Production of Active Chimeric Pediocin AcH in *Escherichia coli* in the Absence of Processing and Secretion Genes from the *Pediococcus* *pap* Operon†

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Minimum requirements have been determined for synthesis and secretion of the *Pediococcus* antimicrobial peptide, pediocin AcH, in *Escherichia coli*. The functional mature domain of pediocin AcH (Lys+1 to Cys+44) is targeted into the *E. coli* sec machinery and secreted to the periplasm in active form when fused in frame to the COOH terminus of the secretory protein maltose-binding protein (MBP). The PapC-PapD specialized secretion machinery is not required for secretion of the MBP-pediocin AcH chimeric protein, indicating that in *Pediococcus*, PapC and PapD probably are required for recognition and processing of the leader peptide rather than for translocation of the mature pediocin AcH domain across the cytoplasmic membrane. The chimeric protein displays bactericidal activity, suggesting that the NH₂ terminus of pediocin AcH does not span the phospholipid bilayer in the membrane-interactive form of the molecule. However, the conserved Lys+1 to Tyr-Tyr-Gly-Asn-Gly-Val+2 sequence at the NH₂ terminus is important because deletion of this sequence abolishes activity. The secreted chimeric protein is released into the culture medium when expressed in a periplasmic leaky *E. coli* host. The MBP fusion-periplasmic leaky expression system should be generally advantageous for production and screening of the activity of bioactive peptides.

Pediocin AcH is a 44-amino-acid antimicrobial peptide that belongs to the class IIa family of nonlanthionine bacteriocins synthesized by several species of lactic acid bacteria (15, 17). The peptide is produced by *Pediococcus acidilactici* H (2, 25) and has an amino acid sequence identical to that of pediocin PA-1, which is produced by *P. acidilactici* PAC1.0 (21). Several other bacteriocins in this family have been characterized, including leucocin A (12) and sakacin P (38). Pediocin AcH displays broad-spectrum bactericidal activity against gram-positive and stressed gram-negative bacteria associated with food spoilage and human pathogenesis (16, 28–30). Bacteriocins also have potential applications in controlling topical infections caused by bacterial pathogens (19, 39). For these reasons, it is important to overproduce bacteriocins such as pediocin AcH in a suitable bacterial host and determine amino acids required for activity.

As is the case for nisin, a class I lantibiotic-type bacteriocin (15, 17), pediocins PA-1 and AcH kill bacteria by forming pore complexes in the cytoplasmic membrane, resulting in dissipation of the membrane electrochemical potential (6, 23, 24). Although binding of class IIa bacteriocins to membranes and nucleation of pore complex assembly may be promoted in vivo by membrane-associated receptor proteins (1), recent biophysical studies show that pediocin PA-1 can form pore complexes in pure *Listeria* phospholipid vesicles in the absence of membrane proteins (6). On the basis of in vitro studies, it is proposed that binding of pediocin PA-1 to membranes is mediated by interactions between positively charged amino acids in the peptide and negatively charged phospholipid head groups (6).

The pre-pediocin AcH structural gene (*papA*, encoding 62 amino acids) is the first gene in the *pap* operon carried on the *P. acidilactici* plasmid pSMB74 (25). Also present in the operon are genes required in the producer strain for immunity (*papB*, encoding 118 amino acids) and membrane translocation (*papC*, encoding 217 amino acids, and *papD*, encoding 178 amino acids) (5, 40). The PapD protein product also is required for removal of the 18-amino-acid leader peptide from the inactive pre-pediocin AcH precursor and generation of the active mature form of the peptide during membrane translocation (5, 40). The sequence of the leader peptide differs markedly from those of signal peptides of gram-positive (35) and gram-negative (41) bacterial standard secretory proteins and is presumed to target the precursor into a specialized secretion machinery composed of PapC and PapD (15). PapC and PapD are homologous to the respective membrane fusion protein (HlyD) and ATPase (HlyB) components of the *Escherichia coli* hemolysin secretion machinery and other ABC export systems (9). PapD shares a double-glycine protease domain with other ABC export proteins active in transport of bacteriocins such as pediocin AcH (12a). Pre-pediocin AcH can be secreted and processed in *E. coli* if the PapC and/or PapD protein is coexpressed in the host (5, 40). The immunity function of PapB is not required for *E. coli* expression (5).

In this article, we report that the mature sequence region of pediocin AcH can be produced and secreted in an active state in *E. coli* without coexpression of PapC and PapD if it is fused to the secretory protein maltose-binding protein (MBP). The MBP-pediocin AcH chimeric protein is released into the culture medium when expressed in a periplasmic leaky host in which the gene encoding the outer membrane protein Braun’s lipoprotein (4) has been disrupted. The implications of the results are discussed with respect to the function of the leader
peptide during pre-pediocin AcH secretion in *Pediococcus* and the structure of the membrane-interactive form of the peptide.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. The *malE* plasmids pPR682 and pH821 were used to construct MBP-pediocin AcH expression plasmids. In both plasmids, transcription of fusion genes is controlled by the isopropyl-β-D-thio-
galactopyranosidase (IPTG)-inducible *lac* promoter. DNA encoding pediocin AcH was obtained by PCR amplification of plasmid pMBR1.0 DNA (5). E. coli E609 (46) and its isogenic derivative E609L, which contains a Tn10 insertion in the *papA* gene, were used to study expression of MBP-pediocin AcH chimeric proteins. E. coli strains were grown at 37°C in Luria Bertani broth or agar, and 12.5 μg of tetracycline per ml was added to the media for strain E609L. Ampicillin (100 μg/ml) was added to the media when E609 and E609L were transformed with expression plasmids. MBP-pediocin AcH activity was tested against the indicator strain *Listeria innocua* Lin 11, grown at 30°C in tryptone-glucose-yeast extract (TGE) broth or agar (5).

**Construction of MBP-pediocin AcH expression plasmids.** A DNA fragment encoding the mature domain of pediocin AcH was obtained by PCR amplification of pMBR1.0 DNA. The sequence of the 5′ primer used for amplification (5′-AAATCTACGGTAAATGGGTCACTTGGT-3′) is identical to codons Lys3 to Cys4 of the *papA* gene (25). The 3′ primer (5′-GGTCTGACCTAG CATATG-3′) begins with the *Eco RI (blunt-end)* and SalI restriction enzyme site (GTGCGAC) and ends with a 19-nucleotide sequence complementary to the stop codon and six codons of *papA*. PCR amplification was performed with 25 U of Taq DNA polymerase (Gibco-BRL) per ml, 0.1 μg of pMBR1.0 template per ml, 6 mM MgCl2, 10 mM each nucleoside triphosphate, and 400 ng of each primer per ml. A 30-cycle repeated protocol consisting of 90 s of strand denaturation (94°C), 60 s of primer annealing (55°C), and 60 s of primer extension (72°C) was used to amplify DNA.

The PCR product was purified by agarose gel electrophoresis (48), subjected to a Klenow polymerase reaction to fill in potentially ragged ends, phosphorylated with T4 polynucleotide kinase, and digested with *Sal I* restriction enzyme. The resulting fragment was gel-purified again and ligated between the *Sal I* and *Eco RI* (blunt-end) and *Sal I* restriction sites within the multiple cloning sites of pPR682 and pH821. Translational fusion genes, in which the *malE* coding regions were confirmed by double-stranded DNA sequencing with Sequenase DNA polymerase (U.S. Biochemicals).

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference(s) or source</th>
</tr>
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<tbody>
<tr>
<td><em>L. innocua</em> Lin 11</td>
<td>Nonpathogenic; pediocin AcH sensitive</td>
<td>Jean Richard, INRA, Paris, France</td>
</tr>
<tr>
<td>E. coli E609</td>
<td>HfrC, pps</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>E609L</td>
<td>pPR682::Tn10; periplasmic leaky; Tc+</td>
<td>New England Biolabs</td>
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<tr>
<td>pMBR1.0</td>
<td>pap gene cluster in pH99; Cm+ Em+</td>
<td>This study</td>
</tr>
<tr>
<td>pPR682</td>
<td>malE plasmid; Ap+</td>
<td>This study</td>
</tr>
<tr>
<td>pH821</td>
<td>malE*, papA plasmid; Ap+</td>
<td>This study</td>
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<td>pH821A</td>
<td>malE::Δ(2-26)-papA plasmid; Ap+</td>
<td>This study</td>
</tr>
<tr>
<td>pH821B</td>
<td>malE::papA(Δ1-7) plasmid; Ap+</td>
<td>This study</td>
</tr>
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**Expression of chimeric proteins.** Expression strain cultures were grown to mid-log phase at 37°C in liquid media plus antibiotics. Preinduction samples were taken, separated by centrifugation into cell pellet and supernatant fractions, and processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis as described below. After preinduction samples, IPTG (1 mM final concentration) was added to the cultures, which were grown for an additional 3 h until postinduction sampling.

Pelleted cells were prepared for SDS-polyacrylamide gel electrophoresis by being solubilized directly in sample loading buffer containing SDS. Proteins in culture supernatants were precipitated with ice-cold 10% trichloroacetic acid. The precipitates were washed once with 5% trichloroacetic acid and once with 80% acetone and then were dried under a vacuum and solubilized in sample loading buffer. Samples were run on 10% acrylamide-bisacrylamide-SDS gels (18), and MBP-pediocin AcH production levels were quantitated by laser scanning densitometry after Coomassie blue dye staining of gels (1a). The same methods and gel system were used to analyze production by Western immunoblotting. MBP-pediocin AcH bands were visualized on immunoblots by staining with rabbit anti-MBP primary antibody and goat anti-rabbit immunoglobulin G–alkaline phosphatase secondary-antibody complex (22).

The bactericidal activity of chimeric proteins was analyzed by polyacrylamide gel electrophoresis and gel overlay screening. Cell pellet and culture supernatant samples were run on 16% acrylamide-bisacrylamide-SDS gels (32). Subsequently, the gels were washed in sterile water for 3 h to remove SDS, placed on precoated TGE agar plates, and covered with a 20-ml TGE soft-agar overlay containing *L. innocua* Lin 11 cells by previously reported methods (2, 45). The plates were incubated at 37°C overnight and examined for zones of growth inhibition associated with proteins in the samples.

**RESULTS**

**Colonial overlay screening analysis of MBP-pediocin AcH production in *E. coli*.** The secretion of translational fusion proteins in which the mature region of pediocin AcH (Lys1 to Cys44) is fused to the COOH terminus of MBP was investigated to determine if the PapC-PapD specialized secretion machinery is required for pediocin AcH production in *E. coli*. Two types of MBP-pediocin AcH chimeric proteins were constructed and analyzed (Fig. 1). In one (designated pre-682-PapA), a wild-type MBP domain (43 kDa) containing a functional signal sequence is joined to pediocin AcH. The mature 682-PapA protein should be secreted to the periplasm after processing of the signal sequence during secretion (7) if the *E. coli sec* machinery can accommodate the pediocin AcH chain. In the second chimeric protein (designated 821-PapA), an MBP domain lacking a signal sequence [MBP::Δ(2-26) (42)] is joined to pediocin AcH. The MBP::Δ(2-26) domain is unable to functionally interact with components of the *E. coli sec* machinery (42), and the 821-PapA protein should be trapped in the cytoplasm (7, 13).

A periplasmic leaky *E. coli* host, strain E609L (Table 1), was used to investigate secretion of the two chimeric proteins. E609L colonies synthesizing the proteins were overlaid with agar containing *L. innocua* Lin 11 and examined for release of antimicrobial activity. Colonies of strain E609L/pPR682 formed large zones of growth inhibition (average diameter = 7 mm) when 682-PapA synthesis was induced with IPTG, whereas smaller zones when synthesis was not induced (Fig. 2A). Smaller zones were formed in the absence of IPTG due to the lower level of expression taking place under noninducing conditions than under inducing conditions (data not shown).

In contrast, only a few colonies of strain E609L/pPH8211 formed small zones of growth inhibition under inducing growth...
conditions (Fig. 2B). Release of the cytoplasmic 821-PapA protein probably was due to lysis of dead cells in these colonies. It has been observed that induction of synthesis of the 821-PapA protein is more toxic to the host than 682-PapA. For example, strain E609L/pIH8211 undergoes a 90% reduction in viable-cell number after 24-h IPTG induction, whereas only a 10% reduction in viability occurs after 24-h IPTG induction of strain E609L/pPR6821 (data not shown).

The 682-PapA protein is secreted via the E. coli sec machinery. The mechanism by which 682-PapA is released from the periplasmic leaky host was investigated by examining the status of processing of the MBP signal sequence. In this regard, processing of the MBP signal sequence is indicative that the protein has been secreted via the sec machinery, because the catalytic domain of the processing enzyme, signal peptidase I, is located in the periplasm (3, 27). A 3-h induction period was selected for these experiments because the maximal level of synthesis of chimeric proteins in both E609L/pPR6821 and E609L/pIH8211 strains is achieved within 3 h, as is release of the 682-PapA protein from the former strain. The toxic effects (loss of viability and cell lysis) of the chimeric proteins on the strains are minimal during this time (data not shown).

Two forms of the 682-PapA protein were present in the cell fraction of the 3-h-induced E609L/pPR6821 strain (Fig. 3, lane 3). The larger of these proteins is the unprocessed precursor, and the smaller is the processed mature form in which the MBP signal sequence has been removed. Band assignments are based on observations that only the mature form of 682-PapA was released into the culture supernatant (Fig. 3, lane 4), and released 682-PapA molecules were the same size as the cytoplasmic 821-PapA protein, which lacks a signal sequence (Fig. 3, lane 6). Because the 682-PapA protein present in the culture supernatant is processed, it has been secreted from the strain via the sec machinery. Minimal amounts of the protein appear to be released by cell lysis, because very little 682-PapA precursor ever is detected in the culture supernatant. In contrast, only a small fraction of 821-PapA molecules was released from cells (Fig. 3, lane 7). As noted above, release is correlated with toxicity and cell death caused by overexpression of the intracellular protein.

It should be noted that not all 682-PapA molecules were secreted, as indicated by the fact that the unprocessed precursor form of the protein also was detected in cells (Fig. 3, lane 3). Some precursors may be trapped in the cytoplasm because the sec machinery is unable to keep pace with fusion protein synthesis under inducing conditions (22). Lastly, secreted mature-form 682-PapA molecules that remain associated with cells (Fig. 3, lane 3) have not leaked out of the periplasm. In this regard, periplasmic leaky strains typically release only 10 to 50% of their periplasmic proteins (20).

MBP-pediocin AcH chimeric proteins possess bactericidal activity. Chimeric proteins were assayed for activity by SDS-polyacrylamide gel electrophoresis and gel overlay screening against L. innocua Lin 11 (Fig. 4). The 821-PapA chimeric protein present in the cytoplasmic fraction of strain E609L/pPR6821 exhibited activity, as indicated by the zone of growth inhibition formed in the high-molecular-weight region of the gel (Fig. 4, lane 6). Zones of growth inhibition attributable to the 682-PapA chimeric protein also appeared in the high-molecular-weight region of the gel in both cell and culture supernatant fractions (Fig. 4, lanes 3 and 4). It should be noted that the unprocessed and processed forms of 682-PapA are not resolved well on the 16% acrylamide gels used to separate chimeric proteins from pediocin AcH-size molecules. These results indicate that pediocin AcH is active when its NH₂ terminus is blocked.
Some 682-PapA molecules in the culture supernatant were degraded, as indicated by the appearance of a growth inhibition zone in the low-molecular-weight region and several zones in the high-molecular-weight region of the gel (Fig. 4, lane 4). The low-molecular-weight active species migrated comparably to wild-type pediocin AcH obtained from *P. acidilactici* (Fig. 4, lanes 1 and 8). Proteolysis of 682-PapA occurred only if it had passed through the periplasm and outer membrane of the host and into the medium. Possibly, a protease(s) residing in the membrane or periplasmic compartments of the cells (14, 36) is responsible for cleaving the secreted chimeric protein.

The NH$_2$ terminus of pediocin AcH is required for bactericidal activity. A truncated derivative of 682-PapA that lacks amino acids Lys$^1$ to Val$^7$ (designated 682-PapA(D1-7)) was constructed to determine if the presence of the NH$_2$-terminal region of pediocin AcH is required for bactericidal activity. The region deleted contains a sequence (Lys$^1$-Tyr-Tyr-Gly-Asn-Gly-Val$^7$-) of unknown function that is conserved in class IIa bacteriocins (15, 17). The 682-PapA(D1-7) protein was synthesized in an amount comparable to that of 682-PapA, and 682-PapA(D1-7) was efficiently processed and released from the leaky host (data not shown). However, the truncated protein did not display activity against *L. innocua* Lin 11 by colony overlay or gel overlay screening. The results show that the deleted pediocin AcH sequence is required for bactericidal activity and suggest that the 682-PapA low-molecular-weight active degradation product discussed above is produced by cleavage of the chimeric protein upstream of the pediocin AcH domain.

The 682-PapA protein is efficiently released from the periplasmic leaky *E. coli* host. To confirm that the periplasmic leaky host is advantageous for pediocin AcH production, we compared the levels of 682-PapA released from strain E609L and the nonleaky wild-type E609 strain (Table 1). As shown in Fig. 5, the two strains synthesized comparable amounts of the protein. However, E609 released only a small fraction of secreted 682-PapA molecules into the culture medium, whereas E609L released about half (Fig. 5, compare lanes 3 and 4 and lanes 7 and 8). The results show that the periplasmic leaky host is better than the wild-type strain for production and release of the 682-PapA protein.

The amounts of 682-PapA synthesized and released from strain E609L were estimated by laser scanning densitometry of Coomassie blue-stained polyacrylamide gel samples (Fig. 6). On the basis of densitometry, the combined level of precursor and processed forms of 682-PapA associated with cells was ~12% of the total cell protein (Fig. 6, lane 3). Because about half of the processed molecules were released into the medium (Fig. 6, compare lanes 3 and 4), the total 682-PapA expression level was on the order of 18%. The 821-PapA protein also was expressed at a high level (~15% of the total cell protein) (Fig. 6, lane 6), but as discussed above, little of this protein was released from the host (Fig. 6, lane 7).
molecular weight standards were loaded in lane 1, and their molecular weights (in thousands) are indicated on the left.

FIG. 3. Western immunoblot analysis of MBP-pediocin AcH synthesis and secretion in strains E609L/pPR6821 (lanes 2 to 4) and E609L/pIH8211 (lanes 5 to 7). Samples were prepared from uninduced cells (lanes 2 and 5), 3-h-induced cells (lanes 3 and 6), and culture supernatants from 3-h-induced cells (lanes 4 and 7). Equivalent amounts of cell pellets and supernatants were loaded in the lanes. Precursor (p) and processed mature (m) forms of 682-PapA are indicated. The 821-PapA protein comigrates with the processed form of 682-PapA. Prestained molecular weight standards were loaded in lane 1, and their molecular weights (in thousands) are indicated on the left.

A potential problem associated with achieving high-level production of native bacteriocins in E. coli is the requirement for balanced coexpression of the specialized secretion machinery needed for recognition and processing of their prepeptides. While powerful systems such as T7 RNA polymerase vectors (37) could be used for expression, it is likely that cellular toxicity will result from high-level synthesis of these integral membrane proteins. For example, the NisT ATPase component of the nisin specialized secretion machinery (9) and other bacterial integral membrane proteins (8, 22) have been shown to interfere with E. coli growth and viability when overexpressed. For this reason, we elected to overexpress and secrete pediocin AcH as a chimeric protein. In this form, pediocin AcH is targeted into the standard E. coli sec machinery, and potential problems associated with overexpression of PapC and PapD can be avoided.

The results demonstrate that this approach eliminates the requirement for coexpression of the PapC-PapD specialized secretion machinery. High-level synthesis of MBP-pediocin AcH proteins was achieved because of the powerful transcription and efficient translation initiation signals present in malE vectors (7). Although the rate of synthesis of the 682-PapA protein exceeded the capacity of the E. coli sec machinery for secretion, about two-thirds of the molecules nonetheless were processed and secreted, and about half of the secreted molecules were released into the culture medium from the periplasmic leaky host. On the basis of the staining intensity of the processed 682-PapA protein in the culture medium, we estimate the level of the protein in the medium to be ~57 mg/g of total cell protein in the cultures. It should be possible to increase the yield by releasing molecules trapped in the periplasm by osmotic shock treatment of cells (7, 26).

Our results demonstrate for the first time that a class IIa bacteriocin can be secreted via the standard sec machinery of a gram-negative bacterium. Apparently, the peptide remains sufficiently unfolded prior to translocation to be accommodated by the core translocation machinery composed of the E. coli SecA, SecE, and SecY proteins (33, 43), for which there are homologs in gram-positive bacteria (35). Only two other, unrelated bacteriocins have been shown to be secreted by the cellular sec machinery. One member of the colicin family of bacteriocins, colicin V, can be secreted in E. coli when fused to the OmpA signal peptide (47). Another bacteriocin, divergicin A, produced by Carnobacterium divergens LV13, contains a standard NH$_2$-terminal signal peptide and is secreted via the sec machinery of this gram-positive bacterium (44).

The results raise the question of why an ABC export system is used for secretion of pediocin AcH in Pediococcus. The peptide chain can be accommodated by the sec machinery, there is no need for a membrane fusion protein (i.e., PapC) to bridge inner and outer membranes, and the peptide need not be targeted into a modification machinery associated with the ABC export system, as occurs with nisin (34). The data also eliminate the formal possibilities that the ABC export machinery, ABC export system, as occurs with nisin (34). The data also eliminate the formal possibilities that the ABC export machinery.
ery participates in catalysis of disulfide bond formation or folding of pediocin AcH after translocation across the membrane. Instead, the data suggest that the *Pediococcus* specialized secretion machinery is required primarily for recognition and processing of the pre-pediocin AcH leader peptide. Why the standard SEC pathway is not used for secretion of this bacteriocin remains unknown.

The finding that MBP-pediocin AcH chimeric proteins retain activity provides insight into the general structural features of the membrane-interactive form of the peptide. First, the results suggest that the NH$_2$ terminus of MBP-pediocin AcH proteins could insert deeply into bilayers, because insertion would require transfer of polar amino acids in the linker and/or the MBP domain into the membrane. Fourth, the NH$_2$-terminal sequence (Lys$^+$ to Val$^+$) is important for the bactericidal activity of the peptide. Deletion of these residues may remove a putative $\beta$ turn formed by amino acids 4 to 7 (6).

In conclusion, the MBP fusion-periplasmic leaky expression system should be generally useful for production and screening of the activity of bioactive peptides such as bacteriocins. The system also should be useful to facilitate purification (7), because relatively few proteins are released into the culture medium along with MBP chimeras. In the future, the system will be used to isolate mutants with alterations in the activity of pediocin AcH and other antimicrobial peptides. Because large amounts of chimeric proteins are released from colonies and zones of growth inhibition in overlays are large, mutants with low specific activities should be detectable.

**ACKNOWLEDGMENTS**

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**REFERENCES**


ERRATA

Trehalose Induces Antagonism towards *Pythium debaryanum* in *Pseudomonas fluorescens* ATCC 17400

A. GABALLA, P. D. ABEYSINGHE, G. URICH, S. MATTHIJS, H. DE GREVE, P. CORNELIS, AND N. KOEDAM

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Volume 64, no. 1, p. 20, reference 40: the reference should read as shown below.


Small-Subunit rRNA Genes and In Situ Hybridization with Oligonucleotides Specific for the Bacterial Symbions in the Larvae of the Bryozoan *Bugula neritina* and Proposal of “Candidatus Endobugula sertula”

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Volume 63, no. 11, p. 4615, column 2, line 10: “AF06607” should read “AF006607.”

Page 4616, column 1, lines 5 and 6: “AF06606,” “AF06607,” and “AF06608” should read “AF006606,” “AF006607,” and “AF006608,” respectively.