Insights into the Functionality of the Putative Residues Involved in Enterocin AS-48 Maturation[▽]

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AS-48 is a 70-residue, α-helical, cationic bacteriocin produced by *Enterococcus faecalis* and is very singular in its circular structure and its broad antibacterial spectrum. The AS-48 preprotein consists of an N-terminal signal peptide (SP) (35 residues) followed by a proprotein moiety that undergoes posttranslational modifications to yield the mature and active circular protein. For the study of the specificity of the region of AS-48 that is responsible for maturation, three single mutants have been generated by site-directed mutagenesis in the *as-48A* structural gene. The substitutions were made just in the residues that are thought to constitute a recognition site for the SP cleavage enzyme (His-1, Met1) and in those involved in circularization (Met1, Trp70). Each derivative was expressed in the enterococcal JH2-2 strain containing the necessary native biosynthetic machinery for enterocin production. The importance of these derivatives in AS-48 processing has been evaluated on the basis of the production and structural characterization of the corresponding derivatives. Notably, only two of them (Trp70Ala and Met1Ala derivatives) could be purified in different forms and amounts and are characterized for their bactericidal activity and secondary structure. We could not detect any production of AS-48 in JH2-2(pAM401-81$_{His-1Ile}$) by using the conventional chromatographic techniques, despite the high efficiency of the culture conditions applied to produce this enterocin. Our results underline the different important roles of the mutated residues in (i) the elimination of the SP, (ii) the production levels and antibacterial activity of the mature proteins, and (iii) protein circularization. Moreover, our findings suggest that His-1 is critically involved in cleavage site recognition, its substitution being responsible for the blockage of processing, thereby hampering the production of the specific protein in the cellular culture supernatant.

Bacteriocins constitute a family of proteins of ribosomal synthesis for microbial defense that show a variable spectrum, mode of action, molecular weight, genetic origin, and biochemical properties (44, 45). Circular bacteriocins comprise a unique group of active posttranslationally modified proteins in which their N and C ends are linked by a peptide bond to form a circular polypeptide chain. Recently, they have been grouped as a new class of bacteriocins (class IV) (28), which has been divided into two subclasses according to their sequences and predicted secondary structures (32): one group including the disparate bacteriocins AS-48 (14, 17), subtilosin A (23), carnocyclin A (31), lactocyclicin Q (50), circularin A (24), and uberolysin (60) and one including butyrivibriocin AR10 (21) and the homologous gassericin A group (gassericin A and reutericin 6, which are identical molecules, and acidocin B) (3, 22, 53, 55). The gene sequences for most of the precursors are known, but putative cleaving and/or circularizing enzymes have not yet been reported. A general trend in the biosynthesis of the circular proteins is the appearance of an N-terminal-extended prepeptide that undergoes posttranslational circularization after proteolytic cleavage to release the mature bioactive molecules (28, 50). Signal sequences containing in-

formation specifying the choice of the targeting pathway, translocation efficiency, cleavage timing, and even postcleavage functions have been proposed (20). Recently, Oman and van der Donk (38) reviewed the different roles for the signal peptides (SPs). The most common function is that of a secretion signal, but these SPs also have been postulated as a recognition motif for the posttranslational modification enzymes or as a *cis*-acting chaperone in which the signal actively assists during the posttranslational modification process. The comparative analysis of the SPs of the circular bacteriocins, however, reflects remarkable differences in length and sequence as well as the absence of conserved motifs among them (28, 50), hindering a consensus sequence between their cleavable sites. These signals are unusually short for subtilosin A, uberolysin, carnocyclin A, circularin A, and lactocyclicin Q formation (8, 6, 4, 3, and 2 amino acids, respectively) compared with the extended signal for AS-48 (35 residues) or acidocin B/gassericin A/reutericin 6 (33 residues).

The AS-48 character (production and immunity) depends upon the coordinated expression of the *as-48A*, *as-48B*, *as-48C*, *as-48C₁*, *as-48D*, *as-48D₁*, *as-48E*, *as-48F*, *as-48G*, and *as-48H* genes (reviewed in reference 27). The structural gene *as-48A* is cotranscribed with *as-48BC* genes, with essential functions in AS-48 biogenesis and immunity (29). The posttranscriptional regulation of *as-48ABC*, recently identified by Fernández et al. (10), provides producer cells with the maximized production of functional AS-48 without deleterious effect before the entire immunity machinery (*as-48D₁* and *as-48EFGH* determinants,

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TABLE 1. Microorganisms used in this work

Microorganism	Relevant characteristic(s)	Reference or source
Escherichia coli JM109	e14-(MrcA-) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *supE44 relA1* Δ(*lac-proAB*) (F' *traD36 proAB lacI^{q}ZΔM15*)	Stratagene
Arthrobacter sp.	AS-48 sensitive	Universidad de Granada collection
Bacillus cereus LWL1	AS-48 sensitive	9
B. megaterium	AS-48 sensitive	CECT 44
Enterococcus faecalis S-47	AS-48 sensitive	15
E. faecalis JH2-2	Plasmid free, Rif^r Fus^r AS-48^s	62
Listeria innocua	AS-48 sensitive	CECT 4030
L. monocytogenes	AS-48 sensitive	CECT 4032
Staphylococcus aureus	AS-48 sensitive	CECT 976

which are located in an independent operon) begins to work (6, 30). In addition, the *as-48* cluster contains an essential ABC transporter, As-48C$_1$D, the absence of which renders a non-producer phenotype, which is devoted to the secretion of newly synthesized enterocin (28, 30). Understanding the precise mechanism underlying the AS-48 maturation and its transport to the cell surface is still our main challenge, because no products with the necessary specific activities and abilities to produce the circular polypeptide backbone have been identified yet. To unravel critical positions of the AS-48 maturation reactions, three single derivatives of AS-48 have been designed: the mutated residues located in the carboxyl end of the SP (His-1Ile) and those at both ends of the proprotein (Met1Ala and Trp70Ala). Comparative results concerning the functional structural features of such residues are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacterial growth conditions. The bacterial strains and plasmids used in this work and their relevant features are shown in Tables 1 and 2, respectively. *Enterococcus faecalis* JH2-2 was used in cloning experiments and production of AS-48 and its derivatives, and it also was an indicator strain. *Enterococcus*, *Listeria*, and *Staphylococcus* were propagated in buffered brain heart infusion (BHI-B) or tryptic soy broth (TSB-B) without aeration at 37°C. The *Arthrobacter* sp. was grown with shaking in BHI-B at 28°C. *Escherichia coli* strains, used as intermediate hosts for cloning, were grown with shaking in Luria-Bertani (LB) medium at 37°C. Culture media were purchased from Scharlau (Chemie, Barcelona, Spain). Agar plates contained 1.5% (wt/vol) agar, and soft agar was made with 0.75% (wt/vol) agar. If necessary, media were buffered in 0.1 M sodium phosphate buffer, pH 7.2. When appropriate, the antibiotics ampicillin (Ap), 50 μg/ml, and chloramphenicol (Cm), 20 μg/ml, from Sigma-Aldrich (Madrid, Spain) were added as selective agents.

Molecular techniques. DNA cloning techniques and transformations were performed according to the standard protocols (46). *E. coli* and *E. faecalis* plasmid DNA were extracted according to Birnboim and Doly (4) and O'Sullivan and Klaenhammer (40), respectively. *E. faecalis* was transformed by electroporation according to Fiedler and Wirth (11). Restriction enzymes, T4, DNA ligase shrimp phosphatase, and other DNA-modifying enzymes are from Fermentas

(Quimigen, Madrid, Spain) and Roche Diagnostics (San Cugat del Vallés, Spain); they were used as recommended by the suppliers. DNA was sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems).

Mutagenesis procedure and pAM401-81$_{Mut}$ plasmid construction. Three single mutations (producing His-1Ile, Met1Ala, and Trp70Ala) were introduced in the structural *as-48A* gene as described elsewhere (10). The oligonucleotides used as primers for site-directed mutagenesis (Table 3) were from Amersham Biosciences Europe (Barcelona, Spain). Once we confirmed the accuracy of the sequences, the 5.4-kb amplified products (pBgD12S$_{Mut}$) were used to transform competent *E. coli* JM109 cells. To restore the *as-48* cluster, each pBgD12S$_{Mut}$ construction containing the required substitution was SphI-BglII digested and cloned into pAM401-76 (8) by replacing the equivalent fragment (pAM401-76$_{Mut}$) according to Fernández et al. (10). The proper orientation of the fragments was established by analysis with EcoRI and PCR analysis using the As48-1- and As48-B4-specific primers (Table 3).

RNA isolation and semiquantitative RT-PCR. Total RNA from cells grown as described above was isolated using the Fast RNA Pro Blue kit (Q-Biogen, MP Biomedicals, European Headquarters, France). DNA was digested with DNase for 1 h at 37°C and heat inactivated at 70°C for 10 min. DNA absence was verified by PCR with specific primers under the conditions described above. Semiquantitative analysis of transcript levels was performed by two-step reverse transcription-PCR (RT-PCR) assays. Primers used for this task were As48-6 and As48-5 (Table 3) for the specific amplification of the *as-48A$_{Mut}$* genes using the wild-type *as-48A* as a positive control and the nonproducer strain JH2-2 as a negative one. Primers W01 and W012 (37) (Table 3) were used for 16S RNA amplification. Moloney murine leukemia virus (M-MuLV) reverse transcriptase was used according to the manufacturer in a reaction mixture containing total RNA (1 μg), 4 μl of cDNA synthesis buffer, 1 mM deoxynucleoside triphosphate mix, 10 μM the appropriate gene-specific primer, and 200 U of enzyme in a final volume of 20 μl. RNA, oligonucleotides, and water were heated at 70°C in a denaturing step prior to the addition of the other components of the mixture, and cDNA synthesis was performed for 60 min at 42°C. For the amplification of each cDNA, 10% of the reaction mixture was used as the template. The cycling conditions were 1 cycle of 94°C for 4 min and 15, 20, 25, and 30 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 1 min. Samples of 5 μl were taken at 15, 20, 25, and 30 cycles.

AS-48 engineered derivative purification. Wild-type and modified AS-48 was obtained from Esprion 300 (E-300; DMV Int., Veghel, Netherlands) plus 1% glucose (E-300-G) cultures of *E. faecalis* JH2-2(pAM401-81) and JH2-2(pAM401-81$_{Mut}$) transformants under the conditions established by Ananou et

TABLE 2. Relevant features of the plasmids used in this work

Plasmid	Characteristics	Reference or source
pBgD12S	Ap^r, *as-48A* cloned into pSL1180 (5.4 kb)	48
pBgC	Ap^r, BglII-BglII (fragment C) from *as-48* cluster cloned into pSL1180 (9.2 kb)	8
pBgD12S$_{Mut}$	Ap^r, pBgD12S with the His-1Ile, Met1Ala, or Trp70Ala substitution in *as-48A* (5.4 kb)	This work
pAM401	Cm^r, Tc^r, cloning expression bifunctional *E. coli-E. faecalis* vector (10.4 kb)	61
pAM401-76	Cm^r, D and B fragments (*as-48A*, *as-48G*, *as-48H* genes) from pMB2 cloned into pAM401 (18.9 kb)	8
pAM401-76$_{Mut}$	Cm^r, pAM401-76 with the His-1Ile, Met1Ala, or Trp70Ala substitution in *as-48A* (18.9 kb)	This work
pAM401-81	Cm^r, *as-48* cluster cloned in pAM401 (25 kb)	8
pAM401-81$_{Mut}$	Cm^r, pAM401-81 with the His-1Ile, Met1Ala, or Trp70Ala substitution in *as-48A* (25 kb)	This work

TABLE 3. Oligonucleotides used in this work[a]

Name	Sequence 5′-3′
His-1Ile-rv	GAACTCTTTAGCCATGATTGCAATCGGCAAAAACTGAAGACTTGC
His-1Ile-fw	TTGCCGATTGCAATCATGGCTAAAGAGTTCGGTATACCAGCA
Met1Ala-rv	CCGAACTCTTTAGCGGCCATGTGCAATCGGCAAAAACTGAAGACTTGC
Met1Ala-fw	TTGCCGATTGCAGCCGCTAAAGAGTTCGGTATACCAGCACCAG
Trp70Ala-rv	TATTGTTAAATTAGGCAGCAATAACTGCCTCTTTTTC
Trp70Ala-fw	AGAGCCAGTTATTGCTGCCTAATTTAACAATATGATAA
As48-5	CCAAGCAATAACTGCTCTTT
As48-6	GAGTATCATGGTTAAAGAAA
As48-1	AATAAACTACATGGGT
As48-B4	AATCCTATTACTACAATAA
W01	AGAGTTTGATCM*TGGCTC
W012	TACGCATTTCACCK″CTACA

[a] The mutations introduced are underlined. M* = A + C; K″ = G + T.

al. (2). When necessary, complex medium (CM) also was used (48). Purified proteins (up to 95% purity) were obtained by reversed-phase chromatography using high-performance liquid chromatography (RP-HPLC) C_{18} columns, as described elsewhere (48). The concentration of the purified samples was determined by measuring UV absorption at 280 nm, which was converted to protein concentration using molecular extinction coefficients (16), which were calculated from the contributions of individual amino acid residues introduced. Mass determinations were obtained by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a model Voyager-DE PRO spectrometer from Applied Biosystems and compared to those for the AS-48 sequence using MassXpert 1.0 software.

Endoproteinase Glu-C digestion and MS/MS analysis. The samples were incubated at 37°C for 18 h with endoproteinase Glu-C from *Staphylococcus aureus* V8 (sequencing grade; Sigma Chemical, St. Louis, MO) at a final concentration of 20 ng/μl in 50 mM ammonium bicarbonate (99.5% purity). Digested samples were analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) (52), using internal mass calibration under the control of flexControl 2.2 software (Bruker Daltonik). Fragment ions generated by the laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after passing the ion reflector to an average of 1,000 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800 to 3,200 *m/z* region. The analysis of mass data was performed using flexAnalysis 2.2 and BioTools 3.0 software (Bruker Daltonik) (51).

CD. Circular dichroism (CD) spectra of the domain were collected on a Jasco J810 spectropolarimeter (Japan) fitted with a thermostated cell holder and interfaced with a Peltier cell unit. The instrument was periodically calibrated with (+)10-camphorsulfonic acid. The steady-state measurements at pH 7.0 (10 mM phosphate) and 25°C were carried out with the same experimental set as that described by Muro-Pastor et al. (35) and Neira et al. (36). To estimate the helical content, the deconvolution of the far-UV CD spectrum of the different AS-48 variants was performed using the DICHROWEB website (57, 58). The thermal denaturation of the purified samples also was carried out as described by Neira et al. (36).

Antimicrobial assays: determination of antimicrobial activities and MICs. The production of AS-48 and derivatives by *E. faecalis* JH2-2 transformants was determined according to Sánchez-Hidalgo et al. (48). The determinations of the range of antimicrobial activity and the MIC for the purified preparations (defined as the minimal concentration of bacteriocin that inhibited the growth of the indicator strain) were carried out essentially as described previously (48).

RESULTS AND DISCUSSION

As a means of gaining insights into the AS-48 maturation reactions, three single-mutation AS-48 derivatives were constructed in residues directly involved in the signal cleavage of the preprotein (His-1Ile and Met1Ala) and the circularization of the proprotein (Met1Ala and Trp70Ala). Supernatants of the JH2-2(pAM401-81$_{Mut}$) transformant exhibiting the modified sequence were obtained in E-300-G by applying the opti-

mized conditions established by Ananou et al. (2) and analyzed regarding the synthesis of the AS-48 derivatives after purification by cation exchange, C_{18}, and RP-HPLC chromatography.

Analysis of the antibacterial activity and resistance conferred by the new AS-48 derivatives. The activity spectra of *E. faecalis* JH2-2(pAM401-81$_{Mut}$) transformants, with that of JH2-2(pAM401-81) as a control, are compared in Table 4 by spotting assays against different Gram-positive bacteria of known AS-48 sensitivity. We noted that both Trp70 and Met1 replacements by Ala showed lower antagonistic activity than that of the reference. This was particularly pronounced for the Met1Ala derivative, which had reduced activity against the majority of the bacteria tested. In contrast, the JH2-2(pAM401-81$_{His-1Ile}$) transformant exhibited no activity against any of the Gram-positive species tested.

It also was found that the introduction of a neutral residue at both ends of the proprotein and at the end of the SP resulted in the same resistance level as that of the parental producer wild-type strain when they were assayed against 5-μl spots containing 18 μg of purified wild-type AS-48 (data not shown). The absence of inhibition halos after incubation indicated that the intact immunity machinery protected these transformants against AS-48, even in the case of JH2-2(pAM401-81$_{His-1Ile}$), where no production could be detected later.

Single-site-directed mutagenesis of the SP. It has long been acknowledged that some specificity of the cleavage reaction

TABLE 4. Diameters of inhibition halos produced by the JH2-2(pAM401-81$_{Mut}$) transformants against different Gram-positive strains, determined using the spot assay technique[a]

Gram-positive strain	Halo diam (mm)			
	AS-48	His-1Ile	Met1Ala	Trp70Ala
Arthrobacter sp.	31	—	17	21
Bacillus cereus LWL1	16.5	—	5	13.5
Bacillus megaterium CECT 44	25	—	11	19
Enterococcus faecalis JH2-2	21	—	13	18
Enterococcus faecalis S-47	21	—	12	17
Listeria monocytogenes CECT 4032	27	—	14	21.5
Listeria innocua CECT 4030	23	—	12	17
Staphylococcus aureus CECT 976	16	—	—	12

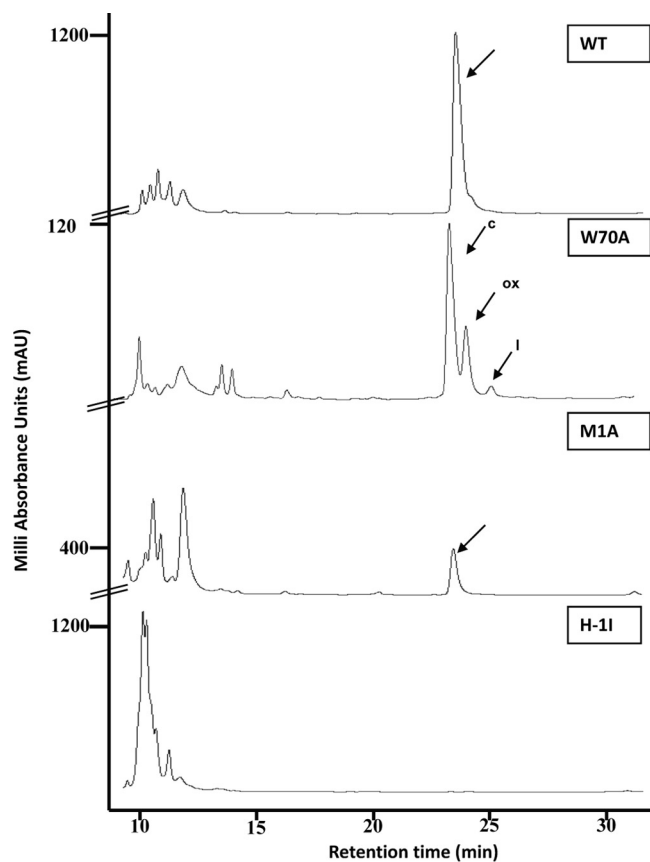[a] The activity of JH2-2(pAM401-81) was determined as a control. —, no activity.

FIG. 1. RP-HPLC spectra found in a C_{18} semipreparative column from E-300-G *E. faecalis* JH2-2 transformants expressing wild-type AS-48 and the three site-directed derivatives. The amounts of protein loaded in the runs were different. The symbols c, ox, and l indicate the circular, oxidized, and linear forms of Trp70Ala, respectively.

resides in the last residue of the signal sequence (43, 54). Moreover, the ability of positively charged amino-terminal residues in the SP to promote protein export seems to be based on their electrostatic interaction with negatively charged phospholipid headgroups (41). Thus, we targeted the expected processing site and mutated the positively charged His-1 to Ile, removing the aromatic side chain to test the function of this unique residue of the AS-48 preprotein, avoiding a charged residue in this position common to some other circular bacteriocins (28). The results, completely unexpected, are depicted in Fig. 1, where no active peaks with characteristic retention times could be isolated during the purification process, either in E-300-G or in complex medium (data not shown). The full loss of the activity of the JH2-2(pAM401-81$_{His-1Ile}$) transformant (Table 4) is consistent with the fact that the His-1Ile substitution hinders a functional production of the AS-48 enterocin as well as of a preprotein truncated product or a lineal proprotein, although the RT-PCR assays confirmed the presence of a specific mRNA transcript (see below). These results also support the absence of potential alternative signal protease cleavage sites adjacent to the normal sites in the preprotein, as has been described formerly for *E. coli* during the maturation of the maltose-binding protein (12). Furthermore, a proper processing requires cleavage sites located in close

proximity to the central hydrophobic and carboxyl-terminal hydrophilic border (reviewed in reference 41). Moreover, those proteins processed by proteases tend to be cleaved between elements of secondary structure rather than within helical, turn, or sheet regions (26). In this sense, the secondary-structure prediction of the native AS-48 preprotein using the PHD algorithm (http://www.predictprotein.org/) suggests that the SP (35 residues) has a propensity to form an α-helical conformation (positions 13 to 31) in wild-type AS-48. However, the His-1Ile mutant was predicted to have a larger transmembrane helical domain (positions 14 to 34), the enlargement of which could affect the cleavage reaction (data not shown). Notably, the occurrence of the His-1Met1 residues is rarely observed as an SP cleavage site; in fact, only capistruin, a 19-residue ribosomally synthesized lasso peptide produced by *Burkholderia thailandensis*, has His-1Gly1 residues, although the replacement of His-1 by Ala did not alter capistruin production (25, 42). It also is remarkable that the M16 family of zinc peptidases has a preference for aromatic residues at P-1 (upstream of the cleavage site) and a broader preference at P1, including hydrophobic/aromatic residues and arginine (7).

Consequences of Trp70 and Met1 residue substitution for AS-48 circularization. Roughly 50% of circular bacteriocins have an aromatic residue (Trp or Tyr) at the carboxyl end of the lineal proprotein, either as the last amino acid (AS-48, lactocyclicin Q, uberolysin, and circularin A) or in a subterminal position (subtilosin A) (28, 50), although none has Met at the P1 position. These data suggest that this type of residue is important during secretion/transport or for acting as a recognition site for the enzymes involved in their maturation. Thus, the effect of both substitutions on AS-48 biosynthesis was evaluated using bioassays for the production of the derivatives against the most-sensitive bacterium, *Bacillus megaterium* CECT44. The two derivatives were quantitatively analyzed according to the total amount of protein obtained from cultures using the usual combination of chromatographic techniques. Indeed, JH2-2 cells harboring pAM401-81$_{Met1Ala}$ or pAM401-81$_{Trp70Ala}$ plasmid harboring the wild-type *as-48BCC$_1$DD$_1$EFGH* genes produced mature and mainly processed AS-48 derivatives, as judged by the appearance of the characteristic peaks after reverse-phase HPLC from supernatant cultures in E-300-G medium (Fig. 1).

Met1 is the N-terminal amino acid of the AS-48 proprotein, and as such it is directly involved in cleavage and propeptide circularization. In this case, a single active fraction with a retention time of 22.4 min (Fig. 1) was purified from supernatant of cells harboring the pAM401-81$_{Met1Ala}$ plasmid. The exact mass of 7,092.23 Da was assigned by MALDI-TOF MS, in agreement with the mass of the circular Met1Ala derivative form. This result also was confirmed by fingerprinting with endoproteinase Glu-C digestion (data not shown). Remarkably, protein yield in culture decreased to values of approximately 2% (0.032 mg · liter^{-1} compared to 1,555 mg · liter^{-1} for wild-type AS-48). The strikingly low expression of this mutant could be caused by an inefficient processing of the preprotein induced by the absence of a Met in such a critical position. It bears noting that no linear forms could be detected under any of the conditions assayed, and in the absence of a Met residue, no oxidized form was predictable. The structure prediction using the Web server http://www.compbio.dundee

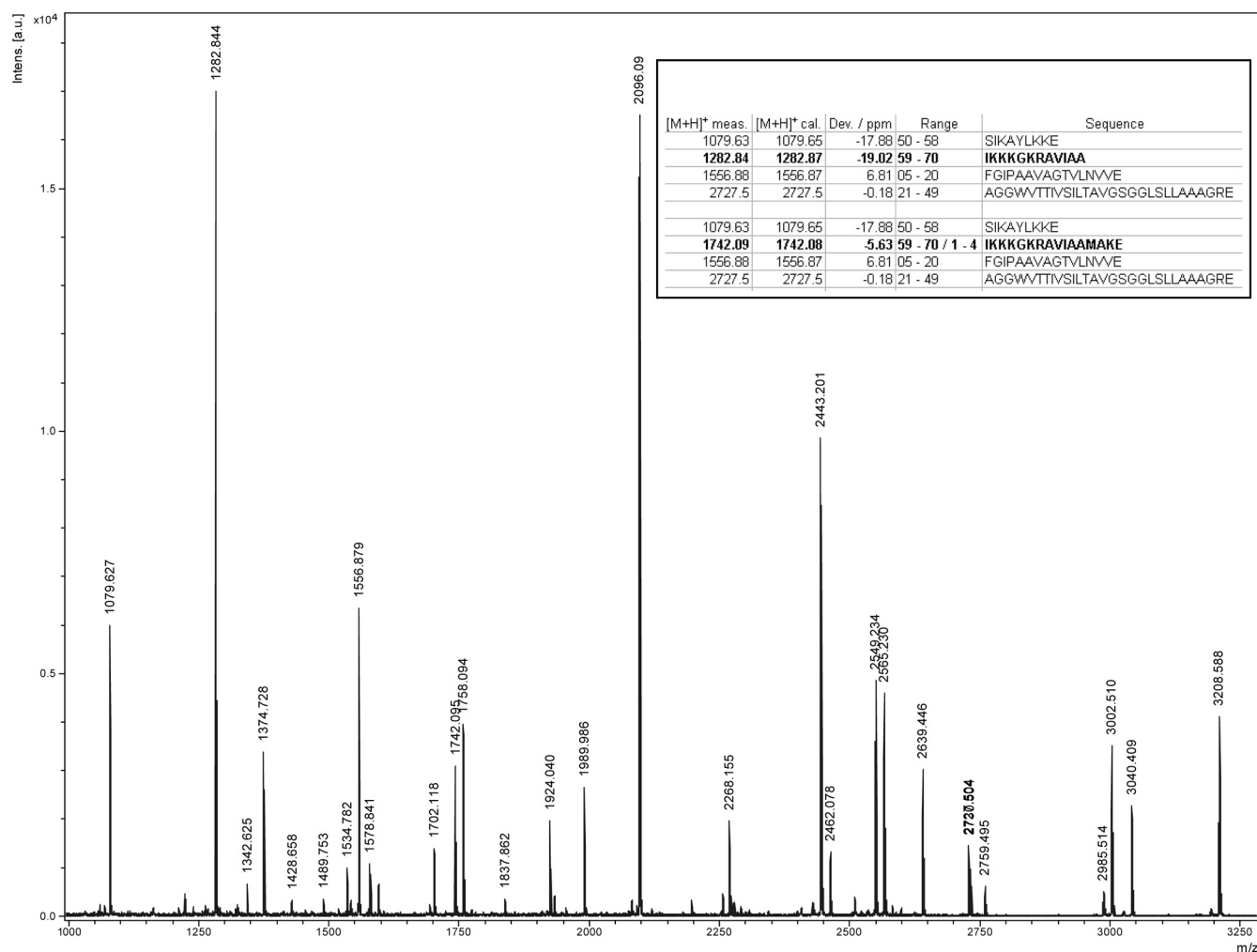| [M+H]+ meas.	[M+H]+ cal.	Dev. / ppm	Range	Sequence
1079.63	1079.65	-17.88	50 - 58	SIKAYLKKE
1282.84	**1282.87**	**-19.02**	**59 - 70**	**IKKKGKRAVIAA**
1556.88	1556.87	6.81	05 - 20	FGIPAAVAGTVLNVVE
2727.5	2727.5	-0.18	21 - 49	AGGWVTTIVSILTAVGSGGLSLLAAAGRE
1079.63	1079.65	-17.88	50 - 58	SIKAYLKKE
1742.09	**1742.08**	**-5.63**	**59 - 70 / 1 - 4**	**IKKKGKRAVIAAMAKE**
1556.88	1556.87	6.81	05 - 20	FGIPAAVAGTVLNVVE
2727.5	2727.5	-0.18	21 - 49	AGGWVTTIVSILTAVGSGGLSLLAAAGRE

FIG. 2. Endoproteinase Glu-C digestion and MS/MS analysis of the linear Trp70Ala derivative. For clarity, each major peak was labeled with the mass and the corresponding single-amino-acid code of the ending residue.

.ac.uk/www-jpred/ (6) shows a minor tendency to fold in the α-helix for this derivative (data no shown). However, it is important to point out that due to the absence of computer programs for circular proteins, we have considered the AS-48 molecule as beginning in the turn connecting the fifth and first helices.

During Trp70Ala mutant purification, three active peaks against *B. megaterium* CECT44, with retention times of 22.6, 23.3, and 24.3 min, were found (Fig. 1). Each fraction was subjected to a second purification step in an analytical RP-HPLC on a C_{18} column. The masses for these active samples were determined by MALDI-TOF (yielding values of 7,035.42, 7,050.93, and 7,053.35 Da) and agreed with the theoretical masses of the circular, oxidized, and linear forms of the Trp70Ala derivative, respectively. The protein yield was in the range of 185 mg liter^{-1} (approximately 12%), which is significantly lower than that of wild-type AS-48. The presence of a naturally oxidized Trp70Ala form (due to the addition of oxygen to Met) is not unusual, because the oxidation of this residue is a common phenomenon in proteins (56). In fact, such oxidized forms currently are arising during the purifica-

tion of wild-type AS-48 in different amounts depending of the conditions of purification used (unpublished results). The subsequent bioassays revealed that the naturally oxidized form had a partial loss of activity (data not shown), as has been described for oxidized nisin (59). However, the similar retention times for native and oxidized forms imply that the hydrophobicity features and the protein structure were practically identical upon oxidation.

The identification of a linear form in the third little peak with a delayed retention time of 24.3 min is noteworthy (Fig. 1). The fragmentation of this peak with endoproteinase Glu-C led to an MS spectrum that shows an ion with an *m/z* value of 1,282.84 (Fig. 2), corresponding to the 12-residue polypeptide patch (IKKKGKRAVIAA) that could be derived only from a lineal Trp70Ala proprotein. Moreover, MS/MS analysis of this fragment unambiguously identifies this peak as linear Trp70Ala. This is the most significant finding of this work, since linear species have never before been described in AS-48 or in other circular proteins. Unfortunately, the specific activity of the linear forms could not be properly established due to the contamination with circular and oxidized species detected in

TABLE 5. MIC values for purified preparations of the circular Trp70Ala and Met1Ala derivatives[a]

Indicator strain	AS-48 MIC (mg/liter) for:		
	WT	Met1Ala	Trp70Ala
Arthrobacter sp.	1.56	6.25	2
B. cereus LWL1	12.5	25	12.5
B. megaterium CECT 44	1.56	3.12	1.56
E. faecalis JH2-2	6.25	12.5	6.25
E. faecalis S-47	6.25	12.5	6.25
L. innocua CECT 4030	2	8	2
L. monocytogenes CECT 4032	1.56	3.12	1.56
S. aureus CECT 976	6.25	12.5	6.25

[a] Samples were serially diluted in sterile 10 mM sodium phosphate buffer at pH 7.0 and assayed against different susceptible strains. The activity of purified wild-type AS-48 (WT) was determined as a control.

the longest polypeptide region (IKKKGKRAVIAAMAKE), with *m/z* values of 1,742.09 and 1,758.09, respectively (Fig. 2).

Another outstanding result was the high activity of mutant Trp70Ala despite the fact that the Trp residues are supposed to be strongly involved in the interaction with the membranes (13). The importance of the Trp70 and Trp24 residues in the activity of AS-48 seems to be influenced by the diverse roles that the different domains have in the approach to and insertion into the membranes of sensitive cells. Thus, it is clear that

the α1-α2 helices, where Trp24 is located, interact with the hydrophobic part of the membrane, leading to its permeabilization (49), whereas the most-amphiphilic helices, α4-α5 in the AS-48 molecule, where Trp70 is situated, are involved in access to the membrane-water interface, being responsible for the union to the host cells (47, 49). Similar results have been found with the genetically modified bacteriocins mesentericin Y105 (34), sakacin P (13), lactococcin G (38), and curvacin A (18). In fact, the determination of the MICs against different indicator strains confirmed that the circular Trp70Ala activity was very similar to that of the wild-type AS-48, whereas the effect of replacing Met with Ala rendered MICs much higher against the different strains assayed (Table 5). Since the mutation is the same (Ala in both cases) and at nearby positions, the effect observed must be due to structural questions, as discussed below.

RT-PCR analysis of the derivative genes. To investigate the reasons behind the negative results found with His-1Ile and to evaluate the different protein levels in the other cases, we performed an RT-PCR experiment. The results using specific primers (Table 3) confirmed the presence and approximate quantity of the transcripts. Notably, the expected 321-nucleotide (nt) amplicons were detected in the transformants after just 15 PCR cycles (Fig. 3B). The controls carried out with rRNAs of the wild type and derivatives were intact, and the
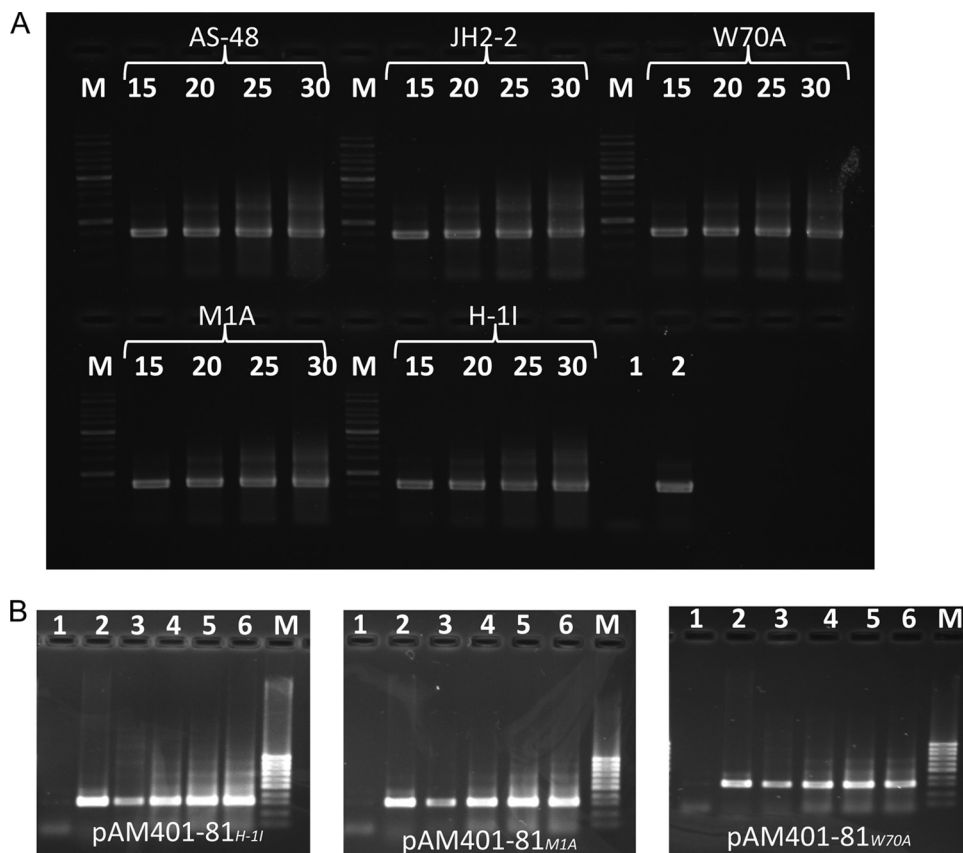

FIG. 3. Semiquantitative RT-PCR analysis of the transcription of the *as-48A* permuted genes. RNA was isolated from *E. faecalis* JH2-2 carrying the recombinant pAM401-81_Mut_ constructions. Samples of 5 μl were taken at 15, 20, 25, and 30 cycles of control rRNA (A) and specific genes (B). Lane 1, negative control; lane 2, positive control; lanes 3, 4, 5, and 6, 15, 20, 25, and 30 cycles. Lanes M indicate the molecular size markers, 1 kb (A) and 100 bp (B).
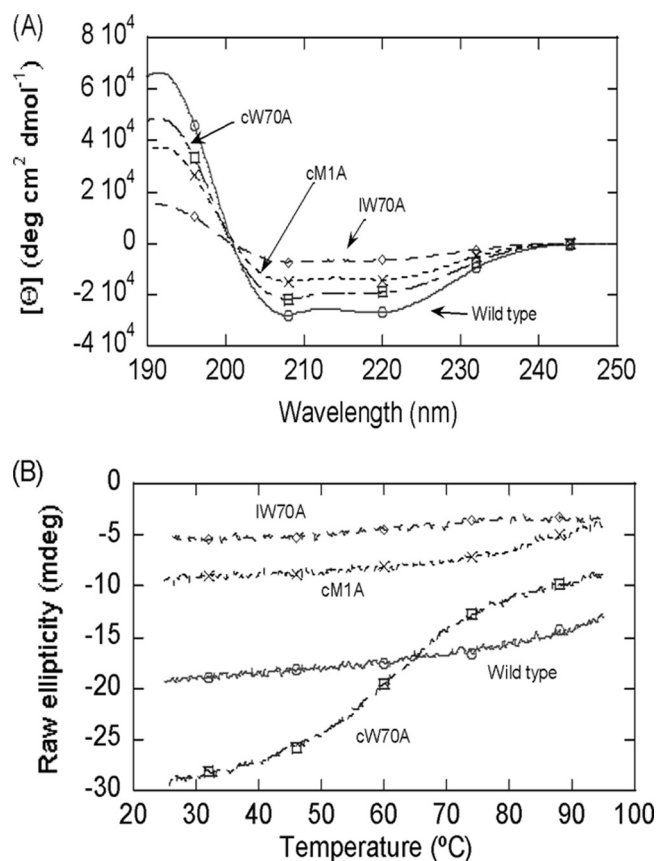
FIG. 4. Far-UV CD spectra of wild-type AS-48 and derivatives. (A) Spectra were acquired in 10 mM phosphate buffer at pH 7.0 and 25°C. (B) Thermal denaturation of wild-type AS-48 and derivatives. The data have been scaled on the *y* axis for ease of reading. The symbols cTrp70Ala and lTrp70Ala are the circularized and linear forms of the Trp70Ala derivative, respectively, and cMet1Ala is the circularized form of the Met1Ala derivative.

amplification occurred in all cases in similar ways (Fig. 3A). Therefore, these results confirmed that no transcriptional block could have been responsible for the lack of protein in the His-1Ile derivative, but they do not explain the differences in the expression levels of proteins found between Met1Ala and Trp70Ala derivatives.

Secondary-structure analysis and stability as measured by CD. Met1Ala and Trp70Ala derivatives were examined by CD

spectroscopy to evaluate the impact of the mutations introduced on the global conformation of these molecules. The far-UV spectra of the wild type and the different forms of the AS-48 derivatives are shown in Fig. 4A. The protein with the largest molar ellipticity values is the wild-type species, and that with the lowest corresponds to the Trp70Ala linear derivative. The ellipticity and the shape of the far-UV CD spectrum of the wild type are identical to those measured previously for the wild type under similar conditions (48). In the same way, the deconvolution of the spectra (Table 6) according to the DICHROWEB website (57, 58) indicates that the highest percentage of helical structure corresponds to wild-type AS-48 (80%). As expected, the linear Trp70Ala species has the lowest percentage of helical structure of all the AS-48 derivatives found so far (48, 49), although this helical percentage is similar to those of the linear derivatives obtained *in vitro* (33). It is worth mentioning that although the two circular Trp70Ala and Met1Ala derivatives have a smaller percentage of helical structure than the wild-type AS-48 molecule, this value was even lower in the Met1Ala derivative (Table 6) despite the absence of the indole moiety in the circular Trp70Ala derivative, which also absorbs at 222 nm (57, 58). Thus, it appears that upon mutation, there is a marked helical fraying toward a less-rigid turn-like structure. This agrees with the fact that the Met1Ala derivative shows weaker membrane-protein interactions, as suggested by the higher MICs shown in Table 5. However, is this lower percentage of helical structure mirrored by a corresponding decrease in stability? To address this question, we conducted thermal denaturizing experiments. As has been described previously, the wild-type protein is extremely stable (5), with no sigmoidal transition between 25 and 95°C (Fig. 4B; Table 6). The circularized Met1Ala species also had high stability without any observable sigmoidal transition, which was similar to the wild type (Fig. 4B), even though its helical content was reduced (Table 6). We suggest that the presence of a higher population of turns (~15% in circular Met1Ala versus 2% in the wild-type protein), which are structurally similar to α-helices, helps to stabilize the protein and thus to increase the melting point (T_m) (Table 6). Notably, the replacement of Trp70 with Ala (situated in the most-amphiphilic helices of the AS-48 molecule) clearly affects the stability of the circular and linear species, with T_ms of 59 and 61°C, respectively, which is in accordance with the decrease in helical content observed in each variant (Fig. 4B; Table 6). This behavior is, however, very different from that shown by Trp24, whose replacement by Ala

TABLE 6. Stability and percentages of secondary structure of wild-type AS-48 and derivatives as deduced from the DICHROWEB[a] site

Structure[c]	% secondary structure[b]											
	WT			Circular Trp70Ala			Linear Trp70Ala			Circular Met1Ala		
	SCM	Contin	k2D	SCM	Contin	k2D	SCM	Contin	k2D	SCM	Contin	k2D
α-Helix	82	69.8	84	62.4	54.7	63	25.2	20.8	27	49.5	44	47
β-Strand	0	3	0	5.1	4.5	6	25.8	29.2	20	9.9	14.1	16
Turn	2	9.6		7.4	16.4		16.0	19.9		15.5	16.8	
Random coil	26	17.6	16	25.1	24.4	31	33	30.1	53	25.1	25.1	37

[a] The following programs used were on the DICHROWEB website: Self-Consistent Method (SCM), Contin, and k2D.
[b] Obtained as described by Neira et al. (36). Percentages of α-helix were also calculated as described elsewhere (36): 72 for WT, 52 for circular Trp70Ala, 18 for linear Trp70Ala, and 39 for circular Met1Ala.
[c] The melting temperatures were as follows: wild type (WT), >95°C; circular Trp70Ala, 59°C; linear Trp70Ala, 61°C; and circular Met1Ala, >95°C.

clearly favored stability, although it was extremely detrimental to the activity of the mutant (49). Thus, the effect of Trp replacements in AS-48 is strongly dependent on their position in the molecule, confirming that these aromatic residues can interact with different environments in the target cell membrane.

Conclusions. In the present study, we have investigated the functionality of three residues involved in the maturation of the AS-48 preprotein by expressing the respective single derivatives. Our results convincingly show that the residue His-1 should not be mutated to Ile, because this change affects the efficiency of a still-unidentified enzyme involved in the cleavage of the SP. In fact, only the His-1Ile mutation categorically restrains the cleavage of the precursor. Moreover, the protein, if synthesized, retains its leading peptide and must be rapidly degraded. Thus, we hypothesize that the proteolytic cleavage might be specific for His-1, and the imidazole ring may facilitate the protein cleavage (63). However, we cannot rule out the requirement of Met1 as a substrate, since its replacement decreases the efficiency for Met1Ala production (approximately 2%).

Another preliminary conclusion from the data presented here is the requirement of a second biosynthetic enzyme or a separate catalytic domain that is responsible for the circularization reaction, the partial specificity of which seems to reside in the last Trp70 residue of the proprotein, since linear forms could be derived only from JH2-2(pAM401-81$_{Trp70Ala}$) transformants. Unmodified pre-AS-48 contains 105 amino acid residues that were targeted to modification machinery, most likely located at the cytoplasmic membrane. Posttranslational AS-48 processing by an enzyme/domain that probably requires the proximity of an NH$_2$ group at the N-terminal end of the proprotein occurs presumably on the extracellular side of the cytoplasmic membrane because of the existence of linear Trp70Ala proteins in the supernatants. This amino group could act as a nucleophile in the circularization reaction with the specific, although not at all essential, C-terminal Trp70 residue, since circular forms also are being produced.

The C-terminal SP sequence appears to be crucial for the machinery involved in processing and transport. The candidate might be the essential ABC transporter As-48C$_1$D, either alone or as a multicomponent system (reviewed in reference 28), able to carry out sequentially the maturation and secretion of AS-48 (involving the removal of the leader peptide and tail-head circularization). In this sense, it has been shown that some ABC exporters are able to remove leader peptides of the double-glycine type concomitantly with export (19). Further research is needed to confirm the biosynthetic maturation pathway and to identify the enzymes involved in this process.

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