Supplementary information to:

Heterologous expression of mersacidin in *Escherichia coli* elucidates the mode of leader processing

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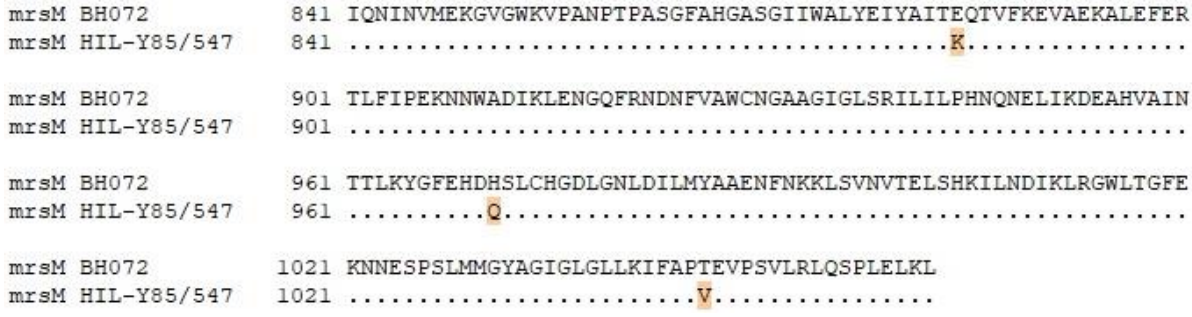
Figure S1 Comparison of *mrsM* from *Bacillus amyloliquefaciens* BH072 and *Bacillus* sp. HIL-Y85/54728

Figure S1 A Alignment of the MrsM nucleotide sequence. *To confirm functionality of the biosynthetic genes from BH072, the nucleotide sequence of the mersacidin gene cluster was compared between known mersacidin producer HIL-Y85/54728 (AJ250862.2)[1,2] and Bacillus amyloliquefaciens BH072 (CP009938.1)[3]. The intergenic sequence was found to be identical for all biosynthetic genes except mrsM, which contains six nucleotide mutations. These mutations in turn result in three amino acids*

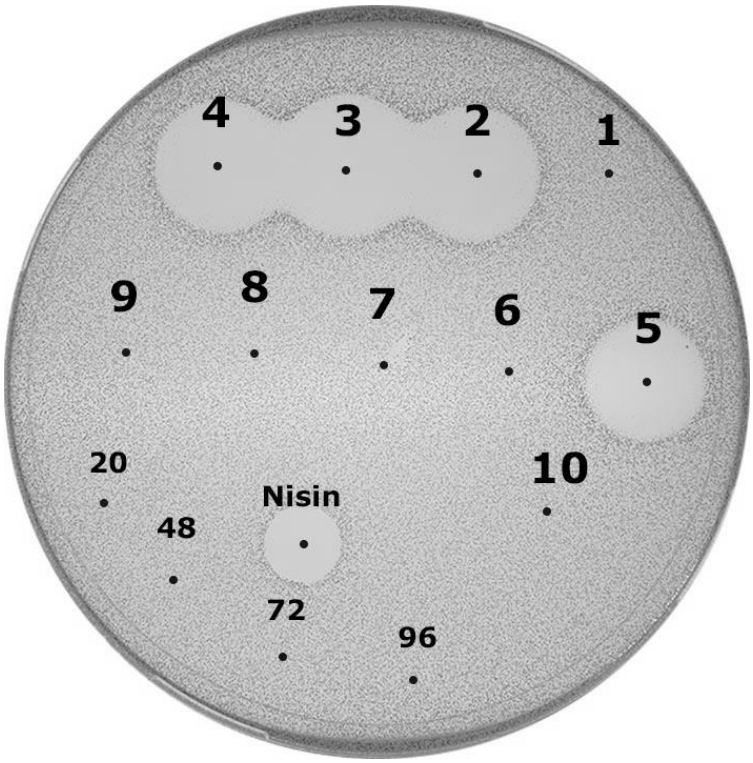


difference: K884E, Q971H, V1046T.

Figure S1 B Alignment of the MrsM amino acid sequences. *The alignment shows the amino acid sequence mutations K884E, Q971H, V1046T. In the process of expression optimization, the effect of these amino acid differences was found to be negligible, if not positive, compared to the HIL-Y85/54728 sequence.*

Figure S2 Antimicrobial activity test of His-MrsA + MrsM + MrsD and BH072 supernatant

Figure S2 Antimicrobial activity test of His-MrsA + MrsM + MrsD. His-MrsA + MrsM + MrsD was *digested by BH072 supernatant from cultures with different growth times and spotted on a Ø140 mm plate. All samples were incubated at 37 °C for 30 minutes, after which 9 µl of each sample was spotted.His-MrsA + MrsM + MrsD can be activated by supernatant of all growth times. The supernatant exhibits no antimicrobial activity.*



	His-MrsA + MrsM + MrsD in 5 mM PO_4 buffer	5 mM PO_4 buffer	BH072 supernatant
1	7 µl	3 µl	
2	7 µl		20 h 3 µl
3	7 µl		48 h 3 µl
4	7 µl		72 h 3 µl
5	7 µl		96h 3 µl
6		10 µl	
7		7 µl	20 h 3 µl
8		7 µl	48 h 3 µl
9		7 µl	72 h 3 µl
10		7 µl	96h 3 µl
20	*B. amyloliquefaciens* supernatant BH072 20 h		
48	*B. amyloliquefaciens* supernatant BH072 48 h		
72	*B. amyloliquefaciens* supernatant BH072 72 h		
96	*B. amyloliquefaciens* supernatant BH072 96 h		
Nisin	Nisin 25 ng / µl		

Figure S3 Antimicrobial activity test of HPLC fractions

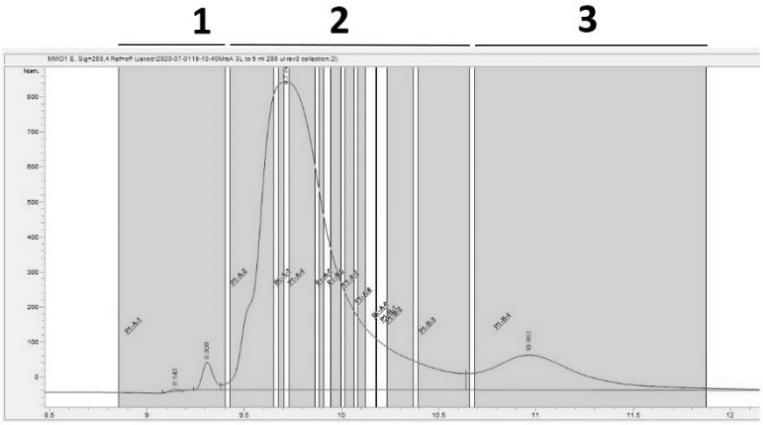
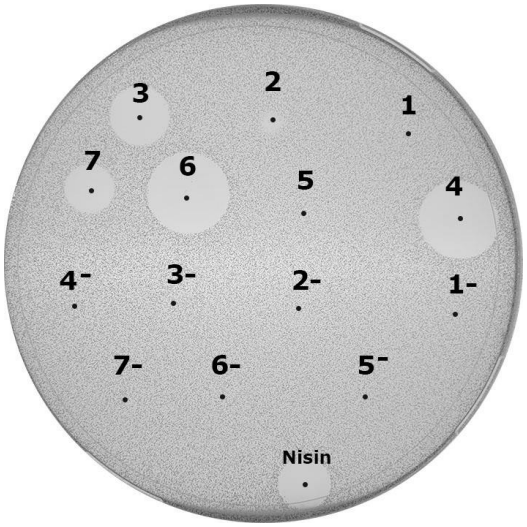


Figure S3 A Separation of His-MrsA + MrsM + MrsD by HPLC. *The freeze-dried samples from open C18 column were dissolved in Milli-Q, and separated in three fractions by HPLC. These fractions were freeze-dried and dissolved in Milli-Q.*



	Sample
1	Milli-Q
2	Peak 2 (4200x concentrated)
3	His-MrsA + MrsM + MrsD pre-HPLC preparation (600x concentrated)
4	Peak 3
5	Peak 1
6	His-MrsA + MrsM + MrsD secundary purification (214x concentrated)
7	His-MrsA + MrsM + MrsD pre-HPLC preparation (300x concentrated)
	Nisin 25 ng / µl

Figure S3 B Activity test of different HPLC fractions*. The activatable fraction was identified by incubating 7 µl of each fraction with 3 µl BH072 supernatant (+) or 3 µl Milli-Q (-). All samples were incubated at 37 °C for 30 minutes, after which 9 µl was spotted on a Ø140 mm plate. Peak 3 (spot 4) contains His-premersacidin. There is a small amount of activity from peak 2. However, this sample is eight times more concentrated than that of peak 3. The activity is most likely from the highly concentrated co-purified tail of peak 3.*

Figure S4 Antimicrobial activity test of MrsT150-His cleaved His-premersacidin

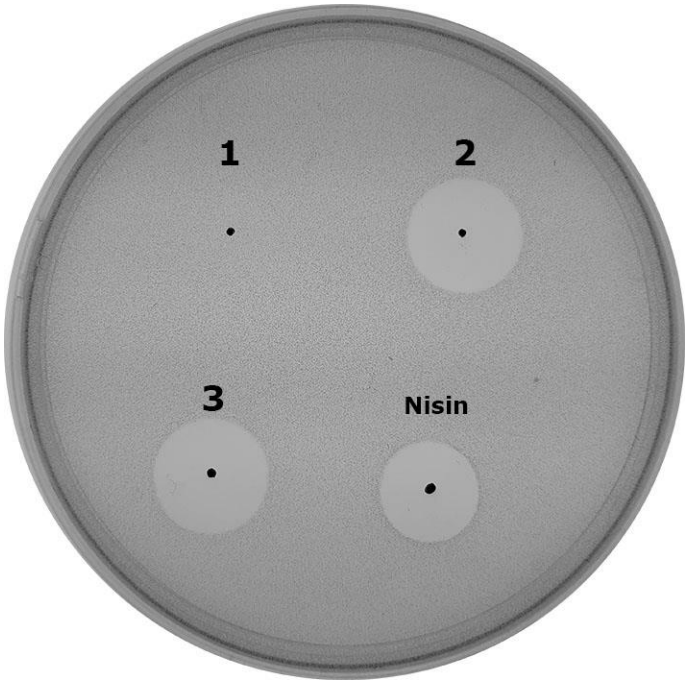
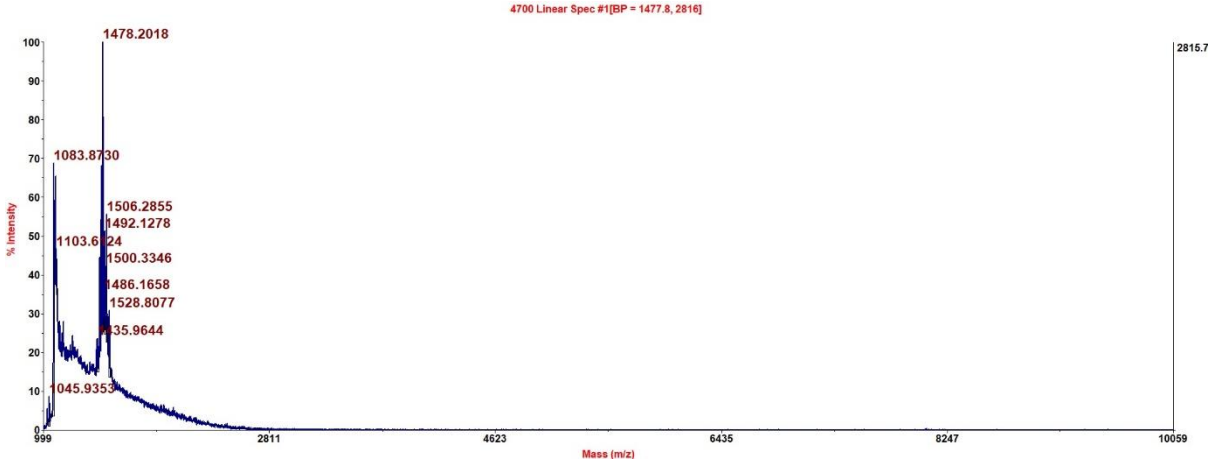


Figure S4 Antimicrobial activity test of MrsT150-His cleaved His-premersacidin. *His-premersacidin sample containing 1,5 µl MrsT150-His His-tag elution was incubated for 3 hours at 37 °C in duplicate. After incubation, 2 µl BH072 supernatant was added to one of the duplicates, and to a sample containing undigested His-premersacidin. All three samples were incubated for another 30 minutes at 37 °C, after which 9 µl was spotted. . MrsT150-His-digested pre-mersacidin and both supernatant digested samples were spotted in equal amounts of initial pre-mersacidin on an antimicrobial activity test-plate against M. flavus in a spotting volume of 9 µl on a Ø 90 mm plate.* ***1.*** *His-premersacidin + MrsT150-His* ***2.*** *His-premersacidin + MrsT150-His + BH072* ***3.*** *His-premersacidin + BH072****.*** *Mersacidin cannot be activated by MrsT150-His alone. After incubation of His-premersacidin with MrsT, it can be activated after incubation with BH072 supernatant. However, incubation with only BH072 supernatant gives a similar result. The partial leader cleavage by MrsT alone does not seem to be crucial for mersacidin activation.*

Figure S5 MALDI-TOF analysis of BH072 supernatant

A Whole spectrum



B Close-up

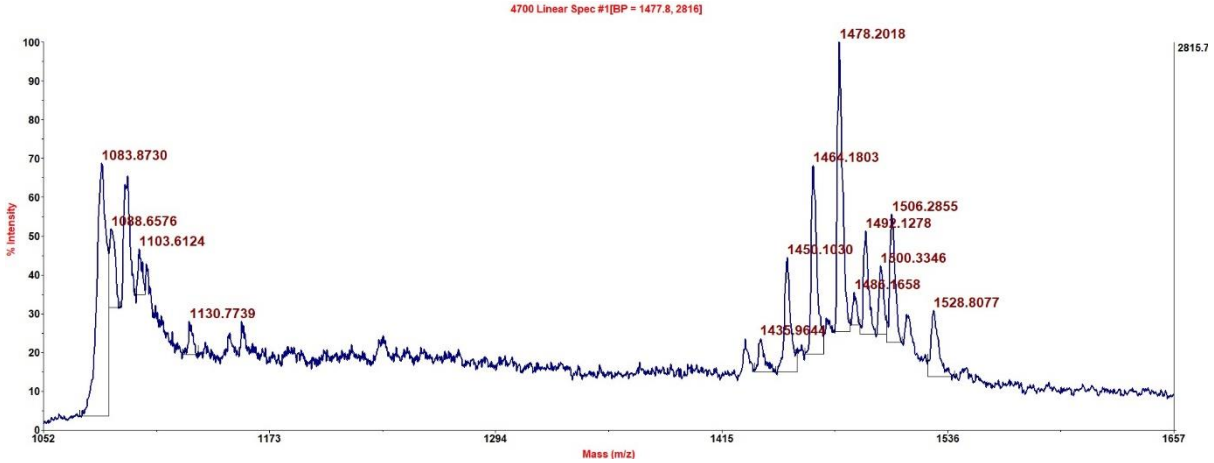


Figure S5 MALDI-TOF analysis of supernatant from a 48-hour *B. amyloliquefaciens* BH072 culture. *There is no mass detected resembling the reported mersacidin mass of 1826 Da[1] (Figure A). There are products present, which are shown in higher resolution below (Figure B). The supernatant was found to have no antimicrobial activity against Micrococcus flavus in any of the bacterial lawn spot tests.*

Figure S6 LC-MS of His-MrsA + MrsM and His-MrsA + MrsM + MrsD fractions

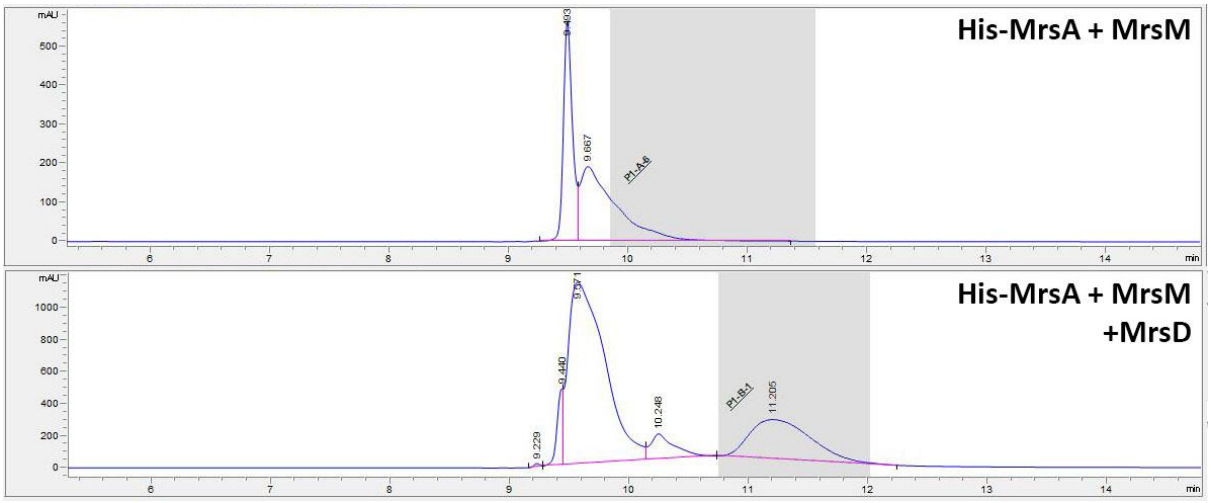


Figure S6 A Selection of HPLC fractions for LC-MS. *To confirm the formation of fully modified His-premersacidin, fractions with the highest rate of modification were looked at. As hydrophobicity of lanthipeptides increases with dehydration and ring formation, the peaks with the highest retention time were selected for analysis by LC-MS. The degree of ring formation was later compared between His-MrsA + MrsM and His-MrsA + MrsM + MrsD through a free-cysteine assay. The products with the highest retention time were collected (grey area).*

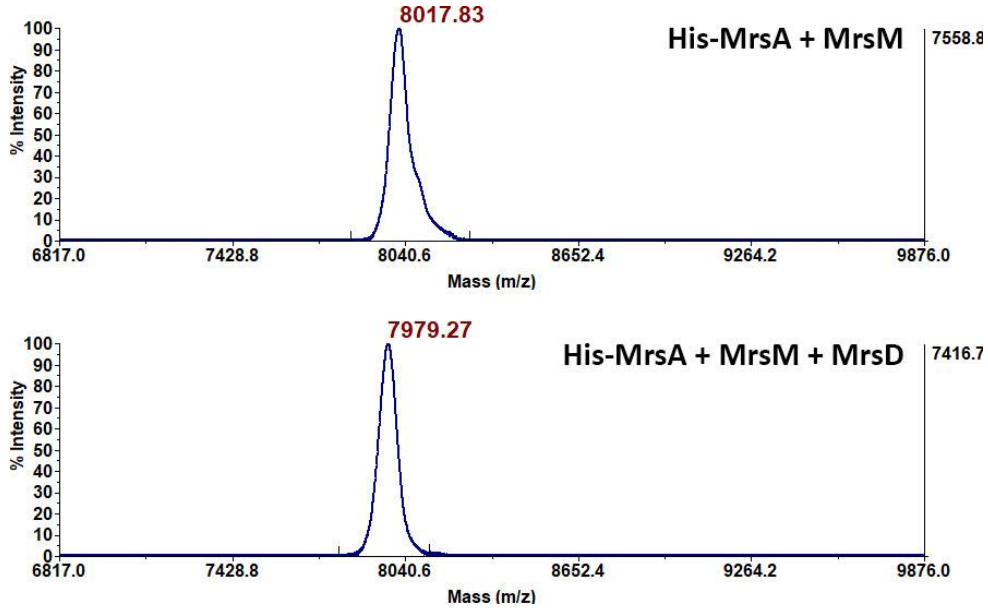


Figure S6 B Analysis of selected fractions by MALDI-TOF. *The purified products were examined by MALDI-TOF. The spectra are similar to that of the pre-HPLC MALDI-TOF spectra.*

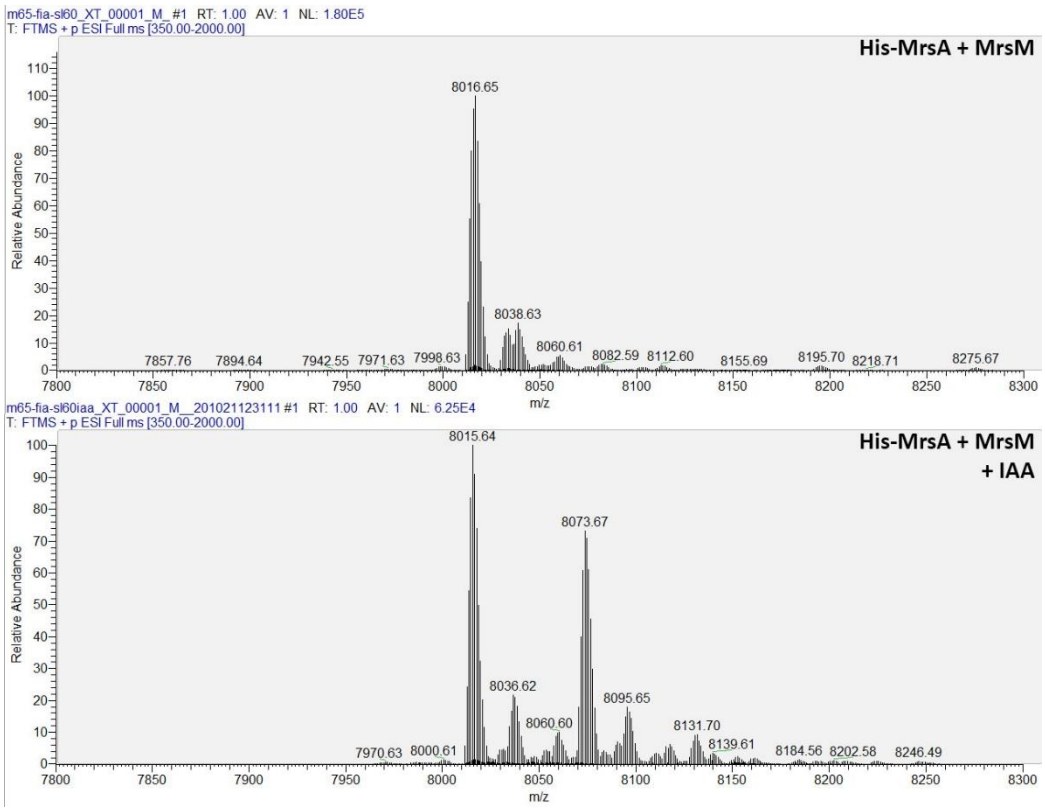


Figure S6 C LC-MS and free-cysteine assay His-MrsA + MrsM. *The exact mass of the HPLC purified fraction of His-MrsA + MrsM was determined by LC-MS, in combination with an iodoacetamide (IAA) free cysteine assay. Part of the His-MrsA + MrsM peak 8016.64 Da peak (five dehydrations) shifts to 8073.67, indicating one derivatisation of cysteine by IAA in ca. 50 % of the product. The remaining 8015.64 Da peak thus has five dehydrations and rings formed. Although complete modification by MrsM is thus possible without MrsD, the fraction studied here is a minor product of the His-MrsA + MrsM pre-HPLC sample. The procedure was performed by the RUG Interfaculty Mass Spectrometry Center (IMSC), as follows: Control - 20 µl sample + 20 µl 100 mM ABC + 20 µl 5 % FA +40 µl Milli-Q (without IAA). Assay - 20 µl sample + 20 µl 55 mM IAA in100mM ABC (incubate at RT for 45 min) then 20 µl 5% FA + 40 µl Milli-Q (with IAA). Of each sample 5 µl was injected*

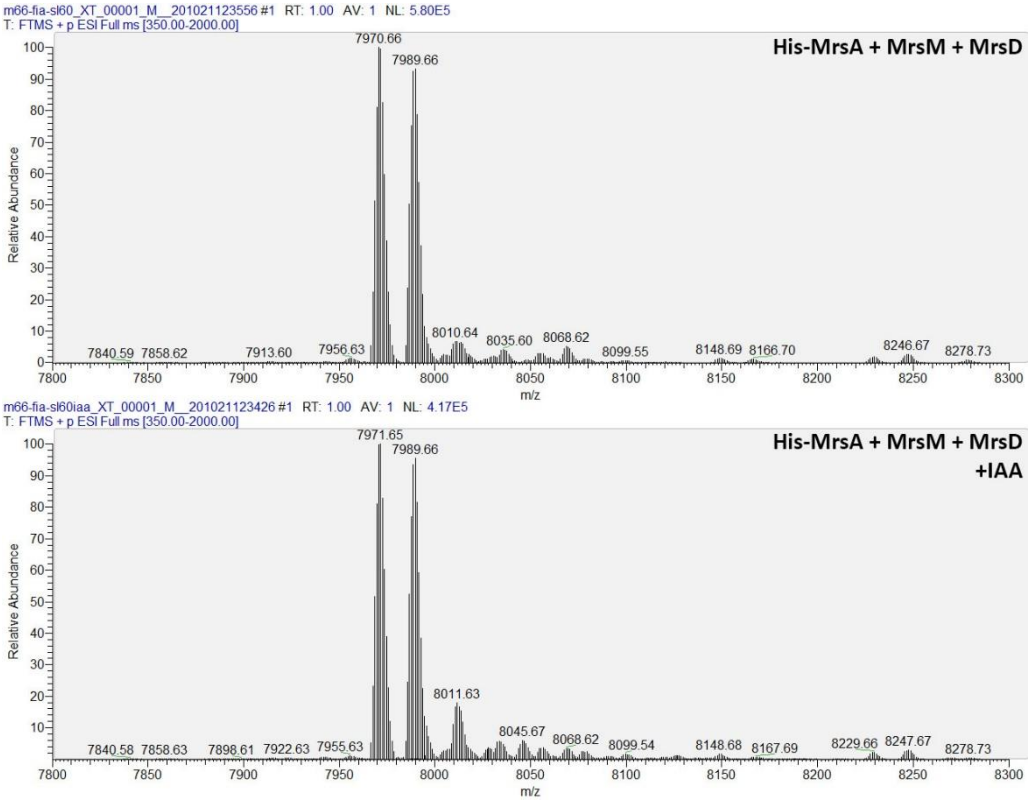


Figure S6 D LC-MS and free-cysteine assay His-MrsA + MrsM + MrsD. *The exact mass of the HPLC purified fraction of His-MrsA + MrsM + MrsD was determined by LC-MS, in combination with an iodoacetamide (IAA) free cysteine assay. The peaks of His-MrsA + MrsM + MrsD do not shift, meaning that the 7971.7 Da and the 7989.7 Da peak have five and four dehydrations and rings, respectively. The 7971.7 Da peak is thus his-premersacidin, while the 7989.7 Da peak lacks one dehydration and subsequent ring formation. The procedure was identical to that described for His-MrsA + MrsM.*

Figure S7 Optimization of the expression protocol

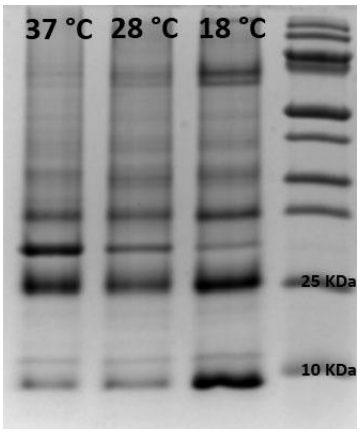


Figure S7-1 A Total yield comparison of 16-hour expression after induction at 37 °C, 28 °C, and 18 °C. *The tricine[4] gel shows an expected ca. 8 KDa band for all samples, most prominent in the sample from 18 °C*.

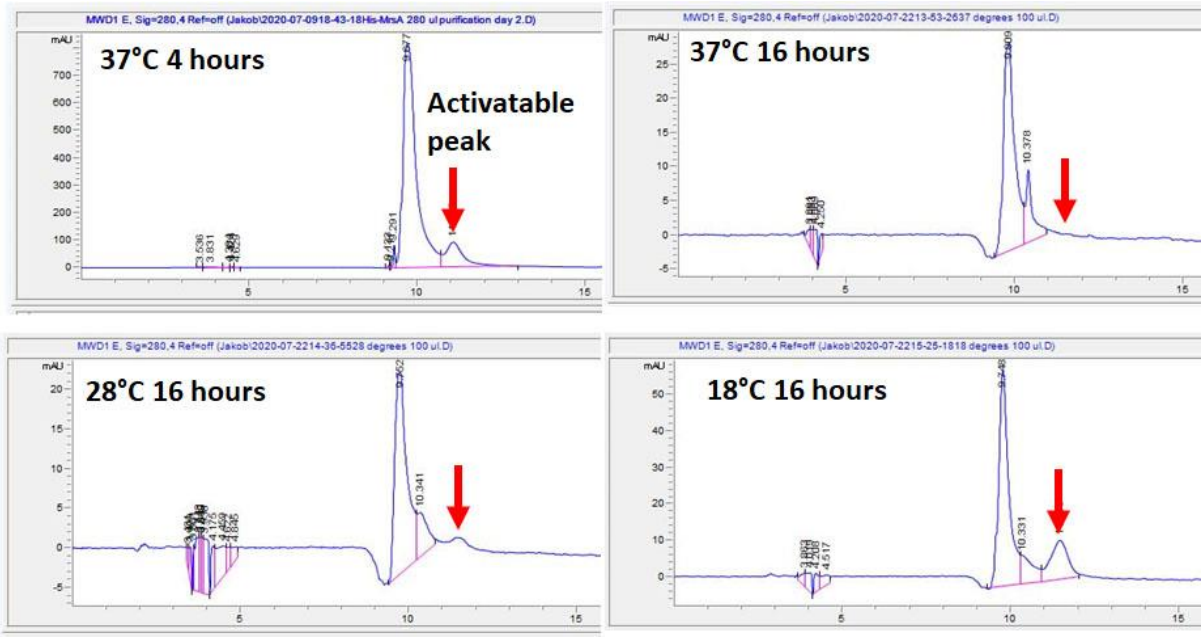
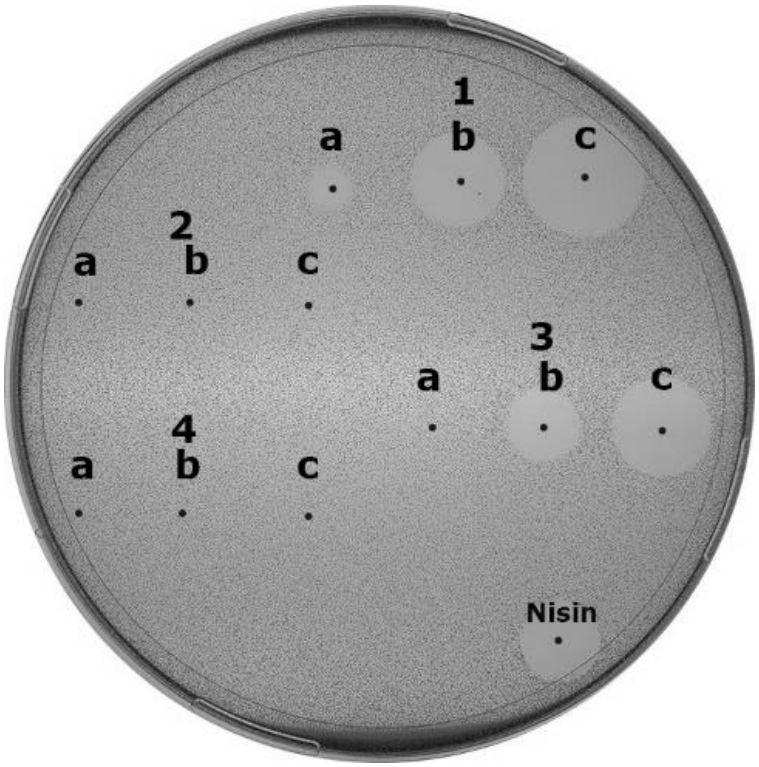


Figure S7-1 B Comparison of modification efficiency by HPLC. *The modification efficiency was compared by the relative size of the peak containing activatable His-premersacidin. The relative amount of activatable peak is highest in the 18 °C sample.*

Figure S7-1 C The higher total yield, and modification efficiency is consistent with the largest halo in the antimicrobial activity test for the 18 °C expression temperature (1C, 3C). For overnight expression, the temperature is negatively correlated with the yield and modification efficiency.



		Peptide from expression at respective temperatures (µl)			BH072 supernatant (µl)	Milli-Q-water (µl)
1000x from medium		37 °C	28 °C	18 °C	SN(48)	MQ
1	a	7			3	
	b		7		3	
	c			7	3	
2	a	7				3
	b		7			3
	c			7		3
3	a	2			3	5
	b		2		3	5
	c			2	3	5
4	a	2				8
	b		2			8
	c			2		8
Of each spot 9 µl, also nisin 25 ng/µl						

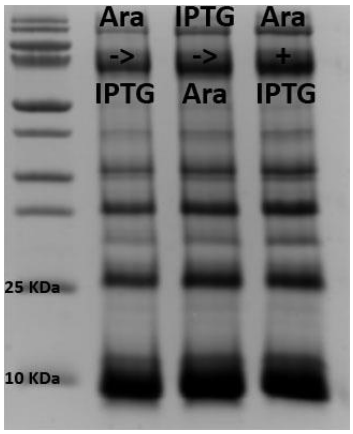


Figure S7-2 A Production yield of different induction order of MrsM and MrsD. *After the initial growth time following dilution of the overnight culture, the cultures were induced at 16 °C with either Arabinose, IPTG, or Arabinose + IPTG. Two hours after the first induction, IPTG was added to the culture initially induced with arabinose, and vice versa. The cultures were grown overnight before purification. The tricine gel shows the initial induction with only IPTG seems to result in a higher yield.*

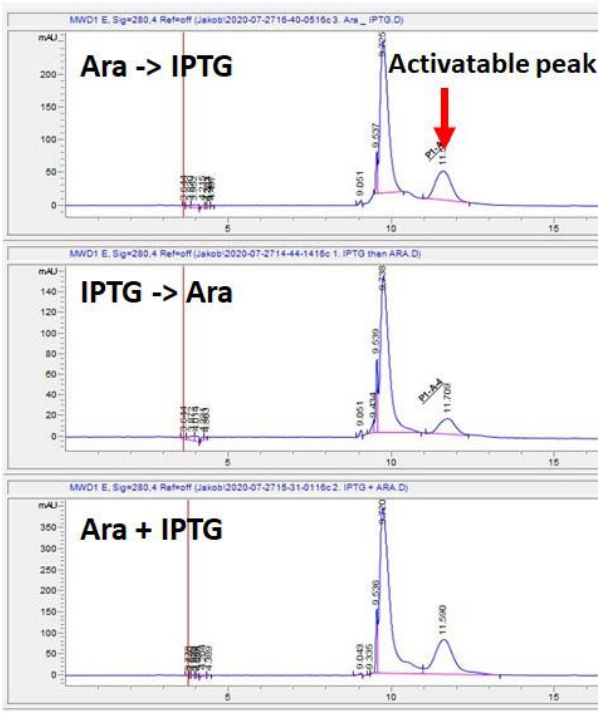
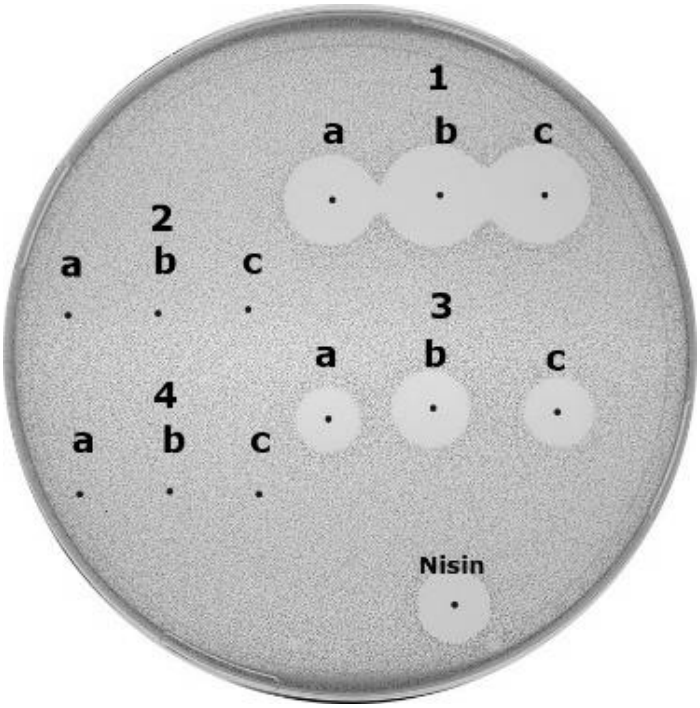


Figure S7-2 B Modification efficiency of different induction order of MrsM and MrsD. *The modification rate of IPTG->Ara was the lowest, as determined by HPLC analysis. Ara -> IPTG and Ara + IPTG appear similar in yield and modification efficiency.*

Figure S7-2 C Antimicrobial activity test of different induction order of MrsM and MrsD*. Here, the sample from Ara + IPTG seems to have a slightly larger halo than Ara -> IPTG, and certainly than IPTG -> Ara. Since this induction protocol is also the most convenient, induction timing was not looked further into*



		Peptide from expression from respective induction times (µl)			**BH072 supernatant (µl)**	**Milli-Q-water (µl)**
1000x from medium		IPTG -> Ara	IPTG + Ara	Ara -> IPTG	SN(48)	MQ
1	a	2.5			3	4.5
	b		2.5		3	4.5
	c			2.5	3	4.5
2	a	2.5				7.5
	b		2.5			7.5
	c			2.5		7.5
3	a	0.5			3	6.5
	b		0.5		3	6.5
	c			0.5	3	6.5
4	a	0.5				9.5
	b		0.5			9.5
	c			0.5		9.5
Of each spot 9 µl, nisin 25 ng/µl						

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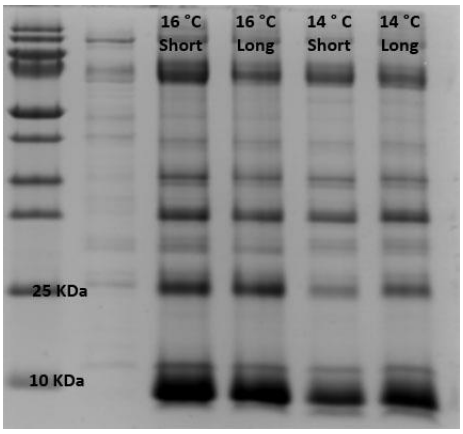


Figure S7-3 A Production yield of 16-hour and 24-hour expression after induction at 16 °C and 14 °C. *The tricine gel shows that the production yields are clearly the highest in the 16 °C samples, for this configuration it is the optimal expression temperature.*

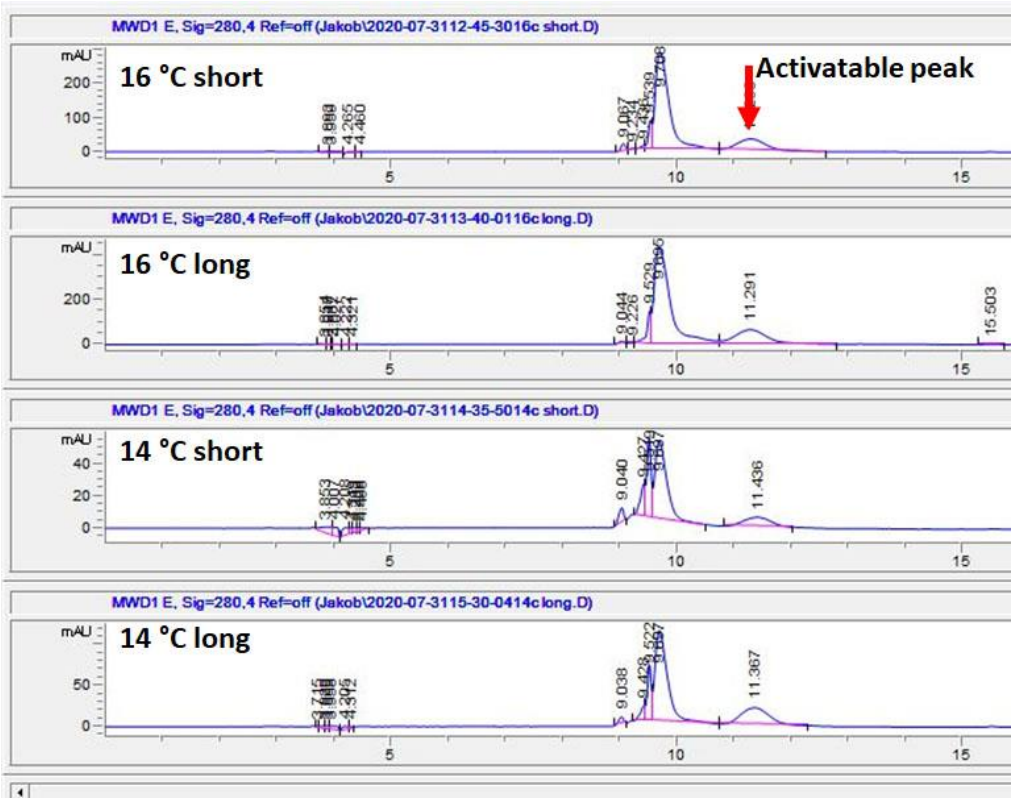
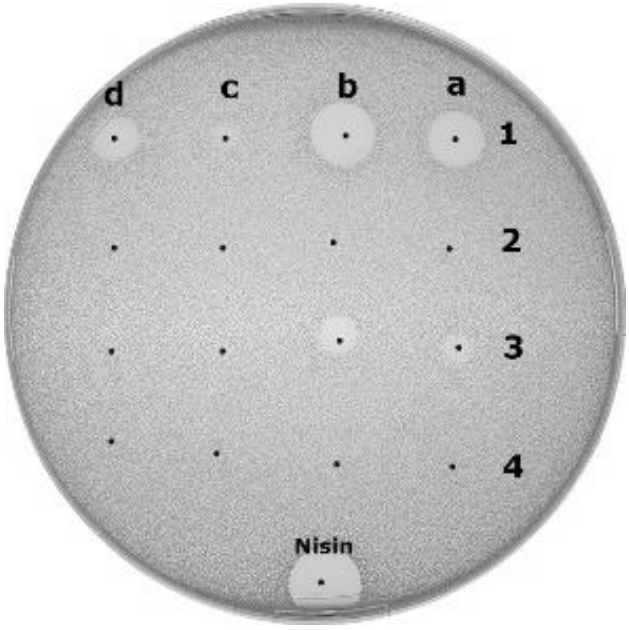


Figure S7-3 B Modification efficiency of 16-hour and 24-hour expression after induction at 16 °C and 14 °C. *HPLC analysis shows that expression time with an additional eight hours significantly increases modification efficiency*

Figure S7-3 C Antimicrobial activity test of 16-hour and 24-hour expression after induction at 16 °C and 14 °C *In the antimicrobial activity test, it can be clearly seen that expression at 14 °C leads to a lower production. The higher production, but more significantly the higher modification rate, of a 24-hour expression time increases the antimicrobial activity.*



		Peptide from expression at respective temperatures (µl)				**BH072 supernatant (µl)**	**Milli-Q-water (µl)**
1000x from medium		16 °C short	16 °C long	14 °C short	14 °C long	SN(48)	Milli-Q
1	a	2,5				3	4,5
	b		2,5			3	4,5
	c			2,5		3	4,5
	d				2,5	3	4,5
2	a	2,5					7,5
	b		2,5				7,5
	c			2,5			7,5
	d				2,5		7,5
3	a	0,5				3	6,5
	b		0,5			3	6,5
	c			0,5		3	6,5
	d				0,5	3	6,5
4	a	0,5					9,5
	b		0,5				9,5
	c			0,5			9,5
	d				0,5		9,5
Of each spot 9 µl, nisin 25 ng/µl							

Figure S7-4 A Modification efficiency of MrsM with an amino acid sequence restored to that of HIL-Y85/54728**.** *When expressed under the optimal conditions determined for the original setup, the modification efficiency when using the HIL-Y85/54728 MrsM is significantly lower than that of BH072. As the HPLC results have consistently matched the activity test-results, the characteristics of modification by HIL-Y85/54728 MrsM were not further looked into.*

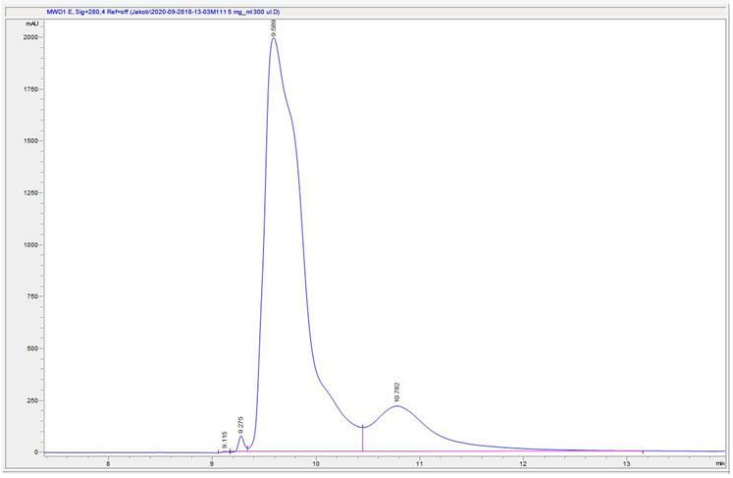


Table S1 List of primers used in this study

Primer Sequence	Goal Plasmid	Function	Template	Site
GATCGGTCTCCGCTCGCCACCGCTGAGCAATAAC	pACYC duet (*thnA-His*) + (m*rsM*)*	Backbone fw	pACYC duet (ThnA-His) + ( ) †	Eco31I
GATCGGTCTCCTCCTTCTTATACTTAACTAATATACTAAG		Backbone rv		Eco31I
GATGGGTCTCCAGGATCAATATGCATACAAAATTCAAACGG		Insert fw	pIL *mrsM*†	Eco31I
GATCGGTCTCAGAGCTCACTCCTAAGAAATAGAGCC		Insert rv		Eco31I
CACTAACTGCAGCAAGCTTG	pACYC duet (*mrsA-His*) + (*mrsM*)*	Backbone fw	pACYC duet (*thnA-His*) + (*mrsM*)	PstI
CATGGGTCTCCCATGGTATATCTCCTTATTAAAG		Backbone rv		Eco31I
CATGGGTCTCCCATGAGTCAAGAAGCTATCATTCG		Insert fw	pNZ8048 m*rsA-His*†	Eco31I
GATCCTGCAGGGTTCAAAGAAAGCTTAAATTAG		Insert rv		PstI
GACGGGTCTCGCATGGTATATCTCCTTATTAAAGTTAAAC	pACYC duet (*His-mrsA*) + (*mrsM*)	Backbone fw	pACYC duet (*mrsA-His*) + (*mrsM*)*	PstI
GACGGGTCTCGATAATTTAAGCTTTCTTTGAACCCTGC		Backbone rv		Eco31I
GACGGGTCTCCCATGCACCATCATCACCATC		Insert fw	pNZ8048 *His-mrsA*†	Eco31I
GACGCTGCAGGGTTCAAAGAAAGCTTAAATTAACAAATAC		Insert rv		Eco31I
GCTGCTGCAGGCATGCAAGC	pBAD (*mrsD)*	Backbone fw	pBAD	PstI
CATGCCATGGTGAATTCCTCCTGCTAGCCC		Backbone rv		NcoI
GACGGGTCTCGCATGAGTATTTCAATATTAAAAGATAAAAAG		Insert fw	BH072 Genomic DNA	Eco31I
GCAGCTGCAGCATCCCATTTATGTTAGTGAGG		Insert rv		PstI
GACGGGTCTCCTAATGCCTTACTTTGTTAAGGCTC	pACYC (*His-mrsA*)	Round PCR fw	pACYC duet (*His-mrsA*) + (*mrsM*)	Eco31I
GACGGGTCTCGATTACTTTCTGTTCGACTTAAGC		Round PCR rv		Eco31I
GGTGTTTCCATGGTATATCTC	pACYC (*mrsT150-His*)	Backbone fw	pACYC duet (ThnA-His) + ( ) †	PstI
GGACATCATCACCATCATCACTAAC		Backbone rv		NcoI
GCCACCTGCAGCAGCGGCCTCAGTGATGATG		Insert fw	pIL (*MrsT150-His*) †	Eco31I
GACGGGTCTCCCATGAGAAGAAGAGTTCCTCTAGTAAG		Insert rv		PstI
GGCGCTATCATGCCATACCGCGAAAG		pACYC sequencing fw		
TTGCACGGCGTCACACTTTG		pBAD sequencing fw		
TACTGCCGCCAGGCAAATTC		pBAD sequencing rv		
GCCGCATAATGCTTAAGTCG		MrsM sequencing 1 fw		
GTATTGGATTCAAAATACTTCTGATCTC		MrsM sequencing 2 fw		
TGCTCGAACAAATAGATTCTTTTAAAGG		MrsM sequencing 3 fw		
ATCGAAAGGGATGAAAACAACG		MrsM sequencing 4 fw		

* Only used for cloning purposes in this study

† Gene originates from, and remains similar to that on BH072 genome, from previous unpublished work

Table S2 HPLC protocol

All samples analyzed or purified by HPLC were run using the following protocol, using a NUCLEODUR C18 HTec, 5 µm, 250x4.6 mm column at ambient temperature.

Time [min]	A [%]	B [%]	Flow [mL/min]
0.00	95.0	5.0	1.000
2.00	95.0	5.0	1.000
3.00	65.0	35.0	1.000
4.00	65.0	35.0	1.000
25.00	59.0	41.0	1.000
26.00	5.0	95.0	1.000
32.00	5.0	95.0	1.000

Table S3 Mersacidin mass tables

All masses in Dalton

Leader + core-peptide

	Mono-isotopic		**Average**	
	His-MrsA	+MrsD	His-MrsA	+MrsD
Dehydrations	8103.7039	8057.6984	8108.9928	8062.9668
1	8085.6934	8039.6879	8090.9823	8044.9563
2	8067.6828	8021.6773	8072.9717	8026.9457
3	8049.6722	8003.6667	8054.9611	8008.9351
4	8031.6617	7985.6562	8036.9506	7990.9246
5	8013.6511	7967.6456	8018.9400	7972.9140

Core peptide

	Mono-isotopic		**Average**	
	His-MrsA	+MrsD	His-MrsA	+MrsD
Dehydrations	1961.8481	1915.8426	1963.3083	1917.2823
1	1943.8375	1897.8320	1945.2977	1899.2717
2	1925.8270	1879.8215	1927.2871	1881.2611
3	1907.8164	1861.8109	1909.2766	1863.2506
4	1889.8059	1843.8004	1891.2660	1845.2400
5	1871.7953	1825.7898	1873.2554	1827.2294

Core peptide + six amino acids (result of cleavage by MrsT150-His)

	Mono-isotopic		**Average**	
	His-MrsA	+MrsD	His-MrsA	+MrsD
Dehydrations	2536.0538	2490.0483	2537.9150	2491.8890
1	2518.0433	2472.0378	2519.9045	2473.8785
2	2500.0327	2454.0272	2501.8939	2455.8679
3	2482.0221	2436.0166	2483.8833	2437.8573
4	2464.0116	2418.0061	2465.8728	2419.8468
5	2446.0010	2399.9955	2447.8622	2401.8362

Table S4 Table of approximate percentage activatable peak of total product as determined by HPLC

Induction protocol	Percentage activatable peak of total
37 °C 4-5 hours	15 %
37 °C 16 hours	<1 %
28 °C 16 hours	5 %
18 °C 16 hours	20,3 %
16 °C 16 hours Ara -> IPTG	27,7 %
16 °C 16 hours Ara + IPTG	27,3 %
16 °C 16 hours IPTG -> Ara	18,3 %
16 °C 16 hours	20 %
16 °C 24 hours	25-30 %
14 °C 16 hours	20 % NB. Total production lower
14 °C 24 hours	25-30 % NB. Total production lower
16 °C 24 hours MrsM HIL-Y85/54728	15 %

From the above, an optimal expression protocol can be distilled, i.e. induction at 16 °C, followed by a 24-hour expression time.

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