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Effect of replacing glutamic residues upon the biological activity and stability of the circular enterocin AS-48

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***Background*: Bacteriocins are antimicrobial peptides produced by bacteria and have a relatively narrow range of activity against closely related strains. AS-48 is a circular bacteriocin produced by *Enterococcus faecalis* that acts against many Gram-positive and some Gram-negative bacteria, and could well serve as a natural food preservative and antimicrobial agent. The structure of AS-48 is a five-helix bundle in which a hypothetical plane containing the C^{α} atoms of E4, E20, E49 and E58 segregates a patch of positively charged residues from the rest of the hydrophobic or uncharged surface residues.**

***Objectives*: The aim of this study is to investigate the significance of the four glutamic residues with regard to the potency, stability and functionality of enterocin AS-48.**

***Methods*: Four genetically engineered variants of AS-48 were obtained by replacing each glutamic residue with alanine by site-directed mutagenesis. Each mutant peptide was purified from *E. faecalis* cultures. The activity of highly concentrated samples and the MIC were determined against nine bacterial strains by the spot-assay method. Structural studies were made with circular dichroism (CD) spectroscopy.**

***Results*: Occasional alterations to the net charge of AS-48 did not significantly affect its activity when high concentrations of bacteriocin were used. Nevertheless, according to the MIC values, three of the four mutated peptides showed weaker activity against the majority of the Gram-positive bacteria tested. CD spectroscopy showed that the derivatives were well structured, in a similar way to those of the native molecule, with no modifications in their helix content.**

***Conclusions*: The spatial location of the Glu residues rather than their negative charge played a critical role in AS-48 target-cell specificity and bactericidal activity, because the replacement of Glu with Ala modify the interactions between neighbouring residues through their side chains and the interaction to the solvent affecting the protein stability and causing variations in the activity levels against identical organisms.**

Keywords: bacteriocins, biological activity, circular antimicrobial peptides, site-directed mutagenesis, lactic acid bacteria, *Enterococcus faecalis*

Introduction

Bacterial antibiotic resistance has become a worldwide public-health problem, which makes necessary the development of new types of antibiotics. In this context, antimicrobial peptides (AMPs) are high on the list in clinical research.[1,2] AMPs produced by bacteria, known as bacteriocins, are a heterogeneous group of ribosomally synthesized, proteinaceous substances, which are extracellularly secreted and to which the producer strain has a specific immunity mechanism. Their mode of activity is primarily bactericidal and directed against closely related strains and species.[3–5] The bacteriocins that will probably have the most immediate potential in food applications will be those produced by food-grade lactic acid bacteria (LAB),[6,7] but attempts are also being made to find applications in veterinary science and medicine.[8]

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AS-48 is a 70 residue, α-helical, cationic bacteriocin produced by *Enterococcus faecalis* S-48,[9,10] and is unique in its circular structure and its broad antimicrobial spectrum against many Gram-positive (including many LAB as well as potentially pathogenic strains of *Listeria, Bacillus, Clostridium* and *Staphylococcus*) and also some Gram-negative bacteria.[11] These characteristics, together with its stability and solubility over a wide pH range, suggest that AS-48 could well be a good candidate for natural food preservative[12–15] and also for veterinary applications.[16] AS-48 acts on the bacterial cell membrane by forming channels or pores,[17] nevertheless the mechanism of its molecular function remains obscure. The molecule consists of a fairly compact globular arrangement of five α-helices[18] and has a remarkable amphipathic surface due to a hypothetical plane containing the C^{α} atoms of E4, E20, E49 and E58, which segregates a patch of positively charged residues from the rest of the hydrophobic or uncharged surface residues.[19] This allows the crystal to contain chains of pairs of molecules linked either by hydrophobic interactions [dimeric form I (DF-I)] or by hydrophilic interactions [dimeric form II (DF-II)] and it has been proposed that a rearrangement from the water-soluble DF-I to the membrane bound DF-II takes place at the membrane surface, followed by membrane insertion and molecular electroporation.[19] To reach a more thorough understanding of the mode of action of this bacteriocin, we have used site-directed *in vitro* mutagenesis to investigate the possibility that each Glu residue present in the AS-48 molecule may influence target-cell specificity.

Materials and methods

Strains, vectors and growth conditions

The bacterial strains and plasmids used are listed in Tables 1 and 2, respectively. The plasmid-free *E. faecalis* JH2-2 strain was used both in cloning experiments and the heterologous production of AS-48 and its variants as well as the indicator strain. Enterococci were grown overnight without aeration in brain heart infusion (BHI) (Scharlau, Barcelona, Spain) at 37°C. *Escherichia coli* DH5α and *E. coli* XL1-Blue were grown in Luria–Bertani medium (Scharlau) at 37°C for use as intermediate hosts for cloning. Agar plates contained 1.5% (w/v) agar, and soft agar was made with 0.75% (w/v) agar. Buffered media were prepared in 0.1 M sodium phosphate buffer, pH 7.2. The antibiotics ampicillin, 50 mg/L, and chloramphenicol, 20 mg/L, both from Sigma-Aldrich (Madrid, Spain) were added as selective agents when appropriate.

Molecular techniques

DNA cloning and *E. coli* transformations were performed according to the standard protocols.[20] Plasmid DNA was extracted as described by Anderson and McKay.[21] *E. faecalis* was electroporated by the method of Fiedler and Wirth.[22] Digested DNA fragments were eluted from agarose gels with the Perfectprep Gel Cleanup Kit (Eppendorf AG, Hamburg, Germany). Restriction enzymes, T4, DNA ligase, calf-intestine phosphatase and other DNA-modifying enzymes came from Fermentas (Barcelona, Spain) and Roche Diagnostics (San Cugat del Vallés, Spain) and were used as recommended by the suppliers. Nucleotide sequences were obtained with an ABI PRISM dye-terminator cycle-sequencing ready-reaction automated sequencer (Perkin Elmer, USA), using 0.6–1 μg of purified DNA and 3.2 pmol of primer at a total volume of 6 μL. Synthetic oligonucleotides were provided by Amersham Biosciences Europe (GmBH, Cerdanyola, Barcelona, Spain) and Genotek (Sabadell, Spain); see Table 3.

Mutagenesis procedure and pAM401-81$_{X\text{-}Mut}$ plasmid construction

We developed a system for site-directed mutagenesis in AS-48. Base substitutions were carried out in the structural gene *as-48A* by inverse-PCR[23] using the *Pwo* polymerase (Roche Diagnostics). To this end, we used pGEM-T$_{as48AX}$ as a template[24] and a pair of primers for each mutation that annealed at their 5′ ends, thus amplifying the complete plasmid (pGEMT$_{as48AX\text{-}Mut}$) (Table 3). Mutagenesis reaction mixtures containing 50 ng of plasmid were used at a total volume of 50 μL, containing 1 U of *Pwo* polymerase, 0.05 mM of each dNTP (Eppendorf) and 10 pmol of each primer. PCR was performed in 15 cycles, each cycle consisting of a denaturing step at 96°C for 50 s, a primer-annealing step at 64°C for 45 s

Table 1. Microorganisms used in this work

Microorganisms	Characteristics	Reference
B. cereus LWL1	AS-48^{s}	53
B. megaterium	AS-48^{s}	CECT 44
E. faecalis JH2-2	plasmid-free, Rif^{r}, Fus^{r}, AS-48^{s}	54
E. faecalis S-47	AS-48^{s}	11
L. innocua	AS-48^{s}	CECT 4030
L. monocytogenes	AS-48^{s}	CECT 4032
S. aureus subsp. *aureus*	AS-48^{s}	CECT 240
E. coli DH5α	*ϕ*80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17* (rk^{-}, mk^{+}), *phoA*, *supE44*, λ^{-}, *thi*-1, *gyrA96*, *relA*	Bethesda Research Lab.
E. coli U-9	AS-48^{s}	11
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tc^{r})]	Stratagene
S. choleraesuis LT2	AS-48^{r}	CECT 722

AS-48^{s}, susceptible to AS-48; AS-48^{r}, resistant to AS-48; Rif^{r}, resistant to rifampicin; Fus^{r}, resistant to fusidic acid; Tc^{r}, resistant to tetracycline; CECT, Spanish Type Culture Collection.

Table 2. Different constructions and plasmids used in this work

Plasmids	Characteristics	Reference
pGEM-$T_{as48A\text{-}X}$	Ap^r, *lacZ*, pGEM-T with *Xho*I site inserted in the 3′ end of the *as-48A* gene (5.1 kb)	this work
pAM401	Cm^r, Tc^r, bifunctional vector *E. coli–E. faecalis* (10.4 kb)	55
pAM401-81	Cm^r, *as-48* cluster cloned into pAM401 (25 kb)	33
pAM401-81_X	Cm^r, pAM401-81 with an *Xho*I site inserted into the 3′ end of the *as-48A* gene (25 kb)	24
pAM401-$81_{X\text{-}E4A}$	Cm^r, pAM401-81_X with the E4A substitution into *as-48A* (25 kb)	this work
pAM401-$81_{X\text{-}E20A}$	Cm^r, pAM401-81_X with the E20A substitution into *as-48A* (25 kb)	this work
pAM401-$81_{X\text{-}E49A}$	Cm^r, pAM401-81_X with the E49A substitution into *as-48A* (25 kb)	this work
pAM401-$81_{X\text{-}E58A}$	Cm^r, pAM401-81_X with the E58A substitution into *as-48A* (25 kb)	this work

Ap^r, resistant to ampicillin; Cm^r, resistant to chloramphenicol; Tc^r, resistant to tetracycline.

and an extension step at 72°C for 10 min. The PCR mixture was *Dpn*I-digested (Fermentas, Quimigen, Madrid, Spain) to eliminate parental DNA. Subsequently, a 1.8 kb *Sph*I/*Xho*I fragment containing the mutated structural gene was obtained from pGEM-$T_{as48AX\text{-}Mut}$ and cloned into pAM401-81_X, which had previously been *Sph*I/*Xho*I digested.[24] These plasmids (pAM401-$81_{X\text{-}Mut}$) were transferred to *E. faecalis* JH2-2 by electroporation.

Purification, chemical cross-linking, SDS–PAGE and immunoblotting of AS-48-engineered variants

Wild-type and modified AS-48 were purified to homogeneity from complex medium[25] cultures of *E. faecalis* JH2-2(pAM401-81_X) and JH2-2(pAM401-81_{XMut}) transformants grown at 37°C for 7 h. The cultures were applied directly onto a CM25 cation exchanger (Amersham Biosciences Europe). To avoid dialysis during the second purification step, eluted fractions from CM25 were applied to a hydrophobic C18 column and finally purified to homogeneity by reversed-phase, high-performance liquid chromatography (RP-HPLC) as described elsewhere.[26] The purity of the bacteriocins was verified to be >95% by analytical reversed-phase chromatography using an HPLC-C18 column (Vydac, The Separation Group, Hesperia, CA, USA) in a chromatography system (Agilent Technologies GmbH, Germany). The antimicrobial activities of mutant AS-48 species were assayed at each step during purification. The concentrations of purified bacteriocins were determined spectrophotometrically at 280 nm using the molecular extinction coefficients of 1.771 $(mg/mL)^{-1}\,cm^{-1}$ for AS-48 wild-type and 1.785 $(mg/mL)^{-1}\,cm^{-1}$ for AS-48 derivatives, calculated according to Gill and von Hippel.[27]

Native and AS-48 derivatives were separated by SDS–PAGE electrophoresis on 15% slab gels as described by Laemmli[28] and transferred to a nitrocellulose (NC) membrane (pore size 0.45 mm; Schleicher & Schuell) according to Towbin *et al.*[29] using a Miniprotean dual-slab cell apparatus (Bio-Rad Laboratories, Madrid, Spain). Immunoblotting was carried out using a Bio-Rad mini trans-blot system. Membranes were developed with specific anti-AS-48 antibodies as described in a previous publication.[30] Bacteriocin solutions (357.5 mg/L) in 10 mM sodium phosphate buffer at pH values of 3.0, 7.0 and 9.0 were cross-linked by incubation with 1% formaldehyde (from paraformaldehyde powder, Taap, Aldermaston, UK) for 1 h at room temperature before being separated by SDS–PAGE.

Bacteriocin assays: determination of antimicrobial activities and MICs

Production of AS-48 and derivatives by *E. faecalis* JH2-2 transformants was determined by spotting 1 μL of liquid overnight cultures onto BHI agar (BHA) plates followed by incubation at 37°C for 16 h. The plates were then overlaid with 6 mL of buffered 0.75% melted BHA soft agar inoculated with the JH2-2 indicator strain (2%) and incubated at 37°C for 12–18 h before the results were read. The range of antibacterial activity of highly purified AS-48 and derivative preparations (1200 mg/L) was determined by the spot-assay method at pH values of 3.0, 7.0 and 9.0. To this end, spots of 5 μL were deposited onto plates of buffered Mueller–Hinton agar (Scharlab) previously overlaid with 6 mL of buffered BHA soft agar inoculated with different Gram-positive and Gram-negative indicator strains (Table 1). Peptide activity was

Table 3. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′–3′)	T_m (°C)
E4A-AS48_fw	GCACATATGGCTAAAG**CT**TTCGGTATACCAGCAGCAGTTGCAGGAACTGTG	80
E4A-AS48_rv	CTGGTATACCGAAA**AG**CTTTAGCCATATGTGCAATCGGCAAAAACTG	76
E20A-AS48_fw	TAATGTAGTTG**CT**GCTGGTGGATGGGTCACTACTATTGTATCAATTCTTACTG	74
E20A-AS48_rv	CATCCACCAGC**AG**CAACTACATTAAGCACAGTTCCTGCAACTGCTG	79
E49A-AS48_fw	CGCTGCAGCAGGAAGAG**CT**TCAATTAAAGCATACCTTAAGAAAGAAATTAAG	75
E49A-AS48_rv	TATGCTTTAATTGA**AG**CTCTTCCTGCTGCAGCGAGTAAAGAAAGACCTCCG	78
E58A-AS48_fw	CATACCTTAAGAAAG**CT**ATTAAGAAAAAAGGAAAAAGAGCAGTTATTGCTTGG	71
E58A-AS48_rv	CTTAAT**AG**CTTTCTTAAGGTATGCTTTAATTGACTCTCTTCCTG	66

The mismatched oligonucleotides with each of the desired mutations are shown in bold.

assessed by measuring the diameter of the inhibition zone after incubation for 18 h at 37°C. To determine the MICs of these preparations, the samples (from 0.72 to 1200 mg/L) were diluted serially in sterile 10 mM sodium phosphate buffer at pH values of 3.0, 7.0 and 9.0 and tested in the same way against the indicator strains.

Haemolytic activity

Haemolytic activity was tested by streaking derivative producer strains (grown for 18 h at 37°C) on Columbia blood agar plates (Oxoid) containing 5% defibrinated horse blood. Blood agar plates were incubated at 37°C for 48 h, after which they were examined for haemolysis.

Effects of heat and pH treatments

Bacteriocin solutions (71.5 mg/L) in 10 mM sodium phosphate buffer were kept at temperatures of 60°C for 30 min, 70°C for 10 min, 80°C for 5 min and 100°C for 5 min at pH values of 3.0, 7.0 and 9.0. After these heat treatments, the samples were rapidly cooled in ice and tested for remaining antimicrobial activity by the spot-assay method against *E. faecalis* JH2-2 strain. Positive controls were kept at room temperature for similar periods of time at the same three pH values. Buffered solutions without bacteriocin were used as negative controls.

In silico analysis

To determine the stability change on AS-48 wild-type due to the glutamic mutations introduced, we used the FOLD-X program,[31,32] which calculates the change in the stability of mutated proteins by computing the changes in the free energy of folding upon mutations. Mutations to Ala were performed without moving any neighbour residues and the analysis were performed on the crystal structures in aqueous solution described by Sánchez-Barrena *et al.*[19] at pH 4.5 (PDB ID 1O82) and pH 7.5 (PDB ID 1O83) and also in the presence of detergent (PDB ID 1O84).

Circular dichroism (CD) measurements

CD studies were made with a JASCO J-810 spectropolarimeter equipped with a PTC-348WI temperature-control unit. CD spectra were recorded in the far-UV region (190 and 260 nm) at 25°C. All measurements were corrected for buffer contributions. Each spectrum represents the average of 10 scans and was recorded at two different bacteriocin concentrations 10 μM (71.5 mg/L) and 40 μM (286 mg/L) to ensure non-dependence on concentration.

Results

Design of the AS-48 glutamic mutants

Four substitutions (E4A, E20A, E49A and E58A) were separately inserted into the *as-48A* structural gene (Figure 1) by site-directed mutagenesis using inverse PCR extension in the *Sph*I–*Xho*I cassette previously created within the pGEMT$_{as48AX}$ plasmid.[24] To express the four AS-48-mutated peptides, we constructed a system that relies on the replacement of the wild-type *as-48A* structural gene by the engineered gene [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The four *as-48A* variants obtained were separately inserted into the pAM401-81$_X$ plasmid by replacement of the equivalent *Sph*I–*Xho*I wild fragment (1.9 kb), thus leading to the different pAM401-81$_{X\text{-}Mut}$ plasmids, which were transferred by electroporation to the JH2-2 enterococcal strain.

Antibacterial spectra, haemolysis and resistance conferred by the AS-48 derivative producer strains

All *E. faecalis* JH2-2(pAM401-81$_{X\text{-}Mut}$) transformants showed lower antagonistic activity than that of the reference, JH2-2(pAM401-81$_X$), particularly the E4A mutant, which lacked activity against all the Gram-positive strains tested, except *Bacillus megaterium* (Figure 2). These results demonstrate that the four mutated peptides were produced by each of the JH2-2(pAM401-81$_{X\text{-}Mut}$) clones, although, as expected, none of them showed antibacterial activity against the two Gram-negative bacteria tested. We also found that the introduction of an uncharged residue in the four AS-48 mutants resulted without exception in the same resistance levels as those of the parental producer wild-type strain when assayed against 5 μL spots containing 18 μg of purified AS-48 derivatives (data not shown). The absence of inhibition halos after incubation indicated that the immunity proteins protected these mutants against their own modified peptide.

As in the case of wild-type AS-48, the derivatives exhibited no haemolytic activity, which suggests that glutamic substitutions do not convey haemolytic activity to this AMP.

Purification and characterization of mutants

To analyse the production capacity and antigenic properties of each AS-48 derivative, the four mutated molecules were purified to homogeneity. Samples of AS-48 mutants were purified in similar quantities to those of the AS-48 wild-type but with shorter HPLC retention times for E4A, E20A and E58A, (19.60, 19.64 and 19.11 min, respectively, compared with 21.47 min for the wild-type molecule) and longer for the mutant E49A (22.48 min). In SDS–PAGE, we observed single bands with identical molecular masses of ~7200 Da (Figure 3a), which were recognized by anti-AS-48 antibodies after being transferred to a NC membrane (Figure 3b), meaning that they all retained the antigenic characteristics of the wild-type AS-48.

To study the influence of pH upon peptide oligomerization, we cross-linked highly purified derivatives (357.5 mg/L) dissolved in 10 mM sodium phosphate buffer at pH values of 3.0, 7.0 and 9.0 with formaldehyde and separated them by SDS–PAGE. At pH values of 7.0 and 9.0, the four samples produced similar degrees of association as the wild-type, a mixture of monomers and oligomers of 2, 3, 4 and 5 molecules, with M_r ranging from 7 to 35 kDa (data not shown). Similarly, at pH 3.0, the behaviour of the four derivatives was comparable to that of the non-fixed solutions, showing a single monomeric band, compatible with their high positive charge under acidic conditions.

Stability against heat and pH

To determine whether the activity of the mutants was affected by heat or pH, highly purified samples of each peptide were buffered and heated as described in the Materials and methods section. The residual activity of the derivatives was examined at the end of the incubation period against *E. faecalis* strain JH2-2,

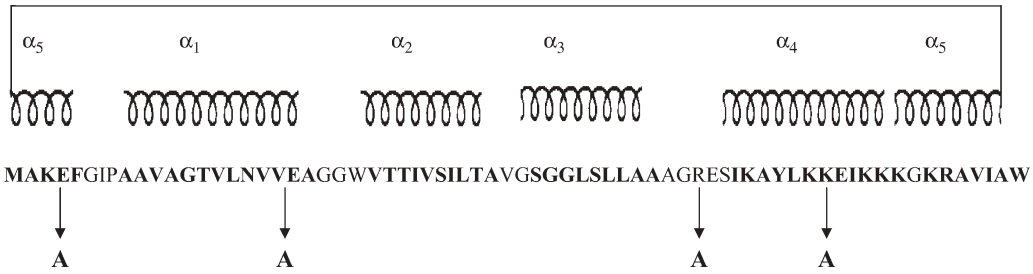

Figure 1. Substitutions introduced into AS-48. The secondary structure is indicated.

selected as the indicator due to its intermediate level of susceptibility (Table 4). We detected no significant loss of activity at any of the temperatures assayed when the proteins, including the wild-type AS-48, were dissolved at pH 3.0. Nonetheless, the activity of E4A and E49 proteins was considerably reduced after treatment at 100°C at pH 7.0, and completely disappeared at pH 9.0. Heating to 100°C for 5 min caused a marked loss of activity (86% and 89%) in the other two mutants at pH 9.0.

Influence of pH and bacteriocin concentration on biological activity

Purified mutant and wild-type AS-48 molecules (1200 mg/L) in 10 mM sodium phosphate buffer at pH values of 3.0, 7.0 and 9.0 were tested on BHA plates inoculated with the different Gram-positive bacteria described above and also against the two Gram-negative strains *E. coli* U-9 and *Salmonella choleraesuis* LT2. The purified derivatives showed no activity against *S. choleraesuis* LT2 and the inhibition detected against *E. coli* U-9 always appeared as cloudy zones, which marks the highest concentration required for complete inhibition. Nevertheless, we found an increase in activity against this bacterium when the samples were assayed at pH 9.0 (data not shown). *Listeria monocytogenes* and *B. megaterium* were the most susceptible bacteria and *Staphylococcus aureus* the most resistant. One noteworthy result was the slight increase in activity of mutant E20A compared with wild-type AS-48 against *Bacillus cereus* LWL1 and the two *Listeria* strains assayed.

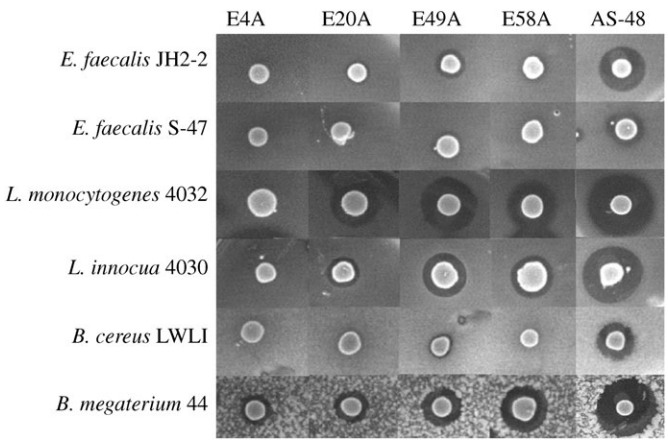

Figure 2. Inhibition halos from AS-48 derivative producer cultures (1 μL) growing on BHA plates against different indicator bacterial strains after 18 h of incubation at 37°C. Activity of JH2-2(pAM401-81$_X$) strain was used as a reference.

We also determined the MIC for the mutant peptides (defined here as the minimum amount of protein necessary to inhibit the growth of the indicator strains in BHA plates), assaying half-decreasing concentrations from RP-HPLC purified samples at different pH values (Table 5). The MIC of E20A proved to vary only slightly from that of the wild-type, except against *S. aureus*, where the concentrations required for inhibition were 5-fold greater at pH 7.0 and 9.0. In the case of mutant E4A, the MIC was very close to that of the wild-type, although between 5 and 10 times the concentrations were required to inhibit the two strains of *E. faecalis* and *S. aureus* 240. The most striking fact was the high value of its MIC against *E. coli* U-9 (5.6-fold more at pH 3.0 and 7.0, and 8.4-fold more at pH 9.0) (Table 5). The behaviour of E49A against *B. megaterium, L. monocytogenes, Listeria innocua* and *E. coli* was similar to that of the wild-type, although double the quantity was required to inhibit the JH2-2 and S-47 strains of *E. faecalis* at pH 7.0 and 9.0, and 5-fold more at pH 3.0. Against *S. aureus*, its MIC was 5-fold higher at pH 3.0 and 10-fold higher at pH 7.0 and 9.0. *B. cereus* LWL1 required only half the quantity of protein at pH 7.0 and 9.0 to be inhibited, although at pH 3.0, it required double. Finally, E58A behaved in a similar way to the wild-type against *B. megaterium* and *L. monocytogenes*, but required double the quantity of protein to inhibit the other indicator strains. As occurred with the E49A derivative, the exception was *S. aureus* 240, which required some 5-fold more at pH 3.0 and 10-fold more at pH 7.0 and 9.0.

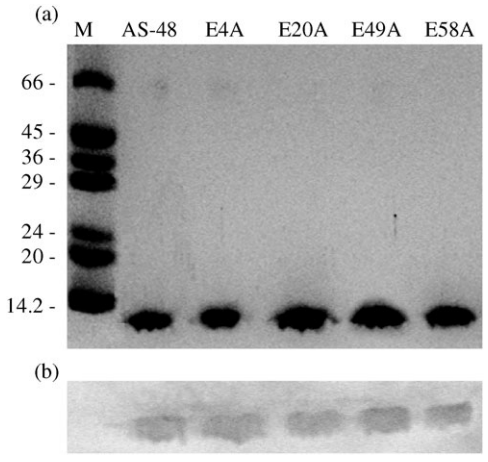

Figure 3. (a) Electrophoretic separation of AS-48 wild-type and derivatives on Coomassie Blue-stained SDS–PAGE gel. Lane 1, standard markers (kDa); lane 2, AS-48 wild-type (7.2 kDa); lanes 3–6, AS-48 derivatives. (b) Immunological detection of wild-type and derivatives by western blotting using specific anti-AS-48 antibodies.

Table 4. Heat and pH resistance of AS-48 and derivatives. Bacteriocin solutions at different pH values were heated for different times to various temperatures. The remaining antimicrobial activity (mm) against *E. faecalis* JH2-2 is shown. C, untreated control; 1, treatment at 60°C for 30 min; 2, 70°C for 10 min; 3, 80°C for 5 min; 4, 100°C for 5 min. (−): no activity. (+) slight inhibition.

AS-48 (71.5 mg/L)	Size of inhibition zone (mm)														
	pH 3.0					pH 7.0					pH 9.0				
	C	1	2	3	4	C	1	2	3	4	C	1	2	3	4
Wild-type	10.0	9.0	9.0	8.0	8.0	9.5	9.5	9.5	8.0	1.0	8.0	7.0	7.0	6.0	1.0
E4A	7.0	7.0	6.0	6.0	6.0	8.0	6.0	6.0	1.0	−	6.0	−	−	−	−
E20A	8.0	8.0	8.0	8.0	8.0	9.0	8.0	8.0	8.0	5.0	9.0	6.0	6.0	7.0	1.0
E49A	7.0	7.0	6.0	6.0	5.0	7.0	7.0	6.0	6.0	−	7.0	+	+	−	−
E58A	8.5	8.0	8.0	7.0	7.0	8.0	7.0	7.0	7.0	5.0	7.0	5.0	5.0	5.0	1.0

Effect on stability of replacing the glutamic residues

We investigated the effect of four substitutions on the stability of AS-48 derivatives according to different arrangements of AS-48 crystal structure[19] (Figure 4). The structural analysis of the dimeric structure DF-II, which is supposed to be involved in membrane insertion,[19] showed that residues E4 and E49, belonging to different chains, are facing each other and a strong repulsive force exists between them (Figure 4b). A hydrogen bond between the different chain backbones of E4 and R48 was also observed.

The theoretical results obtained with FOLD-X (Table 6) suggested a decrease in stability for the four mutants analysed on the crystal structure at pH 4.5 (where the glutamic must be partially protonated), being less patent in E58A mutant, which is less solvent exposed. At pH 7.5, the structure was more destabilized in E49A mutant in comparison with E4A and E20A derivatives (the most solvent exposed residues), and there was not stability change for E58A mutant. On the contrary, the dimeric structure DF-II suffered a strong stabilization upon mutations E4A and E49A, and it was also observed stabilization for E58A. The positive free-energy value for E20A pointed to a destabilization effect.

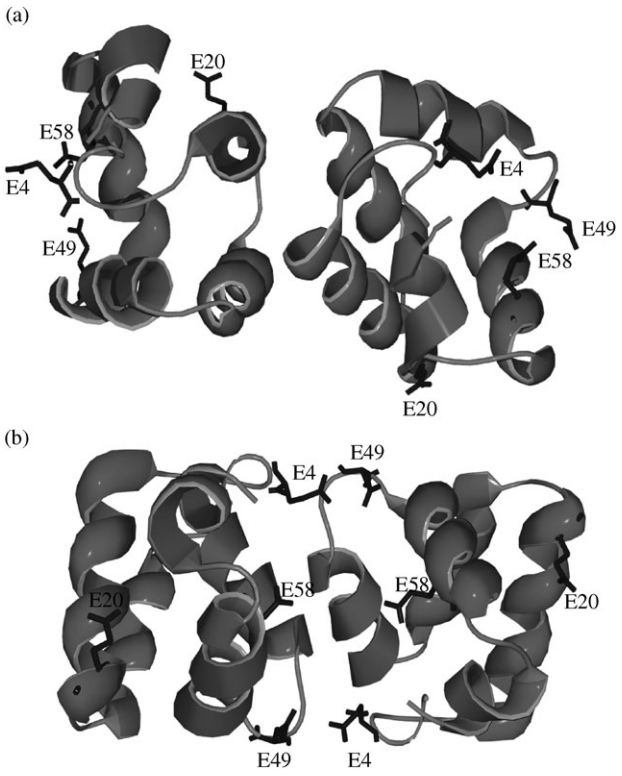

Figure 4. Three-dimensional model of AS-48 (Glu side chains are labelled). Representations of AS-48: (a) DF-I and (b) DF-II structures.[19]

Secondary structure analysis by CD

We evaluated the secondary structure of AS-48 wild-type molecule and its derivatives by CD spectroscopy. The far-UV CD spectra were obtained at 25°C using a concentration of 10 μM (71.5 mg/L) for every sample in 10 mM sodium phosphate buffer at pH 3.0 to guarantee that all the samples were in the monomeric state. No conformational changes could be seen in the structure of the folded state in the CD spectra at 222 nm [Figure S2, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]; all the curves presented ellipticity values typical of highly helicoidal molecules and could be superimposed upon each other, suggesting that the AS-48 wild-type and its glutamic mutants were well structured. When the assay was repeated at four times the concentration (286 mg/L), the spectra showed no dependency upon protein concentration (data not shown).

Discussion

On the basis of the X-ray structure of AS-48, it has been suggested that a plane containing the C^{α} atoms of the glutamic residues located in helices α_5 (E4), α_1 (E20), α_4 (E58) and in the loop connecting the α_3–α_4 helices (E49) of the AS-48 molecule may separate a patch of positively charged residues from the rest of the hydrophobic or uncharged surface residues.[19] To investigate the roles of these glutamic residues in biological activity, we replaced each Glu residue with Ala by site-directed mutagenesis.

To ensure the successful production and immunity of each mutated peptide, we cloned the engineered gene within the *as-48* cluster by a gene replacement strategy. We purified the

Table 5. MICs of highly purified preparations serially diluted in sterile 10 mM sodium phosphate buffer at pH values of 3.0, 7.0 and 9.0 and assayed against different susceptible strains

	MIC (mg/L)														
	AS-48 wild-type			E4A			E20A			E49A			E58A		
	pH			pH			pH			pH			pH		
Indicator strain	3	7	9	3	7	9	3	7	9	3	7	9	3	7	9
E. faecalis JH2-2	3.57	3.57	7.15	14.2	35.5	35.5	3.55	3.55	3.55	14.2	7.1	14.2	7.1	7.1	14.2
E. faecalis S-47	7.15	7.15	14.3	35.5	35.5	35.5	7.1	7.1	7.1	35.5	14.2	14.2	14.2	35.5	35.5
B. cereus LWL1	3.57	7.15	7.15	7.1	14.2	14.2	3.55	3.55	7.1	7.1	3.55	3.55	7.1	14.2	14.2
B. megaterium 44	3.57	3.57	3.57	3.55	3.55	7.1	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55
L. innocua 4030	3.57	3.57	7.15	7.1	7.1	14.2	3.55	3.55	3.55	3.55	3.55	3.55	7.1	7.1	7.1
L. monocytogenes 4032	3.57	3.57	3.57	3.55	3.55	7.1	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55
S. aureus 240	14.3	7.15	7.15	35.5	70.9	70.9	14.2	35.5	35.5	70.9	70.9	70.9	70.9	70.9	70.9
E. coli U-9	71.5	71.5	71.5	397	397	596	70.9	70.9	70.9	70.9	70.9	70.9	70.9	70.9	70.9

Activity of the wild-type AS-48 was determined as control.

same quantities of each mutated peptide to compare their antibacterial activity against different susceptible bacteria and to characterize their stability and secondary structure. None of the replacements introduced altered the immunity of the mutant producer strains against either AS-48 or any of the E→A mutant peptides, indicating that these changes did not compromise their auto-immunity, the complex mechanism of which resides in several determinants from the *as-48* gene cluster,[24,33,34] which had not been altered in these constructions.

During the purification process of the four variants by CM25, C18 and RP-HPLC, we detected inhibitory activity in the samples when the concentration factor ranged from 10 to 24 times that of the crude supernatants. We found slightly different retention times in the active fractions of the mutant peptides, which might be related to their intrinsic hydrophobicity and also to the amphipathicity of the peptides.[35] The derivatives were recognized by the specific anti-AS-48 antibodies used in the western blotting, and no variations in the oligomerization tendency were found compared with the wild-type molecule, which suggests that the changes introduced did not modify the surface and antigenic characteristics of the derivatives to any great extent.

Table 6. Stability analysis of AS-48 derivatives compared with AS-48 wild-type, using the FOLD-X algorithm on the AS-48 crystal structures at pH 4.5 (PDB ID 1O82) and pH 7.5 (PDB ID 1O83) and on the DF-II structure crystallized in the presence of detergent (PDB ID 1O84)

	$\Delta\Delta G$ (kcal/mol)		
AS-48 derivative	PDB 1O82	PDB 1O83	PDB 1O84
E4A	1.220	0.760	−1.660
E20A	1.160	1.290	0.800
E49A	1.280	2.050	−0.950
E58A	1.800	0.000	−1.110

The antimicrobial activity of the purified samples was mainly influenced by the concentration of the bacteriocin, the pH of the solution and the position of the mutation in the molecule. We found that the change in the net charge of the variants favoured the inhibition of *E. coli* strain U-9, especially at pH 9.0 and at the highest concentration of 1200 mg/L. There were, however, no significant variations in activity against the Gram-positive strains analysed, which is in accordance with the antibacterial spectra shown by AS-48RJ, a natural variant of AS-48 produced by *E. faecium* RJ16 with an E20V mutation.[36] On the whole, our results indicate that when high bacteriocin concentrations are assayed (1200 mg/L), the change in the net charge of the mutant peptides does not confer any loss in activity. As with other bacteriocins,[37] AS-48 may perturb the lipid bilayers at the high concentrations used, at which the peptide exists in the form of multimeric units. Interestingly, the four mutants were quite resistant to heat at low pH, but their activity decreased concomitantly with an increase in temperature at higher pH values. Our results indicate that stability was strongly influenced by the pH of the solution, mainly in the E4A variant. It is well known that the heat-induced unfolding of AS-48 at high pH values results in irreversible aggregation,[38] thus explaining the loss of activity observed for the four derivatives, in particular for the most destabilized ones (Table 6, PDB 1O83) against the indicator strain *E. faecalis* JH2-2 under experimental conditions of pH 9.0 and high temperature.

The values obtained for the MICs clearly demonstrated the importance of the concentration used when judging the activity of proteins with a tendency to oligomerize, which consequently are limited in their capacity to diffuse within the agar. This reveals the limitations of the normal method used to evaluate activity. Furthermore, the use of several indicator strains to evaluate the MIC gives a more exact idea about the behaviour of these variants because, as could be seen, the inhibitory effect depended upon the indicator strains used. On the whole, the MIC values indicated that the loss of a negatively charged Glu residue, particularly at positions 4, 49 and 58, had a somewhat more negative effect (MIC values 2- to 10-fold higher than that

of wild-type AS-48) than the corresponding mutation at position 20, which had no detectable consequences against the majority of the Gram-positive bacteria or *E. coli* U9 (the exception was *S. aureus*, which required five times more). In all cases, the most susceptible bacteria (*B. megaterium* and *Listeria* species) had MIC values very similar to that of the native AS-48. Variations in susceptibility and even in the inhibitory action spectra have also been noted after the genetic modification of several cationic peptides due to differences in the net charge on the bacterial surface or in the lipid composition and the existence of capsules or an S layer. In addition, the influence of pH on activity could be interpreted as being due to changes in the oligomerization of the active peptides as well as to changes in the surface charge of the target bacteria.

A correlation has been described between amphipathicity and the haemolytic activity of some cationic AMPs.[39] In the case of AS-48, glutamic substitutions neither modified the amphipathicity of the molecule nor determined haemolytic capacity, although they did influence its biological activity.

Analysis with FOLD-X indicated that some side chains of negatively charged residues may interact in a structurally specific and restricted manner with a polar group or groups on target-cell membranes. In fact, the importance of E58 within DF-II in the crystal structure was suggested in a previous paper where ionic interactions of TyrB54, GluB58, GluD58 and LysD62 were proposed.[19] The free-energy values obtained from the PDB files analysed (Table 6) show a significant stabilization effect for E4A and E49A derivatives in the DF-II dimer, probably due to the fact that they are facing each other and the substitutions may reduce the strong repulsive forces existing between them (Figure 4b). It is thus possible to put down the decrease in antibacterial activity demonstrated with E49A, even more pronounced with E4A, to the stabilization effect of the replacement of the glutamic residues on DF-II. The destabilization effect observed for E20A may well be due to its external position on the surface of DF-II, which might be in contact with the internal face of the biological membrane.

The interaction of AS-48 with the membrane might appear to be mainly electrostatic, but our results suggest that the DF-II interaction may facilitate penetration of most of the domain into the membrane interface in spite of the fact that the constrained helical and circular AS-48 structure confers a certain rigidity upon it, which may reduce significant conformational changes upon meeting its target.[19] All this is in accordance with Shental-Bechor *et al.*[40] who have confirmed that amphipathic and cationic peptides adopt two main membrane-associated states: in the first, the peptide resides mostly outside the polar head-group region, whereas in the second, which is energetically more favourable, the peptide assumes an amphipathic helix conformation, in which its hydrophobic face is immersed in the hydrocarbon region of the membrane and the charged residues are in contact with the surface of smeared charges. In addition, it is interesting to consider that the local pH at the membrane–water interface is lower than in the bulk solution, and the protonation and burial of the acidic side chain in the membrane interior is not energetically costly.[41] In this way, the behaviour of the AS-48-mutated peptides could be explained in the light of the typical distribution of amino acid residues from membrane-inserted proteins at the membrane–water interface, and the more complex chemical nature of this area, which includes charged phosphate heads and ester bonds of phospholipids. Given that the replacement of Glu with Ala might modify interactions between neighbouring residues, it is not surprising that some of the changes introduced affect the stability of the molecules in solution and cause variations in levels of activity against identical organisms according to their precise spatial location, suggesting that the exact spatial position of the side chains of such residues is critical for activity.

These results confirm our hypothesis and agree in general with Jiménez *et al.*,[42] who have proposed that the mechanism of membrane disruption is not simply one of molecular electroporation driven by a deposition of positively charged molecules on the plane of the bacterial membrane, but requires the involvement of hydrophobic regions of AS-48 for partial insertion. In addition, detrimental consequences brought about by mutagenesis of the negatively charged amino acids have also been described for lacticin 3147,[43] sakacin P[44] and mersacidin.[45]

Nevertheless, studies into the relationship between structure and activity in cationic AMPs must take several important factors into account simultaneously: the presence of both hydrophobic and basic residues, the amphipathic nature of the peptides and their secondary structure. For this reason, we obtained the CD spectra from wild-type AS-48 and its mutants at pH 3.0; the molecules were monomeric, as indicated by their non-dependency on protein concentration, and were well structured with no conformational changes in the structure of the folded state. This behaviour is very different from that of the majority of the α-helical linear peptides, which exist as disordered structures in aqueous media and only become helical upon interaction with hydrophobic solvents or phospholipid vesicles.[46]

A considerable number of engineered peptides from Gram-positive bacteria belonging to lantibiotics[45,47,48] or class IIa bacteriocins[44,46,49–51] have been designed and characterized, although there have been no circular peptides among them. To our knowledge, this is the first characterization of derivatives from a circular bacteriocin such as AS-48, belonging to the new class IV.[52] The development of a system for site-directed mutagenesis in AS-48, allowing the targeted exchange of amino acids within its structure, represents an important step towards the design of novel molecules with improved activity spectra and/or different degrees of hydrophobicity, both of which are properties of great interest for biotechnological food and clinical applications.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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