Analysis of the Promoters Involved in Enterocin AS-48 Expression

Rubén Cebrián, Sonia Rodríguez-Ruano, Manuel Martínez-Bueno, Eva Valdivia, Mercedes Maqueda*, Manuel Montalbán-López†*
Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, Granada, Spain

Abstract

The enterocin AS-48 is the best characterized antibacterial circular protein in prokaryotes. It is a hydrophobic and cationic bacteriocin, which is ribosomally synthesized by enterococcal cells and post-translationally cyclized by a head-to-tail peptide bond. The production of and immunity towards AS-48 depend upon the coordinated expression of ten genes organized in two operons, as-48ABC (where genes encoding enzymes with processing, secretion, and immunity functions are adjacent to the structural as-48A gene) and as-48C,DD1EFGH. The current study describes the identification of the promoters involved in AS-48 expression. Seven putative promoters have been here amplified, and separately inserted into the promoter-probe vector pTLRI, to create transcriptional fusions with the mCherry gene used as a reporter. The activity of these promoter regions was assessed measuring the expression of the fluorescent mCherry protein using the constitutive pneumococcal promoter P4 as a reference. Our results revealed that only three promoters P8, P2(2), and P9 were recognized in Enterococcus faecalis, Lactococcus lactis and Escherichia coli, in the conditions tested. The maximal fluorescence was obtained with P8 in all the strains, followed by the P2(2) promoter, which level of fluorescence was 2-fold compared to P8 and 4-fold compared to P9. Analysis of putative factors influencing the promoter activity in single and double transformants in E. faecalis JH2-2 demonstrated that, in general, a better expression was achieved in presence of pAM401-81. In addition, the P2(2) promoter could be regulated in a negative fashion by genes existing in the native pMB-2 plasmid other than those of the as-48 cluster, while the pH seems to affect differently the as-48 promoter expression.

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* E-mail: mmaqueda@ugr.es (MM); m.montalban.lopez@rug.nl (MM-L)
† Current address: Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands

Introduction

AS-48 is a 70-residue alpha-helical cyclic cationic bacteriocin ribosomally produced by diverse Enterococcus strains, with antimicrobial activity against food-borne pathogenic and food-spoilage bacteria. These characteristics, together with its stability and solubility over wide pH and temperature ranges, confer a clear potential to be used as food biopreservative [1]. Besides this, AS-48 could have veterinary and clinical applications [2] currently under investigation, underscoring its potential as an antibacterial agent with biotechnological potential to be developed as protective agent in food preservation.

According to Martínez-Bueno et al. [4] and Díaz et al. [5] the full expression of the as-48 cluster depends on the co-ordinated expression of ten genes (as-48A, B, C, C1, D, D1, E, F, G, and H) (GenBank accession number KJ146793, Y12234.1 and AJ438950.1), although only nine (bacA, B, C, D, E, F, G, H, and I) were identified in the bac cluster [6] (Genbank D85752.1) (Figure 1). However, in the physical and genetic map published by each group there are some differences (Figure 1A). The main discrepancy is that in the bac cluster a protein homologous to As-48D1, the proposed immunity determinant against AS-48, was not considered. However, the mutants that were described in that work show that the deletion of the region where the immunity
protein As-48D1 is encoded, clearly makes a difference in the phenotype in terms of resistance against AS-48 [6]. This is consistent with the existence of a small ORF encoding an immunity determinant as was shown later [9]. There are some other variations, in addition to the nomenclature used, related to the predicted initiation codons for bacC and bacD genes (homologous to as-48C and as-48C1, respectively), and also on a putative promoter proposed for the bacC gene (Figure 1A).

Transcriptional analysis of the as-48 cluster revealed the existence of two polycistronic mRNAs, T1 (3.5 kb) and T2 (6.4 kb), corresponding to the expression of the two operons as-48ABC and as-48C2DD1EFGH, respectively (Figure 1A). A post-transcriptional regulation mechanism was elucidated for T1 that undergoes endonucleolytic processing into two smaller fragments with different half-life in order to ensure the optimal stoichiometry of each gene product [10]. Furthermore a second and shorter mRNA (T3, 5.4 kb), possibly transcribed from an internal promoter, encodes at least the last four genes (as-48EFGH) [5].

All the commented features are in agreement with the general trend in bacteria, where numerous genes are organized in operons transcribed from the same promoter into a single polycistronic mRNA molecule, although many genes could also be transcribed from internal promoters located at intergenic regions or within adjacent genes [11]. Nevertheless, it has been suggested that T1 and T2 are constitutively expressed, while transcription from putative internal promoters might be regulated [5]. In other circular bacteriocins like uberolysin, circularin A and butyrivibriocin AR10 there are regulatory elements encoded in the same gene cluster [12], whereas the production of subtilosin A is controlled by external regulators in response to environmental factors [13–15].

Our group has provided valuable information regarding the impact of the amino acids in the propeptide sequence that are involved in the head-to-tail peptide bond formation [16] and the impact of circularization in the activity and structure of AS-48 [17,18]. In this moment, we are interested in unravelling the interactions between the proteins encoded in the as-48 gene cluster and in elucidating the regulation of the gene expression. Thus, an accurate identification of the promoters is crucial. Such information could also help to explain the failure in the heterologous expression of AS-48 in other lactic acid bacteria, especially in Lactococcus lactis [19], a GRAS (generally recognized as safe) bacterium of great biotechnological interest. Additionally, the identification of promoters, particularly those strong and inducible, provides a potent biotechnological tool for research and industry [20–23]. However, the identification of promoter regions is problematic when dealing with bacterial genomes that have a high A+T content such as E. faecalis (ca. 60% A+T). In these genomes, stretches resembling ~10 elements (5’TATAAT-3’) are frequent and, therefore, the definitive identification of promoters from sequence information remains more difficult [11]. For these reasons, we have investigated the activity of the several putative as-48 promoter regions identified in silico measuring their different expression level in diverse strains. For this, we carried out transcriptional fusions of each putative promoter fragment to drive the expression of a synthetic mCherry gene codon-optimized for Enterococcus into the pTLR1 vector [24]. We isolated seven putative promoter fragments from the as-48 cluster according to two software analyses and cloned each fragment to drive the expression

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**Figure 1.** (A) Schematic representation of the as-48 (black) and bac21 (grey) gene clusters. Solid black arrows represent the proposed promoter regions and dotted arrows indicate the mRNAs detected by Fernandez et al. [10], Diaz et al. [5] and Martinez-Bueno et al. [9]. Solid grey arrows represent the promoter regions proposed by Tomita et al. [6]. (B) Promoters identified in silico (dashed arrows) and their location according to AS-48 nomenclature (Genebank KJ146793 and Y12234.1): P_A (nt 1105-nt 1396), P_C (nt 2129-nt 2477), P_2(1) (nt 2788-nt 3010), P_D1 (nt 3721-nt 4160), P_3(1) (nt 4353-nt 4544) and P_3(2) (nt 4188-nt 4544). Predicted terminators according to BPROM [32] in as-48 gene cluster are pointed with a T. doi:10.1371/journal.pone.0090603.g001
of the mCherry reporter gene in three bacterial strains, E. faecalis, L. lactis and E. coli, using the previously characterized promoter Px of Streptococcus pneumoniae for comparison [24].

Materials and Methods

Bacterial strains, vectors and culture conditions

Bacterial strains and vectors used in this work are listed in Table 1. Escherichia coli was grown at 37°C with shaking in Luria broth (LB; Scharlau, Barcelona, Spain) and selected with erythromycin (Em 250 μg/ml, Sigma-Aldrich, Madrid, Spain) for cells harboring the pTLR1-derivatives. E. faecalis and L. lactis were routinely grown in brain heart infusion (BHI; Scharlau) at 37°C and M17 (Scharlau) plus glucose (0.5%), GM17, at 30°C, respectively. Erythromycin at 10 μg/ml or/or chloramphenicol at 20 μg/ml (Sigma-Aldrich, Madrid, Spain) were added to the culture medium for cells harboring pTLR1 derivative plasmids or pAM401-81 plasmid, respectively.

For fluorescence detection, several culture media were assayed: the chemically defined media CDM-PC [25] and CDM-BP [26], the semi-defined complex medium supplemented with 0.8% glucose (CM-G) [27] and the complex media GM17 and LB.

General DNA manipulation and transformation

The plasmid-free strain E. coli TOP10 was used in cloning experiments. The preparation of chemocompetent cells to be transformed with plasmid DNA and ligation products was done by the calcium chloride protocol as described by Seidman et al. [28]. Electroporation of L. lactis and E. faecalis was performed according to the methods described by Holm and Nes [29] and Friesenegger et al. [30], respectively. Plasmid DNA was isolated from E. coli using the Plasmid Mini I kit from Omega bio-tek (VWR International, USA). PCR products were purified with the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea) and sequenced. Restriction enzymes were obtained from Thermo Scientific (Madrid, Spain), TaqDNA polymerase from MBL Scientific (Madrid, Spain), ligase from Invitrogen (Life Technologies, Madrid, Spain), TaqDNA polymerase from MBL (MBL International Corporation, Woburn, USA); and used as recommended by the suppliers. DNA was sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Applied Biosystems, USA).

In silico analysis

Putative promoter regions from as-48 or bac regions [GenBank KJ146793 and Y12234.1, and D85752.1, respectively (9,6)] were analysed with the bioinformatic programs Promoter Prediction by Neural Network (NNPP) [31] (http://s.fruitfly.org/seq_tools/promoter.html) and BPROM (Softberry Inc., Mount Kisco, NY, USA; http://linux1.softberry.com) [32].

Construction of the pTLR1-derivative plasmids with the mCherry reporter gene

pTLR1 (KitMygen, Madrid, Spain) is a vector for promoter analysis that contains the strong promoter Px from S. pneumoniae upstream of mCherry [24]. The plasmid pAM401-81 was used as a template for PCR amplification of the different predicted promoters [5,9]. All primers used in PCRs (listed in Table 2) were synthesized by Biomedal S.L. (Sevilla, Spain) and were based on the published DNA sequence of the as-48 locus of E. faecalis (Genebank KJ146793, Y12234.1 and AJ438950.1). The PCR conditions were the same for all the amplifications performed: 96°C 2', 30× (96°C 30", 50°C 30", 72°C 30"), 72°C 2'. The amplified DNA fragments containing the presumed promoter regions were cut with BglII and BamHI and ligated into pTLR1 previously digested with the same enzymes, obtaining the pTLR1-derivative constructions shown in Table 1. The ligation mix was transformed into E. coli TOP10. The desired orientation of the fragments was determined by colony PCR using the forward primer of each promoter and the pTLR1-rev primer, which anneals in the vector backbone (Table 2). The verified plasmid isolated from E. coli was used to transform L. lactis LM2301 and E. faecalis JH2-2 or JH2-2 harboring either pMB-2 or pAM401-81 plasmids. pTLR1d lacking the polylinker but containing the mCherry gene

Table 1. Strains and plasmids used in this study. CmR chloramphenicol resistant, EmR erythromycin resistant.

| Bacteria | Characteristics | Source |
|----------|----------------|--------|
| Escherichia coli TOP10 | F- mcrA Δ(mrr-hdRMS-mcrBC) Δ800azΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL Δ (StrR) endA1 Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1 Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1 Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1 Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1Δ(mrr-hsdRMS-mcrBC) (mrr-hsdRMS-mcrBC) (StrR) endA1 | Invitrogen |
| Enterococcus faecalis JH2-2 | Plasmid free, RifR, FusR, A8-48 | [42] |
| Enterococcus faecalis JH2-2 (pMB-2) | JH2-2 transconjugant with pMB-2 plasmid | [5] |
| Lactococcus lactis LM2301 | Plasmid free, host strain for cloning | [43] |
| Plasmid | Characteristics | Source |
| pAM401-81 | CmR, as-48 gene cluster cloned in pAM401 (25 kb) | [5] |
| pTLR1d | Obtained from pTLR1 vector after digestion with BglII and BamHI (lacking the polylinker but containing the mCherry gene) | This work |
| pTLR1 | pTLR derivative containing the promoter Px; EmR (8.3 kb) | [24] |
| pTLR1-Px | EmR, Px promoter cloned in substitution of Px, controlling mCherry expression (8.0 kb) | This work |
| pTLR1-Px | EmR, Px promoter cloned in substitution of Px, controlling mCherry expression (8.1 kb) | This work |
| pTLR1-P2x | EmR, P2x promoter cloned in substitution of Px, controlling mCherry expression (8.0 kb) | This work |
| pTLR1-P2x | EmR, P2x promoter cloned in substitution of Px, controlling mCherry expression (8.2 kb) | This work |
| pTLR1-Pd | EmR, Pd promoter cloned in substitution of Px, controlling mCherry expression (8.2 kb) | This work |
| pTLR1-Pd | EmR, Pd promoter cloned in substitution of Px, controlling mCherry expression (8.0 kb) | This work |

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without promoter was here obtained from pTLR1 vector after digestion with BglII and BamHI enzymes and religation.

Detection and quantification of fluorescence emission in microtiter plates

Each assay was repeated in triplicate in a 96-well optical flat-bottom microplate (Nuclon Delta Surface, Thermo Scientific, Roskilde, Denmark) and monitored with an Infinite 200 Pro microplate spectrophotometer (Tecan Group Ltd., Mannedorf, Switzerland). Briefly, each transformant was separately inoculated into three wells with a final volume of 100 µl/well of the CM-G medium with the appropriate antibiotics at an OD600 nm 0.05 and then grown for 18 h at 30 °C for Lactococcus or 37 °C for E. coli and Enterococcus. During cultivation the spectrophotometer simultaneously provided quantitatively online data every 10 minutes of cell density (OD600) and the growth. In addition the values of fluorescence shown were normalized by the OD to avoid differences in the growth that may lead to erroneous conclusions.

In order to determine the influence of the pH, the medium was adjusted at different pH values (6, 6.5, 7.0, 7.5 and 8.0) using 0.1 M phosphate buffer according to Gomori [33]. All the experiments were performed in triplicate. The background fluorescence of the control strains (harboring the pTLR vector without promoter) was here obtained from pTLR1 vector after digestion with BglII and BamHI enzymes and religation.

Statistical analysis

The statistical analysis of the data was performed using the IBM SPSS statistics 20 (IBM, Spain). Data relating to microbiological density and fluorescence under different conditions were subjected to ANOVA. Tukey was used as a post-hoc test to determine significant differences between promoters and a 0.05 significance level (p value) was considered. The average data from duplicate trials ± standard deviation was determined.

Fluorescence microscopy

Cells were grown overnight in 1 ml of the appropriate medium and harvested by centrifugation. After 3 washes in sterile PBS (Sigma-Aldrich, Madrid, Spain), 5 µl of cells were placed on slides and observed in an Olimpus BX51 microscopy (model BX51TF with a power supply unit Olimpus-U-RFL-T SN 1101008) using a TRITC filter (excitation 590 nm and emission 620 nm). The images were taken at 250 ms of exposure.

Results and Discussion

Bioinformatic location of promoter regions in the as-48 gene cluster

In previous works (e.g. [4–6]) several promoter regions triggering the as-48/bac21 gene cluster expression have been proposed (Figure 1). To unambiguously define the promoter regions involved in AS-48 expression, putative −10 and −35 hexamers were located by their resemblance to the previously defined Enterococcus consensus sequences, using the Promoter Prediction by Neural Network (NNPP) [31] and the BPROM programs (Softberry Inc., Mount Kisco, NY, USA; http://linux1.softberry.com) [32]. Preference was given to motifs that matched to the consensus sequence at the most conserved positions of the hexamers and gave rise to a −35/−10 with a 17±1 nt spacer according to both programs. Therefore, a set of seven putative promoters was predicted (Table 3 and Figure 1B). Detection of these promoters in the as-48 gene cluster would not be achieved using this software. In general, the −10 sequences are

**Table 2. Oligonucleotides used in this study.**

| Target | Primer   | Sequence* 5’→3’ | Product size (bp) |
|--------|----------|-----------------|------------------|
| Pₐ     | ForPₐ    | ACAAGATCTGCGATGATTGATGAAAAAAA | 256 |
|        | RevPₐ    | TTTGGAATCTGCGATGATTGATGAAAAAAA | 347 |
| Pc     | ForPc    | ACGTAGATGCGATGATTGATGAAAAAAA | 263 |
|        | RevPc    | TTTGGAATCTGCGATGATTGATGAAAAAAA | 356 |
| P₃(1)  | RevP₃(1) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 439 |
| P₃(2)  | RevP₃(2) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 191 |
|        | ForP₃(2) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 356 |
| P₁(1)  | RevP₁(1) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 439 |
|        | ForP₁(1) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 356 |
| P₁(2)  | RevP₁(2) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 191 |
|        | ForP₃(2) | TTTGGAATCTGCGATGATTGATGAAAAAAA | 356 |
| pTLR   | pTLR-rev | GTTGAACCTGCGATGCGATCCC CGGG | 9 |

*Restriction enzyme sites are depicted in italics.
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better conserved in all the proposed regions while the −35 sequences were substantially less conserved (Table 3). A characteristic TG motif of Gram-positive bacteria promoters, often found 1 bp upstream of the −10 sequence (the −16 region) [34], was found in 4 of the 7 promoters studied (Table 3). Also an AT-rich region located upstream from the −10 and −35 hexamers was identified. Such an AT-rich region may activate the promoter by DNA bending [35] or form an UP element that stimulates transcription through a direct interaction with the C-terminal domain of the RNA polymerase α subunit [36]. In general, the distance between the −10 and −35 hexamers ranged from 11 to 18 bp (Table 3).

As a result, we have selected the following promoter regions putatively involved in the expression of the AS-48 character (Figure 1 and Table S1):

i) The PA promoter: it is a region with canonical −10 and −35 regions, separated by a correct spacing that could allow for the binding of the vegetative σ factor of the bacterial RNA polymerase without the need for an activator [10].

ii) The PC promoter: Fernández et al. [10] reported that as−48BC genes overlapped and they had a coupled transcription from the PA promoter in absence of a specific promoter for as−48B. This fact does not invalidate, however, the possibility that as−48C could have its own promoter (PC) as it had been proposed for its counterpart bacC [6]. In such case, bacC would encode a shorter protein (57 residues), starting 246 nt downstream from bacB, which does not overlap with bacB as, in fact, it was proposed for its homologous as−48C in the as−48ABC operon [9]. To address this point, we have amplified the region located 347 nt upstream the as−48C gene. It contains motifs that match the consensus sequence at the most conserved positions of the hexamers and an appropriate spacer in accordance with the two predictive programs used (Table 3).

iii) The P2 promoter: according to Diaz et al. [5], this promoter drives the expression of the as−48C2DD1EFGH operon. However, there are discrepancies between Martínez-Bueno et al. [9] and Tomita et al. [6], who place the origin of as−48C1 or bacD 400 bp and 500 bp, respectively, from the previous predicted ORF. Additionally, the software used in the predictions also shows two possible promoters in the region. Therefore, we decided to clone the two putative promoters, namely P2(1) and P2(2), to clarify this possible promoters in the region. Therefore, we decided to clone the two putative promoters, namely P2(1) and P2(2), to clarify this point (Table 3 and Figure 1B).

iv) The P31 promoter: the possibility of an internal promoter in the as−48C2DD1EFGH operon, from which the gene as−48D1 might be transcribed has been also investigated. This new promoter might be located in a 439 nt fragment upstream from the start codon of as−48D1 containing a −10 and a −35 consensus sequence with 4 out of 6 matches (Table 3). This putative internal promoter, which was not considered in the bac21 cluster [6], would confer some degree of immunity to the producer strain (reviewed by [3,12]).

v) The P3 promoter: the putative P3 promoter firmly postulated by both, Diaz et al. [5] and Tomita et al. [6], driving the expression of the four overlapped as−48EFGH genes should be located in the 204 nt intergenic region identified between the as−48D1 and as−48E1 genes, where a plausible −10 and −35 region separated by a correct spacing was found (promoter P3(1)) (Table 3). Additionally, a larger region including part of as−48D1 and a series of conserved sequences separated by 9 nt (named promoter P3(2)) has been also cloned according to the prediction.

### Construction of pTLR1-derivatives containing the promoter regions fused to the mCherry gene

To map more precisely the promoter regions driving the expression of as−48ABC, as−48C, as−48C2DD1EFGH, as−48D1 and as−48EFGH genes, the presumed promoter fragments were amplified and separately inserted into the promoter-probe vector pTLR1, creating several transcriptional fusions with the mCherry gene, which is codon optimized for expression in LAB [24]. To amplify such regions, we used the pAM401-81 plasmid as template and specific pairs of primers (Table 2) discarding the putative RBS of each promoter in the amplifications. In this way, the RBS is common in all the constructions and therefore the amount of mCherry produced will correlate more accurately with the strength of each promoter without any effect of the individual RBSs. The advantage that this expression system provides is the easy monitoring of the mCherry expression as autofluorescence emitted after an excitation pulse of light with a wavelength of 590 nm. As positive and negative controls, the strong PX promoter of S. pneumoniae, which in the absence of the pneumococcal MalR regulator is constitutively expressed [24] and the pTLR1d vector here constructed were used. The recombinant pTLR1-derivative plasmids were separately constructed and cloned into E. coli TOP10, and transferred to the LAB hosts L. lactis LM2301 and the well-characterized laboratory strain E. faecalis JH2-2.

### Conditions for the evaluation of the promoter regions

The mCherry expression can be detected in different ways [20]. As a first indication, a distinct colour of the colonies on agar plates is observed according to the host used: the strongest colour appeared in E. coli, being paler in the LAB hosts indicating roughly the strength of the promoters cloned. This result was confirmed by microscopic analysis during both exponential and stationary phase

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Table 3. Nucleotide sequences of the predicted promoters from the as−48 gene cluster.

| Promoter | −35 | Spacing and TG motif | −10 | RBS and +1 |
|----------|-----|----------------------|-----|-----------|
| consensus | TTGACA | TATAAT | AGGAGG |
| P_A | TTGcat | CAAAATAAACTACATGGG | TATAAT | (30 nt)AGGAGGA(5 nt)ATG |
| P_C | TT(C) | GGGGATTTGTAAGG | TATAAT | (246 nt)AGGA(14 nt)ATG |
| P2(1) | TTGGaA | TAGGAACTATATTC | TATAAT | (56 nt)AGGAGGA(6 nt)TTC |
| P2(2) | T(A) | ATTTTTTTGTTTCA | TATAAT | (51 nt)AGGAGG(20 nt)ATG |
| P31 | TGGAG | AATATTTTGCAAA | TATAAT | (17 nt)AGGAGG (16 nt)ATG |
| P3(1) | TGACAT | ATATCCAGAAGGAGGAT | aATAAT | (108 nt)AGGAGG(9 nt)ATG |
| P3(2) | TTTT | TCTCCCCAT | TATAAT | (220 nt)AGGAGG(9 nt)ATG |

1The putative ribosome binding sites (RBS), TG motif and the distance to −10 boxes and +1 position are shown.

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(data not shown). However the most quantitative results were obtained with E. faecalis, L. lactis and E. coli transformants by measuring the simultaneous cell growth and fluorescence during prolonged cultivations using a microplate reader spectrophotometer. To optimize the cultural conditions, we assayed two chemically defined media (CDM-PC and -BP), a semi-defined complex medium (CM-G) specifically designed for AS-48 production and purification purposes, and two complex media (GM17 and LB). The basal arbitrary units of fluorescence (AU) detected before inoculation for CDM-PC (315.8), CDM-BP (316.3), CM-G (401.0), LB (695.8) and GM17 (2943.0), revealed that although CM-G had higher background fluorescence than the CDMs, this medium allows the growth of the different strains to higher OD. Therefore, we chose CM-G for the fluorescence assays.

Identification of functional promoters in the as-48 gene cluster

Taking together our results about the fluorescence emitted in CM-G medium by the transformants with the different promoter regions cloned (Figure 2), we can confirm the existence of PA and the suitability of the P2(2) proposed by Tomita et al. [6]. These two promoters are functional and active in E. coli and in the two lactic acid bacteria investigated. Apart from this, our results confirm the existence of the internal PD1 promoter suggested by Martínez-Bueno et al. [9]. The absence of fluorescence observed in the micrographs and during the growth curves, unequivocally confirms that those fragments cloned as PA, P2(2), P1(15) and P2(2) did not express the mCherry protein in any of the culture conditions assayed, being for this reason discarded (data not shown). PA, P2(2) and PD1 contain the typical 16 region, reinforcing the interest of this region for the promoter activity. The absence of P15 is in accordance with the expression pattern observed by Fernández et al. [10] for the as-48ABC operon. In this operon, the PX promoter controls the normal expression of as-48-ABC genes rendering the transcript T1 that undergoes a post-transcriptional processing, and arises two different transcripts Tα and TβC with distinct half-life and stability, ensuring the appropriate stoichiometry of the different gene products (Figure 1A). Additionally, the existence of the promoter P22 proposed by [6] would mean that bacC is an ORF shorter than as-48C and would not match with the typical DUF95 domain found in circular bacteriocin gene clusters.

The absence of mCherry expression driven from PX suggests that if P15 does not exist, the second and shorter mRNA (Tα, 5.4 kb) identified by Diaz et al. [5] that encodes the last four genes (as-48EFGH), could only be explained by transcription from the internal PD1 promoter here identified. This conclusion is in accordance with the loss of transcription observed in JH2-2 (pAM401g4) transformants where the as-48A-D1 genes were deleted [5]. These results also indicate that most likely there is no monocistronic mRNA encoding for As-48D1 as it was suggested by [9] and that the immunity determinants As-48D1EFGH are transcribed together since no P3 promoter could be associated to this region (Figure 2). This result is in accordance with the location of the colonies in solid media and the fluorescence of the cells observed in the fluorescence microscopy (data not shown). As expected, the transcriptional fission of PA with the mCherry gene displayed fluorescence in the LAB species, although surprisingly it was more efficient in lactococcal cells (633 AU versus 466 AU). Finally, the PD1 promoter shows a basal and maintained expression, of around 25% compared with that of P2(2) in LAB or 20% in case of E. coli. This expression level must be enough to ensure its protective functional role in the cells (Figures 2 and 3), together with the expression of the additional determinants As-48EFGH [3], and with As-48C [19], which contains a DUF95 domain recently suggested to be involved in both, production of and immunity, against the circular bacteriocin lactocycin Q [37]. In general, the level of fluorescence reached by LAB strains containing pTLR1-PD1 was 2-fold compared to pTLR1-PA and 4-fold compared to pTLR1-P2(2). Although the level of expression was lower in LAB strains than in E. coli, the ratio between each promoter was maintained. Our cumulative results indicate that the mCherry fluorescence increased in parallel with OD600 during the exponential phase of growth reaching the maximal values during the transition to stationary phase.

Induction of mCherry expression

It is likely that the promoters here identified could be regulated in the native E. faecalis S-48 strain by the presence of genes harbored in its genome or in pMB-2 (the native plasmid found in this strain) or even to be influenced by the presence of pAM401-81 (with only the as-48 gene cluster cloned). Furthermore, an adapted response to the cultural conditions cannot be discarded in whichever condition. To address these questions, we have designed different experiments in E. faecalis to compare the fluorescence emitted during the growth of the JH2-2 transformants containing PA, P2(2) or PD1 cloned into pTLR1, with that of the double transformants containing, additionally, either pAM401-81 or pMB-2, both of them compatible with pTLR1. In the results exposed in Figures 3 and 4, it could be observed that the presence of pAM401-81 or pMB-2 affects the expression of P2(2) and PA promoters (Figures 3 and 4). Thus, in presence of pAM401-81, P2(2) reaches values of 1065 AU at 22 h, which are higher (p = 0.018) than those obtained for the single JH2-2 (pTLR1-P2(2)) transformants, although the most noticeable result is the remarkably reduced fluorescence (p values between 0.004 and 0.000) repeatedly observed in presence of pMB-2. These results are also in accordance with the minor amounts of secreted AS-48 observed by JH2-2 (pMB-2) compared to that of JH2-2 (pAM401-81) transformants (Figure S1). In relation to PA, we found that the levels of fluorescence emitted by E. faecalis JH2-2 (pTLR1-P4) are slightly higher in presence of pAM401-81 but more reduced when pMB-2 is present, with significant differences after 14 h of growth (p value of 0.014) according to the statistical analysis performed.

Otherwise, the pH is an outstanding factor in LAB bacteria because of the production of lactic acid during the fermentative metabolism leads to the acidification of the media and the arrest of cell growth and, consequently, to the beginning of stationary phase. The influence of the pH in the production of different bacteriocins, including the circular sacitpeptide subtilosin A, has been reported [15,38–40]. Consequently, we have investigated the influence of the pH on the levels of mCherry expression relative to the cell mass, in the single and double transformants during prolonged cultivation in CM-G broth buffered at pH values of 6.0, 6.5, 7.0, 7.5 and 8.0. The growth curves showed a similar profile in all the conditions tested. The highest OD values were achieved at the highest starting pH of the culture, reaching the stationary phase between 6 h and 10 h after inoculation (Figures S2, S3, S4). In overall, the mCherry expression driven
from P_A and P_{2(2)} promoters confirmed that the highest expression levels are achieved at the high pHs (ca. 8) with significant results (p values of 0.000) from 16 and 18 h of growth (Figure 5 A and B), respectively, while P_{D1} seems to perform better at low pH (Figure 5C). Therefore, it is worth to emphasize that due to the presence of glucose in the medium the initial pH is only maintained during the first 6 h of growth and then, when the exponential growth commences, declined between 1.5–2 units in each case. These results justify the growth curves obtained and are in accordance with the importance of the pH stabilization at 6.5 during the growth as a key factor influencing the AS-48 production [41].

As above, the response of the as-48 promoters showed an improved expression of the mCherry protein in presence of pAM401-81, particularly at the most alkaline pH (Figure 5). The most outstanding result was once more detected with P_{2(2)} in the presence of pMB-2 (Figure 5B). Promoter P_{2(2)} controls the expression of the as-48C1D,DD,EFGH genes encoding two ABC transporters for secretion (as-48C1D) and self-protection (as-48EFGH), in addition to the immunity determinant (as-48D1) against AS-48. As it can be seen in Figure 5B, expression of mCherry from P_{2(2)} was retarded in E. faecalis JH2-2 (pMB-2) and the fluorescence levels were visibly lower at any pH assayed. These results, that have been several times repeated, confirm the above suggestion on the P_{2(2)} promoter, in the sense that it could be regulated in a negative fashion by genes existing in the native pMB-2 plasmid different from those of the as-48 cluster.

**Conclusions**

The current study analyses the functionality of seven promoter regions (namely P_A, P_C, P_{D1} and two regions for P_2 and for P_3) putatively involved in the full expression of the AS-48 character, which is dependent on the co-ordinated expression of the as-48ABC,CC1DD1EFGH genes. Identifying promoters in this locus is relevant to understand how AS-48 is produced, and how to engineer strains to more effectively produce AS-48. The corresponding amplified regions were cloned into the promoter-probe vector pTLR1 by transcriptional fusions with the mCherry gene. The fluorescence emitted by the transformants with the pTLR1-derivatives during a prolonged incubation in CM-G medium, allowed us to ratify the existence of the P_A promoter (driving the expression of the as-48ABC operon) and, more importantly, to definitively localize the P_{2(2)} promoter (involved in the transcription of the second operon as-48CC1DD1EFGH) and the internal P_{D1} promoter, presumably responsible for the transcription of the
last four genes (as-48EFGH) together with the immunity determinant as-48D1. The other promoter regions studied, included the P3 promoter reiteratively proposed [4–6,9], could be discarded for the absence of functionality in the current assay.

Remarkably, the strongest promoter of the as-48 cluster in LAB strains was the P2(2) promoter here identified, which seems negatively regulated by genes present in pMB-2 plasmid and up-regulated by high pHs and genes of the as-48 cluster, although having a strong basal activity in the absence of regulators. It is tempting to speculate with a membrane stress caused by AS-48 as the trigger for an increased transcription from P2(2) that could involve alternative extracellular function factors. This stress could be controlled in cells containing pMB-2 by additional mechanisms encoded in this plasmid whereas in the cells transformed with pAM401-81 this function is overtaken by the two ABC-transporters coded in the as-48C1DD1EFGH operon. Additionally, the pMB-2 plasmid is a pheromone-responding plasmid involved in conjugation process between enterococcal communities in natural environments. Cells harboring pMB-2 have the capability to elicit a complex response to sex pheromones secreted by the receptor cells, inducing coordinated responses among members of a community and resulting in the formation of cell aggregates that allow clumping of cells to facilitate efficient conjugal transfer of this plasmid. Expression of genes involved in secretion of the bactericidal AS-48 bacteriocin, rapidly kill conventional recipient enterococcal cells preventing the conjugation process, being a disadvantage relative to the transfer of the pMB-2-plasmid from bacteriocin-producing donors, as it has been already demonstrated in a recent study about the transferability of R-Plasmid in bacteriocin-producing Enterococcus coli donors [44]. Interestingly, PA and PD1 promoters have a strength

Figure 3. Maximal fluorescence values of the mCherry protein reached during prolonged growth in CM-G medium normalized by the OD 600 nm in single and double transformants bacteria, harbouring the functional derivatives pTLR1-PX (dark blue), pTLR1-PA (purple), pTLR1-P3 (sky blue) pTLR1-P01 (orange), and pTLR1d used as negative control (red). Standard deviation bars for the different replicates are included.
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that is one half and one quarter, respectively, compared to P2(2).
Both promoters drive a constitutive transcription in all the assay
conditions, although the presence of the as-48 genes and the pH
seem to enhance their expression.

We also conclude that the strength of the as-48 promoters is
organism dependent. Thus, the strength of all these promoters was
highest in E. coli, while in LAB strains only minor differences could
be observed. Surprisingly the three promoters of the as-48 cluster
perform slightly better in lactococcal cells (Figure 2), indicating
that this is not the reason that can justify the inability for the
heterologous expression of AS-48 described by Fernández et al.
[19] and supporting the idea of an incorrect processing of the
mRNA or an inefficient production of the modification machinery
involved in AS-48 maturation.

Supporting Information

Figure S1  Antibacterial activity of JH2-2(pAM401-81) and JH2-2(pMB-2) against JH2-2 used as indicator strain.
(TIF)

Figure S2  Influence of pMB-2 and pAM401-81 plasmids in the expression of the as-48 cluster promoters during prolonged growth in CM-G medium normalized by the OD 600 nm in E. faecalis JH2-2 containing the pTLR1-derivatives (low panels). The growth of cultures was monitored at a wavelength of 600 nm (upper panels). Fluorescence emission of mCherry was recorded at 620 nm after excitation at a wavelength of 590 nm (medium panels). pTLR1d (red), pLTR1-Pa (purple), pLTR1-P2(2) (sky blue), pLTR1-PD1 (orange). Standard deviation bars for the different replicates are included.
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Figure S3  Influence of pMB-2 and pAM401-81 plasmids in the expression of P2(2) promoter during prolonged growth in CM-G medium at different pH values normalized by the OD 600 nm in E. faecalis JH2-2 (pTLR1-P2(2)) (low panels). The growth of cultures was monitored at a wavelength of 600 nm (upper panels). Fluorescence emission of mCherry was recorded at 620 nm after excitation at a wavelength of 590 nm (medium panels). pH 6 (red), pH 6.5 (green), pH 7.0 (purple), pH 7.5 (sky blue), and pH 8.0 (orange). Standard deviation bars for the different replicates are included.
(TIF)
Figure 5. Expression of fluorescence of the mCherry normalized by the OD600 during prolonged growth in CM-G medium of *E. faecalis* JH2-2 containing the pTLR1-derivatives as indicated or double transformants containing pTLR1-derivatives and pMB-2 or pAM401-81 plasmids. Fluorescence emission of mCherry was recorded at 620 nm after excitation at a wavelength of 590 nm. The growth of cultures was monitored at pH 6 (red), pH 6.5 (green), pH 7.0 (purple), pH 7.5 (sky blue), and pH 8.0 (orange) at a wavelength of 600 nm. Standard deviation bars for the different replicates are included.

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Table S1  Sequence of promoter regions studied in this work. The predicted -10 and -35 sequences are underlined and depicted in bold.

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Author Contributions
Conceived and designed the experiments: MM MM-L. Performed the experiments: RC SR-R. Analyzed the data: MM RC. Contributed reagents/materials/analysis tools: EV. Wrote the paper: MM MM-L. Statistical analysis: MM-B.
