[Frontiers In Bioscience, Scholar, 10, 238-247, January 1, 2018]

Genetic organization of *Streptococcus salivarius* 24SMBc *blp*-like bacteriocin locus

Maria Santagati[1], Marina Scillato[1], Stefania Stefani[1]

[1]LabMMAR, Department of Biomedical and Biotechnological Sciences, Section of Microbiology, Via Santa Sofia 97, University of Catania, Italy

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1. ABSTRACT

In this paper, we describe, for the first time, the genetic organization of the *blpU*-like cassette in *Streptococcus salivarius* 24SMBc by entire genome sequencing. This strain has recently been found useful and widely applied as an oral probiotic in the prevention of recurrent otitis media. The 24SMBc *blpU*-like cassette is 8,023 bp in length, organized in 11 *orfs,* of which *orf* 8 encodes for the pore-forming peptide bacteriocin, belonging to class IIc, with a double-glycine leader peptide. The first characterization of *blp* locus was described in *Streptococcus pneumoniae,* showing a crucial role in interspecies competition within the nasopharynx. The salivarius *blpU*-like cassette is inserted upstream of the *pep*X gene in the chromosome. A hypervariable region between *pepX* and o*rf1* was found and used as a specific target able to distinguish *S. salivarius* 24SMBc from all other streptococci. All *orfs* carried by the *blp*-like cassette are functionally expressed (qPCR assays). Our results contribute to elucidate the microbial interactions in the nasopharynx, underlining the potential role of the *blp* locus in human nasopharyngeal colonization.

2. INTRODUCTION

S. salivarius, *S. vestibularius*, and *S. termophilus* make up the group of streptococcal salivarius, these are genetically similar species and are particularly important for humans (1). The first species is found only in humans and is predominant in the oropharyngeal and gastrointestinal tracts (2, 3, 4). In recent years, several publications have indicated *S. salivarius* as a microorganism acting positively on the oral and digestive tract ecology exerting its impact on human health through different effects on the stability of microbiota composition, generally categorized as: i) capability to adhere to epithelial cells of the host, interfering with surface colonization of other microorganisms thus limiting pathogen emergence, and ii) ability to apply a competition mechanism through the elaboration of ribosomally synthesized antimicrobial peptides, known as bacteriocins, able to compete for the same environment, both in inter- and intra-species ways (5,6). Bacteriocins (small antimicrobial peptides) have many properties that suggest their potential as an alternative to antibiotic therapy, possessing a different degree of potency, an antibacterial spectrum – both broad and narrow – the possibility to be delivered *in situ* and the possibility to be manipulated by bioengineering techniques (7,8).

In Gram-positive bacteria, there are two main classes of bacteriocins: i) lantibiotics (class I), which contain lanthionine and β-methil lanthionine

residue as well as dehydrated amino acids; and ii) unmodified bacteriocins (class II), ribosomally synthesized peptides with minor modifications except for cleavage of leader peptides during peptide export. This latter class of bacteriocins produces a prepeptide containing a leader peptide of 14-30 amino acids with a conserved processing site of two glycine residues. The heterogeneous nature of class II bacteriocins makes a rational classification difficult. However, this class includes five subclasses: IIa, pediocin-like bacteriocins; IIb, two-peptide bacteriocins; IIc (formally class V) cyclic bacteriocin; IId non-pediocin singular linear peptide and IIe, non-ribosomal siderophore-type post-translation modification at the serine-rich carboxy-terminal region (9,10). Class II bacteriocins of lactic acid bacteria (LAB) permeabilize the target cell membrane by the formation of poration complexes that leads to the dissipation of the proton motive force (8).

S. salivarius is one of the major bacteriocin producers among all lactic acid bacteria and has been proposed as a probiotic in various clinical applications (11). A recent review of the literature found 5 different formulations containing diverse bacteriocin-producing strains: with the only exception of the TOVE-R strain, which has an unknown bacteriocin, and the K58 strain, which possesses the pantothenate inhibitor enocin, all the others produced different types of bacteriocins belonging to the salivaricin group, namely A, B, A2, 9 and M (12). Salivaricin A2 and B were the first bacteriocins characterized in *Streptococcus salivarius* K12, localized on a large, transmissible plasmid responsible for its inhibitory activity (13,14). Salivaricin peptides also belong to a lantibiotic subclass of which the prototype is nisin (15), but it is an atypical lantibiotic since it contains no dehydrated residues in its biologically active propeptide form and shows bacteriostatic rather than bactericidal activity versus a target bacterium.

Our studies were focused on *Streptococcus salivarius* 24SMBc, selected from a healthy child for its probiotic characteristics and for its remarkable ability to interfere with URTIs, mainly AOM pathogens i.e. *S. pyogenes* and *S. pneumoniae* (11, 16, 17)

Previous studies failed in the identification of the bacteriocin produced by *S.salivarius* 24SMBc while this study aimed at characterizing this unknown bacteriocin through the sequence of its genome. A *blpU*-like bacteriocin, belonging to a class IIc type with characteristics similar to peptides produced by *S. pneumoniae* and *S. termophilus* (8, 19, 20) was found. In this paper we report the genetic characterization of the *blpU*-like bacteriocin cassette in *S. salivarius* 24SMBc, its expression by qRT-PCR and a strain-specific chromosomal marker useful for its detection among all streptococcal isolates conferring a strong diagnostic significance in terms of its detection and quantification also in biological samples.

3. Materials and methods

3.1. Bacterial strains and growth conditions

S. salivarius 24SMBc and all the strains used in this study were grown in Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) alone and/or with 1.5.% agar and 5% horse blood. Cultures were incubated overnight at 37 °C in 5% CO_2 in air atmosphere. All strains were frozen at –70°C in BHI broth (Oxoid, Basingstone, UK) with 20% glycerol until the time of their use.

3.2. Sequencing of the *blp*-like bacteriocin cassette and strain-specific targeting identification

The *S. salivarius* 24 SMBc genome was sequenced by using Illumina sequencing technology at BMR Genomics (Padova, Italy). A preliminary assembly of the genome demonstrated 375 contigs covering 1,893,903 bp (data not shown). During this preliminary analysis the *blpU*-like bacteriocin locus was identified. The locus sequence was determined through assembling neighboring contigs (contigs 00333-00334-00335-00336) by Long PCR using *Takara LA Taq* (Takara) (21) by MS482 (5'-CCAAATACCGTGTCATCACCAAA-3') located on the left junction and MS489 (5'-GGTGGCACTAGGTGTCTACCGC-3') at the right end. The strain-specific targeting sequence of 24SMBc *S. salivarius* was amplified using MS442 (5'-GCCCTAAGCCAAAGTCAGATGA-3') and MS443 (5'-GGTATGGCTCACCCTTTTATGTG-3'). All primers used in this study were designed by the Vector NTI software program. PCR and long PCR fragments were sequenced by direct automated methods (21).

To evaluate the specificity of the target and the lack of cross reaction with the other streptococcal species, 20 strains belonging to different streptococcal species i.e. *S. salivarius* (n.5), *S. mitis* (n.3), *S. oralis* (n.3), *S. sanguis* (n.3), *S. pneumoniae* (n. 2), *S. agalactiae* (n.2), *E. faecalis* (n.1), and *S. aureus* (n.1), were included. qPCR was performed using QuantiNova™ Probe PCR Kit mix in an AriaMx Real-Time PCR System (Agilent Technologies). Each qPCR mixture contained 0.4.μM of each primer and 0.2. μM TaqMan probe. Thermal cycler conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Each reaction was run in triplicate. Standard curves were generated by dilution series of purified DNA from 24 SMBc *S. salivarius* cells (10^7–10 genome copies).

3.3. Sequence analysis

The preliminary sequence analysis of the *S. salivarius* 24SMBc genome (data not shown) was performed by PRODIGAL (Prokaryotic Dynamic Programming Gene finding Algorithm; prodigal.ornl.gov) and MyRast server (Rapid Annotations using Subsystems Technology; http://blog.theseed.org/servers/). The software BLAST (22). was used to conduct homology searches of the GenBank database and the microbial genome databases available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) and at the WIT Computational Biology Group at the Argonne National Laboratory website (http://wit.mcs.anl.gov./WIT2/CGI). Protein domains were identified searching the protein family database (Pfam) available at the Wellcome Trust Sanger (http://pfam.xfam.org/).

3.4. RNA extraction and qPCR assays

For RNA isolation, *S.salivarius* 24SMBc was inoculated in BHI broth and incubated at 37 °C in 5% CO_2 overnight. Cells were harvested in two different phases: i) the stationary phase (12 h) at an optical density of 600 nm (OD_{600}) of 0.8.; ii) the exponential phase with an OD of 0.4. Total RNA was isolated from the cell pellets by an RNeasy kit (Qiagen, Valencia, CA, USA) treated with RNase-free DNase (Qiagen, Hilden, Germany), and eluted in RNase-free water (Ambion, Austin, TX, USA) according to the manufacturers' instructions. RNA concentration was determined spectrophotometrically and the quality was determined by analysis of the $A_{260/280}$ ratio. Contaminating genomic DNA was removed from each RNA sample using Turbo DNase (Ambion) and verified by PCR. In addition, each sample was analyzed at least three times.

3.5. RT-PCR and qRT-PCR

Total RNA (10 µg) was converted into complementary DNA (cDNA) using hex nucleotide primers ImPRO-II Reverse Transcriptase Kit (Promega) according to the manufacturer's instructions. Quantitative real-time PCR assays were performed using QuantiNova™ Probe PCR Kit mix in an AriaMx Real-Time PCR System (Agilent Technologies). Quantitative real-time PCR (qRT-PCR) was used to evaluate the expression level of all *orfs* (from *orf*1 to *orf*11) carried by the bacteriocin locus. The change in the quantification cycle (ΔC_q) of each sample was normalized by the *sod*A gene. TaqMan primers and probes were designed by Beacon Designer™ 8.0. and are listed in Table 1. Each qPCR mixture contained 0.4.µM of each primer and 0.2. µM TaqMan probe. Thermal cycler conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Expression analysis was evaluated as the increment/decrement (fold changes) of the *S. salivarius* strain at exponential phase versus the stationary phase. Each reaction was run in triplicate. For each analysis, three distinct biological replicates were carried out. Statistical expression analyses were performed using the Relative Expression Software Tool (REST) (23).

3.6. GenBank accession number

The nucleotide sequence of the *blp-U*-like of *S. salivarius* 24 SMBc has been submitted under accession number KY347796.

4. Results

4.1. Genetic organization of the blpU-like bacteriocin cassette

In order to characterize the streptococcal bacteriocin responsible for the 24SMBc antagonism activity, we sequenced the entire genome of this strain. A preliminary assembly demonstrated 375 contigs covering 1,893,903 bp, a GC content of 40.3.% and 1,736 coding sequences (data not shown). The genome sequence analysis was evaluated by Rast and Prodigal software to detect all coding sequences (CDS) present in the genome. We found only one *blpU*-like genetic locus of 8,023 bp carrying the *blpU*-like, which was involved in the bacteriocin production. In our strain, the *blp* cassette is organized in 11 *orfs*, as shown in Fig1(A) and function prediction of the eleven *orfs* is reported in Table 2. The *blp* cassette is inserted close to an adjacent gene (*pep*X) encoding for x-propyl-dipeptidyl aminopeptitase at 1,863,862 bp, as referred to the *S.salivarius* NCTC 8618 genome (GenBank accession no. NZ_CP009913.1.). The bacteriocin cassette in the 24SMBc strain shows a genetic structure similar to that of the other *blp* operon already well characterized in *S. pneumoniae* and *S. thermophilus* (18, 19, 20); particularly, it is made up of three main modules: i) the ABC-transporters that recognize the N-termini of bacteriocins and transport these peptides out of the cell with the cleavage of the double–glycine motif, ii) the bacteriocin immunity module to protect the producer bacteria from the effects of their own bacteriocins, and iii) the peptide pheromone with a typical leader peptide containing a double-glycine motif. *orf*8 (180 bp) appears to be a structural gene, *blpU*-like, encoding 59 amino acids and a pore forming peptide belonging to bacteriocin class II with a double-glycine leader that could be important for its processing to its shorter mature peptide form. The amino acidic analysis by the Pfam domains tool revealed that the *blp* peptide is part of the clan *GG-leader* CL0400 that carries a distinctive GG-cleavage motif including 5 families: bacteriocin_IIc, ComC, L_biotic_typeA, Antimicrobial17 and Lactococcin.

Table 1. Sequences of primers and probes used in this study

Target	Primer/probe name	primer and TaqMan probe (5' to 3')
orf*1*	*orf1*-up *orf1*-rew *orf1*-Tq	AGTGGAACAAGACAAAGATA TTGGGTGATAAAGAAATATGG FAM-TTAAGAAGACGCTATGGCTCACC- BHQ1
orf2	*orf2*-up *orf2*-rew *or2*-Tq	GCGTAAATTTGTTTAGGAGTA GGTTCTTCTTTCTTATTTTGG FAM-ACTGTCGTTCCTCAAGTGGAC- BHQ1
orf3	*orf3*-up *orf3*-rew *orf3*-Tq	CATCCTGCTTAAGATATAGG CGATGAAGTAATTGAAACTATC FAM-CGAGAGTCTGCAAGTTCCGTTC-BHQ1
orf**4**	*orf4*-up *orf4*-rew *orf4*-Tq	ATGGCTATAGGTCCTAAATG GGAGTAGTCATTGGGAATATAG FAM-TCTCTATCTAGTCAGCATCCAATCTGG-BHQ1
orf**5**	*orf5*-up *orf5*-rew *orf5*-Tq	GCAAGTAAGCTAGAAAATCA GTGCTTTGTGTAGAGTTTTAC FAM-ATACCACAGACCGCATAGCACT-BHQ1
orf**6**	*orf6*-up *orf6*-rew *orf5*-Tq	AGGCTATAAAGAAGCAAAG CCTCGCTAATCTATTTAGAAG FAM-AGTCCACCATACCATTAACTTGCTG-BHQ1
orf**7**	*orf7*-up *orf7*-rew *orf7*-Tq	TCGCTCCCATTTACTAAA CTAGCAATTTTCCTTTTAGG FAM-AAAGTGATTAATGCCAACCCTACCAAA-BHQ1
orf**8**	*orf8*-up *orf8*-rew *orf8*-Tq	GTAGCATAGCCAACTAAAGC GTGGATGGGTTAAGTGTTATG FAM-CACAATTGGCTCTGCTCTAGTAGGATC-BHQ1
orf**9**	*orf9*-up *orf9*-rew *orf9*-Tq	CTGACTATAATCGCGAATATC CCTTCTCCTATGATGGTAAG FAM-CACCGTCAAGAAGAACTCGTCCT-BHQ1
orf**10**	orf10-up orf10-rew or10-Tq	GACCATCTTGCATGACTAAG GACAGAACATGCTATTATTGAC FAM-CACCACCGCACTAAGACGATG-BHQ1
orf11	orf11-up orf11-rew or11-Tq	TGCCTCACTGAGATAGCGAC GCAGATACTTATCAAAGCGGAC GCCCTCTTTCTCGCATAATATCGGCATCA
sodA	*sodA-up* *sodA-rew* *sodA-Tq*	GCTTGTTGTTAACGATGA ACGGTAGTTTAGGTAGTAAG FAM-TTCAACTGCTAACCAAGACACTCCTAT-BHQ1

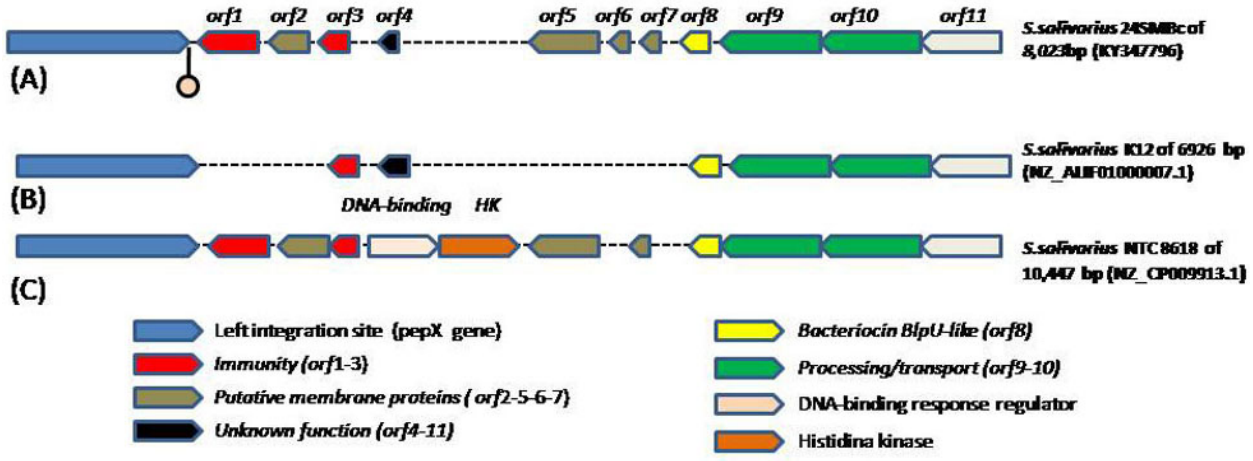

Figure 1. Genetic organization of the *blp*-like cassette of *S. salivarius* 24SMBc and comparison with the corresponding locus in other *S. salivarius* strains. (A) schematic representation of the *blp-U like* locus of *S. salivarius* 24SMBc, the presumed functional designations are indicated by the color of the ORF. The left integration site, pepx gene, is in the blue box. The DNA region between *pepX* and orf1 is a strain specific target (pink). (B) genetic organization of the blp locus of *S.salivarius* K12 (B) and NTC 8618 (C).

Table 2. Homologies of the *blp* cassette ORFs

ORF(aa)	Homologous gene (accession no)	Origin	Proposed function of gene product	Pfam domains	E-value	Amino acid identity
PepX (755 aa) Integration site	x-prolyl-dipeptidyl aminopeptidase (KEO44511)	*S. salivarius*	X-prolyl-dipeptidyl aminopeptidase	PepX_N (pfam09168) PepX_C (pfam08530)	0.0.	731/755 (97%)
orf1 (126 aa)	blpX_2 (CVX48085)	*S. pneumoniae*	Putative bacteriocin self-immunity protein		3e-77	116/126 (92%)
orf2 (108 aa)	putative membrane protein(CVX48107)	*S.pneumoniae*	Putative membrane protein		1e-50	94/104 (90%)
orf3 (98 aa)	EntA_Immun (CDF03895)	*S.salivarius*	Immunity protein	Enterocin A Immunity pfam08951	3e-62	96/98 (98%)
orf4 (59 aa)	hypothetical protein (ALR80594)	*S.salivarius*	Hypothetical protein		3e-26	51/54 (94%)
orf5 (133 aa)	hypothetical protein (AJFW01000020.1.)	*S.salivarius*	Putative integral membrane protein	Putative integral membrane protein (DUF2391)	3e-71	120/133 (90%)
orf6 (55 aa)	hypothetical protein (LRQS02000062.1.)	*S.salivarius*	Hypothetical membrane protein		4e-30	54/55 (98%)
orf7 (55 aa)	putative membrane protein (FDNI01000011)	*S.pneumoniae*	Putative membrane protein		3e-29	54/55 (98%)
orf8 (59aa)	bacteriocin class II with double-glycine leader peptide (JJM T01000019.1.)	*S.salivarius*	Pore-forming peptide, peptide (blpU –like)	COMC family; pfam03047 Bacteriocin_IIc pfam10439	8e-33	59/59 (100%)
orf9 (581 aa)	MdlB; multidrug ABC transporter ATP-binding protein (CP009913.1.)	*S.salivarius*	ABC-type multidrug transport system, ATPase and permease component	ABC transporter transmembrane region; pfam00664 ATP-binding cassette domain of glucan transporter, cd03254	0.0.	579/581 (99%)
orf10 (585 aa)	multidrug resistance-like ATP-binding protein mdlA (FR873481.1.)	*S.salivarius*	ATP-binding cassette domain of iron-sulfur clusters transporter, subfamily C	ABC transporter transmembrane region; pfam00664 P-loop containing Nucleoside Triphosphate Hydrolases; cl21455	0.0.	579/585 (99%)
orf 11 (150 aa)	hypothetical protein CP009913.1.	*S.salivarius*	Hypothetical protein		6e-105	144/148 (97%)

The multiple sequence alignment of the *blpU*-like peptide from three different strains, i.e. *S. salivarius* 24SMBc, *S. salivarius* K12 (NZ_ ALIF01000007.1.) and *S. salivarius* NCTC 8618 (NZ_ CP009913.1.) and *blp*O of *S.pneumoniae* (CEY55673) by *clustalW* showed the presence of a consensus and conserved region (amino acids 2 to 25) of a peptide belonging to the *com*C family (pfam03047) (Fig 2 A, B). It is worth stressing that *orf8* shared homology also with *S. pneumoniae,* which would explain the strong inhibitory activity of *S. salivarius* 24 SMBc against *S. pneumoniae*, thus mediating interspecies competition. In the region downstream of *orf8,* two transporter genes encoding for different multidrug ABC transporter binding proteins were found: *orf9,* showing 99% identity at the amino acid level to the MdlB protein (581aa) and *orf10,* homologous (99%) to the MdlA protein (585 aa), both ABC transporter ATP-binding proteins belonging to the super family SNT encoded for ABC-type bacteriocin/lantibiotic exporters, and contain an N-terminal double-glycine peptidase domain that could be involved in the export of drugs, peptides and many other compounds (24).

At the left end of *orf8*, two more *orfs* were found: *orf*1, encoding the *blpx*_2-like protein, and o*rf*3, belonging to EntA_Immun (pfam08951): both *orfs* are involved in a specific immune system responsible for

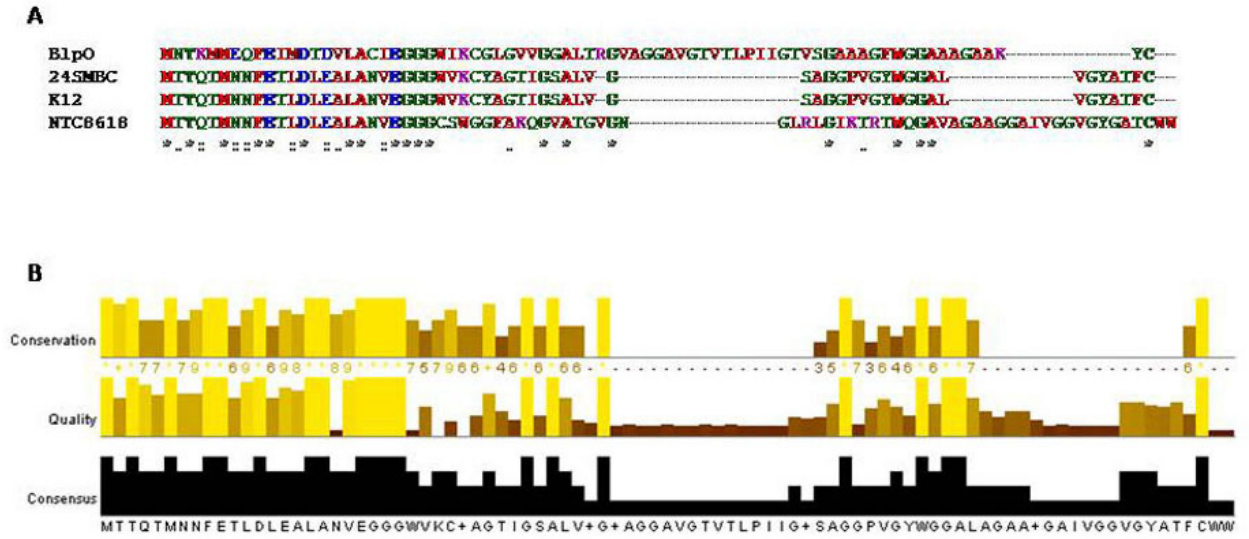

Figure 2. Amino acid alignment of putative bacteriocins by clustalW showing the homology between predicted structural peptides: *blpU*-like from *S. salivarius* 24SMBC, K12 and NTC8618 and *blpO S. pneumoniae* (A) proving the consensus and conserved region of a peptide belonging to the *com*C family (amino acids 2 to 25) (B).

the protection of the bacterium itself (shown in Fig 1 (A). Unlike other *blp* cassettes found in streptococcal strains, *S. salivarius* 24 SMBc lacks the histidine kinase genes of a two-component regulatory system controlled by a quorum-sensing mechanism.

From the few data published until now, the length of the *blp* bacteriocin gene cassette is variable in *S. salivarius* strains, ranging from 6.9. kb to 10 kb as shown in Fig1 (A, B, C) in which the genetic organization of the *blp*–cassette characterized in 24SMBc (8,023 kb in size) is compared with the other two *S. salivarius* strains, *S. salivarius* K12 (6,926 bp) and NTC 8618 (10,447 bp), whose genomes have been deposited in GenBank (*S. salivarius* 24SMBc bp accession n KY347796; *S.salivarius* K12 accession n NZ_ALIF01000007.1. and *S.salivarius* NTC 8618 accession n NZ_CP009913.1.). The genetic element of our strain appeared to be more related to the *blpU*-locus present in *S.salivarius* NTC8618 except for the absence of regulatory genes – DNA-binding response regulator and histidina kinase – however, *orf1,orf3, orf5, orf8, 9, 10* and *orf11* are conserved in all structures. The sequence analysis of the *blp*-structure identified the specific target that distinguishes our strain from other *S. salivarius* strains. This DNA sequence was 241 bp in size (from 2233 bp to 2504) located between the x-propyl-dipeptidyl aminopeptitase gene and orf1. To evaluate the inter- and intra-species cross reaction, 20 strains belonging to *S. salivarius, S. mitis, S. oralis, S. sanguis, S. pneumoniae, S. agalactiae, E. faecalis and S. aureus* were also included in this study.

Strain-specific targeting was evaluated by the absolute genomic copy number using high-quality standard curve parameters in terms of R2 (coefficient of correlation), M (slope) and E (efficiency) of ≥ 0.9.9, -3.1./-3.6., 90-100%, respectively. We found high sensitivity and specificity (from 84% to 100%), able to identify and quantify the presence of our strain among many different Gram-positive bacteria evaluated in three limits of detection (LOD): 10^4,10^3, 10^2 genome copies.

4.2. Expression study of ofrs carried by the blp-U cassette

The expression of all *orfs* present in the *blp* bacteriocin cassette, evaluated by qPCR at the exponential and stationary phases of growth, showed that all *orfs* were transcribed – at different levels – in both growth phases. The relative quantitative expressions of *orfs* are shown in Fig3 expressed as fold changes of the exponential phase compared with the stationary phase. It is noteworthy that only two *orfs*; *orf*1 and 5, encoding, respectively, for bacteriocin self-immunity and integral membrane proteins, appeared to be unregulated in the stationary phase with respect to other *orfs* that showed a high transcription level during the exponential phase.

5. Discussion and Conclusion

blp bacteriocin cassettes provide an interesting mechanism for competition among different streptococcal species including pathogens and commensal strains, contributing to the changing

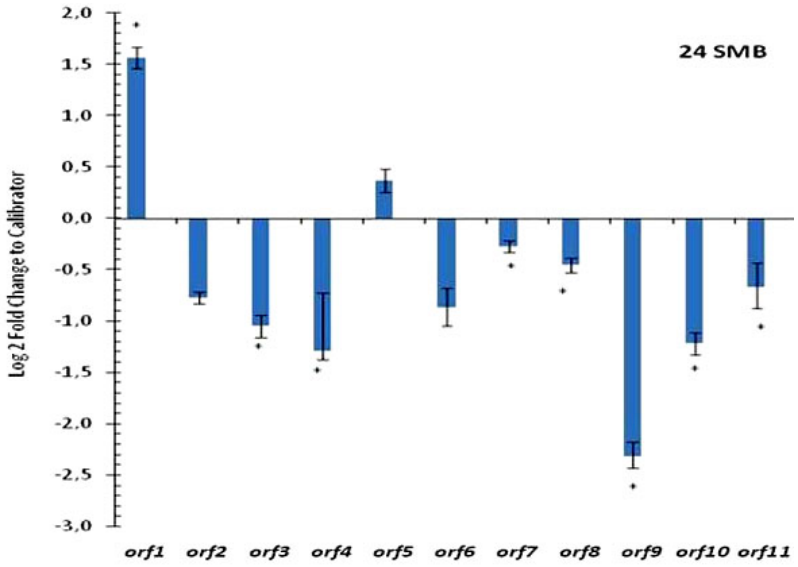

Figure 3. Relative quantitative expression of all *orfs* characterized in the 24SMBc strain evaluating the expression level in overnight culture versus exponential growth.

microbial environment of the nasopharyngeal microbiota. To date, this locus has been characterized in detail only in *S. pneumoniae* and *S. thermophilus* showing a high heterogeneity between isolates as demonstrated by the available genome sequences. This phenomenon suggests that there is an ongoing positive selective pressure exerted on this locus so that it always remains effective and beneficial to the success of the organism (18, 19, 25). This work describes, for the first time, the genetic organization of the *blpU-like* bacteriocin cassette in *Streptococcus salivarius* 24SMBc and its genetic expression. The cassette contains the genes required for inhibitory activity against *S.pneumoniae, S.pyogenes* and other closely related Gram-positive bacteria. This genetic structure shows strong similarity to other *blp* operons already characterized in *S.pneumoniae* and *S.thermophilus,* having the same genetic organization except for the HK regulator and response systems that are absent in our strain. The genome sequence analysis of *Streptococcus salivarius* 24SMBc showed the presence of only one locus involved in bacteriocin production, characterized by carrying *orf8* encoding for a pore forming peptide belonging to bacteriocin class IIc. Furthermore, in our strain other bacteriocin producing loci were absent, such as those belonging to the group of salivaricins commonly described in *S.salivarius*. Our data, for the first time, highlight the functionality of the *blp* locus present on the *S.salivarius* genome that could be masked by the strong activity of other bacteriocins, such as S. *salivarius* K12. (14).

Using real-time gene expression, it was found that all *orfs* are expressed during the exponential phase while only *orf* 1 and 5, encoding respectively for bacteriocin self-immunity and integral membrane proteins, appeared to be upregulated in the stationary phase with respect to other *orfs.* Further expression data are necessary to determine exactly what factors stimulate over expression of bacteriocin self-immunity at the stationary phase considering that this cluster appears to not be regulated by a classic quorum sensing two-component regulatory system lacking histidine-kinase and regulatory proteins (BlpH-R) in 24SMBc. It could be argued that the increased expression of the proteins involved in the immune system at the late phase could ensure increased protection against the strain's production of bacteriocin. The studies to understand the cause of such changes of gene expression in relation to growth stages are ongoing in our lab.

Since the left end of the *blp*-cassette showed DNA variability among *S.salivarius* strains, this DNA region was used by qPCR as a strain specific genomic marker capable of discriminating the 24SMBc strain from other streptococcal strains. We also demonstrated the high sensitivity and specificity of this genomic target against different strains and species i.e. *S.salivarius*, *S.mitis*, *S.oralis*, *S.sanguis, S.pneumoniae*, *S.agalactiae*, *E.faecalis* and *S.aureus,* showing the lack of inter- and intra-species cross reaction up to 10^3 and 10^4 genome copies. The use of a strain specific qPCR assay for probiotic strains, as well as for 24SMBc, which is already being used as an oral probiotic, can provide probiotic strain monitoring, by detection and quantification in various sources including biological samples, mixed cultures or environments harboring hundreds of species.

It is remarkable that there are many genetic methods available to successfully identify one strain

or probiotic bacteria from among other strains, such as randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis, PCR amplification of repetitive bacterial DNA elements (rep - PCR) or ribotyping (26). However, all these assays do not allow rapid detection and quantification of specific strains in different samples and, furthermore, they are applied to pure isolates or DNA extract from pure culture and the main problem in various studies is to evaluate the efficiency of probiotic strains by bacterial detection/ quantification in biological samples. In this context, our strategy could detect the probiotic strain and measure its efficacy and effectiveness, which are required to prove that these strains can confer specific disease reduction or clinical treatment benefits.

It is well known that bacteriocins play a fundamental role in the intra- and inter-species competition between the normal oral streptococcal species and pneumococci, contributing to maintain the complex equilibrium between a healthy state and the progression toward disease within the normal range (20, 27) *Streptococcus salivarius* species is one of the pioneer strains, colonizing the oropharyngeal and gastrointestinal tracts in newborns, remaining predominant throughout the human life span, and capable of improving health-associated oral and gut microbiota just by interfering with potential pathogens (28).

Many studies have highlighted the close correlation between the reduction of potential pathogens and the presence of commensal streptococci. The alteration of the nasopharyngeal microbiota and the absence or reduction of α-streptococci may correlate with the pathogenesis of URTIs and, in particular, with recurrent acute otitis media (rAOM), acting as a reservoir for mainly respiratory pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (29, 30).

In conclusion, our probiotic strain, *S.salivarius* 24SMBc, harbors a *blp*-like bacteriocin carrying a cassette with high genetic variability, similar to constructs found in other streptococcal species, i.e. *S.pneumoniae* and S.*thermophilus*. Furthermore, all genes are expressed in this strain in both the exponential and stationary phase of growth even if they lack the regulator system found in *S.peneumoniae*. Our data on the *blp* cassette of *S.salivarius* can provide additional information about intra- and inter-species competition altering and/or rebalancing the nasopharyngeal microbiota.

4. Acknowledgements

We thank the Scientific Bureau of the University of Catania for the language support. This study was supported by a grant from DMG Italia and FIR2014(A4CA57).

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Key Words: *blp*-like cassette, *S. salivarius*, Bacteriocin, Antagonist Activity

Send correspondence to: Maria Santagati, LabMMAR, Department of Biomedical and Biotechnological Sciences, Via Santa Sofia 97, University of Catania, Tel 39 –95-4781245, E-mail: m.santagati@unict.it