JOURNAL OF BACTERIOLOGY, Aug. 2010, p. 4251–4255 Vol. 192, No. 16
0021-9193/10/$12.00 doi:10.1128/JB.00492-10


# Identification of the Biosynthetic Gene Cluster for the *Pseudomonas aeruginosa* Antimetabolite L-2-Amino-4-Methoxy-*trans*-3-Butenoic Acid▽†

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Received 30 April 2010/Accepted 27 May 2010

**L-2-Amino-4-methoxy-*trans*-3-butenoic acid (AMB) is a potent antibiotic and toxin produced by *Pseudomonas aeruginosa*. Using a novel biochemical assay combined with site-directed mutagenesis in strain PAO1, we have identified a five-gene cluster specifying AMB biosynthesis, probably involving a thiotemplate mechanism. Overexpression of this cluster in strain PA7, a natural AMB-negative isolate, led to AMB overproduction.**

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that causes a wide range of human infections and is considered the main pathogen responsible for chronic pneumonia in cystic fibrosis patients (7, 23). *P. aeruginosa* also infects other organisms, such as insects (4), nematodes (6), plants (18), and amoebae (20). Its ability to thrive as a pathogen and to compete in aquatic and soil environments can be partly attributed to the production and interplay of secreted virulence factors and secondary metabolites. While the importance of many of these exoproducts has been studied, the antimetabolite L-2-amino-4-methoxy-*trans*-3-butenoic acid (AMB; methoxyvinylglycine) (Fig. 1) has received only limited attention. Identified during a search for new antibiotics, AMB was found to reversibly inhibit the growth of *Bacillus* spp. (26) and *Escherichia coli* (25) and was later shown to inhibit the growth and metabolism of cultured Walker carcinosarcoma cells (28). AMB is a γ-substituted vinylglycine, a naturally occurring amino acid with a β,γ-C═C double bond. Other members of this family are aminoethoxyvinylglycine from *Streptomyces* spp. (19) and rhizobitoxine, made by *Bradyrhizobium japonicum* (16) and *Pseudomonas andropogonis* (15) (Fig. 1). As inhibitors of pyridoxal phosphate-dependent enzymes (13, 17, 21, 22), γ-substituted vinylglycines have multiple targets in bacteria, animals, and plants (3, 5, 10, 21, 22, 29). However, the importance of AMB as a toxin in biological interactions with *P. aeruginosa* has not been addressed, as AMB biosynthesis and the genes involved have not been elucidated.

**Identification of the *ambABCDE* biosynthesis cluster.** To identify the AMB biosynthetic genes, we searched a Tn*5*Gm mutant library of *P. aeruginosa* PAO (9) for mutants that lacked the ability to produce AMB using a bioassay based on growth inhibition of *E. coli* K-12 (25). Bacteria were spotted onto minimal medium E (MME) (30) plates amended with 0.5% glucose as a carbon source and 1 mM threonine and grown at 37°C for 14 h. The bacteria were then killed by a 5-min UV exposure, and the plates were overlaid with a mixture of 3 ml 0.5% bacteriological agar no. 1 (Oxoid) and 0.3 ml of an *E. coli* K-12 culture grown overnight in MME and adjusted to an optical density at 600 nm ($OD_{600}$) of 0.5 with 0.9% NaCl. The plates were incubated at 37°C and were scored for zones of clearance after 1 day. As shown in Fig. 2 (left panel), growth inhibition of the *E. coli* indicator resulted in a clearing zone around *P. aeruginosa* PAO1 as well as around a filter disk soaked with chemically synthesized AMB (2). In both cases, inhibition was reversible by the addition of methionine (data not shown), which acts as an AMB antagonist in *E. coli* (25). Among 4,500 Tn*5*Gm mutants analyzed using this assay, four AMB-negative mutants were identified and the location of

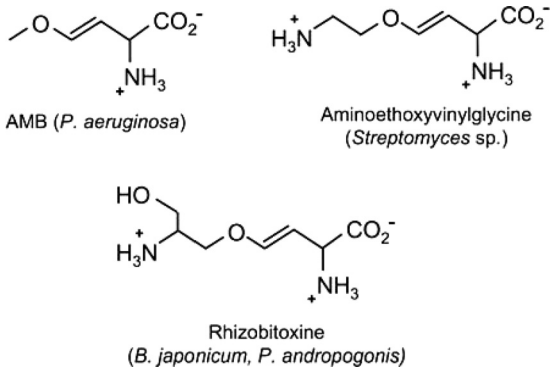


FIG. 1. Chemical structures of the γ-substituted vinylglycines AMB, aminoethoxyvinylglycine, and rhizobitoxine.

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▽ Published ahead of print on 11 June 2010.

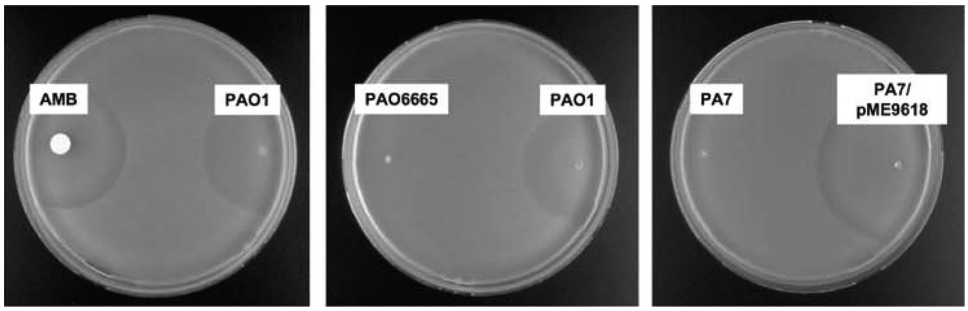



FIG. 2. Biotest for AMB production by different *P. aeruginosa* strains. AMB is detected by a typical clearing zone around the *P. aeruginosa* colonies resulting from growth inhibition of the *E. coli* K-12 indicator strain. As a positive control, 20 μl of chemically synthesized AMB at 0.2 mM was deposited onto a filter disk of 6 mm in diameter.

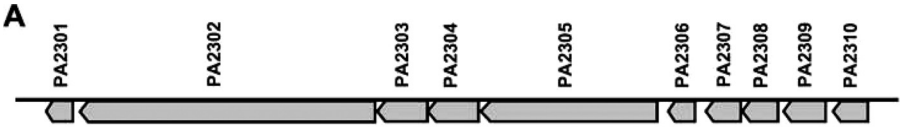
their Tn*5*Gm insertion was determined by sequence analysis, as done previously (9). All four insertions mapped to the same gene cluster; three had occurred in PA2305, and one was found in PA2302.

To confirm the importance of PA2302 and PA2305 in AMB production and to evaluate the potential implication of adjacent genes (Fig. 3A), we constructed several in-frame deletion mutants of strain PAO1 by reverse genetics (34) using the suicide plasmids listed in Table 1. The ability of these mutants to produce AMB was subsequently analyzed using the bioassay described above. A typical example of the results of these tests is given in Fig. 2 (middle panel), which shows that the PA2302 deletion mutant PAO6665 (Table 1) no longer inhibits *E. coli* growth. In summary (Fig. 3B), mutations in PA2306, PA2305, PA2304, PA2303, and PA2302 (hereafter named *ambA*, *ambB*, *ambC*, *ambD*, and *ambE*, respectively) abolished AMB production, whereas mutations in PA2301 and PA2310 had no effect.

Bioinformatics analysis revealed that the protein encoded by *ambA* belongs to the LysE superfamily of transmembrane solute translocators (1, 32) and could thus be responsible for AMB export. The *ambB*, *ambC*, *ambD*, and *ambE* genes are predicted to form an operon encoding the AMB biosynthetic proteins. AmbB and AmbE have a modular structure typical of nonribosomal peptide synthetases (8), whereas AmbC and AmbD are potential α-ketoglutarate-dependent non-heme iron oxygenases of the TauD family (11). Comparison of the *ambBCDE* DNA and deduced protein sequences with sequences available in the NCBI database did not reveal any related gene clusters in other vinylglycine-producing bacteria, such as *B. japonicum*, suggesting that AMB biosynthesis might follow a different route.

**Identification and quantification of AMB in bacterial culture supernatants by tandem MS.** Given the chemical structure of AMB, the implication of two nonribosomal peptide synthetases in biosynthesis was unexpected and called for confirmatory experiments. A tandem mass spectrometry (MS) method was developed to identify and quantify AMB. Bacteria

A




B

| Locus tag | Gene name | Gene product | Deletion mutant | AMB production in deletion mutant |
|---|---|---|---|---|
| PA2301 | - | Hypothetical protein | PAO6664 | + |
| PA2302 | *ambE* | Non-ribosomal peptide synthetase | PAO6665 | - |
| PA2303 | *ambD* | TauD-like protein | PAO6692 | - |
| PA2304 | *ambC* | TauD-like protein | PAO6697 | - |
| PA2305 | *ambB* | Non-ribosomal peptide synthetase | PAO6694 | - |
| PA2306 | *ambA* | Putative LysE-type translocator | PAO6691 | - |
| PA2307 | - | Permease component of ABC transporter | nd | nd |
| PA2308 | - | ATP-binding component of ABC transporter | nd | nd |
| PA2309 | - | Substrate binding component of ABC transporter | nd | nd |
| PA2310 | - | TauD-like protein | PAO6663 | + |

FIG. 3. The *ambABCDE* gene cluster in *P. aeruginosa* PAO1. (A) Schematic representation of locus tags PA2301 to PA2310 (given at http://www.pseudomonas.com). (B) Putative gene products and their implication in AMB production, as deduced from bioassays. nd, not done.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristic(s)[a] | Source |
|---|---|---|
| *E. coli* K-12 | Wild type | 27 |
| *P. aeruginosa* strains | | |
| LESB58 | Liverpool epidemic strain | 33 |
| PAO1 | Wild type | ATCC 15692 |
| PAO6663 | ΔPA2310 | This study |
| PAO6664 | ΔPA2301 | This study |
| PAO6665 | Δ*ambE* (PA2302) | This study |
| PAO6691 | Δ*ambA* (PA2306) | This study |
| PAO6692 | Δ*ambD* (PA2303) | This study |
| PAO6694 | Δ*ambB* (PA2305) | This study |
| PAO6697 | Δ*ambC* (PA2304) | This study |
| PA7 | Multidrug-resistant clinical isolate | 24 |
| PA14 | Wild-type *P. aeruginosa* strain with increased virulence | 14 |
| Plasmids | | |
| pME3087 | Suicide vector for gene replacement experiments; $Tc^r$ | 31 |
| pME6032 | $lacI^q$-$P_{tac}$ expression vector; $Tc^r$ | 12 |
| pME9601 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs 2301-1/2301-2 and 2301-3/2301-4; used to delete codons 7 to 176 of PA2301 | This study |
| pME9602 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs 2302-1/2302-2 and 2302-3/2302-4; used to delete codons 8 to 2122 of *ambE* | This study |
| pME9604 | pME3087 with 1.7-kb PAO1 insert generated by PCR with primer pairs 2310UpFor/2310UpRev and 2310DownFor/2310DownRev; used to delete codons 3 to 291 of PA2310 | This study |
| pME9613 | pME3087 with 0.7-kb PAO1 insert generated by PCR with primer pair 2301-1/2301-2Eco; used for *ambABCDE* assembly | This study, Fig. 4 |
| pME9614 | pME3087 with 0.7-kb PAO1 insert generated by PCR with primer pair 2306-1/2306-2Bam; used for *ambABCDE* assembly | This study, Fig. 4 |
| pME9618 | pME6032 with *ambABCDE* under $P_{tac}$ control | This study, Fig. 4 |
| pME9701 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs 2306-1/XL002 and XL003/XL005; used to delete codons 7 to 203 of *ambA* | This study |
| pME9707 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs XL019/XL020 and XL021/XL022; used to delete codons 6 to 337 of *ambD* | This study |
| pME9709 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs XL027/XL028 and XL029/XL030; used to delete codons 7 to 1245 of *ambB* | This study |
| pME9721 | pME3087 with 1.4-kb PAO1 insert generated by overlap extension PCR with primer pairs XL023/XL024 and XL048/XL026; used to delete codons 3 to 355 of *ambC* | This study |

[a] Tc, tetracycline. For the primer sequences, see Table S1 in the supplemental material.

were grown in GYP medium (1% [vol/vol] glycerol, 0.4% Bacto peptone [Difco], 0.4% Bacto yeast extract [Difco]) for 24 to 72 h. To 1 ml of cell-free culture supernatant, 10 μl of 100 μM d8-valine (labeled amino acid standards set A; Cambridge Isotope Laboratories, Andover, MA) and 10 μl of formic acid (98 to 100% [wt/vol]) were successively added, before centrifugation for 15 min. The analytes were purified and concentrated by micro-solid-phase extraction using an Oasis MCX μElution plate (Waters, MA). The eluates were dried, resuspended in 90% (vol/vol) acetonitrile, and analyzed by high-pressure liquid chromatography at 24°C on an Atlantis HILIC silica column (2.1 by 150 mm; Waters) using a Rheos 2200 CPS_LC pump (Flux Instruments, Switzerland) and a PAL system autosampler (CTC Analysis, Switzerland). A reverse-phase gradient elution was carried out with the following: solvent A was water, solvent B was acetonitrile, and solvent C was 200 mM ammonium formate, pH 3. The flow rate was 0.3 ml/min, and solvent C was held at 5% throughout the 35-min run. Solvent B was held at 90% for the first 2 min, followed by a 10-min gradient from 90% to 80% solvent B, a decrease to 50% over the next 8 min, a rest at 50% for 5 min, and then an increase again from 50% to 90% over the next 1 min. Solvent B was then held at 90% for a further 9 min. The column affluent was monitored using a triple-quadrupole Finnigan TSQ Quantum Discovery Max apparatus (Thermo Fisher, CA). The instrument was equipped with an electrospray interface and was controlled by Xcalibur software (Fisher Scientific). Samples were analyzed in the positive ionization mode operating at a cone voltage of 4 kV. The tandem mass spectrometer was programmed using the selected reaction monitoring mode to allow the $[MH]^+$ ions of AMB (eluted at 9.1 min; *m/z* 132) and of the internal standard, d8-valine (eluted at 9.0 min; *m/z* 126), to pass through the first quadrupole (Q1) and into the collision cell (Q2). The daughter ions for AMB and d8-valine were *m/z* 115 and 80, respectively. Quadratic log-log calibration curves with equal weighting were computed using the ratio of the peak area of the analytes and known AMB standards.

AMB production was measured from triplicate cultures and reached concentrations of 2.2 ± 0.8 μM, 15.3 ± 2.9 μM, and 25.5 ± 5.6 μM in culture supernatants of strain PAO1 grown for 24 h ($OD_{600}$ = 2.7 ± 0.4), 48 h ($OD_{600}$ = 3.7 ± 0.7), and 72 h ($OD_{600}$ = 4.8 ± 0.4). In contrast, no AMB-specific peaks were detected (detection limit = 0.05 μM) in samples from the

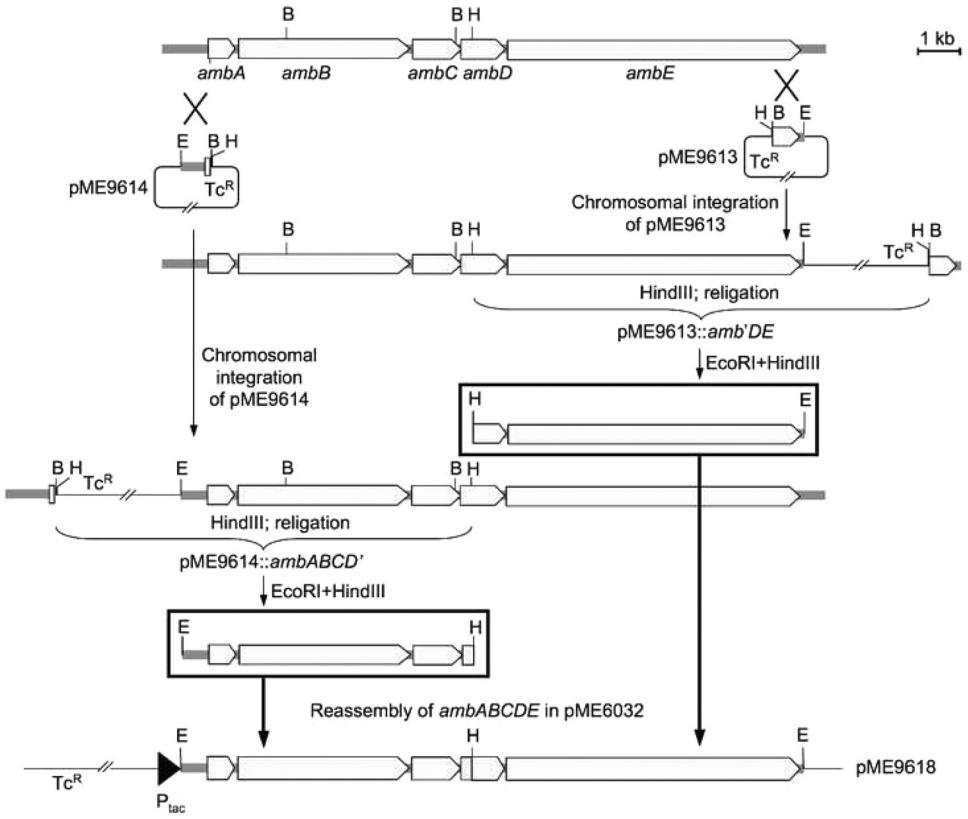



FIG. 4. Reassembly of the *ambABCDE* genes in the pME6032 expression plasmid. See Table 1 and Table S1 in the supplemental material for details of construction.

*ambE* deletion mutant PAO6665 grown under identical conditions. (For typical chromatograms of these experiments, see Fig. S1A, B, and D in the supplemental material). These data confirm and extend the bioassay results and demonstrate that the *ambABCDE* gene cluster is required for AMB production in *P. aeruginosa*.

**Transfer of *ambABCDE* to *P. aeruginosa* PA7.** Sequence comparison reveals that the *ambABCDE* genes are conserved in strains PA14 (14) and LESB58 (33) but that strain PA7 (24) is devoid of all locus tags between PA2302 and PA2313, with the exception of PA2306. We thus predicted that AMB is made by strains PA14 and LESB58 but not by PA7. This was indeed the case (data not shown). To further corroborate that the *ambABCDE* genes specify AMB production, we cloned all five genes downstream of the *tac* promoter on pME9618 (Fig. 4). Transfer of pME9618 to PAO6691, PAO6694, PAO6697, PAO6692, and PAO6665 (Table 1) restored the AMB production to these AMB-negative mutants (data not shown), demonstrating that all *amb* genes on pME9618 were expressed and complemented the corresponding gene function missing from the different mutants. We then tested if pME9618 would enable the naturally AMB-negative *P. aeruginosa* strain PA7 to make AMB. As shown in Fig. 2 (right panel), a large clearing zone was observed around PA7/pME9618, which suggested that AMB was produced in considerable amounts. Tandem MS analysis of culture supernatants from PA7/pME9618 grown for 24 h ($OD_{600}$ = 2.2 ± 0.4), 48 h ($OD_{600}$ = 4.4 ± 0.3), and 72 h ($OD_{600}$ = 4.6 ± 0.2) revealed AMB concentrations of 86.4 ± 45.8 μM, 418.5 ± 16.9 μM, and 682 ± 48.1 μM, respectively (see also Fig. S1C in the supplemental material for the amount of AMB production after 48 h), demonstrating that transcriptional overexpression of the *amb* gene cluster results in the >25-fold overproduction of AMB. We conclude from this that the *ambABCDE* genes specify AMB production in *P. aeruginosa* and that the putative ABC transport genes PA2307 to PA2309 (Fig. 3) are not required.

**Conclusions.** In this work we have identified the biosynthetic gene cluster *ambABCDE* for the antimetabolite AMB in *P. aeruginosa* and developed a straightforward analytical method to identify and quantify this metabolite in bacterial culture supernatants. AMB biosynthesis appears to occur by a thiotemplate mechanism. We propose that AmbB and AmbE generate a larger precursor peptide which may be hydroxylated during assembly by AmbC and AmbD and subsequently processed to yield AMB.

We thank Bertrand Rochat for performing initial MS experiments identifying AMB and Alexandre Beguin for help with tandem MS.

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