

Chapter 2

From the Producer Microorganisms to the Lasso Scaffold

2.1 Producer Microorganisms and Genetic Systems

Currently known lasso peptides are produced by bacteria in the phyla of Actinobacteria and Proteobacteria with frequent occurrence in the classes of *Alpha*- and *Beta*-proteobacteria (Table 2.1). The producer strains occupy very diverse ecological niches distributed across large geographical locations. They can be found in diverse ecological niches such as soils and marine sediments (e.g. *Streptomyces* sp.), aquatic environments including seawater (e.g. *Sphingopyxis alaskensis*), freshwater (e.g. *Asticcacaulis excentricus*) and contaminated water (e.g. *Caulobacter* sp. K31 and *Rubrivivax gelatinosus*) and particle surfaces (e.g. *Rhodanobacter thiooxydans*). Some producers live in association or symbiosis with eukaryotic hosts such as *Escherichia coli* from the mammalian intestines, *Burkholderia rhizoxinica* as an endosymbiont of the fungus *Rhizopus microsporus* (Partida-Martinez et al. 2007) and *Phenylobacterium zucineum* isolated from a human erythroleukemia cell line (Zhang et al. 2007). Only two *Xanthomonas* producers are plant pathogens, whereas most of them are non-pathogenic. An overview of the producing organisms can give us a glimpse of lasso peptides' large distribution in nature, a notion that is lately reinforced by bacterial genome mining studies (see Chap. 5). This also hints that lasso peptides may play diverse roles in the environment.

Microcin J25 (MccJ25) is considered the archetype of lasso peptides and is used as a model. It was isolated in 1992 from the culture supernatant of a faecal isolate *E. coli* AY25 while searching for microcins, the antimicrobial peptides from *Enterobacteria* (Salomón and Fariás 1992). Its genetic system was the only characterized gene cluster of lasso peptides until 2008. The 4.8 kb cluster is encoded by a low-copy-number plasmid (Solbiati et al. 1996). It contains four genes necessary for the production and export of MccJ25. The gene products are a precursor peptide (McjA), two maturation enzymes (McjB and McjC) and an ATP-binding cassette (ABC) transporter (McjD), which ensures active export of the peptide to the environment and consequently confers self-immunity to the producer by pumping the toxin out of the cell (Solbiati et al. 1999). Homology search of McjB and McjC revealed a similar gene locus in *Burkholderia thailandensis* E264 that led to the identification of capistruiin in 2008 (Knappe et al. 2008). The *mcj* and *cap* systems

Table 2.1 Bacterial strains producing known lasso peptides. The rows highlighted in grey correspond to lasso peptides discovered by genome mining approaches. The order of the peptides follows the alphabetical order inside the sub-classes that are defined in Sect. 2.3 and presented in Tables 2.4 and 2.5

Peptide	Producing strain	Source	References
ACTINOBACTERIA			
Aborycin/ RP 71955	<i>Streptomyces griseoflavus</i> Tü472 <i>Streptomyces</i> sp. SP9440	Soil sample, Alice Springs, Australia Soil sample	(Potterat et al. 1994) (Helynck et al. 1993)
Siamycin I/ MS-271/NP-06	<i>Streptomyces</i> sp. MS-271	Soil sample collected under a pine tree, Hinatamura, Machida-city, Tokyo, Japan	(Yano et al. 1996)
Siamycin II Sviceucin/ SSV-2083	<i>Streptomyces</i> sp. MS-271 <i>Streptomyces sviceus</i> ATCC 29083	Soil sample (Hanka and Dietz 1973)	(Ducasse et al. 2012a) (Kersten et al. 2011)
Anantín	<i>Streptomyces coeruleus</i> DSM 4777/4778	Soil sample taken near Salt Lake City, Utah, U.S.A	(Weber et al. 1991)
Lariatín A^a Lariatín B^a	<i>Rhodococcus jostii</i> K01-B0171	Soil sample, Yunnan, China	(Iwatsuki et al. 2006; Iwatsuki et al. 2007)
Propeptín	<i>Microbisporasp.</i> SNA-115	Soil sample, Misakiguchi, Miura-city, Kanagawa Prefecture, Japan	(Kimura et al. 1997)
RES-701-1^b RES-701-3^b	<i>Streptomyces</i> sp. RE-701 and RE-896	Soil sample, Aichi Prefecture, Japan	(Morishita et al. 1994; Ogawa et al. 1995)
SRO15-2005	<i>Streptomyces roseosporus</i> NRRL 15998	Soil sample from Mount Ararat, Turkey (Eaton et al. 1989)	(Kersten et al. 2011)
Sungsanpin	<i>Streptomyces</i> sp. SNJ013	Deep-sea marine sediments (138 m depth) off the coast of Sungsanpo on Jeju Island, Korea	(Um et al. 2013)
BI-32169	<i>Streptomyces</i> sp. DSM 14996	Soil sample, Los Gigante, Teneriffa, Spain	(Potterat et al. 2004)
PROTEOBACTERIA^c			
Astexin 1(23)*** Astexin 2 Astexin 3	<i>Asticcacaulis excentricus</i> CB48 DSM 4724 (alpha)	Pond water, North Carolina USA (Poindexter 1964)	(Maksimov et al. 2012; Zimmermann et al. 2013)
Burhizin	<i>Burkholderia rhizoxinica</i> HKI454 DSM 19002 (beta)	Endosymbiont of fungus <i>Rhizopus microsporus</i> van Tieghem var. <i>chinensis</i> ATCC 62417 (Partida-Martinez et al. 2007a)	(Hegemann et al. 2013b)
Caulonodin I^d Caulonodin II^d Caulonodin III^d	<i>Caulobacter</i> sp. K31 (alpha)	Low temperature, chlorophenol-contaminated groundwater from Karkola, Finland	(Hegemann et al. 2013b)

Table 2.1 (continued)

Peptide	Producing strain	Source	References
PROTEOBACTERIA^c			
Capistruin	<i>Burkholderia thailandensis</i> E264 DSM 13276 (beta)	Environmental sample, Thailand (Brett et al. 1998)	(Knappe et al. 2008)
Caulosegnin I Caulosegnin II Caulosegnin III	<i>Caulobacter segnis</i> DSM 7131 (alpha)	Soil sample	(Hegemann et al. 2013a)
Microcin J25	<i>Escherichia coli</i> AY 25 (gamma)	Feces of a newborn infant, Argentina	(Salomón and Farías 1992)
Rhodanodin^d	<i>Rhodanobacter thiooxydans</i> LCS2 DSM 18863 (gamma)	Biofilm on sulfur particles (Lee et al. 2007)	(Hegemann et al. 2013b)
Rubrivinodin	<i>Rubrivivax gelatinosus</i> IL44 NBRC 100245 (beta)	Food wastewater in Nagano, Japan (Hoshino and Satoh 1985)	(Hegemann et al. 2013b)
Sphingonodin I^d Sphingonodin II^d	<i>Sphingobium japonicum</i> UT26 DSM 16413 (alpha)	Soil sample (Pal et al. 2005)	(Hegemann et al. 2013b)
Sphingopyxin I^d Sphingopyxin II^d	<i>Sphingopyxis alaskensis</i> RB2256 DSM 13593 (alpha)	Seawater, Gulf of Alaska (Vancanneyt et al. 2001)	(Hegemann et al. 2013b)
Syanodin I^d	<i>Sphingobium yanoikuyae</i> XLDN2-5 (alpha)	Petroleum-contaminated soils (Gai et al. 2007)	(Hegemann et al. 2013b)
Xanthomonin I^d Xanthomonin II^d	<i>Xanthomonas gardneri</i> DSM19127/ATCC 19865	Plant pathogen (Jones et al. 2004)	(Hegemann et al. 2014)
Xanthomonin III^d	<i>Xanthomonas citri</i> pv <i>mangiferae</i> LMG241 ATCC11637 (gamma)	Fruit pathogen (Doidge 1915)	
Zucinodin	<i>Phenylobacterium zucineum</i> HLK1 (alpha)	Intracellular bacterium isolated from human erythroleukemia cell line (Zhang et al. 2007)	(Hegemann et al. 2013b)

- Lariat A is the 18 amino acid form of lariat B (now termed lariat), truncated at the Cterminus.
- RES-701-2 and RES-701-4 are oxidized forms of RES-701-1 and RES-701-3, respectively. They are thus not considered here as individual lasso peptides.
- The class of the proteobacterial strains is indicated in brackets after the name of the strain.
- The lasso peptides isolated correspond to truncated forms of the peptides predicted from the precursor gene sequence.

represent a prototype of lasso gene clusters. Therefore, the nomenclature ABCD is recommended by the RiPPs community to describe these clusters (Arnison et al. 2013). The first gene cluster from Actinobacteria was characterized for lariatins in *Rhodococcus jostii* K01-B0171 (Inokoshi et al. 2012), in which the B-like protein was split and encoded by two genes. The genome-wide survey that followed, revealed widespread distribution of *mcj/cap*-like clusters in microbial genomes as well as deviations in the gene organization (Severinov et al. 2007; Maksimov et al. 2012; Hegemann et al. 2013b). Currently 25 out of 35 known lasso peptides have

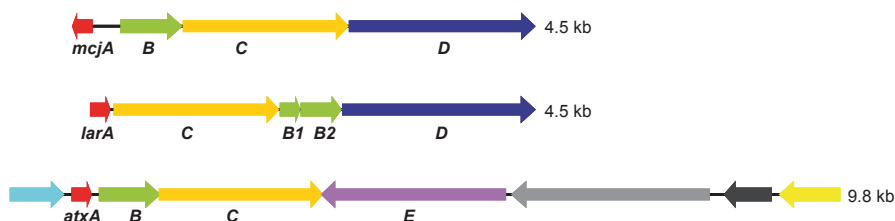


Fig. 2.1 Gene organization of lasso peptide clusters. Examples of known lasso peptide gene clusters representing: (1) core cluster with ABC transporters: *mcj* (MccJ25 cluster from *Escherichia coli*) and *lar* featuring split-B genes (lariatins from *Rhodococcus jostii*) and (2) core cluster with isopeptidases: *axt* featuring accessory genes (astexin 1 from *Asticcacaulis excentricus*). Validated genes are labelled with standard names. Genes coding for precursors (red), full length or split B proteins (green), C proteins (orange), transporters (dark blue), isopeptidases (purple), GntR-like transcription regulators (light blue), TonB-dependent receptors (grey), sigma factors (yellow) and anti-sigma factors (black) are shown

their gene clusters validated. A global picture of lasso gene architectures emerged from these functional and genomic data. The *A*, *B* and *C* genes are conserved in all characterized and putative clusters comprising the core cluster essential for lasso peptide biosynthesis. In most cases they are arranged in the same direction. The *B* and *C* genes are generally transcriptionally coupled. Putative transcription terminators are present in some instances in the intergenic region between *A* and *BC* genes (Severinov et al. 2007). For MccJ25, *mcjA* is transcribed in an opposite direction to the *mcjBCD* operon and is under the control of its own promoter. The main deviations in the core cluster include the presence of multiple *A* genes (up to three) and split of *B* genes (*B1* and *B2* genes). Three types of lasso gene architectures can be discerned: (1) core cluster only, (2) core cluster with ABC transporter genes and (3) core cluster with an isopeptidase gene (Fig. 2.1). The other accessory genes potentially involved in the regulation or further post-translational modifications (e.g. phosphorylation, sulfonation, acetylation, methylation) can be found clustered with some ‘core cluster/ABC transporter’ and ‘core cluster/isopeptidase’ architectures (Hegemann et al. 2013b; Maksimov and Link 2014); however, they have not been experimentally validated yet. Currently, 6 out of 24 known lasso peptides with characterized genes are from the ‘core cluster/ABC transporter’ type, while the rest are products of the ‘core cluster/isopeptidase’ type. No lasso peptides from the ‘core cluster only’ type have yet been isolated. A summary of characterized lasso gene clusters is given in Table 2.2.

2.2 Production and Purification

Early discovery of lasso peptides used a classical approach relying on the search for antibiotic, antiviral or other biological activities of bacterial extracts, followed by purification of the active fractions. The last lasso peptide discovered following

Table 2.2 Gene cluster organisation of identified lasso peptides and core cluster gene products. The peptides are presented according to the producing bacteria and following the alphabetical order inside sub-classes as defined in Sect. 2.3 and Table 2.1. The protein accession number is indicated behind the protein name when available. The type I peptide is highlighted in grey

Name	Gene cluster type	Protein A /Accession no.	Protein B/Accession no.	Protein C/Accession no.
ACTINOBACTERIA				
Sviceucin (SSV-2083)	core cluster+ABC transporter	SvicA (56 aa) WP_007385812	SvicB1 (86 aa) WP_007385814 SvicB2 (165 aa) WP_007385815	SvicC (607 aa) WP_007385813
Lariatrin	core cluster+ABC transporter	LarA (46 aa) BAL72546	LarB1 (84 aa) BAL72548 LarB2 (147 aa) BAL72549	LarC (604 aa) BAL72547
SRO15-2005	core cluster+ABC transporter	A (61 AA) EFE76491.1	B1 (83 aa) EFE76493.1 B2 (147 aa) EFE76494.1	C (312 aa) EFE76492.1
PROTEOBACTERIA				
Astexin-1	core cluster + isopeptidase	AtxA1 (51 aa) YP_004088035	AtxB1 (209 aa) YP_004088036	AtxC1 (572 aa) YP_004088037
Astexin-2/3	core cluster + isopeptidase	AtxA2 (49 aa) YP_004088250 AtxA3 (49 aa) YP_004088249	AtxB2 (283 aa) YP_004088248	AtxC2 (606 aa) YP_004088247
Burhizin	core cluster+ABC transporter	BurhA (50 aa) YP_004028910	BurhB (276 aa) YP_004028969	BurhC (580 aa) YP_004028967
Caulonodins	core cluster + isopeptidase	Ck31_A1 (44 aa) YP_001676628 CK31_A2 (44 aa) YP_001676627 CK31_A3 (44 aa)	Ck31_B (219 aa) YP_001676626	Ck31_C (579 aa) YP_001676625
Capistruin	core cluster+ABC transporter	CapA (47 aa)	CapB (221) WP_011402376	CapC (582 aa) WP_011402377
Caulosegnins	core cluster + isopeptidase	CsegA1 (42 aa) YP_001676628 CsegA2 (37 aa) YP_001676627 CsegA3 (37 aa)	CsegB (216 aa) YP_001676626	CsegC (617 aa) YP_001676625
Microcin J25	core cluster+ABC transporter	McjA (58 aa) AAD28494	McjB (208 aa) AAD28495	McjC (513 aa) Q9X2V9
Rhodanodin	core cluster + isopeptidase	RhotA (54 aa)	RhotB (222 aa) ZP_10205116	RhotB (584 aa) ZP_10205117
Rubrivinodin	core cluster + isopeptidase	RugeA (43 aa) YP_005435543	RugeB (241 aa) YP_005435542	RugeC (617 aa) YP_005435541
Sphingonodin I	core cluster + isopeptidase	Sjap1_A (39 aa)	Sjap1_B (218 aa) YP_003546448	Sjap1_C (583 aa) YP_003546449
Sphingonodin II	core cluster + isopeptidase	Sjap2_A (46 aa) YP_003547071	Sjap2_B (217 aa) YP_003547070	Sjap2_C (581 aa) YP_003547069
Sphingopyxin I	core cluster + isopeptidase	Sala1_A (44 aa)	Sala1_B (213 aa) YP_617574	Sala1_C (570 aa) YP_617573
Sphingopyxin II	core cluster + isopeptidase	Sala2_A (44 aa)	Sala2_B (219 aa) YP_617642	Sala1_B (578 aa) YP_617641
Syanodin	core cluster + isopeptidase	Syan1_A (44 aa)	Syan1_B (220 aa) ZP_09906533	Syan1_B (581 aa) ZP_09906534
Xanthomonins	core cluster + isopeptidase	XgaA1 (46 aa) ZP_08185022 XgaA2 (44 aa) ZP_08185023	XgaB (224 aa) ZP_08185024	XgaC (577 aa) ZP_08185025
Zucinodin	core cluster + isopeptidase	Pzuc_A (39 aa)	Pzuc_B (212 aa) YP_002131237	Pzuc_C (571 aa) YP_002131236

this classical strategy is sungsanpin that was isolated from a deep sea *Streptomyces* sp. (Um et al. 2013). The era of lasso peptides discovery following genome mining approaches started in 2008 with the characterization of capistruin from *B. thailandensis* (Knappe et al. 2008), and then extended to many novel lasso peptides described mainly from Proteobacteria. Genome mining approaches using, either protein homology-based (Knappe et al. 2008; Ducasse et al. 2012a; Hegemann et al. 2013a, b, 2014; Zimmermann et al. 2013), or precursor-centric (Kersten et al. 2011; Maksimov et al. 2012) strategy, proved extremely efficient. Out of the 35 currently known lasso peptides, 23 have been identified following this method, out of which 21 are from Proteobacteria (Knappe et al. 2008; Maksimov et al. 2012; Hegemann et al. 2013a, b; Zimmermann et al. 2013, 2014) and 2 from Actinobacteria (Kersten et al. 2011; Ducasse et al. 2012a).

2.2.1 Production in the Natural Producer

The antimicrobial peptides produced by bacteria (bacteriocins, microcins) have been proposed to act a major role in microbial competitions and as such to be produced essentially in stress conditions. Nutritional deficiency is a main factor that triggers the production of an arsenal of toxic peptides and proteins by some bacterial and archaeal strains, which are actors in these mechanisms. Nutrient depletion is thus a stress that can be more directly correlated to the synthesis of such compounds by bacteria. However, relying on the rock-paper-scissors model (Riley and Wertz 2002) and the strong impact of the spatial structure of the environment on the interactions between bacterial populations (Hibbing et al. 2010), the cost of peptide production is in most of the cases unfavourable to a producing strain that can be outcompeted by a resistant strain capable of resistance to the toxin, but unable to produce it. Therefore, production of such antimicrobial peptides remains difficult to anticipate and control.

Although some lasso peptides have been found to be antimicrobial, others have not been shown to exert this activity in nature. However, most of them, particularly when produced by Proteobacteria, have been observed as produced in nutrient depletion conditions. Therefore, poor culture media are generally preferred to more complex ones for cultivating natural producers in order to enhance lasso peptide production. Moreover, minimal media (such as M9 minimal medium) have the additional advantage to simplify further purification procedures compared to rich media. In the case of Actinobacteria, more complex culture media are required. The production yields of lasso peptides are extremely variable. Some of them are secreted in very good yields in the supernatants, such as anantin (5–10 mg/L culture; Weber et al. 1991) or MccJ25 (1–5 mg/L culture; Blond et al. 1999), while others are very minor compounds that require intense production optimization (capistruin; Knappe et al. 2008) or heterologous production (caulosegnins (Hegemann et al. 2013b); astexins (Maksimov et al. 2012; Zimmermann et al. 2013); xanthomonins, (Hegemann et al. 2014), for further studies. The physical parameters (pH and temperature in particular) have to be optimized too, in order to select the best

conditions for peptide production. The optimization of these parameters must be strongly connected to the growth phase of the producing bacteria, which is also a critical parameter for metabolite production. Depending on the peptides and the producing strains, lasso peptides are produced either in early exponential phase, such as capistruin (Knappe et al. 2008), or in stationary phase, as observed for MccJ25 (Chiuchiolo et al. 2001). Therefore, duration of the fermentation has to be determined in accordance with bacterial growth curves measured in parallel with lasso peptide production.

When culture media and fermentation conditions have been optimized for maximum lasso peptide production, large-scale fermentation procedures can be applied in order to obtain appropriate amounts of peptides for further structural studies and biological assays. Nearly, all known lasso peptides from Actinobacteria were isolated from large-scale fermentations. When this approach fails and insufficient production yields are obtained, heterologous production (see Sect. 2.2.2 below) is the best way for permitting the characterization of novel lasso peptides.

2.2.2 Gene Cluster Engineering and Heterologous Production

It is not uncommon to observe that natural producers produce very low amounts of lasso peptides, even when the culture conditions have been scrutinized and optimized. Large-scale fermentation was generally required to obtain enough peptides for structure elucidation, as described in Sect. 2.2.1. The last 5 years have seen a new era of lasso peptide discovery, which is guided by genome-mining approaches. As lasso peptide clusters are small and amenable to manipulation, heterologous expression is an obvious choice. Indeed, heterologous expression in *E. coli* was successfully applied for lasso peptides from Proteobacteria since the discovery of capistruin in 2008 (Knappe et al. 2008). This strategy circumvents the inconvenience of large-scale fermentation and in many cases is indispensable, as the natural producers do not produce at all the concerned peptide under laboratory conditions (e.g. caulosegnin III from *Caulobacter segnis* (Hegemann et al. 2013a)).

As the first example, Knappe et al. cloned the capistruin gene cluster (*capABCD*) into pET-41a vector in a way that the transcription of the four genes was under the control of a T7 promoter (Knappe et al. 2008). A vector-borne ribosome binding site (RBS) was in front of *capA*, while *capBCD* was transcribed from an intrinsic RBS from *B. thailandensis*. The resulting construct was transformed into *E. coli* BL21(DE3) for expression and led to the production of capistruin with a yield of 0.2 mg/L culture in the defined M20 medium. Link and coworkers further improved the yield of capistruin to 1.6 mg/L culture by engineering a new construct, in which *capA* was under the control of a tetracycline-inducible promoter and the transcription terminator-containing intergenic region between *capA* and *capBCD* operon was replaced by an optimized *E. coli* RBS sequence (Pan et al. 2011). Thus, the modification of the intergenic region between the precursor gene and the processing enzyme genes could be the key for successful heterologous expression. For this, either the optimized *E. coli* RBS or a combination of terminator and the *mcjBCD* pro-

Table 2.3 Conditions for heterologous expression of lasso peptides. The order of the peptides follows the alphabetical order inside sub-classes as defined in Table 2.1. The type I lasso peptide is highlighted in grey

Name	Optimal medium	Conditions for optimal production	Yield (mg/L culture)
Sviceucin	GYM	5 d, 30°C	14
Astexin 1	M9	1 d, 37°C	nd
Astexin 2	M9	1 d, 37°C	nd
Astexin 3	M9	1 d, 37°C	nd
Burhizin	M9	1 d, 37°C	nd
Caulonodin I	M9	3 d, 20°C	3.4
Caulonodin II	M9	3 d, 20°C	2.2
Caulonodin III	M9	3 d, 20°C	0.5
Capistruin	M20	2 d, 37°C	0.2
Caulosegnin I	M9	3 d, 20°C	0.3
Caulosegnin II	M9	3 d, 20°C	0.15
Caulosegnin III	M9	3 d, 20°C	0.1
Rhodanodin	M9	3 d, 20°C	nd
Rubrivinodin	M9	1 d, 37°C	0.5
Sphingonodin I	M9	3 d, 20°C	0.9
Sphingonodin II	M9	3 d, 20°C	nd
Sphingopyxin I	M9	3 d, 20°C	3.4
Sphingopyxin II	M9	3 d, 20°C	0.4
Syanodin I	M9	3 d, 20°C	5.2
Zucinodin	M9	3 d, 20°C	nd

motor from MccJ25 system was used. The utility of the latter was demonstrated for caulosegnin I, II and III whose production was increased 16-, 24-, 50-fold compared to the original cluster, respectively (Hegemann et al. 2013a). In cases of clusters with multiple *A* genes, such as those of caulosegnins and caulonodins (Hegemann et al. 2013a; b), single precursor constructs resulted in higher production of the corresponding peptides. Usually, the yields of peptides from multiple precursors in one cluster were not equal. For caulosegnin II and III, it was demonstrated that hybrid precursor genes with the leader peptide from caulosegnin I encoded improved their production. Caulosegnin I showed the greatest yield among the three peptides, which might be attributed to its longer leader sequence. *E. coli* culture conditions play essential roles for expression of proteobacterial lasso peptides. Minimal media are required and frequently used, such as M9 and M20 with vitamin supplements. Generally, very little or no peptide production can be observed in rich LB medium as exemplified by capistruin (Knappe et al. 2008). With regards to the growth temperature, some peptides require 37°C for 1 day for optimal production, while the others prefer 20°C for 3 days. A summary of the conditions for heterologous production of lasso peptides is given in Table 2.3.

Worth of note, MccJ25 is one exception of proteobacterial lasso peptides, which is produced from the natural gene cluster in the native host with high yields (1–5 mg/L culture). Nevertheless, Link and coworkers engineered MccJ25 gene clusters on the pQE60 vector in an attempt to further increase MccJ25 titer (Pan et al. 2010). In these constructs, *mcjA* was placed under the control of its own promoter or a strong T5 promoter, whereas the *mcjBCD* operon was either behind the

natural or an arabinose-induced promoter. None of these constructs resulted in significant improvement, indicating that Mcc25 natural cluster is already well-tuned for optimal production. However, the engineered Mcc25 gene cluster allowed facile cloning to generate variant libraries, and served as a robust platform for MccJ25 engineering in the follow-up studies (Pan and Link 2011).

Heterologous expression of lasso peptides from Actinobacteria in *E. coli* is not an appropriate choice, as work in our lab showed little success of such strategy (unpublished data). It is likely due to the differences of codon usage and gene regulation mechanisms between the natural and heterologous host. A more suitable host that is closer to the production strain should be considered. Our laboratory produced successfully svieceucin from *Streptomyces sviceus* in *Streptomyces coelicolor* for NMR study (Ducasse et al. 2012a). However, this strategy did not bring success for all actinobacterial lasso peptides studied in our laboratory (unpublished data). It thus appears to us that deciphering the regulation mechanism is the key to access lasso peptide production. Screening various antibacterial hosts and genetic engineering of the cluster may be required for successful heterologous expression.

Other ways to improve or facilitate production of lasso peptides, which can be used in addition to heterologous production or independently, are the use of immobilization techniques of the bacterial strains and optimized continuous production conditions (Scannell et al. 2000), or the exploitation of regulation mechanisms in the native hosts similar strategy being applied for bacteriocins from gram-positive bacteria (Diep et al. 1995, 1996; Kuipers et al. 1995). These approaches are currently underway in our laboratory.

2.2.3 Purification Procedures

The first lasso peptides to be characterized were isolated from fermentation broths of wild-type strains of Actinobacteria or Proteobacteria. Since the use of genome mining approaches, heterologous expression in *E. coli* for proteobacterial and in *S. coelicolor* for actinobacterial lasso peptides, became the essential mode of production of the recently isolated representatives. The purification process starts with centrifugation to separate cell pellets and culture supernatants. Lasso peptides produced by natural producers from Proteobacteria, such as MccJ25 or capistruin (Blond et al. 1999; Knappe et al. 2008), are most often isolated from supernatants due to their export in the culture media. When expressed heterologously in *E. coli* under controlled conditions, they are detected and isolated from cell pellets (e.g. astexins, caulosegnins, caulonodins, caulosegnins, sphingopyxins etc.) (Hegemann et al. 2013a, b; Zimmermann et al. 2013). Lasso peptides from Actinobacteria are mainly isolated from mycelia. Being generally hydrophobic in nature (Table 2.4), lasso peptides from the supernatants are first submitted to solid phase extraction from the aqueous phase using C8 or C18 reversed phase cartridges. A size exclusion chromatography step is often introduced, mainly using Sephadex LH20. A second purification step is applied using high performance liquid chromatography (HPLC) on C18 reversed-phase columns, in a preparative or semi-preparative scale in order

Table 2.4 Primary structures of characterized lasso peptides. The macrolactam rings are indicated with an elliptic line (7, 8 and 9 amino acids rings are shown in *green*, *blue* and *red*, respectively), while the disulfide bridges are indicated with *black* right-angled lines. The sequences within brackets correspond to the C-terminal sequence deduced from the precursor genes, when only truncated forms of the predicted peptides were detected

Lasso peptides from actinobacteria		Lasso peptides from proteobacteria	
Class I			
Aborycin/ RP 71955	CLGIGSCNDFAGCGYAVVCFW		
Siamycin I/ MS-271/ NP-06/BMY29304	CLGIGSCNDFAGCGYAIVCFW		
Siamycin II BMY-29303	CLGVGSCNDFAGCGYAIVCFW		
Sviceucin/ SSV-2083	CVWGGDCTDFLGCCTAWICV		
Class III			
BI-32169	GLPWGCPSPDIPGWNTPWAC		
Class II			
Anantin	GFIGWGNDIFGHYSGDF	Astexin 1	GLSQGVPEPDIGQTYFEESRINQD
Lariatin	GSQLVYREWVGHSNVIKPGP	Astexin 2	GLTQIQALDSVSGQFRDQLGLSAD
Propeptin	GYPWWDYRDLFGGHTFISP	Astexin 3	GPTPMVGLDSVSGQYWDQHAPLAD
RES-701-1	GNWHGTAPDWWFFNYW	Burhizin	GGAGQYKEVEAGRWSDRIDSDE
RES-701-3	GNWHGTSPDWWFFNYW	Caulonodin I	GDVLNAPEPGIGREPTG (LSRD)
SRO15-2005	GYFVGSYKEYWSRII	Caulonodin II	GDVLFAPEPGVGRPPMG (LSED)
Sungsanpin	GFGSKPIDSFGLSWL	Caulonodin III	GQIYDHPEVGIGAYGCE (GLQR)
		Capistruin	GTPGFQTPDARVISRFGFN
		Caulosegnin I	GAFVGQPEAVNPLGREIQG
		Caulosegnin II	GTLTFGLPEDFLPGHYMPG
		Caulosegnin III	GALVGLLLEDITVARYDPM
		Microcin J25	GGAGHVPEYFVGIGTPISFYG
		Rhodanodin	GVLPIGNEFMGHAATPG (ITE)
		Rubrivinodin	GAPSLINSEDNPAFPQRV

Lasso Peptides

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Entangled Scaffolds

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