SUPPLEMENTARY

Simple rules govern the diversity of bacterial nicotianamine-like metallophores

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SUPPLEMENTARY MATERIALS

Chemical synthesis of yNA

All solvents and reagents for the synthesis were purchased from Sigma Aldrich, Fluka and Alfa Aesar in gradient grade or reagent quality. All reactions involving air-sensitive reagents were performed under nitrogen or argon. Purifications were performed with column chromatography using silica gel (Merck 60, 230-400 mesh). Nuclear magnetic resonance NMR spectra were recorded on a Bruker spectrometer Avance 300 at 600 MHz. Chemicals shifts (δ , PPM) are reported from tetramethylsilane with the solvent resonance as internal standard. LC/MS system consisted in a Waters Alliance 2690 HPLC, coupled to a ZQ spectrometer (Manchester, UK) fitted with an electrospray source operated in the positive ionization mode (ESI⁺). All the analyses were carried out using a C18 Chromolith Flash 25 x 4.6 mm column operated at a flow rate of 3 ml/min. A gradient of 0% of 0.1% aqueous TFA (solvent A) to 100% of acetonitrile containing 0.1% TFA (solvent B) was developed over 3 min. Positive-ion electrospray mass spectra were acquired at a solvent flow rate of 100-200 μ L/min. Nitrogen was used for both the nebulizing and drying gas. The data were obtained in a scan mode ranging from 200 to 1700 m/z in 0.1 s intervals. A total of 10 scans was summed up to get the final spectrum. Compounds 1 and 2 were purified using a gradient composed of water/acetonitrile with 0.1% TFA at 50 mL/min flow rate performed on a Gilson PLC 2250 preparative apparatus equipped with a C18 Deltapak column (100 mm x 40 mm, 15µm, 100 Å). Purity was determined by RP-Analytic HPLC performed on an Agilent 1220 using a 50 x 4.6 mm Chromolith® High Resolution column. Compounds were separated using a linear gradient system (0 to 100% solvent B in 10 min) using a constant flow rate of 3mL.min⁻¹. High-resolution mass spectra (HRMS) were performed by the "Laboratoire de Mesures Physiques" of Montpellier University on a Micromass Q-Tof spectrometer equipped with electrospray source ionization (ESI), using phosphoric acid as internal standard.

Compound 1: (*S*)-*tert*-butyl 4-(((*S*)-3-(1*H*-imidazol-5-yl)-1-methoxy-1-oxopropan-2-yl)amino)-2- ((*tert*-butoxycarbonyl)amino)butanoate



Compound 2: (*S*)-2-(((*S*)-4-(*tert*-butoxy)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutyl)amino)-3-(1*H*-imidazol-5-yl)propanoic acid



A suspension of H-(L)-His-OMe-2 HCl (305 mg, 1.26 mmol) in MeOH (10 mL) was neutralized with finely powdered NaOH (100.8 mg, 2.52 mmol). After 10 min at room temperature, 300 mg of anhydrous MgSO₄ were added followed by Boc-(L)-Asa-OtBu (345 mg, 1.26mmol). After stirring the solution for 30 min at room temperature, the reaction was cooled with an ice bath and NaCNBH₄ (237 mg, 3.78 mmol) was added in portion over 30 min. The reaction mixture was stirred for 3 hours at room temperature then filtered and evaporated under vacuum to remove the solvent. The residue was purified by silica gel chromatography (DCM/MeOH from 98:2 to 96:4) to give the title compound (393 mg, 73%) as a colourless oil.

ESI-MS: 427.0 $[M+H]^+$, RP-LC: $t_R = 0.97$ min



Figure S1: 1H NMR spectra of compound **1** (600 MHz, CDCl₃) δ 8.41 (s, 1H), 7.29 (s, 1H), 5.47 (s br, 1H), 4.23-4.20 (m, 1H), 4.14-4.06 (m, 1H), 3.90 (s, 3H), 3.66-3.54 (m, 2H), 3.53-3.40 (m, 1H), 3.04-2.99 (m, 1H), 2.52-2.43 (m, 1H), 1.81-1.72 (m, 1H), 1.45 (s, 9H), 1.33 (s, 9H).



Figure S2: ¹³C NMR spectra of compound **1** (150 MHz, CDCl₃). δ 170.04, 166.76, 133.99, 125.18, 119.23, 83.83, 82.15, 60.08, 53.86, 50.94, 44.69, 30.13, 28.06, 27.75, 25.51.

Compound 1 (155 mg, 0.36 mmol) was dissolved in THF (10 mL) and a solution of LiOH·H₂O (22.6 mg, 0.54 mmol) in water (3 mL) was added. After 5 h, 0.5 eq. of LiOH·H₂O were added and the reaction was stirred until completion. The reaction mixture was neutrilized with 1M HCl in dioxane and solvent were removed under vacuum. The residue was purified over preparative HPLC (Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile, from 0% to 10% of B in 5 min, then to 40% of B in 35 min) and freeze dried to obtain compound **2** as a white powder (91.6 mg, 61%).

ESI-MS: 413.1 [M+H]⁺, RP-LC: t_R = 1.09 min



Figure S3: 1H NMR spectra of compound **2** (600 MHz, CDCl₃). δ 8.38 (s, 1H), 7.46 (s, 1H), 5.45 (s br, 1H), 4.12 (br, 1H), 4.06 (br, 1H), 3.6-3.55 (m, 2H), 3.47 (br, 1H), 3.06 (br, 1H), 2.45 (br, 1H), 1.82 (br, 1H), 1.45 (s, 9H), 1.33 (s, 9H).



Figure S4: ¹³C NMR spectra of compound **2** (150 MHz, CDCl₃). δ 170.23, 157.25, 157.19, 133.16, 126.08, 119.45, 83.71, 81.61, 61.14, 51.05, 45.03, 29.97, 28.06, 27.73, 25.48.

Compound **2** (55 mg, 0.13 mmol) was dissolved in 1:1 mixture of TFA/DCM (4 mL/mmol) and TIS (3% volume) was added. The mixture was stirred for 3 h at room temperature. Volatiles were removed under reduced pressure. The compounds was dissolved in HCl 0.01 N and freeze dried. This procedure was repeated twice to obtain the hydrochloridric salt **yNA** as a white solid in quantitative yield.

yNA*3HCl: 5-((*S*)-2-(((*S*)-3-ammonio-3-carboxypropyl)ammonio)-2-carboxyethyl)-1*H*-imidazol-1-ium chloride

Figure S5: 1H NMR spectra of yNA (600 MHz, D₂O). δ 8.53 (d, *J* = 1.4 Hz, 1H), 7.28 (d, *J* = 1.2 Hz, 1H), 4.07 (dd, *J* = 7.5, 5.3 Hz, 1H), 3.97 (dd, *J* = 8.2, 5.1 Hz, 1H), 3.40-3.16 (m, 4H), 2.35-2.10 (m, 2H).



Figure S6: ¹³C NMR spectra of xNA (150 MHz, D₂O). δ 171.31, 170.24, 134.11, 126.28, 118.06, 59.74, 51.04, 43.70, 26.45, 24.64.



Figure S7: HRMS spectrum of yNA (ESI). $C_{10}H_{17}N_4O_4$ calculated [M+H]: 257.1250, measured: 257.1247.

SUPPLEMENTARY TABLES AND FIGURES

Name	Sequence (5'-3')
PmCntL-pET-TEV Forward	GGCATTCCATATGATGAAGACGAAGACACAAGACC
PmCntL-pET-TEV Reverse	CCGGAATTCTTAAAGCTGCTCATCATAGGAAC
PmCntM-pET-TEV Forward	GGCATTCCATATGATGATGAGCAGCTTTAACCG
PmCntM-pET-TEV Reverse	CCGGAATTCTCATGTAAGTTCCCCCGAC
SaCntM:R33H Forward	GATATGGTTGGAC <u>AC</u> GCCTCAACATC
SaCntM:R33H Reverse	GATGTTGAGGC <u>GT</u> GTCCAACCATATC
SaCntM:D150A Forward	TATCTTGGCG <u>C</u> TACACGTATT
SaCntM:D150A Reverse	AATACGTGTA <u>G</u> CGCCAAGATA
PaCntM:A153 Forward	CAGCTACTACGCGG <u>AC</u> ACCAAGGTGATCG
PaCntM:A153 Reverse	CGATCACCTTGGT <u>GT</u> CCGCGTAGTAGCTG

 Table S1: Oligonucleotides used in this study.

Protein- vector	Growth conditions	Buffer A Buffer B		Imidazole-free buffer B	Reference	
	conditions	$20 \text{mM} \text{N}_{2} 2 \text{HPO} 4$	$20 \text{mM} \text{N}_2 2 \text{HPO} 4$			
SaCntM-	37°C without	2000000000000000000000000000000000000	2000000000000000000000000000000000000	20mM Henes 500mM		
pET- induction		15mM Imidazole	500mM Imidazole	NaCl pH-8	[1]	
SUMO	overnight	pH=8	pH=8	Nucl, pH=0		
SaCntM:	2700 11	20mM Na2HPO4,	20mM Na2HPO4,			
D150A-	37°C without	300mM NaCl,	300mM NaCl,	20mM Hepes, 500mM	This work	
pET-	induction	15mM Imidazole,	500mM Imidazole,	NaCl, pH=8		
SUMO	overnight	pH=8	pH=8			
	16°C	20mM Na2HPO4,	20mM Na2HPO4,			
SaCntM-	with	500mM NaCl,	500mM NaCl,	500 M NL CI T : 11 9 5	[2]	
pET-101	induction	15mM Imidazole,	250mM Imidazole,	500mM NaCI-Tris, pH=8.5		
-	overnight	pH=7.5	pH=7.5			
SaCntM: R33H- pET-101	16°C	20mM Na2HPO4,	20mM Na2HPO4,			
	with	500mM NaCl,	500mM NaCl,	500m M NoCl Tris all-95	This work	
	induction	15mM Imidazole,	250mM Imidazole,	500mm NaCI-Tris, pH=8.5		
	overnight	pH=7.5	pH=7.5			
PaCntM-	16°C	20mM Na2HPO4,	20mM Na2HPO4,	50mM potassium		
	with	500mM NaCl,	500mM NaCl,	phosphate 150mM sodium	[3,4]	
рыт- тем	induction	15mM Imidazole,	250mM Imidazole,	aitrata 20% alveerel pH-9		
IEV	overnight	pH=7.5	pH=7.5	citiate, 20% giyceioi, pn-o		
PaCntM:	16°C	20mM Na2HPO4,	20mM Na2HPO4,	50mM potassium		
A153D-	with	500mM NaCl,	500mM NaCl,	phosphate 150mM sodium	n This work	
pET-	induction	15mM Imidazole,	250mM Imidazole,	citrate 20% glycerol pH-8		
TEV	overnight	pH=7.5	pH=7.5	citate, 20% givenoi, pri-8		
PmCntM -pET- TEV	16°C	20mM Na2HPO4,	20mM Na2HPO4,			
	with	with 500mM NaCl, 500mM NaCl,		20mM Na2HPO4, 300mM	This work	
	induction	15mM Imidazole,	250mM Imidazole,	NaCl, pH=8	THIS WORK	
	overnight	pH=7.5	pH=7.5			
DmCntI	16°C	20mM Na2HPO4,	20mM Na2HPO4,			
nFT-	with	500mM NaCl,	500mM NaCl,	20mM Na2HPO4, 300mM	M This work	
TEV	induction	n 15mM Imidazole, 250mM Imidazole		NaCl, pH=8		
	overnight	pH=7.5	pH=7.5			

 Table S2: Growth conditions and buffers used in this study.







Figure S8: SDS-Page gels following the purification steps on a Ni-NTA column of SaCntM WT and D150A mutant, PaCntM WT and A153D mutant, and PmCntL and PmCntM. Mw: Molecular weight markers, L: Load, FT: Flow through, W: Wash, E: Elution.



Figure S9: Titration of NADPH binding to SaCntM WT (black circle) or to SaCntM:D150A (white circle) followed by fluorescence energy transfer between tryptophan excitation (280 nm) and NADPH emission (450 nm). The signals were measured between 300 and 800nm. We then calculated the mean fluorescence between 400 and 545nm (which correspond to the entire peak of NADPH emission) for a concentration range of NADPH.



Figure S10: Activity profile of PmCntM using variable concentration of xNA (black circle) and yNA (white circle) with fixed concentrations of others substrates: 0.2 mM of NADPH and 0.2 mM of α -ketoglutarate. The data points are means of three replicates with standard deviations. The fits are made using the Michaelis-Menten model.

Protein	xNA vs yNA	$K_m (\mu M)$	k_{cat} (s ⁻¹)	$k_{cat}/K_m (M^{-1}s^{-1})$
PmCntM	xNA	12 ± 1	0.40 ± 0.02	33 383
PmCntM	yNA	7 ± 1	0.48 ± 0.01	72 572

Table S3: Kinetic parameters of PmCntM activities established for a concentration range of xNA and yNA with fixed concentrations of others substrates: 0.2 mM of NADPH and 0.2 mM of α -ketoglutarate. The data and the standard errors associated with were generated by SigmaPlot according to the Michaelis-Menten model.

SUPPLEMENTARY REFERENCES

- Hajjar, C., Fanelli, R., Laffont, C., Brutesco, C., Cullia, G., Tribout, M., Nurizzo, D., Borezée-Durant, E., Voulhoux, R., Pignol, D., et al. (2019) Control by Metals of Staphylopine Dehydrogenase Activity during Metallophore Biosynthesis. J. Am. Chem. Soc. 141, 5555–5562.
- 2 Ghssein, G., Brutesco, C., Ouerdane, L., Fojcik, C., Izaute, A., Wang, S., Hajjar, C., Lobinski, R., Lemaire, D., Richaud, P., et al. (2016) Biosynthesis of a broad-spectrum nicotianamine-like metallophore in *Staphylococcus aureus*. Science **352**, 1105–1109.
- 3 Lhospice, S., Gomez, N. O., Ouerdane, L., Brutesco, C., Ghssein, G., Hajjar, C., Liratni, A., Wang, S., Richaud, P., Bleves, S., et al. (2017) *Pseudomonas aeruginosa* zinc uptake in chelating environment is primarily mediated by the metallophore pseudopaline. Sci. Rep. 7.
- 4 McFarlane, J. S. and Lamb, A. L. (2017) Biosynthesis of an Opine Metallophore by *Pseudomonas aeruginosa*. Biochemistry **56**, 5967–5971.