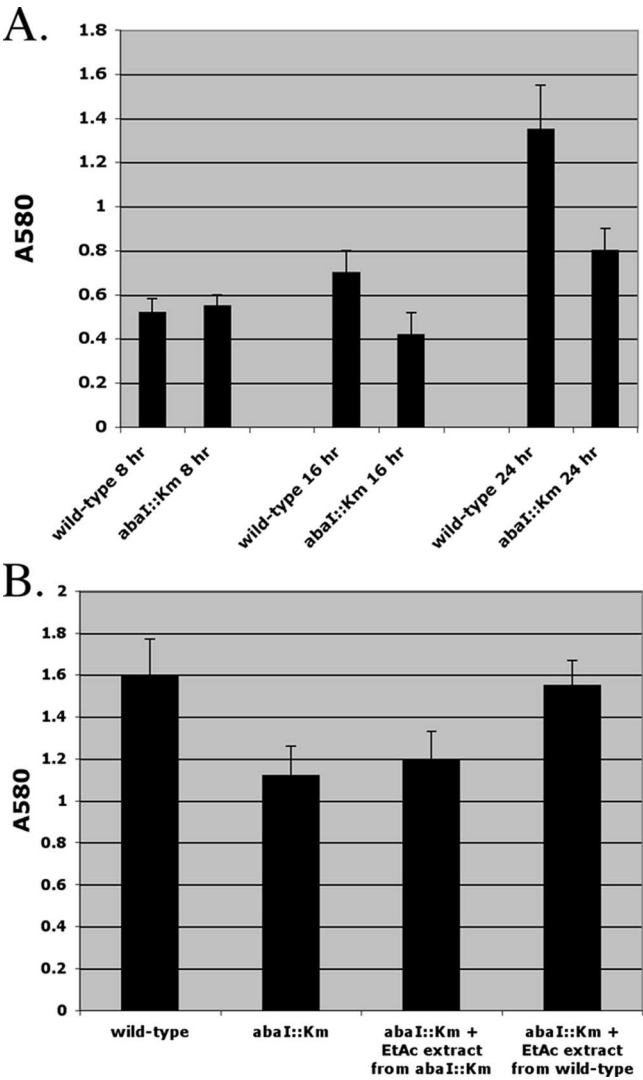


FIG. 4. Biofilm formation of the wild type and an *abaI* mutant. Biofilm assays were done according to method a described previously by O'Toole and Kolter (30). Exponential-phase cultures of M2 (wild type) and the *abaI::Km* mutant were diluted in LB broth to an OD₆₀₀ of 0.02, and 100 μ l of each culture was applied to wells of a microtiter plate (Fisher). After incubation at 30°C for various times, growth at an OD₆₀₀ was measured. Ten microliters of 0.4% crystal violet was then added to each well and incubated for 15 min, and wells were rinsed thoroughly with sterile H₂O to remove nonadherent cells. After rinsing, 100 μ l of 33% acetic acid was added into each well, and plates were vortexed for 30 s to solubilize the crystal violet. Biofilm formation was measured by reading the absorbance at an OD₅₈₀. The values reported in both panels represent the averages of 12 individual wells. (B) Ethyl acetate (EtAc) extracts of wild-type and the *abaI::Km* mutant wells at a 5 $\times$ concentration relative to the original volume of spent culture supernatant that was extracted. For the growth of the wild type and the *abaI::Km* mutant, the same volume of ethyl acetate alone was added.

directly demonstrate that this biofilm defect was due to a loss of AHL signal production directed by AbaI, we assayed the biofilm formation of the *abaI*::Km mutant in the presence of exogenously added AHL signal provided as an ethyl acetate extract from wild-type cells or the *abaI*::Km mutant. Ethyl acetate alone was added in negative control samples. The addition of ethyl acetate extracts from wild-type cells restored the ability of the *abaI*::Km mutant to form normal biofilms (Fig. 4B). However, the addition of ethyl acetate extracts from the *abaI*::Km mutant did not rescue the defect in biofilm formation exhibited by the *abaI*::Km mutant (Fig. 4B).

Concluding remarks. The gene for an autoinducer synthase, designated *abaI*, has been cloned and characterized in this study. The deduced AbaI protein was highly similar to members of the LuxI family. Analysis of the recently completed *A. baumannii* 17978 genome sequence (36) did not reveal additional genes that encode members of the LuxI or LuxM/AinS family of autoinducer synthases. Therefore, based on the AHL profiles determined in Fig. 3, and the fact that AbaI is probably the only autoinducer synthase, the primary AHL signal in *A. baumannii* M2 is 3-hydroxy-C₁₂-HSL. However, since AHL signals can be produced by acyltransferases that do not have similarity to LuxI or LuxM/AinS (34), it cannot be ruled out that additional AHL signals are present in *A. baumannii* M2. To determine if AHL quorum sensing was utilized by other clinical isolates of *A. baumannii*, we tested 19 additional isolates (10 from military infections in Iraq and 9 from U.S. hospitals) for AHL production. All isolates produced AHL signals that activated the *A. tumefaciens* *traG-lacZ* biosensor (data not shown). However, in two cases, TLC analysis indicated that a unique AHL signal not produced by M2 was present. The nature of this signal is under investigation.

The *abaI* gene was activated by ethyl acetate extracts of culture supernatants and by synthetic 3-hydroxy-C₁₂-HSL (Table 1). Therefore, *abaI* is regulated by a positive-feedback loop involving self-produced AHL signal and a cognate R protein. The activation of *abaI-lacZ* by the addition of AHL signal in the *abaI* null background was greater in cells at high density (Table 1). This indicates that an inhibitor of quorum sensing is present in LB broth and depleted during cell growth. A similar situation was previously reported for *Pseudomonas aeruginosa* (45). The role of the LuxR homolog AbaR in the activation of *abaI* in the presence of 3-hydroxy-C₁₂-HSL is unknown at present, as repeated attempts to create an *abaR* null allele have failed for unknown reasons. However, given that the only predicted LuxR protein encoded in *A. baumannii* is AbaR, it is likely that this is required for the activation of *abaI*. Although the *abaI* promoter has not yet been identified, a putative Lux box (CTGTAAATTCTTACAG) is centered 67 bp upstream of the putative ATG start for AbaI and may represent a binding site for AbaR.

The AbaI autoinducer synthase was required for normal biofilm development (Fig. 4). The differences between the wild type and the *abaI*::Km mutant were evident after 16 and 24 h of development, but there was no difference at 8 h. This suggests that the *abaI*-directed quorum-sensing pathway is required for the later stages of biofilm maturation. At present, the only known determinants required for biofilm formation in *A. baumannii* are the *csu*-encoded chaperone-usher pilus assembly system (39) and the Bap protein (25). It is possible that

quorum sensing influences the expression of these genes, but this remains to be determined. The identification of *abaI* and the corresponding AHL signals will now allow the identification of signal antagonists that inhibit biofilm development. These antagonists may also reduce the ability of *A. baumannii* to survive on environmental surfaces for extended periods, a key component of its ability to persist in intensive care wards (20, 42).

Nucleotide sequence accession number. The *abaI* sequence from *A. baumannii* M2 has been deposited in the GenBank database under accession number EU334497.

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