Processing of *as-48ABC* RNA in AS-48 Enterocin Production by *Enterococcus faecalis*[▽]

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Enterocin AS-48 production and immunity characters are encoded by 10 genes (*as-48ABCC₁DD₁EFGH*) of the pMB2 plasmid from the *Enterococcus faecalis* S-48 strain. Among these, *as-48A*, encoding the AS-48 peptide, and the *as-48BC* genes constitute a cluster required for AS-48 biogenesis and full immunity. In this study, the levels of expression of this cluster have been altered by insertion and site-directed mutagenesis as well as by expression coupled to *trans* complementation. Phenotypic studies of the mutants have indicated cotranscription of the three genes and revealed that the inactivation of *as-48B* prevents the production of AS-48, thus confirming its essentiality in AS-48 biogenesis. These studies have also supported the involvement of *as-48C* in enterocin immunity. In addition, they established that the intergenic region between the *as-48A* and *as-48B* genes is decisive for AS-48 expression, since a 3-bp substitution, which should disrupt a potential 47-nucleotide complex secondary structure, resulted in a hypoproducing phenotype. Transcriptional analyses of the *E. faecalis* wild-type and mutant strains supports the possibility that the *as-48ABC* genes are transcribed from the P$_A$ promoter located upstream of *as-48A*. Moreover, analysis and bioinformatic predictions of RNA folding indicate that *as-48ABC* mRNA is processed at the secondary structure located between *as-48A* and *as-48B*. Thus, synthesis of the AS-48 peptide appears to be controlled at the posttranscriptional level and is uncoupled from *as-48BC* translation. This mechanism of genetic regulation has not been previously described for the regulation of bacteriocin expression in enterococci.

The production of antimicrobial peptides (bacteriocins) directed against competitive microorganisms is widespread among gram-positive bacteria (21). A large variety of these are secreted by lactic acid bacteria, and the peptides have been extensively studied due to their potential use as natural preservatives in food applications (21). Enterocin AS-48 is a broad-spectrum, circular peptide of hydrophobic and cationic nature, which is ribosomally synthesized and posttranslationally circularized by a head-to-tail peptide bond (19). The well-defined, three-dimensional structure of AS-48, recently solved by nuclear magnetic resonance and X-ray diffraction techniques (reviewed in reference 30), is a reference for numerous studies of molecules structured in α helices (39, 42). As is usual for bacteriocin determinants, the genes encoding processing, secretion, and immunity functions are clustered. In fact, the *as-48* gene cluster encoding the AS-48 trait was located on the 68-kb pMB2 conjugative pheromone response plasmid identified in *Enterococcus faecalis* S-48 (31) and functionally transferred to *E. faecalis* JH2-2 (34). The full expression of AS-48 and immunity depends on the coordinated expression of the *as-48ABCC₁DD₁EFGH* genes (12, 34). Among these, *as-48A* is the structural gene and *as-48D₁*, which encodes a small cationic hydrophobic peptide, provides some degree of bacteriocin resistance by itself and has thus been identified as the immunity determinant (34). Interestingly, two overlapping genes (*as-*

48BC) are located 73 nucleotides (nt) downstream of the TAA termination codon of *as-48A* and the intergenic region includes a long inverted repeat (IR). This repeat was previously proposed to be a transcriptional terminator or attenuator, since Northern hybridization revealed an abundant RNA species containing the structural gene (T$_A$) and a minor species designated T$_1$, which could include either *as-48ABC* or *as-48BC* (34). The *as-48* cluster also contains two ABC transporters encoded by the *as-48C₁D* and *as-48EFGH* genes, which are cotranscribed in a polycistronic mRNA, together with *as-48D₁* (12). As-48C₁D seems to be devoted to exporting the newly synthesized bacteriocin and provides low levels of immunity. The function of this transporter cannot be replaced by the second multicomponent ABC transporter, As-48EFGH, which is involved in self-protection against exogenously administered AS-48 and works as a complementary immunity mechanism (12). We have postulated that As-48B, alone or in association with the ABC transporter As-48C₁D, may form a pore that is specific for the secretion of AS-48 molecules. The ultimate activity of the multicomponent proteins, As-48BC₁D, would be the removal of the leader peptide promoting head-to-tail circularization concomitantly with the export of the molecule (34). However, the mechanisms underlying AS-48 processing and its transport to the cell surface remain unknown.

The expression of most of the bacteriocin systems so far characterized (lantibiotics [reviewed in reference 28] or non-lantibiotics [reviewed in reference 15]) is controlled by a so-called, three-component regulatory system, which is an auto-regulatory model that includes the secreted bacteriocin as peptide pheromone and the signal transduction by the corre-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
E. faecalis JH2–2	Plasmid-free derivative of *E. faecalis* JH-2	49
E. coli DH5α	*supE44 ΔlacU169*(φ80*lacZΔM15*)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
E. coli RYC1000	*araD139 ΔlacU169 rpsL Δrib7 thiA gyrA recA56*	7
pAM401	Bifunctional *E. coli-E. faecalis* cloning vector; Cm^r Tc^r	17
pMB2	AS-48$^+$ Imm$^+$; 68-kb *as-48* cluster (*as-48ABCC1DD1EFGH*)	32
pMG36e	Lactoccocal cloning and expression vector, replicative in *E. coli*; Ery^r	48
pGEM-3Zf(+)	Cloning vector; Amp^r	Promega
pSL1180	Cloning vector; Amp^r	Pharmacia
pAM401-76	AS-48$^-$, partially Imm$^+$; 1.9-kb SphI-BglII (*as-48A* promoter (P$_A$) and *as-48A* structural gene) and 6.6-kb BglII (*as-48H*) DNA fragments from pMB2 cloned into pAM401; Cm^r	12
pAM401-81	AS-48$^+$ Imm$^+$; complete 25-kb *as-48* region (P$_A$ and *as-48ABCC1DD1EFGH*) from pMB2 cloned into pAM401; Cm^r	12
pAM401-52	AS-48$^+$ partially Imm$^+$; 7.8-kb SphI-PstI (P$_A$ and *as-48ABCC1DD1EF*) DNA fragment from pMB2 cloned into pAM401; Cm^r	34
pAM401-58	AS-48$^-$ and Imm$^+$ 0.16.7 kb of the *as-48* region (carries the 3′ region of *as-48A*, the intergenic region between *as-48A* and *as-48B*, and *as-48BCC1DD1EF*); derivative of pAM401-52 by deletion of the 1.7-kb SphI-PstI DNA fragment; Cm^r Tc^r	34
pAM401-81:: Tn5P$_A$	Tn5 insert in P$_A$ (*as-48A* promoter); Cm^r Tc^r	This work
pAM401-81::Tn5$_B$	Tn5 insert in *as-48B* gene; Cm^r Tc^r	This work
pAM401-81::Tn5$_C$	Tn5 in *as-48C* gene; Cm^r Tc^r	This work
pMG36-A	P$_A$-*as-48A* cloned into pMG36e; Ery^r	This work
pMG36-BC	*as-48BC* cloned into pMG36e under control of P$_{32}$ promoter; Ery^r	This work
pAM401-76$_X$	pAM401-76 containing XhoI restriction site 22 nt downstream of *as-48A* gene; Cm^r	This work
pBgD12S	1.9-kb SphI-BglII D fragment from pMB2 cloned into pSL1180; Amp^r	This work
pAM401-81$_X$	pAM401-81 containing XhoI restriction site 22 nt downstream of the *as-48A* gene; Cm^r	This work

[a] Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Amp^r, ampicillin resistant; Ery^r, erythromycin resistant; AS-48$^+$, AS-48 production; AS-48$^-$, no AS-48 activity detected; AS-48^r, AS-8 resistant; AS-48^s, AS-48 sensitive.

sponding two-component regulatory signaling system. We have previously shown that genes encoding transduction signals do not exist in the *as-48* cluster and that the cloning of this DNA region into the pAM401 plasmid results in full expression of the AS-48 character (production and immunity) (12). Here we present a detailed transcriptional analysis of *as-48ABC* genes reporting the posttranscriptional mechanism that regulates their expression and the essential role that *as-48B* plays in the biogenesis of AS-48.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study, together with the Tn5 insertion mutants of the pAM401-81 enterocin determinant, are listed in Table 1. Enterococcal cells were grown without aeration at 37°C in brain heart infusion broth (BHI-B; Oxoid). *Escherichia coli* strains were grown with shaking in Luria broth at 37°C. The plasmid-free strain *E. faecalis* JH2-2 (49) was used in cloning experiments of the as-48ABC cluster and as an indicator strain to detect enterocin activity. When necessary, the indicated antibiotics (Sigma-Aldrich, Madrid, Spain) were added as follows: ampicillin (Ap), 50 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; erythromycin (Ery), 200 μg ml^{-1} for *E. coli* and 6 μg ml^{-1} for *E. faecalis*; and kanamycin (Km), 30 μg ml^{-1}.

Antimicrobial activity assays. AS-48 production by different *E. faecalis* transformants was assayed by spotting 2 μl from a liquid overnight culture onto BHI agar (1.5%), followed by incubation at 37°C for 16 h. We then overlaid the plate with 6 ml of BHI soft agar (0.75%, with or without antibiotics) containing a 2% inoculum of indicator strain and incubated at 37°C for 12 to 18 h before reading the results. To test AS-48 sensitivity, 5-μl spots containing different quantities of purified AS-48 were applied to lawns seeded with *E. faecalis* JH2-2 (harboring different plasmids) and the plates were examined for inhibition halos after 18 h of incubation at 37°C.

Prediction genetic analysis. Mfold (50) and Fold programs (Genetics Computer Group package; Wisconsin University), based on energy minimization,

were used for predicting RNA secondary structures and folding, respectively. Calculation of the predicted termination efficiency of the secondary structure located between *as-48A* and *as-48B* was performed by using the algorithm developed by d'Aubenton Carafa et al. for *E. coli* (10) and optimized by De Hoon et al. (11) for low G+C content bacteria, in which the *d* value for a given terminator is calculated from the following equation: $d = 7.9(\Delta G/n_{SL}) + 2.67T - 14.61$, where ΔG is the Gibbs free energy of stem-loop formation in kilocalories per mole (calculated with the Mfold program), n_{SL} is the number of nucleotides of the entire stem-loop structure, and T is a parameter that depends on the number of consecutive U residues in the 14-nt stretch adjacent to the 3′ end of the stem-loop and for which an exponential decay weight from the 5′ to 3′ direction is applied. The condition $d > 0$ establishes a threshold that separates transcriptional terminators from intracistronic structures and shows that a positive value for *d* can be taken as strong indication of a ρ-independent terminators. This *d* score has a sensitivity of 96.93% for the detection of the predicted terminators in the *E. faecalis* V583 entire genome, and these terminators have average values for ΔG, stem length, and uracil stretch of −18.2, 11.9, and 9.9, respectively (11), which yield an average ***d*** value of 3.07. An analysis of different regions of structure I (see Fig. 5A) revealed that the entire structure (47 nt) has a *d* value of −11.85, and the best score corresponds to its central region (t_A), which has a negative value of −1.2. t_A and includes a stem of 11 nt, followed by a UUUUUUAAUU stretch close to the average for *E. faecalis* terminators, but has a ΔG of −7.5, which would minimize its formation efficiency and, consequently, its attenuation effect on transcription by the RNA polymerase in this host.

Plasmids and DNA manipulations. DNA cloning and *E. coli* transformations were performed according to standard protocols (44). *E. faecalis* plasmid DNA was extracted according to the method of Anderson and McKay (4). *E. faecalis* cells were transformed by electroporation according to the method of Fiedler and Wirth (16). Antibiotic-resistant transformants were screened for AS-48 production by replica plating and overlaid with a sensitive strain. DNA was sequenced with the ABI Prism Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems). Synthetic oligonucleotides were from Amersham Biosciences Europe GmBH (Cerdanyola, Barcelona, Spain).

PCR and DNA sequencing. All primers used in PCRs were synthesized by Amersham Pharmacia Biotech (Uppsala, Sweden) and were based on the published DNA sequence of the *as-48* locus of *E. faecalis* (EMBL X79542). Total DNA was prepared as described elsewhere (38) and used as a DNA template for PCR amplifications carried out with Biotools DNA polymerase (B&M Labs, Madrid, Spain) in an iCycler 170-8720 thermal cycler (Bio-Rad Laboratories, Madrid, Spain). The presence and molecular size of the PCR products were determined by electrophoresis on 1% (wt/vol) agarose gels using λ DNA digested with HindIII and/or the 100-bp ladder (Gibco-BRL, Invitrogen, United Kingdom) as the molecular weight standard. All PCR products were purified with the Perfectprep Gel Cleanup kit (Eppendorf AG, Hamburg, Germany) and sequenced.

Generation of transposon insertional mutants. The transposition of Tn5 into pAM401-81 was performed by introducing the plasmid into *E. coli* RYC1000 according to the method of Martínez-Bueno et al. (34). Selected pAM401-81::Tn5 mutagenized DNAs were transferred to *E. faecalis* JH2-2 by electroporation, and the new recombinant strains were screened for AS-48 production and immunity.

Site-directed DNA mutagenesis. An XhoI restriction site in the intergenic region between the *as-48A* and *as-48B* genes was generated by overlap extension PCR mutagenesis (24). The external oligonucleotides used were the M13 universal (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-AACAGCTATGA CCAT-3′) primers. The internal primers, mutXh3 (5′-TTTAACAATATGATA AAAAAAC**CTCGAG**ATTTTCTAGAGATATTCTGTTTT-3′) and mutXh4 (5′-AAAACAGAATATCTCTAGAAAAT**CTCGAG**TTTTTTTTATCATATTGTT AAA-3′) (underlined sequences show the added restriction sites, and bold nucleotides are the point mutations), exhibited three mismatches relative to the wild-type sequence, generating the new XhoI site. The template for mutagenesis was the pBgdΔ12S construction, a pSL1180 derivative bearing the D (SphI-BglII) fragment from pMB2 (Table 1). Amplification conditions were as described by Higuchi (23), and the resulting 2-kb amplified fragment containing the XhoI restriction site was SphI-BglII digested and cloned into pAM401-76 (12) by replacing the equivalent SphI-BglII fragment [pAM401-76$_X$]. Finally, ligation in the frame of the C fragment (BglII) of 6.4 kb from pMB2 into pAM401-76$_X$, previously digested with the same enzyme, yielded the plasmid pAM401-81$_X$ (25 kb). The proper orientation of the D, C, and B fragments was established by restriction analysis, and the presence of the XhoI restriction site was confirmed by DNA sequencing.

Complementation plasmid constructions and compatibility test. The lactococcal expression vector pMG36e (Ery^r) was chosen to construct complementation plasmids. This broad host range plasmid is a derivative of pWV01, which replicates via rolling circle (48), and consequently, in *E. faecalis* it should be compatible with pAM401 (Cm^r), a very stable theta-replicating plasmid able to accommodate large inserts (such as the 14.8 kb of the *as-48* cluster) while maintaining high segregational stability. To test this compatibility, the JH2-2[pAM401-81] and JH2-2[pAM401] strains were electroporated with pMG36e plasmid and transformants were selected on plates containing chloramphenicol and/or erythromycin. Several transformants of each transformation were grown in either the presence or the absence of selective antibiotics over several generations, and the stability of both plasmids was confirmed by electrophoretic DNA plasmid analysis (data not shown). On the basis of these experiments, pMG36e was used to clone the *as-48A* and *as-48BC* genes independently. To construct pMG36-A, a 644-bp EcoRV/EcoRI fragment containing the structural gene and its P$_A$ promoter was obtained from pAM401-81. This fragment was cloned in pMG36e, previously digested with SmaI/EcoRI enzymes, which delete the P$_{32}$ promoter. Plasmid pMG36-BC was constructed by PCR amplification of the coding regions of the *as-48BC* overlapping genes without a promoter by using appropriate primers as-48BXma (5′-CG**CCCGGG**GATGAATCTCTTTGGAA TTCTA-3′) and as-48AC2 (5′-CTGCA**GCATGC**GGATCCGATATCGTAAT ACCGATGCACTTTTTCAA-3′). The PCR product (2,620 bp) was digested with SmaI/SphI and cloned into the pMG36e vector, previously digested with the same restriction enzymes, downstream of the P$_{32}$ promoter (18). Therefore, pMG36-BC carries a P$_{32}$–*as-48BC* transcriptional fusion. These constructions were made in *E. coli* DH5α and then transferred to *E. faecalis* JH2-2, harboring different plasmids.

RNA isolation. Cultures of *E. faecalis* JH2-2, harboring the indicated plasmids (see Fig. 2, 3, and 4), were grown to an optical density at 600 nm (OD$_{600}$) of 0.4 for semiquantitative reverse transcription-PCR (RT-PCR) and primer extension analysis or to OD$_{600}$ values of 0.4 and 0.9 for Northern hybridization analysis. Cells were harvested by centrifugation, and their total RNAs were extracted by use of the Fast RNA Pro Blue kit (Q-Biogen, MP Biomedicals, Illkirch, France), following the manufacturer's instructions. For RT, total RNAs were treated with 5 U of RNase-free DNase I (Roche Diagnostics, San Cugat del Vallés, Spain) for 1 h at 37°C. The total RNA preparations were checked for the integrity and yield

of the rRNAs by analysis in agarose gels stained with ethidium bromide. The total RNA concentrations were determined by UV spectrophotometry.

Semiquantitative RT-PCR. Semiquantitative analysis of RNA levels containing regions of *as-48A*, *as-48AB*, *as-48BC*, and 16S genes was performed by two-step RT-PCR assays. For RT assays, total RNAs (500 ng) were added to 20-μl RT reaction mixtures containing 4 μl of cDNA synthesis buffer, 5 mM dithiothreitol, 40 U of RNaseOUT (Invitrogen), 1 mM deoxynucleoside triphosphate mix, a 10 μM concentration of the appropriate gene-specific primer, and 15 U of ThermoScript RT (Invitrogen) and then incubated for 30 min at 56°C (*as-48A*), 60°C (*as-48BC*), or 61°C (*as-48AB*). Reaction mixtures containing RNA instead of cDNA were included in the analysis to confirm the absence of DNA contamination in RNA preparations. The primers used were R-16S (5′-C TGCTGCCTCCCGTA-3′), R-AsA (5′-CTGCTCTTTTTCCTTTTTTCTTAAT TTCTTT-3′), R-AsAB (5′-CGTGTGTTCTATAACAATTTGATGCTCCTTG AACA-3′), and R-AsBC (5′-GGTATCTAATCGCATGTACAACAAAAATTC CTTTC-3′) for synthesis of 16S, *as-48A*, *as-48AB*, and *as-48BC* cDNAs, respectively. RT was terminated by incubation at 37°C for 20 min in the presence of 2 U of RNase H (Invitrogen).

For the amplification of either cDNA, 10% of the cDNA synthesis reaction mixture, 50 pmol of each primer, 500 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 3.5 mM MgCl$_2$, 20 mM Tris-HCl (pH 8.4), and 50 mM KCl were used for each 50-μl PCR, which was performed with 2 U of *Taq* DNA polymerase (Invitrogen). The cycling conditions were 1 cycle (96°C for 2 min) and 15, 20, 25, and 30 cycles (94°C for 1 min, 56°C, 60°C, or 61°C for 1 min for *as-48A*, *as-48BC*, or *as-48AB*, respectively, and 72°C for 1 min). Six microliters of the RT-PCR product was analyzed by 1% agarose gel electrophoresis and visualized with the Quantity One Gel Doc 2000 software (Bio-Rad Laboratories). The primers used for PCR amplification were F-AsA (5′-TTTTGAGGA GGAGTATCATGGTTAAAGAAAA-3′) and R-AsA for synthesis of a 321-nt amplicon containing the 100 first codons of *as-48A* for the detection of *as-48A* mRNA (T$_A$), F-AsAB (5′-TGCAGCAGGAAGAGAGTCAATTAAAGCATA CCT-3′) and R-AsAB for synthesis of a 420-nt amplicon containing the last 26 codons of *as-48A* and the first 88 codons of *as-48B* for the detection of *as-48AB* mRNA, F-AsBC (5′-CGCTTCTAAGATCGTCTCCTAATGGTTTGA-3′) and R-AsBC for synthesis of a 492-nt amplicon containing the 164 last codons of *B* and the 5 first codons of *C* for the detection of *as-48BC* mRNA, and F-16S (5′-AGAGTTTGATCCTGGCTCAG-3′) and R-16S for synthesis of a 368-nt amplicon containing the 5′ region of the gene encoding the 16S rRNA.

Northern blot hybridization. For the Northern blot analysis, 5 μg of total RNAs were denatured in 30 μl of MOPS (morpholinepropanesulfonic acid) (40 mM MOPS, 10 mM sodium acetate, 2 mM EDTA) buffer containing 50% deionized formamide, 17% formaldehyde, and 0.4% bromophenol. Then they were fractionated on a 1% agarose-formaldehyde gel run at 23 V at room temperature for 14 h. Staining of the gel with ethidium bromide allowed us to check the integrity of rRNAs after fractionation and their proper transfer to the membrane after blotting. Nucleic acids were transferred to a nylon membrane (HybondN+; Amersham Pharmacia) by capillary blotting for 16 h using 20× SSC (150 mM NaCl plus 15 mM sodium citrate) buffer. The RNAs were fixed to the membrane by exposing the filters to UV light for 4 min. The DNA probes used were those described above: the 321-nt amplicon (containing the first 100 codons of *as-48A*) and the 492-nt amplicon (containing the last 164 codons of *B* and the first 5 codons of *C*). The amplicons were PCR synthesized as described above and purified by use of the FavorPrep kit (Favorgen Biotech Corp., Kaohsiung, Taiwan). Then they were used as substrates for radioactive synthesis and labeling of the probe in a thermocycling reaction mixture using 20 ng of template DNA, 25 pmol of each primer, 1.5 mM MgCl$_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 150 μM of each deoxynucleoside triphosphate (dATP, dGTP, and dTTP), 50 μM dCTP, and 150 nM of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). Free [α-^{32}P]dCTP was removed by filtration on a MicroSpin G25 column (Amersham Pharmacia). Hybridization was performed overnight at 50°C in ULTRAhyb buffer (Ambion, Applera Hispania S.A., Madrid, Spain) that contains 50% formamide. For autoradiography, the blotted filter was exposed to Agfa CURIX RP2 Plus films. The radioactivity present in the filter bands was quantified directly with a PhosphorImager (Fujifilm, Barcelona, Spain).

Primer extension analysis and DNA sequencing. Primer extension analysis was performed as previously described (20). The primer used to detect the start site of *as-48ABC* mRNA was F-5′ mRNA (5′-TCTTTAACCATGATACTCCTCC-3′), which is complementary to the transcript from nucleotides 46 to 25. One picomole of primer was annealed to 3.8 μg of total RNA from the JH2-2[pAM401-81] or JH2-2[pAM401-81X] strain. The primer used to determine the processing site of *as-48ABC* mRNA was R-Processing (5′-TCTTGATTTGCTC TTAACTTC-3′), which is complementary to the mRNA from 470 to 450. One picomole of primer was annealed to 7.5 μg or 15 μg of total RNA from the
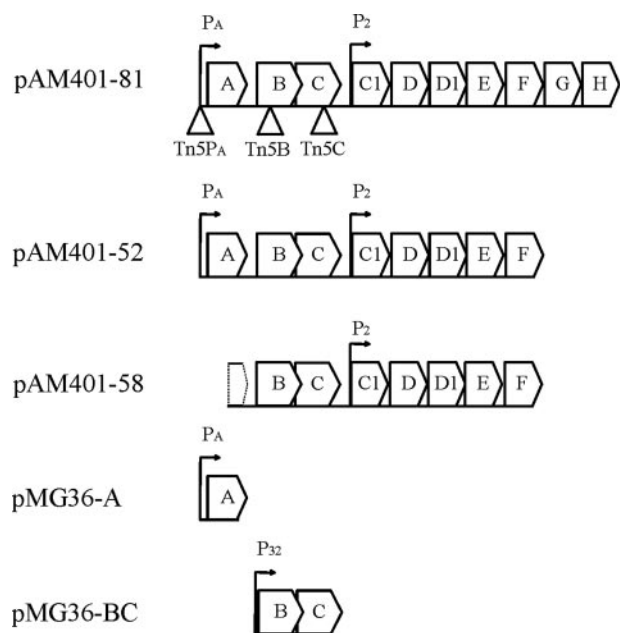
FIG. 1. Physical map of plasmids carrying *as-48* genes. A schematic representation of the inserts present in the plasmids is depicted. The locations of the Tn*5* insertions in the mutants constructed in this work are indicated in pAM401-81. Putative promoters (P_A, P_2, and P_{32}) are shown for each plasmid.

JH2-2[pAM401-81] or JH2-2[pAM401-81X] strain. The primers were previously labeled at their 5′ ends by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Primer extension reactions were performed by incubating the annealing mixture with 15 U of ThermoScript RT (Invitrogen) at 50°C for 30 min. The sizes of the reaction products were determined by using an 8% polyacrylamide gel containing 7 M urea. Bands labeled with ^{32}P were detected by autoradiography on Kodak X-Omat S film. The length of the extended products was inferred by the use of an unrelated DNA sequence ladders. The double-stranded plasmid pFS21 (47), which harbors the lactococcal *citQRP* operon heterologous sequence, and unlabeled primer R-Pcit (5′-CGGGTATCAAGTCATGG-3′) were used for sequencing reactions. The nucleotide sequence of the lactococcal *citQRP* operon has the accession number S77101 in the EMBL data bank. DNA sequencing was determined by the dideoxy chain terminator method (45) by using the T7 polymerase sequencing kit (Amershan-Pharmacia) and labeled [α-^{32}P]dCTP.

Accession numbers for nucleotide sequences are as follows: *as-48A*, X79542 (33); *as-48ABCC₁DD₁*, Y12234 (34); and *as-48EFGH* from fragment B BglII cloned in plasmid pAM401-81, AJ438950 (12).

RESULTS

Mutational insertion analysis of the *as-48ABC* cluster. *E. faecalis* JH2-2 is a plasmid-free strain sensitive to AS-48 enterocin, and we have previously constructed, and transferred to it, the pAM401-81 recombinant plasmid (12) carrying the *as-48ABCC₁DD₁EFGH* wild-type cluster from pMB2 and its derivatives pAM401-52 and pAM401-58 (Fig. 1 and Table 1). Both of these deletion plasmids lack the last two coding genes of the ABC transporter As-48EFGH (34). In addition, pAM401-58 lacks the 5′ region of the *as-48A* structural gene and its P_A promoter (Fig. 1) (34). Consequently, this plasmid cannot express the structural *as-48A* gene, but still carries the 3′ region of *as-48A*, the intergenic region between the *as-48A* and *as-48B* and *as-48BCC₁DD₁EF* genes. Here, to characterize expression of the *as-48ABC* genes, different Tn*5* insertions were randomly introduced into

pAM401-81 and, subsequently, insertions at three different locations were selected, namely, three nucleotides upstream of the −10 region of the putative P_A promoter of *as-48A* (Tn5P_A) or within either the *as-48B* (Tn5$_B$) or the *as-48C* (Tn5$_C$) gene (Fig. 1). The new insertion plasmids were transferred independently to the JH2-2 strain, and subsequently, the bacteriocin production and immunity of the transposition mutants were analyzed in comparison with those of JH2-2[pAM401-81], JH2-2[pAM401-52], and JH2-2[pAM401-58] (Table 2).

The JH2-2[pAM401-81] transformant produces AS-48 and has resistance levels to this enterocin similar to those of the original isolate S-48[pMB2] (12) and the recombinant bacteria JH2-2[pMB2] (32, 34) (Table 2). Nonproducer phenotypes were detected in JH2-2[pAM401-81::Tn5P_A], JH2-2[pAM401-81::Tn5$_B$], and JH2-2[pAM401-58]. By contrast, the interruption of *as-48C* in pAM401-81::Tn5$_C$ only decreased the levels of AS-48 production (hypoproducing phenotype) in the JH2-2 background. These results supported an essential function for not only *as-48A* but also *as-48B* in the expression of the AS-48 character (production and immunity). Moreover, the results suggested that P_A is a transcriptional promoter and, indeed, primer extension experiments (see below) showed this to be the case.

Unexpectedly, the three insertion mutants displayed the same reduced levels of resistance to AS-48 that JH2-2[pAM401-81] did. In addition, their levels of immunity were higher than that of JH2-2[pAM401-58] (resistance to 6 μg 5 μl^{-1} versus 1 μg 5 μl^{-1}). Interestingly, JH2-2[pAM401-58] showed higher sensitivity to AS-48 than JH2-2[pAM401-52] (Table 2) did, despite the fact that the only difference between them was the absence of a functional *as-48A* gene and of its putative P_A promoter (Fig. 1; Table 1). Therefore, these results

TABLE 2. AS-48 production and resistance from *E. faecalis* JH2–2 harboring the plasmids used in this work

Plasmid	AS-48 production zone of inhibition (mm)[a]	Sensitivity to indicated amt (μg 5 μl^{-1}) of AS-48[b]					
		0.5	1	3	6	9	18
pMB2 (positive control)	16	R	R	R	R	R	R
pAM401 (negative control)	ND	S	S	S	S	S	S
pAM401-81	16	R	R	R	R	R	R
pAM401-81::Tn5P_A	ND	R	R	R	R	S	S
pAM401-81::Tn5$_B$	ND	R	R	R	R	S	S
pAM401-81::Tn5$_C$	8	R	R	R	R	S	S
pAM401-81$_X$	11	R	R	R	R	R	R
pAM401-52	11	R	R	R	S	S	S
pAM401-58	ND	R	R	S	S	S	S
pAM401-81::Tn5P_A/pMG36-A[c]	7	R	R	R	S	S	S
pAM401-58/pMG36-A[c]	ND	R	R	S	S	S	S
pAM401-81::Tn5$_B$/pMG36-BC[c]	11	R	R	R	R	R	R

[a] AS-48 production was assayed in plates against the indicator strain *E. faecalis* JH2–2[pAM401] by using AS-48[pMB2] as a control (wild-type phenotype). ND, no detectable production.

[b] To test AS-48 sensitivity, 5-μl spots containing different amounts of purified AS-48 were applied on lawns seeded with a standard inoculum of *E. faecalis* JH2–2 harboring different plasmids. The plates were examined for inhibition halos after 18 h of incubation at 37°C. R, resistant; S, sensitive.

[c] Erythromycin was present during growth of the strains in liquid and solid media, and JH2–2[pAM401/pMG36e] was used as an indicator for AS-48 production.
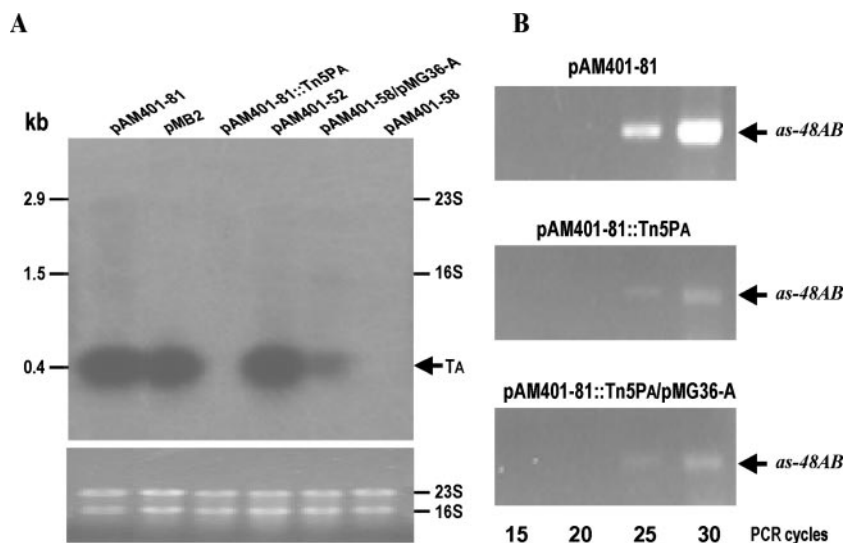
FIG. 2. Analysis of the *as-48AB* cotranscription. (A) Northern blot analysis. RNA was isolated from *E. faecalis* S-48[pMB2] and JH2-2 carrying the indicated pAM401-derived plasmid at the end of the exponential phase. The upper panel shows an autoradiogram of the blotting. The lines labeled 16S rRNA and 23S rRNA indicate the locations of these rRNA species, which are known to trap smaller RNA species (25). The lower panel shows the 23S rRNA and 16S rRNA loaded in the gel and stained with ethidium bromide. T_A, RNA species containing the *as-48A* gene (see the legend of Fig. 5 for details). (B) Semiquantitative RT-PCR detection. RNA was isolated from *E. faecalis* JH2-2 carrying pM401-81 wild-type and mutant plasmids. The specific primers used and reaction conditions are described in Materials and Methods. The plasmid carried by the JH2-2 strain and the number of cycles of each sample are indicated.

suggested that *as-48C* could be involved in immunity and that this gene is cotranscribed with *as-48AB* from P_A.

Complementation analysis between genes from the *as-48ABC* cluster. To test the above hypothesis, plasmid *trans* complementation experiments were performed after independently cloning the P_A-as-48A transcriptional unit and the *as-48BC* cluster, which lacks a known promoter. Independent cloning of *as-48B* and *as48C* was not attempted, since the overlapping of the genes (see Fig. 4) and the lack of an apparent RBS for *as-48C* indicated a coupled translation of both genes. For cloning, the pMG36e (Ery[r]) plasmid was used (48). This expression vector carries the strong constitutive lactococcal P_{32} promoter (18), which could support the expression of *as-48BC*, and it was shown to be compatible in *E. faecalis* with pAM401-81 (Cm[r]), which harbors the *as-48* cluster (see Materials and Methods for details). Two recombinant plasmids, pMG36-BC and pMG36-A, were generated (Fig. 1); the first carries the *as-48BC* genes under the control of P_{32}, whereas the second lacks this promoter and harbors the as-48A structural gene coupled to its own natural P_A promoter. Subsequently, the JH2-2[pAM401-58/pMG36-A], JH2-2[pAM401-81::Tn5P$_A$/pMG36-A], and JH2-2[pAM401-81::Tn5$_B$/pMG36-BC] strains were constructed by plasmid transfer and their production of AS-48 as well as immunity to the enterocin were investigated (Table 2).

Remarkably, the expression of *as-48A* in *trans* from pMG36-A in the deletion and insertion mutants had different effects. The introduction of pMG36-A in JH2-2[pAM401-58] did not result in an AS-48-proficient producer phenotype, whereas the presence of pMG36-A in JH2-2[pAM401-81::Tn5P$_A$] partially restored the AS-48-producing capacity (7 versus 16 mm of halo in the isogenic wild-type strain) of the enterococcal cells that had been lost due to the Tn5 insertion

into the P_A. This differential complementation in the two mutants did not appear to be due to gene dosage effects, as the transcription of *as-48A* (the expected T_A RNA of approximately 400 nt) from pMG36-A was detected by Northern blot hybridization in both, although at lower levels than in JH2-2[pAM401-81] (Fig. 2A and data not shown). These results suggested that the nonproducer AS-48 phenotype of JH2-2[pAM401-58/pMG36-A] was due to a lack of *as-48BC* expression, and indeed, it was undetectable in the pAM401-58 background by RT-PCR (data not shown). They results also indicated that the transcriptional read-through from the Tn5 carried in pAM401-81::Tn5P$_A$ allowed low levels of synthesis of As-48B, which was detectable by RT-PCR analysis of the intergenic region between *as-48A* and *as-48B* (Fig. 2B). Consequently, it seems that in the wild-type situation, *as-48B* cotranscribed with *as-48A* from P_A was involved in AS-48 biogenesis. Supporting this hypothesis, in JH2-2[pAM401-81::Tn5$_B$/pMG36-BC], expression from pMG36-BC under the P_{32} promoter partially restored the AS-48-producing capacity (Table 2).

In addition, the presence of pMG36-A did not increase the immunity levels of either JH2-2[pAM401-81::Tn5P$_A$] or JH2-2[pAM401-58], suggesting the absence of the necessary expression of the *as-48BC* accompanying genes. However, expression from pMG36-BC in JH2-2[pAM401-81::Tn5$_B$/pMG36-BC] conferred wild-type levels of resistance to the strain. This total complementation of the immunity phenotype, coupled with the overall results demonstrated that indeed, the low resistance against AS-48 of Tn5$_B$ and Tn5$_C$ insertion mutants is not due to any polar effects in expression of the *as-48D$_1$EFGH* downstream genes involved in the double-immunity mechanisms previously described (12, 34). Consequently, *as-48C* must constitute a third determinant involved in that process.
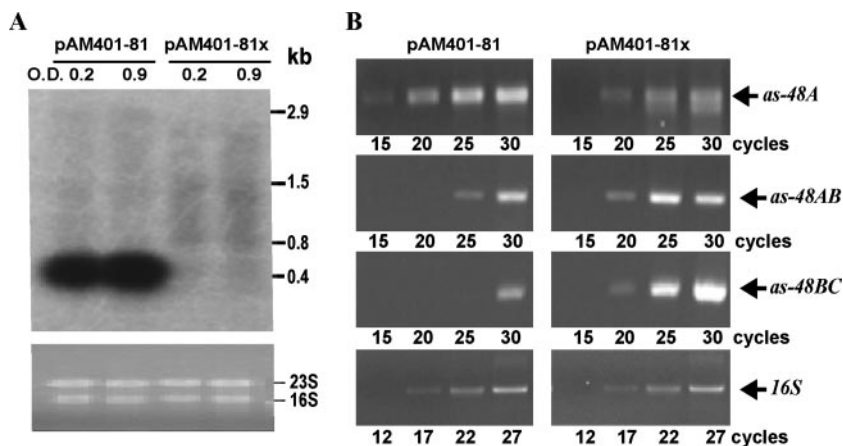
FIG. 3. Transcriptional analysis of the *as-48ABC* genes in JH2-2[pAM401-81] and JH2-2[pAM401-81$_X$] strains. (A) Northern blotting detection using specific probes derived from the *as-48A* gene. RNA was extracted at the indicated OD$_{600}$. O.D., optical density. (B) Semiquantitative RT-PCR analysis of *as-48A* (upper panel), *as-48AB* (middle panel), and *as-48BC* (lower panel) mRNAs in the indicated strains. The plasmid carried by the JH2-2 strain and the number of cycles of each sample are indicated.

Transcriptional analysis of the *as-48ABC* region. The expression of the *as-48ABC* gene cluster in JH2-2, carrying the natural pMB2 plasmid or its pAM401-based derivatives, was investigated by Northern blot analysis, and the results obtained were corroborated by RT-PCR analysis. Total RNA was extracted at the end of the exponential growth phase and hybridized with specific probes derived from the *as-48A* and *as-48B* genes (see Materials and Methods for details). The detection of RNA hybridization with the *as-48A* probe by autoradiogram and of rRNAs by ethidium bromide stain is depicted in Fig. 2A. A single and prominent band corresponding to the *as-48A* transcript (T$_A$) was detected in RNA preparations from S-48[pMB2] and JH2-2 carrying either pAM401-81 or pAM401-52 (Fig. 2A), correlating with their AS-48 producer phenotype. As expected, in JH2-2[pAM401-58] or JH2-2[pAM401-81::Tn5P$_A$] lacking a functional P$_A$ promoter, T$_A$ was not detected in the blotting. In addition, T$_A$ was revealed (albeit at low levels) in RNA samples from JH2-2[pAM401-58/pMG36-A], in which expression of the structural gene is driven from the P$_A$-*as-48A* transcriptional unit carried by pMG36-A (Fig. 2A). Furthermore, two faint and smeared bands larger than T$_A$ were also detected only in strains carrying a functional P$_A$ promoter (Fig. 2A and 3A and data not shown). However, when an *as-48B* probe was used, no clear hybridization bands could be detected in any of the blottings obtained; only weak marks could be detected just below the 23S (2,905 nt) and 16S (1,522 nt) rRNAs (data not shown). The overall results suggest that transcripts carrying *as-48B* had a very short life span, which impaired its detection by Northern hybridization.

Therefore, to confirm the cotranscription of *as-48A* and *as-48B* genes, their 73-nt intergenic region was analyzed by RT-PCR using specific primers (F-AsAB and R-AsAB) (Fig. 4) deduced from the coding regions of these genes. The expected 420-nt amplicon was detected in the three strains analyzed (Fig. 2B), JH2-2[pAM401-81], JH2-2[pAM401-81::Tn5P$_A$], and JH2-2[pAM401-81::Tn5P$_A$/pMG36-A]. The existence of RNA substrate for the RT-PCR amplification in the Tn5P$_A$ mutant at low level but not affected by the presence of pMG36-A, demonstrated transcriptional read-through from

pAM401-81::Tn5P$_A$, presumably driven from the aminoglycoside phosphotransferase promoter of Tn5. The detection of this amplicon in JH2-2[pAM401-81] and the fact that *as-48B* and *as-48C* overlap (Fig. 4A) suggested that the *as-48ABC* genes constitute a transcriptional unit driven from the P$_A$ promoter. Moreover, the results indicated that T$_A$ could be a specific and stable degradation product of the unstable T$_1$ transcript (T$_{ABC}$) previously detected in JH2-2[pAM401-52] as a minor band, migrating at the position of the 23S RNA and encompassing the *as-48ABC* genes (34).

Analysis of the intergenic region between *as-48A* and *as-48BC* genes. No obvious promoter sequences were observed in the 73-nt intergenic region between *as-48A* and *as-48B*. Moreover, the AS-48 nonproducer phenotype of JH2-2[pAM401-58], not complemented by pMG36-A and its reduced immunity (Table 2), rules out the existence of a promoter for the *as-48BC* genes in this region. However, analysis of this region with the Mfold program revealed the existence of a long IR with potential to form a complex secondary structure having an estimated ΔG free energy of -22.4 (Fig. 4A and 5A). This kind of structure is characteristic of substrates for endoribonucleases, suggesting that it could be implicated in mRNA processing and be decisive in the expression of the *as-48BC* genes (1, 13, 26, 31). To test this hypothesis, we analyzed the effect of replacing three bases within the IR in a site-directed mutagenesis experiment, creating an XhoI restriction site 21 nt downstream of the *as-48A* gene (Fig. 4A). For this purpose, the pAM401-81$_X$ plasmid, exhibiting three mismatches relative to the wild-type sequence, was constructed by overlap extension PCR mutagenesis. This plasmid should encode an mRNA with an altered secondary structure (Fig. 5B). When this plasmid was transferred to the JH2-2 strain, the transformants produced smaller inhibition zones than those of the wild-type strains (11 versus 16 mm), but they were fully resistant to 18 μg per 5 μl of pure AS-48 added exogenously (Table 2).

A comparative analysis by Northern blotting of *as-48A* expression in JH2-2[pAM401-81$_X$] and its isogenic JH2-2[pAM401-81] wild-type control during the exponential phase of growth revealed that the mutation introduced in the inter-

A


```
                                                                        R-5′ mRNA
                                                          CCTCCTCATAGTACCAATTTCT
                                                              F-AsA
TTGcatCAAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTCAAAAATATTTTGAGGAGGAGTATCATGGTTAAAGAAAA
    -35              -10        +1                          RBS      as-48A
```

```
        F-AsAB                                       R-AsA
--221 nt-TGCAGCAGGAAGAGAGTCAATTAAAGCATACCT    TAAGAAAGAAATTAAGAAAAAAGGAAAAAGAGCAGTTATTGC
```

```
                            TCGGG                    ↓↓
TTGGTAATTTAACAATATGATAAAAAAACAGGATATTTTCTAGAGATATTCTGTTTTTTAATTAAAAAAAGGGGGGCGCTCATGAATCTC
as-48A       →        →      →            ←         ←         RBS      as-48B
```

```
            R-Processing              R-AsAB
        CTTCAATTCTCGTTTAGTTCT    ACAAGTTCCTCGTAGTTTAACAATATCTTGTGTGC
TTTGGAATTCTAATGAAGTTAAGAGCAAATCAAGA--186 nt--TGTTCAAGGAGCATCAAATTGTTATAGAACACACG--930 nt-
```

```
                               R-AsBC
                     CTTTCCTTAAAAACAACATGTACGCTAATCTATGG
F-AsBC
CGCTTCAAGATCGTCTCCTAATGGTTTGA--427 nt--TTAGGAAAGGAATTTTTGTTGTACATGCGATTAGATACCATTAA
                                                      as-48C        as-48B
```
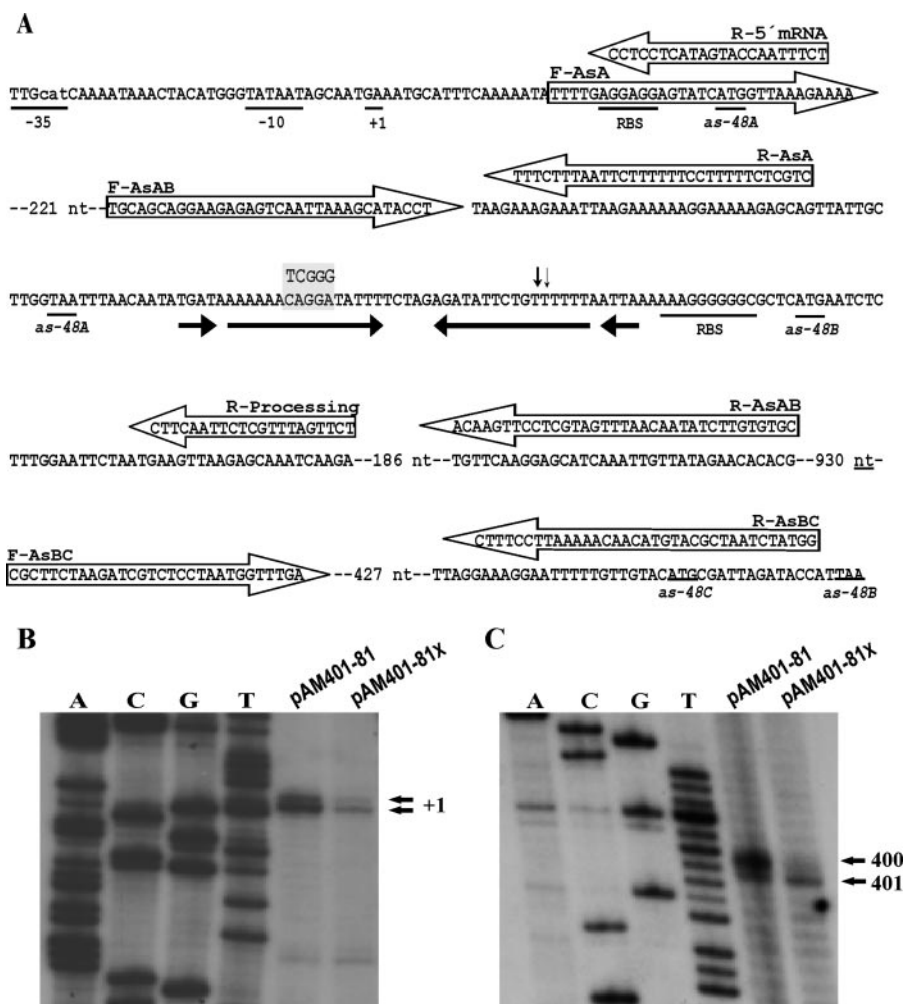
B

C

FIG. 4. Relevant DNA sequences for transcription and translation of the *as-48ABC* operon and determination of the initiation of transcription and mRNA processing. (A) The P_A promoter of the *as-48ABC* operon and RBSs, as well as the initiation and termination translational codons, are underlined. The long IR is marked by arrows. The three bases changed to generate the XhoI site are in the gray box. Transcription initiation sites of *as-48A* (inferred from results presented in panel B) are underlined and labeled as +1. The processing sites in the mRNA (inferred from results presented in panel C and corresponding to positions 401 and 402 of the mRNA) are indicated by arrowheads. Also indicated are the location, sequence, and polarity of all primers used for RT-PCR experiments depicted in Fig. 2 (F-AsAB and R-AsAB) and 3 (F-AsA, F-AsAB, F-AsBC, R-AsA, R-AsAB, and R-AsBC) and for primer extensions depicted in panel B (R-5′ mRNA) or in panel C (R-processing). F, forward; R, reverse. Determination of the initiation of transcription from P_A (B) and mRNA processing (C) by primer extension in the wild-type and pAM401-81$_X$ mutant strains. Autoradiograms of the gels are depicted, and the detected extended products are labeled with the corresponding coordinates in the *as-48ABC* mRNA. The coordinates were inferred from the length of the products (95 nt for +1 in panel B and 70 and 69 nt for 400 and 401 in panel C) that were estimated by the use of unrelated DNA sequence (A, C, T, and G) ladders.

genic region between *as-48A* and *as-48B* had important consequences at the mRNA level. The most significant was the almost undetectable amounts of T_A present in RNA preparations of the mutant (Fig. 3A), which is most likely related to the conformational changes provoked by the mutation at the mRNA level (see below). In fact, a degraded mRNA, visualized as faint and smeared bands above the T_A position, was observed for the mutant and not for the wild-type strain (Fig. 3A), in spite of the fact that rRNAs of both strains were intact (Fig. 3A, bottom panel). Furthermore, when levels of RNA species carrying *as-48A* were analyzed by RT-PCR (Fig. 3B) using the F-AsA and R-AsA primers (Fig. 4A), higher levels of *as-48A* mRNA were detected in the wild type than in the mutant, correlating with the results obtained by Northern blot-

ting and in accordance with the hypoproducer phenotype of the mutant strain (Table 2). By contrast, the opposite situation was observed for *as-48AB* and *as-48BC* mRNAs, since their levels were higher in the mutant, in accordance with its full resistance against AS-48 and correlating with the detection of hybridization bands longer than *as-48A* by Northern blot analysis that could be a substrate for RT-PCR amplification. These results strongly indicated that the intergenic region between *as-48A* and *as-48B* plays an important role in the fate of the unstable *as-48ABC* mRNA transcribed from the P_A promoter.

Therefore, the initiation of transcription from P_A (Fig. 4B), as well as the processing of mRNA (Fig. 4C), was investigated in the wild-type and pAM401-81$_X$ mutant strains by primer extension. The detection of the 5′ end of the mRNA revealed

FIG. 5. Prediction of the secondary structures present in the processing region of *as-48ABC* mRNAs and of their 5′ end processed species folding. Secondary structures present between the TAA translation termination codon of *as-48A* and the fifth codon of *as-48B* of the *as-48ABC* mRNAs encoded by pAM401-81 (A) and pAM401-81$_X$ (B). Prediction was made with the Mfold program. Folding of the 5′ RNA species generated from pAM401-81 (C) and pAM401-81$_X$ (D) transcripts by mRNA processing at the major cleavage sites detected in Fig. 4C and indicated here in panels A and B by arrowheads. The folding was performed with the Fold program.

in both strains, two extended products, corresponding to transcript starts with an adenosine and a guanidine residue located 38 and 39 nt upstream of the start codon of *as-48A* and at the correct position if transcription is driven from the P$_A$ promoter (Fig. 4A). This promoter (Fig. 4A) has a canonical TATAAT −10 region and a TTGctA (lowercase letters indicate bases that do not conform to the consensus sequence) −35 region that are separated from each other by 17 nt, which is the correct spacing for binding of the vegetative sigma factor of the bacterial RNA polymerase without the need of an activator. Moreover, inverted or direct repeats characteristic of transcriptionally regulated operators are present neither within nor surrounding the P$_A$ promoter. Consequently, under physiological conditions, transcription from P$_A$ in the wild type occurs during exponential growth (Fig. 2 to 4). Unexpectedly, a lower amount of the extended DNA fragments was detected in the pAM401-81$_X$ mutant (Fig. 4B). This result could be due to a shorter half-life of the T$_A$ in the mutant than in the wild-type strain, since lower levels of *as-48A* mRNA were also detected in the mutant by Northern blot and RT-PCR analyses (Fig. 3)

and DNA sequencing of this region revealed no rearrangements in the mutant plasmid.

In addition, the use of a primer located proximal to the 3′ end of the secondary structure revealed that in the wild-type strain, there exist two RNA species (one major and one minor) (Fig. 4C) having in their 5′ ends two adjacent uridine residues located within the right arm of the secondary structure (Fig. 5A). Interestingly, only the minor RNA species was detected in the mutant strain, which is predicted to encode an mRNA with an altered secondary structure (Fig. 5B). Moreover, folding of the processed RNA species carrying *as-48A* in the wild-type strain (Fig. 5C) revealed protective secondary structures at the 5′ and 3′ ends that were not present in the mutant RNA species (Fig. 5D). These predictions, together with the results of the transcriptional analysis (Fig. 3), indicated that alteration of the 3′ end of the processed T$_A$ species in the mutant should result in its being unstable and being a good substrate for fast degradation by 3′ exonucleases without accumulating specific degradation products.

Finally, the overall results from RT-PCR and primer exten-

sion experiments (Fig. 3B and 4) indicate that the mutation at the secondary structure in pAM401-81$_X$ causes a decrease of mRNA processing in the JH2-2 background. However, this decrease is accompanied by an increase of species longer than T_A but not T_{ABC} (Fig. 3A), indicating that degradation from the 3′ end of T_{ABC} is still very efficient in the mutant and that the detected degradation products could be the substrate for the increased RT-PCR amplification of as-48BC observed in JH2-2[pAM401-81$_X$] (Fig. 3B).

DISCUSSION

In this work, we present evidence that a constitutive P_A promoter drives transcription of the as-48ABC operon that includes the as-48A bacteriocin structural gene, the as-48B gene with an essential function for AS-48 biogenesis, and a putative cognate immunity gene (as-48C). Moreover, we have shown that the expression of as-48ABC is controlled at the posttranscriptional level.

RT-PCR analysis showed that as-48A and as-48B, as well as as-48B and as-48C, could be linked (Fig. 3B). Primer extension analysis revealed that an RNA species exists, carrying as-48B and having the 5′ end located in a putative secondary structure (Fig. 4 and 5A). Northern blotting analysis suggests that the as-48ABC operon mRNA transcript, T_{ABC}, is rapidly degraded by endonucleases, leading to a stable mRNA product, T_A, and a T_{BC} with a short life span, which makes its detection almost impossible (Fig. 2 and 3). The detection of RNA species containing the overlapping 3′ end of as-48B and 5′ end of as-48C by the more sensitive RT-PCR assay (Fig. 2 and 3) supports this hypothesis.

The computation of the secondary structure of the as-48AB intergenic region and the first five codons of as-48B with the Mfold program predicted two stem-loop secondary structures (I and II) (Fig. 5A). Structure I includes the two detected processing sites. The putative RBS of as-48B is located in the stem of structure II, which could modulate the translation of As-48B. The processing sites are two adjacent uracils located in a double-stranded region of structure I. The ubiquitous endoribonuclease RNase III identified in lactic acid bacteria (3) cleaves in double-stranded regions located in stem-loop structures (14, 43), and it is therefore possible that it is responsible for the processing of the as-48ABC transcript. Interestingly, the specificity of RNase III of E. faecalis may also depend on the mRNA sequence, since the T_{ABC} transcript encoded by the JH2-2[pAM401-81$_X$] mutant is processed at the second uracil residue (Fig. 4 and 5B). The computation predicted that this U is also located in a double-stranded region of the mutant mRNA, but in structure III rather than structure I (Fig. 5B). The ΔGs predicted for the formation of the secondary structure of the intergenic regions of the wild type (-22.4) and the mutant (-16.6) indicate that processing should occur in the latter with less frequency, as was indeed detected (Fig. 4).

Here, we have reported that mRNA processing yields an abundant T_A in the wild-type strain. The prediction of the folding of this T_A (Fig. 5C) revealed that its 3′ end is located in a small secondary structure similar to the ρ-independent transcriptional terminators, which can act as a barrier to 3′-5′ exoribonucleases. Consequently, the prominent 0.4-kb T_A RNA species could result from a combination of endoribonu-

cleolytic cleavage of the primary T_{ABC} transcript in the region between as-48A and as-48B and the subsequent resistance of the resulting product to 3′-5′ exoribonucleolytic degradation (6, 22). Supporting this proposed role of the small secondary structure, the T_A encoded by the pAM401-81$_X$ mutant is much less abundant than the wild type (Fig. 3) and does not seem to possess this structure at its 3′ end (Fig. 5D). The high level of T_A in the wild type may be due to the synthesis of an independent as-48A transcript from P_A, because the upper part of structure I (t_A) includes a 3-nt loop and an 11-nt stem, followed by the sequence UUUUUUAAUU, as occurs in the weak transcriptional terminators that act as attenuators (36). This resembles the model described for the ltnA1A2 transcript in the production of lacticin 3147 and its immunity (37). However, the predicted ρ-independent terminators of E. faecalis have an average d value of 3.07 (see Materials and Methods for details) (11), whereas t_A has a negative d score of -1.2, which makes it very unlikely that t_A acts as an attenuator. Nevertheless, even if the termination of transcription occurs at low efficiency at this location, it is predicted that as in the case of processing, it will yield a stable as-48A transcript with a protective secondary structure at its 3′ end (data not shown).

Thus, it appears that RNA processing is the main mechanism that uncouples the transcription and translation of as-48A and as-48BC (8). The presence of a stem-loop structure between the structural gene and the downstream biosynthetic genes is a feature of a number of regulated bacteriocinogenic systems, such as mersacidin (2), sakacin A (5), sakacin P (35), nisin (29), or lacticin 3147 (9), where notable differences in concentrations between transcripts of the structural gene and of those genes involved in biosynthetic machinery have also been reported. It has been established that in addition to allowing partial read-through to downstream synthetic genes, the stem-loop structure in Pep5 is also required for mRNA stability (40). Our results demonstrate that the mutagenesis of such structures is not a reliable way to improve read-through without adversely impacting mRNA stability and thus reducing bacteriocin production.

With regard to as-48ABC operon translation, as-48A and as-48B are preceded by putative RBSs (5′-tgAGGAGGag-3′, where lowercase letters indicate bases that do not conform to the consensus sequence, and 5′-AAAGGGGGGC-3′) complementary to the 3′ end of the E. faecalis 16S rRNA (3′-UUU CCUCCCGAGUG-5′) (41) and at the correct spacing from their AUG translation initiation codons. These RBSs have computed ΔG free-energy values of -12.1 and -14.2, respectively, for a strong binding to the 3′ end of the enterococcal 16S rRNA according with the rules of Schur et al. (46). By contrast, as-48C does not possess sequences that correspond to a competent RBS and the overlapping of its 5′ end with the 3′ end of as-48B suggests that coupled translation of both genes occurs, constituting a second mechanism of posttranscriptional control. This is also the case for sakacin Q, in which the translation of the structural and the immunity genes are coupled (35).

It is frequently postulated that the bacteriocin-encoding and the immunity protein-encoding genes must be cotranscribed and expressed to ensure that the producer strain is not killed by its own bacteriocin. The results presented here support the fact that transcription of as-48BC takes place only from the P_A linked to the as-48A structural gene. The precise function of

As-48B and As-48C is still unknown, although both have a significant homology with CirB (58%) and CirC (65%), which are required for the production of circularin A (27). The reason why the genes of *as-48ABC* are cotranscribed must be investigated through the functional analysis of their gene products in the cell, and according to our results, As-48B is indispensable for AS-48 biogenesis, while the integral membrane protein As-48C, with four postulated transmembrane domains (34), could be involved in protection functions, together with the other immunity determinants *as-48D₁* and *as-48EFGH*, which are located in an independent operon (12, 34).

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