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

Rubén Cebrián, Mercedes Maqueda, José Luis Neira, Eva Valdivia, Manuel Martínez-Bueno, and Manuel Montalbán-López

Departamento de Microbiología. Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain
Istituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Elche (Alicante), Spain
and Instituto de Biocomputación y Física de los Sistemas Complejos, 50009 Zaragoza, Spain

Received 13 May 2010/Accepted 28 August 2010

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) 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. 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, subtilosin A, carno- nocyclin A, lactocyclicin Q, circularin A, and uberolysin and one including butyrivibiocein AR10 and the homologous gasserin A group (gasserin A and reuterinica 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 prepeptide that undergoes posttranslational circularization after proteolytic cleavage to release the mature bioactive molecules (28, 50). Signal sequences containing information 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 to the extended signal for AS-48 (35 residues) or acidocin B/gasserin A/reuterinica 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, 2010, American Society for Microbiology. All Rights Reserved.
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 1D, 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 Enterobacter sp. was grown with shaking in Luria-Bertani (LB) medium at 37°C. The E. coli strains, used as intermediate hosts for cloning, were grown with aeration at 37°C. The producer phenotype, which is devoted to the secretion of newly produced enterocin, 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 I 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 as48a-6 and as48-5 (Table 3) for the specific amplification of the as-48A gene (9.2 kb) and as48a-5 (Table 3) for the specific amplification of the as-48A gene (485 bp) 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’s instructions.

TABLE 1. Relevant features of the plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAM401</td>
<td>Cloning and expression vector</td>
<td>This work</td>
</tr>
<tr>
<td>pAM401-76</td>
<td>Cloning vector</td>
<td>This work</td>
</tr>
<tr>
<td>pAM401-81</td>
<td>Cloning vector</td>
<td>This work</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAM401</td>
<td>Cloning and expression vector</td>
<td>This work</td>
</tr>
<tr>
<td>pAM401-76</td>
<td>Cloning vector</td>
<td>This work</td>
</tr>
<tr>
<td>pAM401-81</td>
<td>Cloning vector</td>
<td>This work</td>
</tr>
</tbody>
</table>

Downloaded from http://aem.asm.org/ on October 15, 2017 by guest
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) C18 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.

Endopeptidase Glu-C digestion and MS/MS analysis. The samples were incubated at 37°C for 18 h with endopeptidase 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). Dige-
staged 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 (Bruker Daltonik) (52), using internal mass calibration under the control of flexControl 2.2 software (Bruker Daltonik). The samples were denatured in residues directly involved in the signal cleavage of the preprotein and at the end of the SP resulted in the same resistance level as that of the parental producer strain when they were assayed against 5-μl spots containing 18 μg of purified wild-type AS-48 (data not shown).

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 strain when they were assayed against 5-μl spots containing 18 μg of purified wild-type AS-48 (data not shown).

The thermal denaturation of the purified samples also was carried out as described by Neira et al. (36). The determi-
tnations of the range of antimicrobial activity and the MIC for the purified preparations (de-
ferred by the new AS-48 derivatives.

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 (de-
Man 3-site-directed mutagenesis of the SP. It has long been acknowledged that some specificity of the cleavage reaction

RESULTS AND DISCUSSION

As a means of gaining insights into the AS-48 maturation reactions, three single-mutation AS-48 derivatives were con-
TABLE 3. Oligonucleotides used in this work*  

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-1Ile-rv</td>
<td>GAACTTCTTATGCCATGTAGGCCAATCGGCAAATAACTGGAAGAATTGTC</td>
</tr>
<tr>
<td>His-1Ile-fw</td>
<td>TTGCGGATTTGCAATCTGGCTAAAGAGTTGCGGATTAACGGC</td>
</tr>
<tr>
<td>Met1Ala-rv</td>
<td>CGAACTTCTTATGCCATGTAGGCCAATCGGCAAATAACTGGAAGAATTGTC</td>
</tr>
<tr>
<td>Met1Ala-fw</td>
<td>TTGCGGATTTGCAATCTGGCTAAAGAGTTGCGGATTAACGGC</td>
</tr>
<tr>
<td>Trp70Ala-rv</td>
<td>TATGTTAAAATATTGCCAGCAATAACTTGCTCTTTTTCA</td>
</tr>
<tr>
<td>Trp70Ala-fw</td>
<td>AGAGCGATTTATGGCTCTAATTAAACAAATATGGATA</td>
</tr>
<tr>
<td>As48-5</td>
<td>CCAAGCAATAACTGCTCTTT</td>
</tr>
<tr>
<td>As48-6</td>
<td>GAGATCATGTTTTAAGAAA</td>
</tr>
<tr>
<td>As48-1</td>
<td>AATAAACTACATGGGT</td>
</tr>
<tr>
<td>As48-B4</td>
<td>ATACCTATTACTACATAA</td>
</tr>
<tr>
<td>W01</td>
<td>AGAGTTTGATCMTGCTGCT</td>
</tr>
<tr>
<td>W012</td>
<td>TACGCAATTTCACCKCTACA</td>
</tr>
</tbody>
</table>

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

tified 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, C18, 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) 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) 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), where no production could be detected later.

TABLE 4. Diameters of inhibition halos produced by the JH2-2 (pAM401-81) transformants against different Gram-positive strains, determined using the spot assay technique

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

* The activity of JH2-2 (pAM401-81) was determined as a control. —, no activity.
loss of the activity of the JH2-2(pAM401-81 in E-300-G or in complex medium (data not shown). The full times could be isolated during the purification process, either in Fig. 1, where no active peaks with characteristic retention riocins (28). The results, completely unexpected, are depicted residue in this position common to some other circular bacte-
unique residue of the AS-48 preprotein, avoiding a charged
processing site and mutated the positively charged His-1 to Ile,
pholipid headgroups (41). Thus, we targeted the expected pro-
resides in the last residue of the signal sequence (43, 54).
proximity to the central hydrophobic and carboxyl-terminal
hydrophilic border (reviewed in reference 41). Moreover,
those proteins processed by proteases tend to be cleaved be-
tween elements of secondary structure rather than within he-
tical, 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 a α-helical
conformation (positions 13 to 31) in wild-type AS-48. How-
ever, the His-1Ile mutant was predicted to have a larger trans-
membrane helical domain (positions 14 to 34), the enlarge-
ment 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 produ-
tion (25, 42). It also is remarkable that the M16 family of
zymepidases 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 subtermi-
nal 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 recogni-
tion site for the enzymes involved in their maturation. Thus,
the effect of both substitutions on AS-48 biosynthesis was eval-
uated 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 pAM401-81ڑM1Ala
or pAM401-81ڑP70Ala plasmid harboring the wild-
type as-48BCC, DD, EFGH genes produced mature and
mainly processed AS-48 derivatives, as judged by the appearance
of the characteristic peaks after reverse-phase HPLC from super-
natant 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
circulation. In this case, a single active fraction with a retentio-
time of 22.4 min (Fig. 1) was purified from superna-
tant cultures using the usual combination of chromatographic
techniques (upstream of the cleavage site) and a broader preference at P1,
including hydrophobic/aromatic residues and arginine (7).

FIG. 1. RP-HPLC spectra found in a C18 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.

WT
W70A
M1A
H-1I

Mill Absorbance Units (mAU)

Retention time (min)

10
15
20
25
30
1200
1200
1200
1200

on October 15, 2017 by guest http://aem.asm.org/ Downloaded from
ac.uk/www-jpred/ (6) shows a minor tendency to fold in the α-helix for this derivative (data not 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 C18 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 purification 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 linear 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...
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 \( \alpha_1-\alpha_2 \) helices, where Trp24 is located, interact with the hydrophobic part of the membrane, leading to its permeabilization (49), whereas the most-amphiphilic helices, \( \alpha_4-\alpha_5 \) 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-Ile 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

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>WT MIC (mg/liter)</th>
<th>Met1Ala MIC (mg/liter)</th>
<th>Trp70Ala MIC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>1.56</td>
<td>6.25</td>
<td>2</td>
</tr>
<tr>
<td><em>B. cereus</em> LWL1</td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td><em>B. megaterium</em> CECT 44</td>
<td>1.56</td>
<td>3.12</td>
<td>1.56</td>
</tr>
<tr>
<td><em>E. faecalis</em> JH2-2</td>
<td>6.25</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td><em>E. faecalis</em> S-47</td>
<td>6.25</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td><em>L. innocua</em> CECT 4030</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> CECT 4032</td>
<td>1.56</td>
<td>3.12</td>
<td>1.56</td>
</tr>
<tr>
<td><em>S. aureus</em> CECT 976</td>
<td>6.25</td>
<td>12.5</td>
<td>6.25</td>
</tr>
</tbody>
</table>

\( ^{\text{a}} \) 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.

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 \( \mu l \) 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).
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-1Ie 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 denaturation 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 (Tm) (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 Tm 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 site

<table>
<thead>
<tr>
<th>Structure</th>
<th>WT</th>
<th>Circular Trp70Ala</th>
<th>Linear Trp70Ala</th>
<th>Circular Met1Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Helix</td>
<td>62.4</td>
<td>54.7</td>
<td>63</td>
<td>25.2</td>
</tr>
<tr>
<td>β-Strand</td>
<td>5.1</td>
<td>4.5</td>
<td>6</td>
<td>25.8</td>
</tr>
<tr>
<td>Turn</td>
<td>27.4</td>
<td>16.4</td>
<td>31</td>
<td>16.0</td>
</tr>
<tr>
<td>Random coil</td>
<td>25.1</td>
<td>24.4</td>
<td>31</td>
<td>25.1</td>
</tr>
</tbody>
</table>

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 restraints 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 here in 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 here in the requirement of Met1 as a substrate, since its replacement decreases the efficiency for Met1Ala production (approximately 2%).

ACKNOWLEDGMENTS 

This work was supported by the Spanish Dirección General de Investigación Científica y Técnica (projects BIO2008-01708, CSD2008-00005, and SAF2008-05742-C02-01) and Grupo de Investigación de la Junta de Andalucía (CIV 016), as well as ACOMP/2010/114 by Generalitat Valenciana and the FIPSE foundation (number 36557/06). Rubén Cebrián is the beneficiary of a grant from the Spanish Ministry of Education.

REFERENCES


