AMB Express

ORIGINAL ARTICLE Open Access

Heterologous expression of lasso peptides with apparent participation in the morphological development in *Streptomyces*

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Abstract
Lasso peptides, ribosomally synthesized and post-translationally modified peptides, are primarily produced by bacteria and some archaea. *Streptomyces* lasso peptides have been known for their antimicrobial, anticancer, and antiviral properties. However, understanding their role in the morphology and production of secondary metabolites remains limited. We identified a previously unknown lasso peptide gene cluster in the genome of *Streptomyces* sp. L06. This gene cluster (LASS) produces two distinct lasso peptides, morphosin-1 and −2. Notably, morphosin-2 is a member of a new subfamily of lasso peptides, with BGCs exhibiting a similar structure. When LASS was expressed in different *Streptomyces* hosts, it led to exciting phenotypic changes, including the absence of spores and damage in aerial mycelium development. In one of the hosts, LASS even triggered antibiotic formation. These findings open up a world of possibilities, suggesting the potential role of morphosins in shaping *Streptomyces*' morphological and biochemical development.

Keywords Lasso peptide, *Streptomyces*, Morphology, BGCs, RiPPs

Introduction

Peptides are a promising avenue for developing new drugs due to their specificity to biological targets and potential for redesign through chemical synthesis or genetic engineering (Rima et al. 2021). Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a diverse group of natural products with broad chemical diversity and bioactivity (Cao et al. 2021). Lasso peptides represent a subset of RiPPs produced by bacteria and some archaea. They feature an N-terminal macrolactam ring formed by an isopeptide bond between the α-amino group of the first residue and the carboxyl group of the side chain of an aspartate or glutamate (Delgado et al. 2001; Adelman et al. 2004; Kodani et al. 2020). The C-terminal end of these peptides is threaded into the ring and held inside by the steric hindrance generated by bulky amino acids and/or disulfide bridges, creating a unique three-dimensional structure (Fig. 1). This fold provides stability to several lasso peptides against various proteases and temperatures up to 120 °C (Hegemann et al. 2015; Hegemann 2020). Lasso peptides are classified into 4 distinct classes based on the number and position of their disulfide bridges. Class II peptides have no

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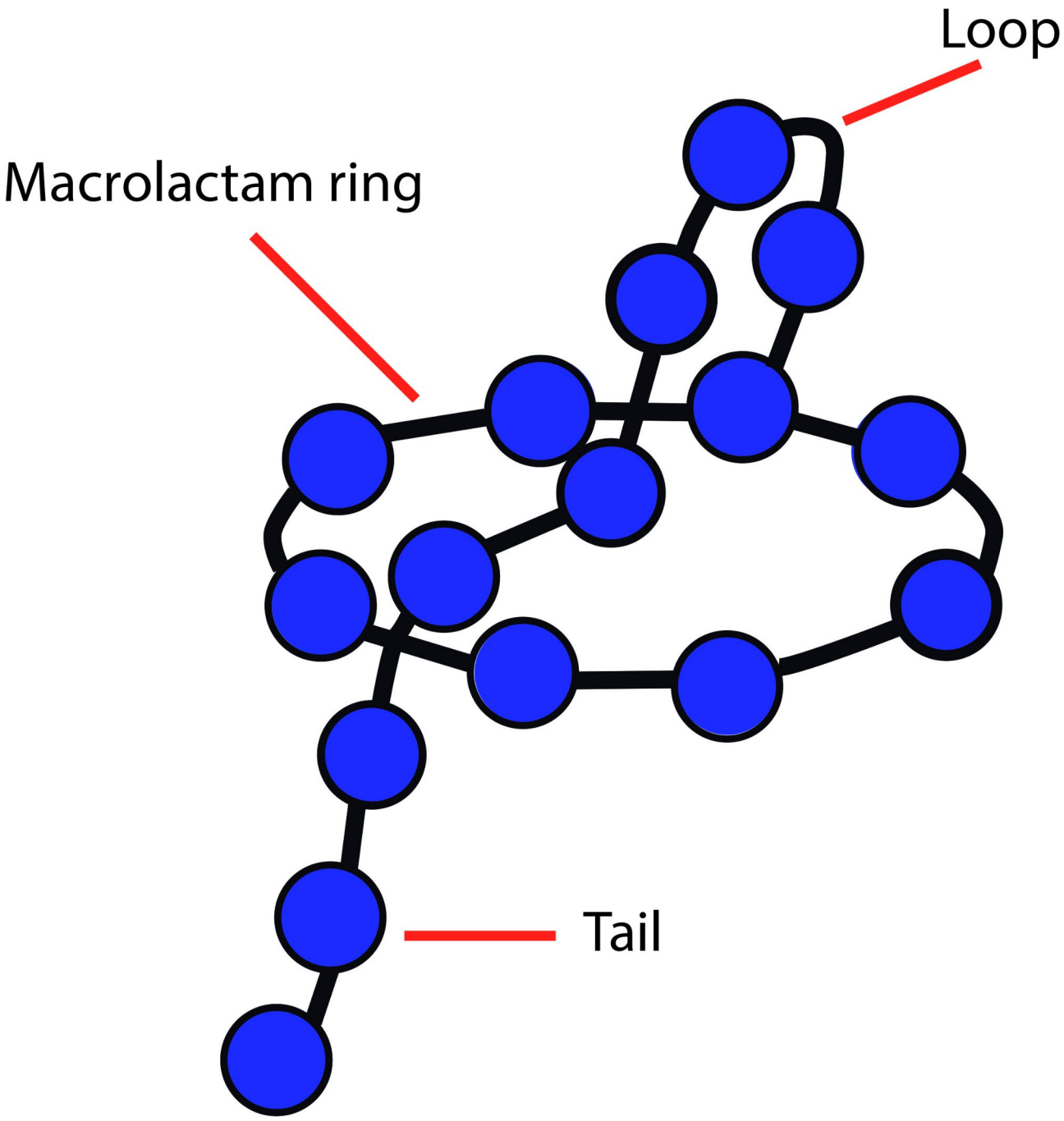

Fig. 1 Schematic representation of a lasso peptide

disulfide bonds and are the most abundant (Tietz et al. 2017; Hegemann et al. 2020).

The biosynthetic gene cluster (BGC) that gives rise to lasso peptides generally comprises a core of three genes. A gene (A) codes for a precursor peptide with a leader sequence and a core sequence (structural sequence), the RiPPs recognition element (RRE) and leader peptidase are encoded as two separated proteins by the genes B1 and B2, respectively, or as a single protein by the gene B1/B2, the gene (C) encoding a lasso cyclase, homologous to asparagine synthetase, which catalyzes the formation of an isopeptide bond to form the macrolactam ring. Sometimes, these BGCs contain additional genes encoding isopeptidases, ABC transporters (D), and other secondary post-translational modification enzymes (Maksimov et al. 2012; Zhu et al. 2016). It is thought that peptides originating from BGCs with ABC transporters could act outside the producing cell due to their requirement to be

exported (Hegemann et al. 2015; Bountra et al. 2017; Beis et al. 2019; Smits et al. 2020).

Of the nearly 80 lasso peptides isolated, at least 30 have reported biological activity (Kodani et al. 2020). Notably, these peptides exhibit antimicrobial activity against both Gram-positive (Metelev et al. 2015; Gavrish et al. 2014; Tan et al. 2019) and Gram-negative bacteria (Delgado et al. 2001; Adelman et al. 2004; Kuznedelov et al. 2011; Metelev et al. 2017). Other lasso peptides have antagonist effects against receptors such as the glucagon receptor (Knappe et al. 2010), type B endothelins (Morishita et al. 1994), or the atrial natriuretic factor (Weber et al. 1991). In addition, peptides with anticancer (Um et al. 2013; Elsayed et al. 2015; Son et al. 2018; Guerrero-Garzón et al. 2020) and antiviral properties have been isolated (Helynck et al. 1993; Tsunakawa et al. 1995; Kaweewan et al. 2018). Knowledge about the ecological role they play in nature is scarce; it has been shown that Microcin J25, the lasso peptide most studied, participates in interspecies competition in the microbial community of the gut, where it was first isolated (Li and Rebuffat 2020; Baquero et al. 2024). However, as far as we know, nothing has been established about lasso peptides' participation in physiology, morphology, and the production of secondary metabolites.

The *Streptomyces* genus has a peculiar life cycle involving vegetative mycelia, aerial hyphae, and spores. Some subsets of RiPPs have been reported to mediate the morphological development in *Streptomyces*. Thus, the lanthipeptides SapB and SapT from *Streptomyces coelicolor* and *Streptomyces lavenduligriseus* Tu 901 are involved in the emergence of aerial hyphae (Kodani et al. 2004, 2005; Sarksian et al. 2022). The class III lanthipeptide AmfS, a SapB counterpart, from *Streptomyces griseus* has a morphogenic activity (Takano et al. 2017).

In the genome of *Streptomyces* sp. L06, we discovered a gene cluster that codes for two lasso peptides, morphosin-1 and −2. Subsequently, these peptides were heterologously produced, and tests were conducted to study their chemical and biological properties. Morphosin-2 is a member of a new lasso peptide subfamily that has not been previously characterized. We observed that the expression of both lasso peptides harmed the morphological differentiation of three different *Streptomyces* species used for their expression.

Materials and methods

Bacterial strains

Streptomyces sp. L06 was previously isolated from *Amphyterigium adstringens*, and deposited in the UNAM-48 WFCC culture collection under accession BM-B-601 (Mexico City) (Caicedo-Montoya et al. 2021). *Streptomyces lividans* TK24 (Rückert et al. 2015), S. *coelicolor* M1152 (Gómez-Escribano and Bibb, 2011), and *Streptomyces avermitilis* SUKA22 (Komatsu et al. 2013) were used as hosts for heterologous expression.

For plasmid propagation, *Escherichia coli* DH5α was selected. *E. coli* ET12567/pUZ8002 was used as a DNA donor in the conjugation process with *Streptomyces* strains. The *E. coli* strains were grown overnight in LB medium at 37 °C at 200 rpm (Gust 2009).

Sporulation

Spores of *S. coelicolor* M1152 and *S. lividans* TK24 were obtained in plate cultures of the MS medium (Kieser et al. 2020), while *S. avermitilis* SUKA 22 sporulated in the YMS medium (Ikeda et al. 1987). All strains were spread onto their respective sporulation media and incubated at 29 °C for at least 5 days, during which spores matured. *Streptomyces* strains were stored as spore suspensions in 20% (v/v) glycerol at -20 °C, as previously described (Rocha-Mendoza et al. 2021), or mycelium suspension in 25% (v/v) glycerol at -80 °C (Shepherd et al. 2010).

Bioinformatics analysis

The LASS BGC of *Streptomyces* sp. L06 was identified using AntiSMASH 7.0 (Blin et al. 2023). We performed alignments of each biosynthetic enzyme within the BGC using Muscle (EBI) (Madeira et al. 2022) to confirm the presence of the preserved residues required for their proper function. We used the two precursor peptides as the query sequence for two individual searches in BLAST-p against the NCBI database of non-redundant protein sequences. All hits were considered potential precursor peptides. To check whether the BLAST-p hits were part of the lasso peptides BGC, we analyzed the genomes available in NCBI GenBank from each hit using antiSMASH 7.0. We also manually analyzed the genes flanking the core region of the BGC to verify their association with other genes.

Sequence Similarity Networks (SSNs) analysis of all precursor peptides and of lasso cyclases present in the LASS-BGCs were generated using Enzyme Similarity Tool (ESI-EST) setting the alignment score threshold 11 and 100, respectively. Both of SSN were visualized with Cytoscape.

Construction of the expression plasmid of the BGC of morphosins

The *Streptomyces* sp. L06 genomic DNA (gDNA) has been extracted according to Caicedo-Montoya et al. (2021). The gDNA was used to amplify a 4,832 bp fragment containing the LASS BGC genes *lassA1*, *lassA2*, *lassC*, *lassB*, and *lassD*. The GC-RICH PCR system (Roche, USA) was employed to amplify this fragment with FW/RV_LassAABCD- primers (Table 1). A *Nde*I cleavage site was introduced into the first forward primer, and an *Eco*RI site was introduced into the reverse

Table 1 Oligonucleotide primers used in this study. All sequences are provided in the 5´ to 3´ directions. Restriction sites are underlined

Name	Sequence
Fw_LassAABCD_*Nde*I	GAA*CATATG*ACGCCGGAGATCCACGACG
Rev_LassAABCD_*Eco*RI	GTG*AATTC*GGTGGAGGACTACTACGCGGTC
Seq-Lass 1 F	GTCATCTCGTTCTCCGC
Seq-Lass 2 F	CGTGTGGGCGGACTCAG
Seq-Lass 3 F	GCCGTACTTCAGCCACC
Seq-Lass 4 F	CAACCGCCACCTCAAAG
Seq-Lass 5 F	GATTGCCTGCGGTGTTGTG
Seq-Lass 6R	CGCCTGCATGGAAATC
Seq-Lass 7R	CGTCATGGTCGTCGCAC
Seq-Lass 8R	GAGGATCTGACCGACG
Int-Lass_*sco*4848	CGTCGTATCCCCTCGGTTG
Int-Lass_pIJ10257	GAGCCGGGAAAGCTCATTCA

primer. The amplicon was cloned into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific, USA), to create the TOPO-LASS vector. LASS, derived from the TOPO-LASS vector, and the expression vector pIJ10257 were digested the *Nde*I and *Xho*I restriction enzymes and then purified using QIAGEN's gel extraction kit. Both were linked employing T4 DNA ligase (New England BioLabs, USA). The ligation reaction was transformed into *E. coli* DH5α cells and transformed colonies were selected with hygromycin (Thermo Fisher Scientific, USA) (50 µg/ml). The final construction, pIJ10257-LASS, was confirmed by sequencing with Seq primers at the Sequencing Unit from our Institute (Table 1).

Heterologous production of morphosins

The construct pIJ10257-LASS was transformed into the non-methylating strain *E. coli* ET12567/pUZ8002, to conjugate with expression hosts, following the method described by Kieser et al. (2000). For conjugation and subsequent analysis of lasso peptide production, the empty plasmid pIJ10257 under the control of the constitutive promoter ermE, was used as a control. Hygromycin (Sigma-Aldrich, USA) was added at a 50 µg/ml concentration and was used as a selection antibiotic for recombinant *Streptomyces* strains. The integration of LASS within the genome was verified by PCR using the IntLASS primers (Table 1). The three *Streptomyces* strains used as expression hosts were grown in their respective sporulation media at 29 °C and monitored for 7–10 days. The control strains were an unmodified strain and a strain with the empty vector pIJ10257.

To prepare a seed culture of each *Streptomyces* strain containing pIJ10257-LASS, 50 ml of YMG medium supplemented with the selection antibiotic was inoculated with a single colony of each organism in a 250-mL baffled flask and incubated at 200 rpm at 29 °C for 3 days. The seed cultures were then used to inoculate 50 ml of YMG medium. The fermentations were carried out for 7 days at 200 rpm at 29 °C; then, cells were harvested by centrifugation (4,000 rpm for 10 min.). The cell pellets were washed with distilled water and extracted with 30 mL methanol by agitation overnight at room temperature. The methanolic extracts of the mycelium were clarified by centrifugation, evaporated at reduced pressure at 40 °C, and resuspended in 60% acetonitrile. Diaion HP20 resin (3 g) (Sigma-Aldrich, USA) was added to the supernatants of the previous cultures and incubated under agitation (1 h at room temperature). The resin was recovered by filtration and washed with distilled water. Supernatant extracts were eluted from the resin with 30 mL methanol before evaporating under reduced pressure at 40 °C and resuspended in 250 µL 60% acetonitrile.

Antimicrobial activity

As previously reported (Balouiri et al. 2016), the agar diffusion method was used to test the antibacterial activity of supernatant and mycelium extracts against *Bacillus subtilis* and *Micrococcus luteus.* For the negative control, 60% acetonitrile was employed. Each extract was used at a concentration of 20 µg/ml.

Effect of supernatant extract containing morphosins on the morphological development of *Streptomyces*

The anti-sporulation activity was assessed following the method outlined by Strahight et al. (2006) with some adjustments. A 1:10 dilution of a spore suspension of Streptomyces strains carrying only pIJ10258 (1×107) was spread as 100 µl on the MS or YMS agar surface in a Petri dish. The culture was then incubated at 29 °C for 48 h until the vegetative mycelium developed. Following this, supernatant extracts containing morphosins were applied to the mycelium. After applying the morphosins sample, the culture was incubated for 48 h to observe the development of *Streptomyces*. Supernatant extracts from *Streptomyces*-pIJ10257 and unmodified *Streptomyces* strains were used as controls. These extracts were obtained as from 250 mL bacterial cultures and suspended in MESS buffer before application.

UPLC/MS analysis

All reagents used in this assay were UPCL/MS grade (J. T. Baker Chemicals USA). 10 µl of each concentrated extract was taken and analyzed using a UPLC/MS AQUITY Xevo G2XSTO (Waters, Mexico). An ACQUITY UPLC C18 BECH 130 A column, 1.7 µm, 2.1 mm x 50 mm (Waters, Mexico), was used at a 300 µl/min flow rate. The gradient was performed using solvent A (water/0.1% formic acid) and solvent B (acetonitrile /0.1% formic acid). The process started with 5% solvent B and was followed by a linear increase of 5–60% solvent B from 1 to 10 min post-injection. From 10 to 11 min, 60-95% solvent B was

used, followed by a linear decrease to 5% solvent B from min 11 to 16 min. The UPLC/MS data was recorded in positive ion mode in a m/z 100-3,000 mass range. The capillary voltage was 3 kV, and the cone voltage was 40 V. The source temperature was 180 °C, and the desolvation temperature was 400 °C. A dry gas flow used was 800 L/h.

Results

Bioinformatics analysis

The genome of *Streptomyces* sp. L06 contains a lasso peptide BGC cluster, which encodes two different lasso peptides, namely morphosin-1 and morphosin-2. These peptides can form a macrolactam ring between G1-E8 and G1-D7 or G1-D9. The cluster also includes a lasso cyclase (*LASSC*), a RiPP recognition element, a peptidase domain (*LASSB*), and a peptidase domain (*LASSB1/B2*, and an ABC-like transporter (*LASSD*) (Fig. 2A and B).

To determine if this cluster is present in other genomes, the sequences of the precursor peptides were used as a Blast-P query against the non-redundant protein sequence database in NCBI. The morphosin-1 analysis did not yield homologous. The morphosin-2 search generated 47 hits with a percentage of identity > 40%. All the resulting sequences belong to the genus *Streptomyces*. Manual inspection of the genetic context of each hit confirmed that most of them are precursor peptides encoded in a lasso peptide BGC (LASS BGCs). A total of 27 putative sequences of lasso peptides homologous to morphosin-2 were identified in 40 different *Streptomyces* strains. The core sequence of morphosin-2 and its 27 homologous sequences were aligned to visualize conserved

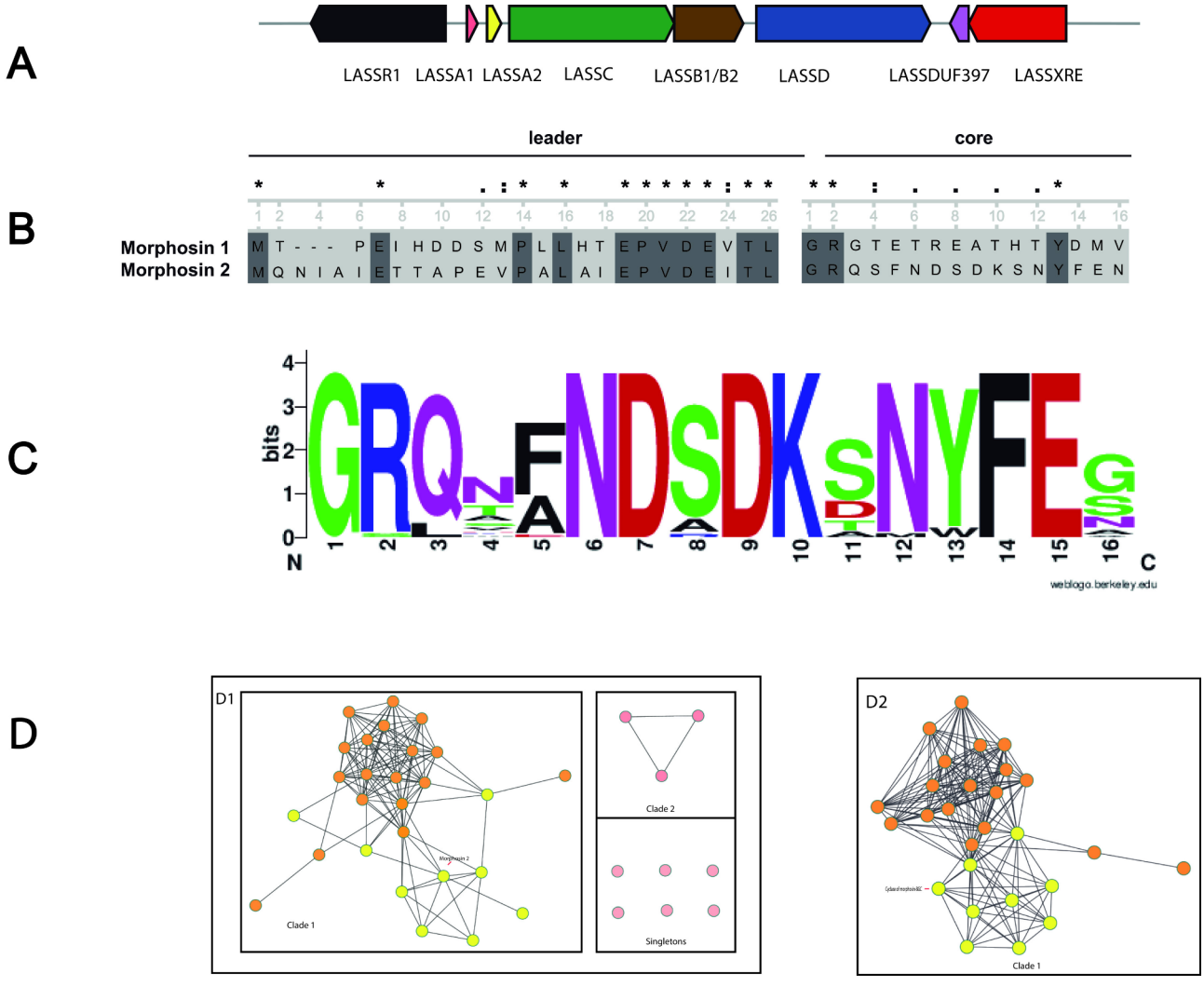

Fig. 2 **(A)** Morphosins BGC from *Streptomyces* sp. L06 and sequence alignment of the precursor peptides. **(B)** Sequence logo of morphosin-2 family with 27 members. **(C)** Sequence similarity network analysis of all precursor peptides (D1) and lasso cyclases (D2) contained in LASS BGCs. Orange nodes represent sequences that are included in LASS BGCs with only one peptide precursor. The sequences that are included in LASS BGCs with more than one precursor peptide are represented by the yellow nodes. The pink nodes indicate the precursor peptides that do not have homology with morphosin 2

motifs. We found that the sequences are highly similar, with a motif sequence (GRX3NDX2DKXNYFEX), as shown in Fig. 2C.

The SSNs analysis of precursor peptides showed that all homologous to morphosin 2 formed a clade. The precursor peptides found in the BCGs containing more than one precursor peptide were identified as the closest neighbors to morphosin 2. Three out of the nine precursor peptides unrelated to morphosin 2 were clustered together, while the rest, including morphosin 1, appeared as individual sequences. The analysis of lasso cyclases showed a similar pattern, with only one cluster being formed. The cyclases from the LASS BGCs with multiple peptide precursors were identified as the closest neighbors to the cyclase in morphosin BGC (Fig. 2D).

The variation between BGCs is mainly due to the presence or absence of secondary modification enzymes, especially acetylornithine deacetylase, and the number of precursor peptides in addition to those homologous to morphosin-2. Another factor is whether the lasso peptide RRE is present as a discrete protein (LASSB1) or fused to the leader peptidase (B1/B2). We found several BGCs with up to 2 more precursor peptides. In certain LASS BGCs, the core genes were flanked by two regulators: a regulator of unknown function (LASSR) downstream and a xenobiotic response element (XRE) followed by a small protein with a DUF397 domain (LASSXRE/LASS-DUF397), upstream (Fig. 3).

Effect of morphosins expression and supernatant extract containing morphosins on morphological development of *Streptomyces*

The morphosin BGC (LASS) was expressed in three different strains of *Streptomyces* (none of which contain a morphosin-like BGC), using the pIJ10257 vector. This plasmid integrates into the single integration site of phage ΦBT, located in the *sco*4848 gene of *S. coelicolor* and its orthologs in other *Streptomyces* species (Gregory et al. 2023). The recombinant strains correctly integrated the pIJ10257-LASS or pIJ10257 (control strain) construct. Differences in phenotype were observed between strains containing LASS and those with unmodified or empty vectors. The latter two showed equivalent development. Strains containing LASS exhibited a phenotype commonly associated with defects in the formation of spores and aerial mycelium (Fig. 4). This was observed as white colonies with a fuzzy appearance. The same figure indicates that *S. lividans* TK24, containing the LASS region, initiated actinorhodin production, in contrast to the non-conjugated strain or the one with the empty vector.

To further support that morphosins could affect the morphological development of *Streptomyces*, we applied

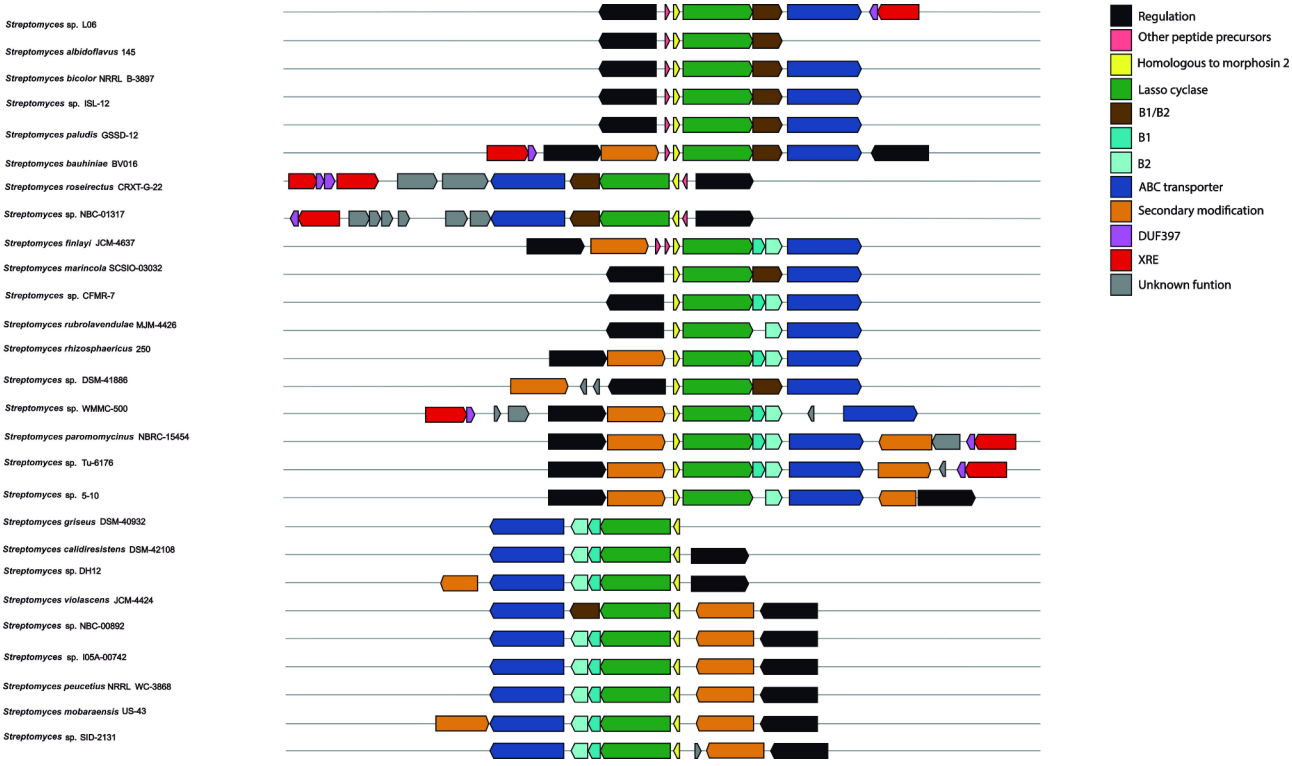

Fig. 3 Comparison of the LASS BGC carrying a peptide precursor homologous to morphosin-2. Arrows indicate genes. Color codes are described in the key

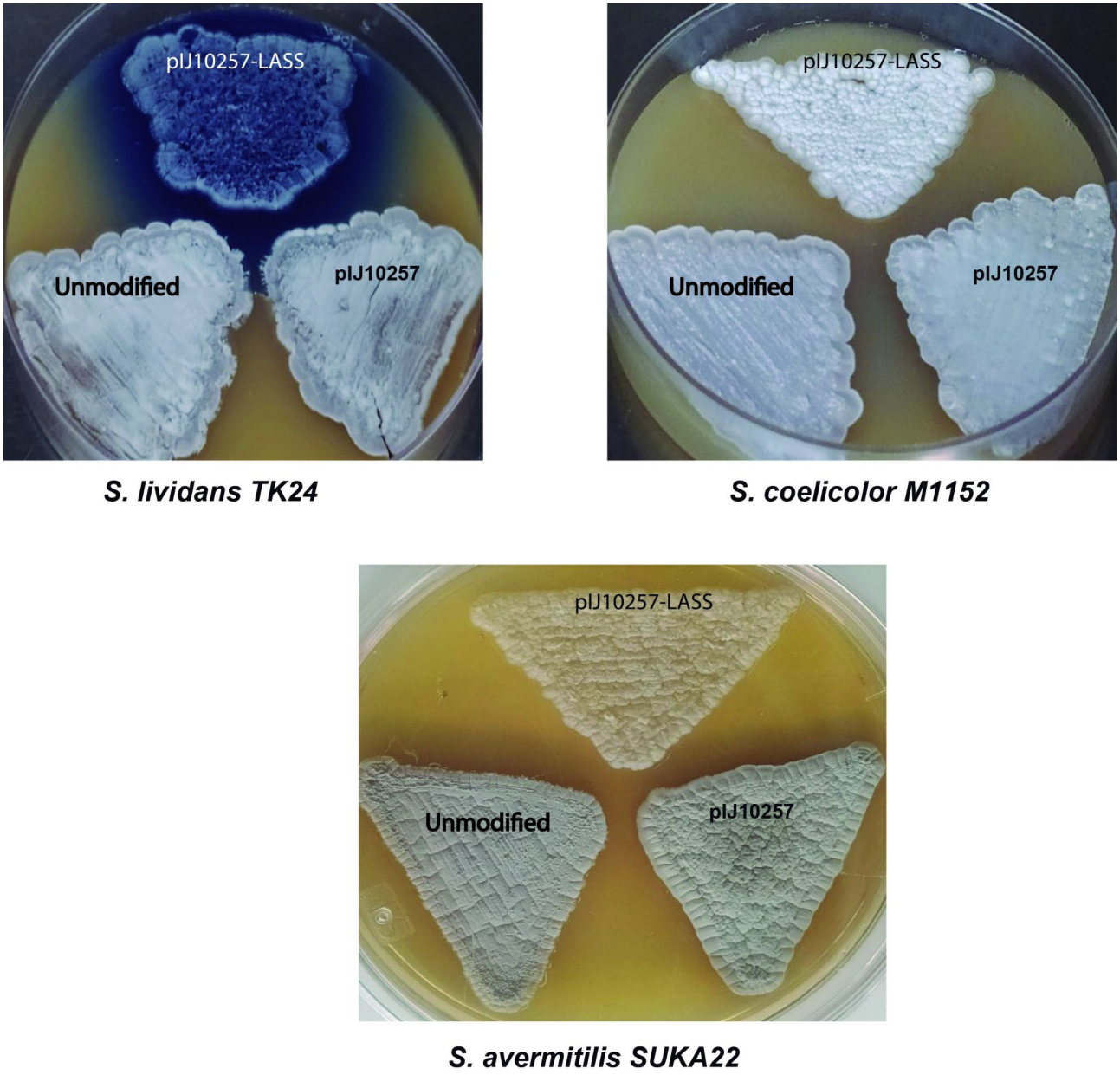

Fig. 4 Heterologous expression of the LASS BGC in three different strains of *Streptomyces* leads to defects in their morphological development. Strains expressing the LASS BGC (pIJ1027-LASS), strains carrying the empty plasmid (pIJ10257), and unmodified strains were grown on their sporulation medium for seven days

a supernatant extract containing morphosins to the plain strains carrying only pIJ10257. In Fig. 5a white ring is visible around the inhibition zone, indicating defective sporulation compared to the gray pigmented spores.

Structural analysis of morphosins

In the UPLC/MS analysis of the supernatant extracts, morphosin-1 was detected at a retention time (RT) of 1.9 min (Fig. 6A). However, morphosin-2, was detected at two different retention times, 1.7 and 2.9 min (Fig. 6B), suggesting that this lasso peptide exists of two distinct conformations, possibly of lasso and branched cyclic. The retention times correspond to hydrophilic peptides, as their primary sequence indicates. The measured m/z ($[M+2\ H]^{2+}$) of both peptides closely matched with the calculated m/z (<2 ppm) for the peptide sequences GRGTETREATHTYDMV and GRQSFNDSDKSNYFEN. This is expected for both lasso peptides, considering the loss of a water molecule. The signals of both compounds were also identified in UPLC/MS analysis of the mycelium extracts (Fig. 7A and B). This evidence, combined with the fact that unmodified strains or those with the empty

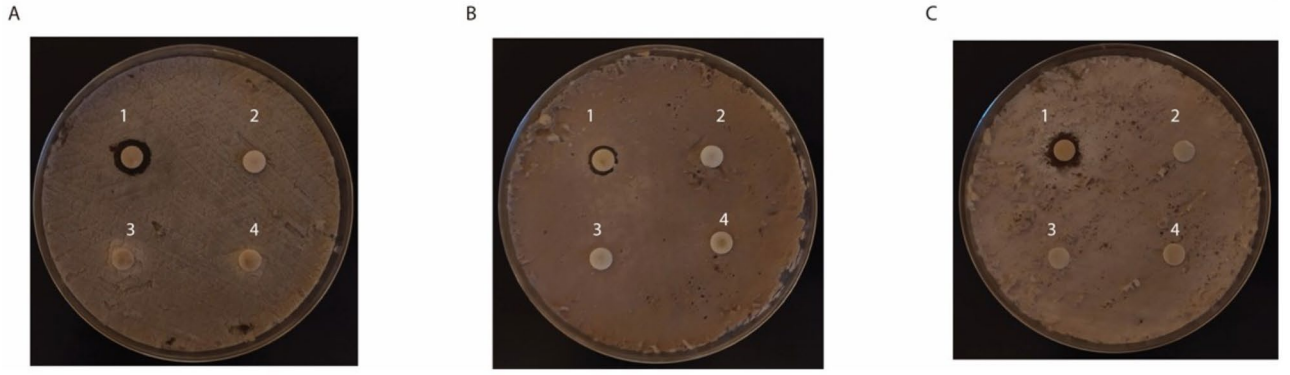

Fig. 5 Effect of the morphosin-containing supernatant extract on the *Streptomyces* strains carrying pIJ10257. (A) *S. avermitilis* SUKA22-pIJ10257. (B) *S. coelicolor* M1152-pIJ10257. (C) *S. lividans* TK24-pIJ10257. **(1)** morphosin-containing supernatant extract. **(2)** MESS buffer **(3)** supernatant extract from *Streptomyces*-pIJ10257. **(4)** Supernatant extract from *Streptomyces* no modified were used

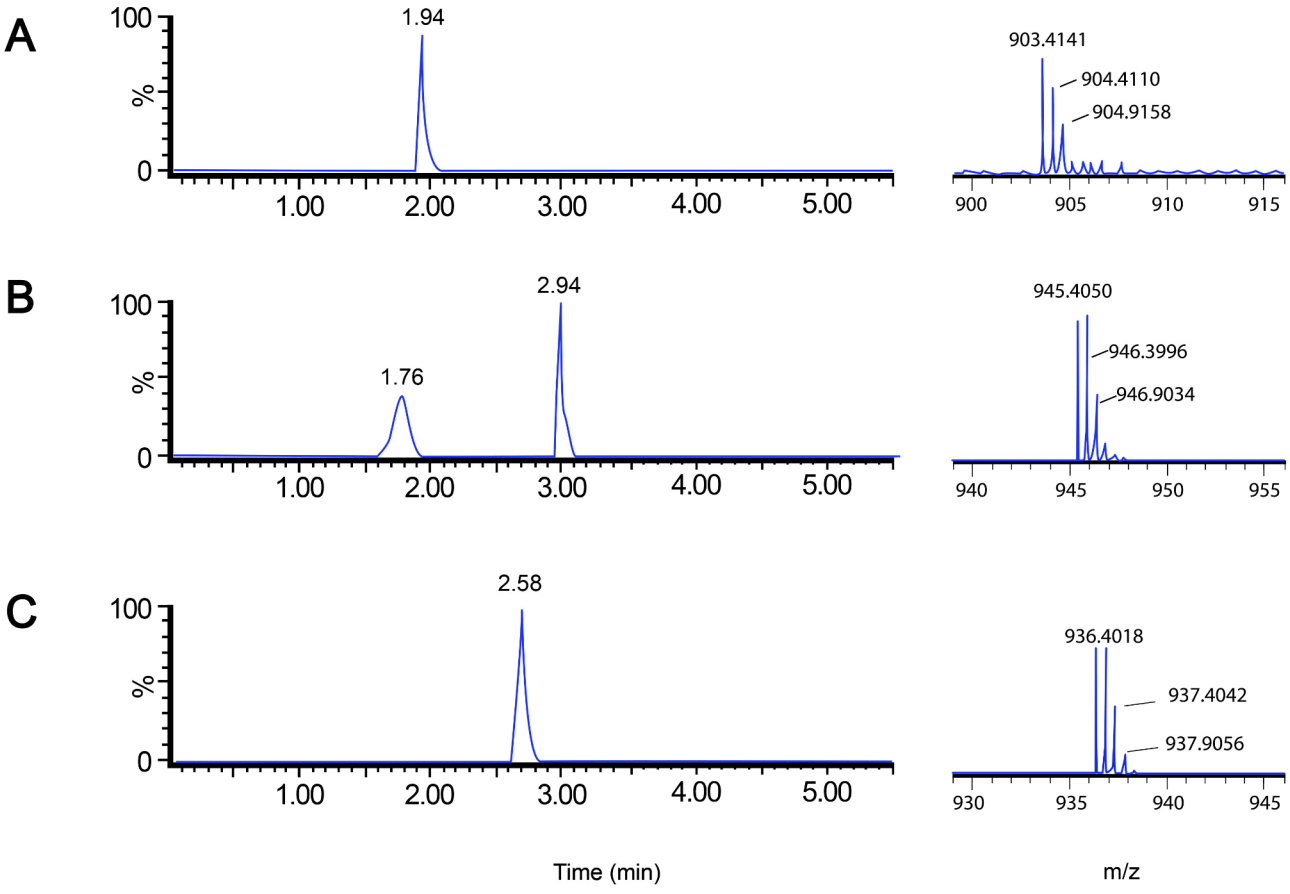

Fig. 6 Mass spectrum (right) and extracted ion chromatogram (left) of the [M+2 H] ions of lasso peptides contained in the supernatant extracts (**A**) Morphosin-1 (RT 1.94 min; HRESIMS m/z 903.4141 $[M+2\ H]^{2+}$; calculated for $C_{74}H_{116}N_{24}O_{27}S$, m/z 903.415399), (**B**) Morphosin-2 (RT 1.76 and 2.94 min; HRESIMS m/z 945.4050 $[M+2\ H]^{2+}$; calculated for $C_{80}H_{112}N_{24}O_{30}$, m/z 945.406086). (**C**) Signal corresponding to a peptide with a molecular mass of 1870.7054 Da (RT 2.58 min)

vector didn't show any changes in their traits, strongly suggests that the observed changes in sporulation in the hosts are directly connected to the presence of lasso peptides within the cells.

In addition, a signal with a RT of around 2.6 min and a m/z ($[M+2\ H]^{2+}$) of 936.4018 Da was detected in the mycelium and supernatant extracts of the LASS-containing strains. This signal corresponds to a peptide with a molecular mass of 1,870.7 Da, which could be

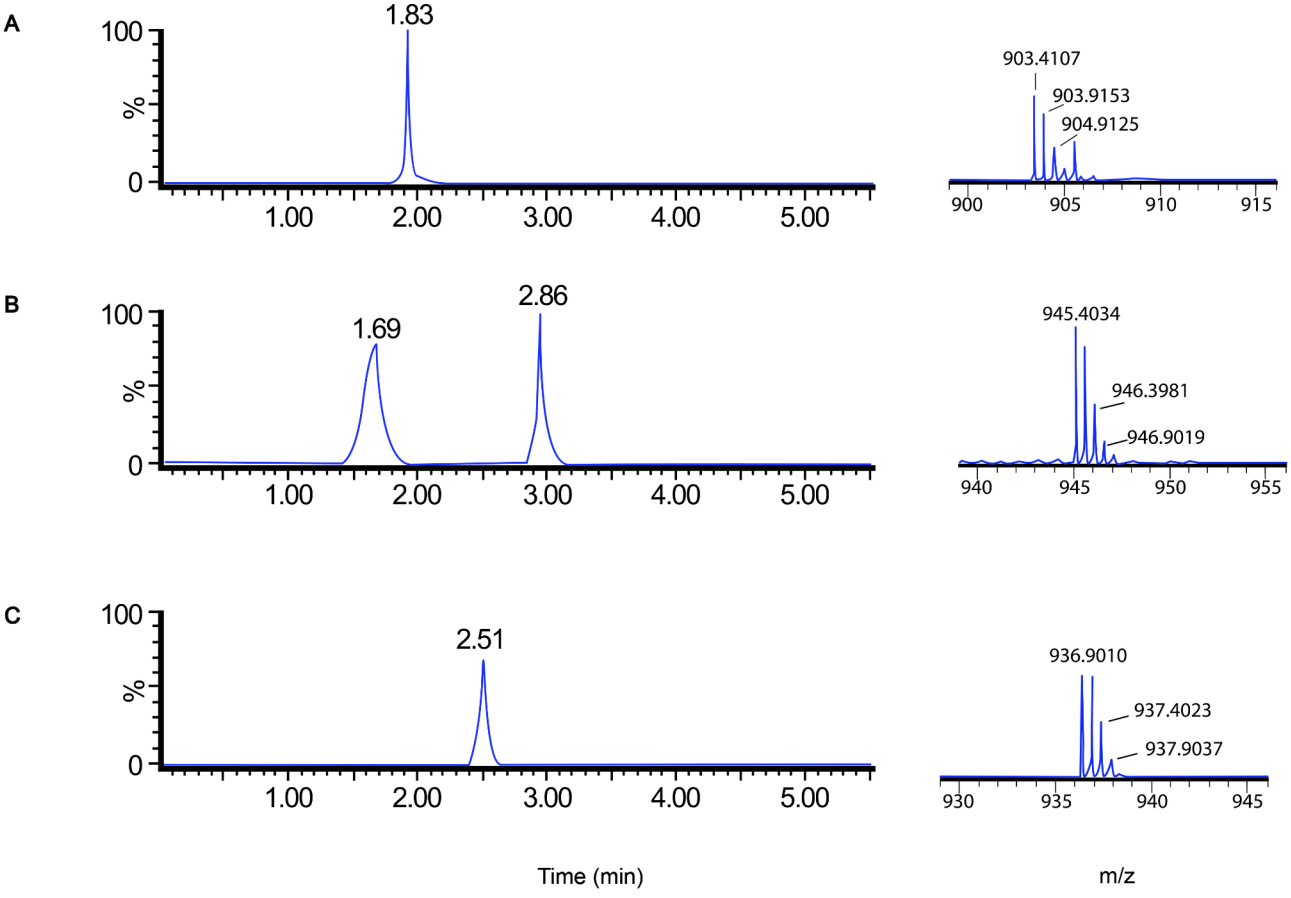

Fig. 7 Mass spectrum (right) and extracted ion chromatogram (left) of the [M + 2 H] ions of lasso peptides contained in the mycelium extracts. (**A**) Morphosin-1 (RT 1.83 min.; HRESIMS m/z 903.4107[M + 2 H]$^{2+}$; calculated for $C_{74}H_{116}N_{24}O_{27}S$, m/z 903.415399). (**B**) Morphosin-2 (RT 1.69 y 2.86 min; HRESIMS m/z 945.4034 [M + 2 H]$^{2+}$; calculated for $C_{80}H_{112}N_{24}O_{30}$, m/z 945.406086). C. Signal corresponding to a peptide with a molecular mass of 1870.7054 Da (RT 2.51 min)

morphosin-2 with the loss of a water molecule (Figs. 6C and 7C).

Antibacterial activity

The antibacterial activity of the mycelium and supernatant extracts of *S. avermitilis* SUKA 22, which produces morphosin-1 and −2, was tested against *B. subtilis*, *M. luteus and E. coli*. However, no antimicrobial effect could be detected with this assay (not shown).

Discussion

Our research has identified a lasso peptide BGC within the genome of the endophyte *Streptomyces* sp. L06. This BGC contains two distinct precursor genes whose products can be turned into two different lasso peptides: morphosin-1 and morphosin-2. Morphosin-2 is a member of a particular subfamily of lasso peptides characterized by the GRX3NDX2DKXNYFEX sequence. Initially, this subfamily was classified as an unknown subfamily with only six different sequences (Tietz et al. 2017). However, in this study, we identified 27 different sequences in this subfamily, and reported, for the first time, the production of a lasso peptide from this subfamily.

According to the SSNs analysis six of the nine precursor peptides that are dissimilar to morphosin 2, have sequences quite different from each other. However, the cyclases that modify every precursor peptide have similar sequences. It also highlights the fact that lasso peptide RRE and leader peptidase are encoded separately by most LASS BGCs that contain only one peptide precursor, and two domain B proteins are encoded in all LASS BGCs that have two precursor peptides.

Lasso peptides are known for their high-temperature stability and protease resistance (Hegemann et al. 2020). Our UPLC/MS analysis of supernatant and mycelium extracts showed a signal corresponding to the molecular mass of the morphosin-2 at two different retention times, one of which could correlate with its threaded version and the other with its unthreaded version. This result suggests that the structure of morphosin-2 is labile and can exist in an unthreaded form. Peptides that unthread at temperatures associated with the solvents used for

extraction (usually 40 °C) have been reported (Hegemann et al. 2020). Moreover, organic solvents like acetonitrile, used for solubilization and purification, can also cause unthreading of lasso peptides (Koos et al. 2019). In our study, we prepared extracts, containing morphosin-1 and morphosin-2, at temperatures of 40 °C and resuspended them in acetonitrile. Therefore, morphosin-2 could be prone to unthreading due to temperature or organic solvents.

Streptomyces undergo a complex life cycle involving aerial hyphae and spore development. Each stage of this process exhibits a distinct phenotype, so defects in any phase can be visually detected (Jani et al. 2015). The phenotypic changes observed in hosts that contain a LASS in their chromosomes correspond to the absence of spores and damage in aerial mycelium development. A similar phenotype was observed when extract containing morphosins was externally applied to the plain strains carrying only the empty vector. These findings suggest that morphosins cause morphological abnormalities in the development of *Streptomyces*.

Some RiPPs play a role in forming *Streptomyces* antibiotics and their morphological development. Two lanthipeptides, SapB and SapT, produced by *S. coelicolor* and *S. lavenduligriseus* Tu 109, respectively, contribute to aerial mycelium formation and restore aerial hyphae formation in bld mutants when supplied extracellularly. These peptides act as surfactants, reducing surface tension (Kodani et al. 2004, 2005; Sarksian et al. 2022), but only SapT shows antibacterial activity. Goadsporin, a thiazole/oxazole-modified microcin peptide (TOMM) synthesized by *Streptomyces* sp., promotes sporulation and/or synthesis of undecylprodigiosin but does not affect the producing strain when applied exogenously (Onaka et al. 2001). Thiostrepton, a thiopeptide produced by a *Streptomyces laurentii*, stimulates the formation of pellets in *S. coelicolor* at sub-inhibitory concentrations, but it reduces the production of actinorhodin and undecylprodigiosin (Wang et al. 2017). The three species of *Streptomyces* tested expressing morphosins exhibited an inhibition of their morphological development. Two of these bacteria are deleted strains for secondary metabolite production (*S. coelicolor* M1152 and *S. avermitilis* SUKA 22). However, in *S. lividans* TK24 grown on MS medium, actinorhodin production was observed only when the LASS fragment was present. Normally, *S. lividans* TK24 does not produce actinorhodin when it is grown on MS medium (Rebets et al. 2018). These findings suggest that the small molecules produced by *Streptomyces* could act as signaling molecules in the morphological development of *Streptomyces* and the production of antibiotics.

In addition, studying the genetic makeup of the core of a BGC can offer valuable insights into the functionality and physiological relationship among the genes of the cluster. This analysis can provide a basis for hypotheses about the function of each gene, the entire cluster, or the compound produced (Kountz et al. 2021). For lasso peptides, regulatory genes that control the production of these compounds are frequently located upstream of the BGC (Mevaere et al. 2018; Guerrero-Garzón et al. 2020). Our research has identified two genes with potential regulatory functions that flank the BGC of interest. One of these regulators, XRE, and a protein bearing the DUF397 domain constitute the XRE/DUF397 system. Recently, 14 different XRE/DUF397 systems from *S. coelicolor* were studied, and they were found to act as regulators of morphological development and the production of actinorhodin and other secondary metabolites (Riascos et al. 2023). These authors report that overexpression of the sco4678(XRE)/sco4679 (DUF397) system leads to a bald phenotype and increased actinorhodin production in *S. coelicolor*. Likely, these effects are unrelated to lasso peptides, as *S. coelicolor* does not produce these compounds. However, this effect is similar to what we observed in *S. lividans* TK24 when expressing morphosins. It is unknown what causes this effect in *Streptomyces hosts*, it is speculated that morphosins may act as ligands, activating the XRE/DUF397 pathway. Although in this study, regulatory genes were not cloned, *Streptomyces* hosts have their own XRE/DUF systems (Riascos et al. 2023). Strains such as *S. coelicolor* M1152 and *S. avermitilis* SUKA 22 cannot produce their main antibiotics because they were engineered to delete their antibiotic gene clusters. Hence, only the morphological effect can be observed when the LASS region is present in their genomes. It has been reported that some chemicals that inhibit sporulation can also act as antibiotics against rod-shaped and coccoid Gram-positive bacteria by interfering with cell division, either directly or indirectly. Specific molecules that target DNA can also cause defects in the sporulation process of *Streptomyces* at subinhibitory concentrations (Jani et al. 2015; McAuley et al. 2019a, b). We tested the antibacterial effect of expressed lasso peptide extracts containing morphosin-1 and −2 on *B. subtilis* and *M. luteus*. However, our results showed no activity against either of the two strains. It is important to note that this does not necessarily mean that lasso peptides are not bioactive. Factors such as low expression, lack of secondary modification enzymes, and purification could have affected the results obtained in our study.

Further research needs to confirm the lasso topology of morphosin-1 and morphosin-2 to establish that *LASSC* gene encodes a lasso-cyclase capable of catalyzing the creation of macrolactam rings with varying sizes (8 and 9 residues) and with different residues G-E in morphosin-1 and G-D in morphosin-2. Besides, understanding the function of the regulatory genes flanking the core genes that produce lasso peptides will shed light on the

role of morphosins in developing morphology and antibiotic production and whether both peptides act together or independently.

In our research, we analyzed the LASS BGC from the genome of *Streptomyces* sp. L06. Our findings revealed the presence of two genes that encode precursor peptides that can be turned out into lasso peptides with different ring sizes and compositions. Specifically, we identified morphosin-2 as a member of a new lasso peptide subfamily comprising 27 additional lasso peptides. This study represents the first investigation of a member of this subfamily.

Furthermore, our research suggested that the heterologous expression of the LASS BGC affects the morphological development of three *Streptomyces* strains and induces actinorhodin production in one of them. This finding is a significant step forward in our understanding of lasso peptides and their role in morphological development and antibiotic production in *Streptomyces* strains.

Acknowledgements
We extend our gratitude to Dr. Silvia Ivonne Mora for her technical support and advice in the UHPLC-Mass analyses. AORC is a doctoral student in the Programa de Ciencias Biológicas, UNAM. She was supported by the doctoral scholarship number 742885, awarded from CONAHCYT, Mexico.

Author contributions
AORC, and SS conceived and designed the study. AORC carried out the experiments and wrote the first version of this manuscript. BRV, MLMR, EL, RRS, and SS, analyzed and monitored the data, contributed with some of the techniques and reagents/materials, and revised and improved the manuscript.

Funding
This project was funded by the grant DGAPA, PAPIIT, UNAM, IN295922 and IN205519. We are grateful for the support provided by the NUATEI institutional program of the Instituto de Investigaciones Biomédicas, UNAM.

Data availability
All data generated or analyzed during this study are included in this manuscript. Requests for any additional information can be made to the corresponding author.

Declarations

Ethics approval
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships.

Consent to participate
All authors have their consent to participate.

Consent for publication
All authors have their consent to publish their work.

Received: 29 May 2024 / Accepted: 22 August 2024
Published online: 03 September 2024

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