

Biosynthesis of Hesperetin, Homoeriodictyol and Homohesperetin in a Transcriptomics-driven Engineered Strain of *Streptomyces albidoflavus*

Álvaro Pérez-Valero^{1,2,3}, Juan Serna-Diestro^{1,2,3}, Albert E. Tafur Rangel^{4,5}, Simona Barbuto Ferraiuolo⁶, Chiara Schiraldi⁶, Eduard J. Kerkhoven^{4,5,7}, Claudio J. Villar^{1,2,3}, Felipe Lombó^{1,2,3} *

¹ Research Group BIONUC (Biotechnology of Nutraceuticals and Bioactive Compounds), Departamento de Biología Funcional, Área de Microbiología, Universidad de Oviedo, Oviedo, Principality of Asturias, Spain

² IUOPA (Instituto Universitario de Oncología del Principado de Asturias), Principality of Asturias, Spain.

³ ISPA (Instituto de Investigación Sanitaria del Principado de Asturias), Principality of Asturias, Spain.

⁴ Department of Biology and Biological Engineering, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden.

⁵ Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

⁶ Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, University of Campania "Luigi Vanvitelli", Via De Crecchio 7, 80138, Naples, Italy.

⁷ SciLifeLab, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden.

* Correspondence: lombofelipe@uniovi.es

Supplementary Materials:

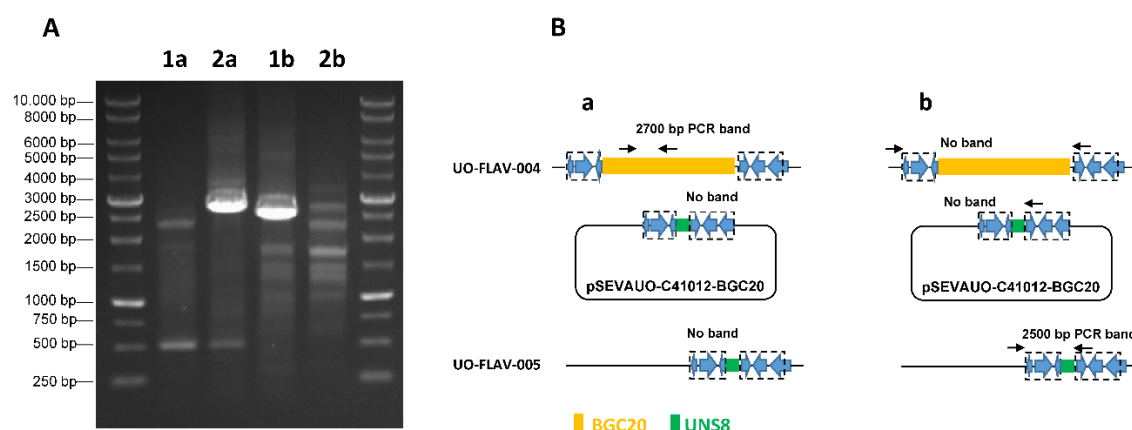


Figure S1. Generation of the *S. albidoflavus* UO-FLAV-005 strain. A) Agarose gel for PCR verification of the BGC20 deletion event, using the primers “JSD UP-checkingOK-BGC20” and “JSD RP-checkingWT-BGC20” on the mutant strain *S. albidoflavus* UO-FLAV-005 (lane 1a) and in the parental strain *S. albidoflavus* UO-FLAV-004 (lane 2a), and also using the primers “JSD UP-checkingOK-BGC20” and “JSD RP-checking-BGC20” on the mutant strain *S. albidoflavus* UO-FLAV-005 (lane 1b) and on the parental strain *S. albidoflavus* UO-FLAV-004 (lane 2b). B) Graphical representation of the expected PCR amplifications shown in the agarose gel picture: (a) expected PCR results on the parental (*S. albidoflavus* UO-FLAV-004) and mutant (*S. albidoflavus* UO-FLAV-005) strains using

the primers “JSD UP-checkingOK-BGC20” and “JSD RP-checkingWT-BGC20” (2700 bp); (b) expected PCR results with primers “JSD UP-checkingOK-BGC20” and “JSD RP-checking-BGC20” (2500 bp). The black arrows indicate the primers and are shown where annealing is possible for one or both of them. UNS8: non-coding DNA region of 40 bp in the pSEVAUO-C41012-BGC20 between the homologous arms. PCR conditions using Terra™ PCR Direct Polymerase: 1 cycle of 98 °C (2 min), and 35 cycles of 98 °C (10 sec) + 68 °C (3 min).

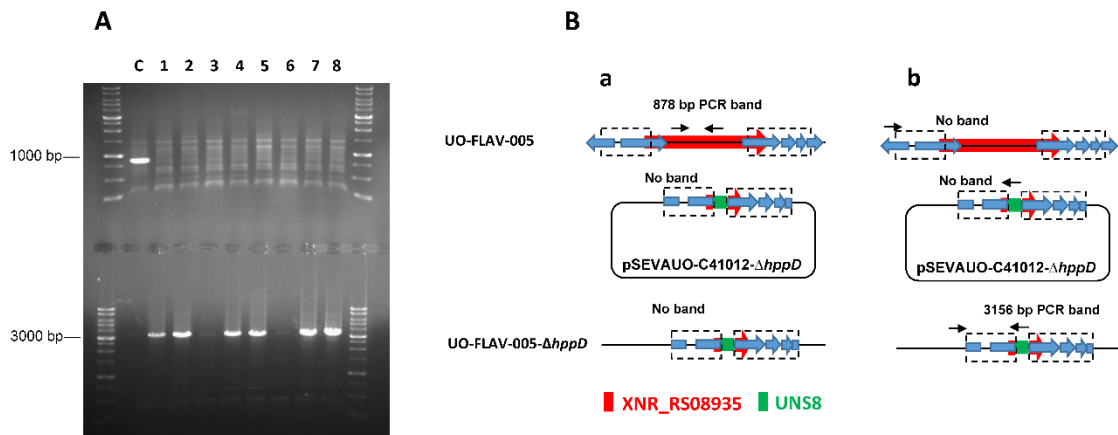


Figure S2: Generation of the *S. albidoflavus* UO-FLAV-005- Δ hppD strain. A) Agarose gel for PCR verification of the hppD knock-out event. Lanes on top are PCR results using the primers “Deletion hppD fw” and “Deletion hppD rev” on the mutant strain *S. albidoflavus* UO-FLAV-005- Δ hppD (lanes 1-8) and in the parental strain *S. albidoflavus* UO-FLAV-005 (lane C). Lanes below are PCR results using the primers “Recombination hppD FW” and “UNS8 REV” on the mutant strain *S. albidoflavus* UO-FLAV-005- Δ hppD (lanes 1-8) and on the parental strain *S. albidoflavus* UO-FLAV-005 (lane C). All the clones checked are correct. B) Graphical representation of the expected PCR amplifications shown in the agarose gel picture: (a) expected PCR results on the parental (*S. albidoflavus* UO-FLAV-005) and mutant (*S. albidoflavus* UO-FLAV-005- Δ hppD) strains using the primers “Deletion hppD fw” and “Deletion hppD rev” (878 bp); (b) expected PCR results with primers “Recombination hppD FW” and “UNS8 REV” (3156 bp). The black arrows indicate the primers and are shown where annealing is possible for one or both of them. UNS8: non-coding DNA region of 40 bp in the pSEVAUO-C41012- Δ hppD between the homologous arms. PCR conditions using Terra™ PCR Direct Polymerase: 1 cycle of 98 °C (2 min), and 35 cycles of 98 °C (10 sec) + 68 °C (3.5 min).

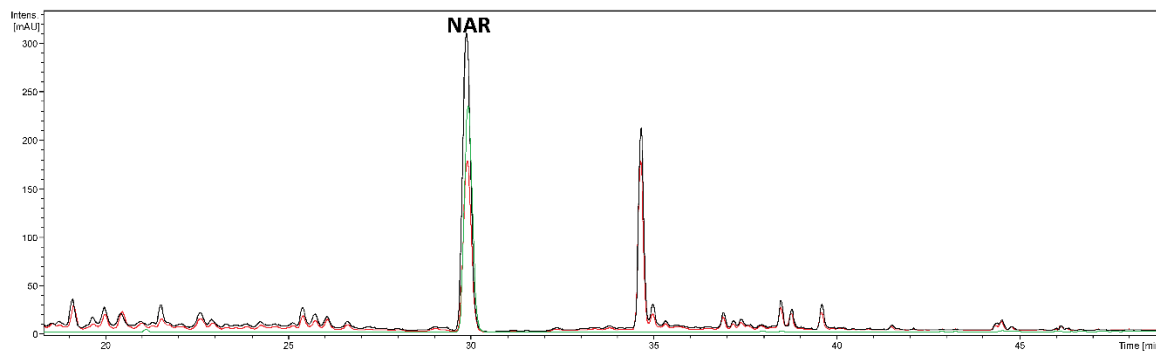


Figure S3: HPLC-DAD chromatograms showing the biosynthesis of naringenin in the strains *S. albidoflavus* UO-FLAV-005-NAR (red) and *S. albidoflavus* UO-FLAV-005- Δ hppD-NAR (black). Naringenin (NAR) pure standard in green.

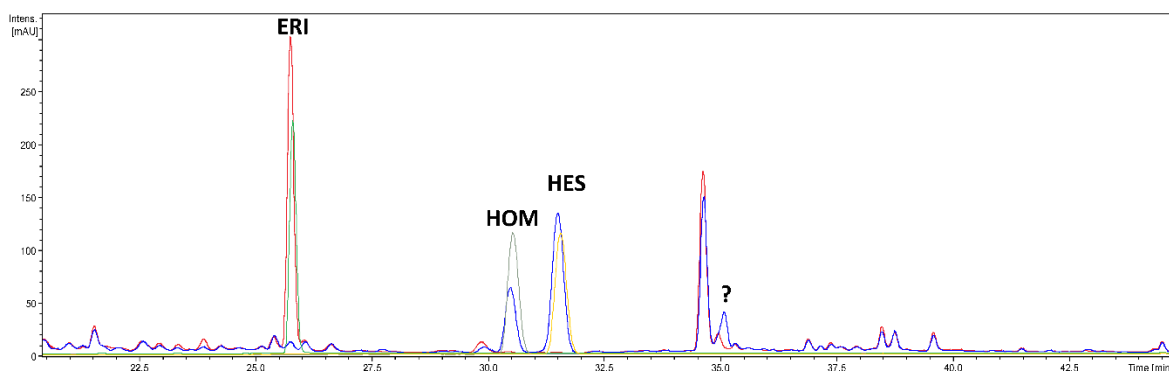


Figure S4: HPLC-DAD chromatograms of *S. albidoflavus* UO-FLAV-005- Δ hppD-ERI (red) and *S. albidoflavus* UO-FLAV-005- Δ hppD-HES/HOM (blue). Eriodictyol (ERI) pure standard in green; Homoeriodictyol (HOM) pure standard in gray; Hesperetin (HES) pure standard in orange; Tentative dimethylated eriodictyol (?).

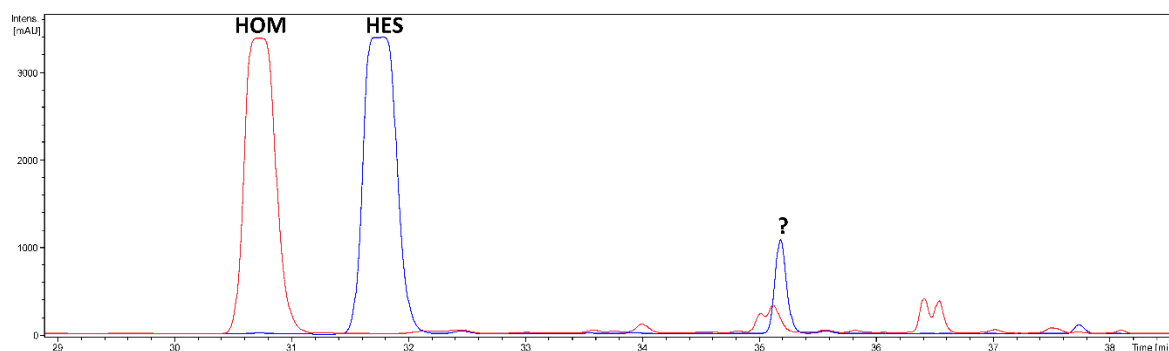


Figure S5: HPLC-DAD chromatograms of *S. albidoflavus* UO-FLAV-005 fed with homoeriodictyol (red) and hesperetin (blue). Homoeriodictyol (HOM); Hesperetin (HES); Tentative dimethyl eriodictyol (?).

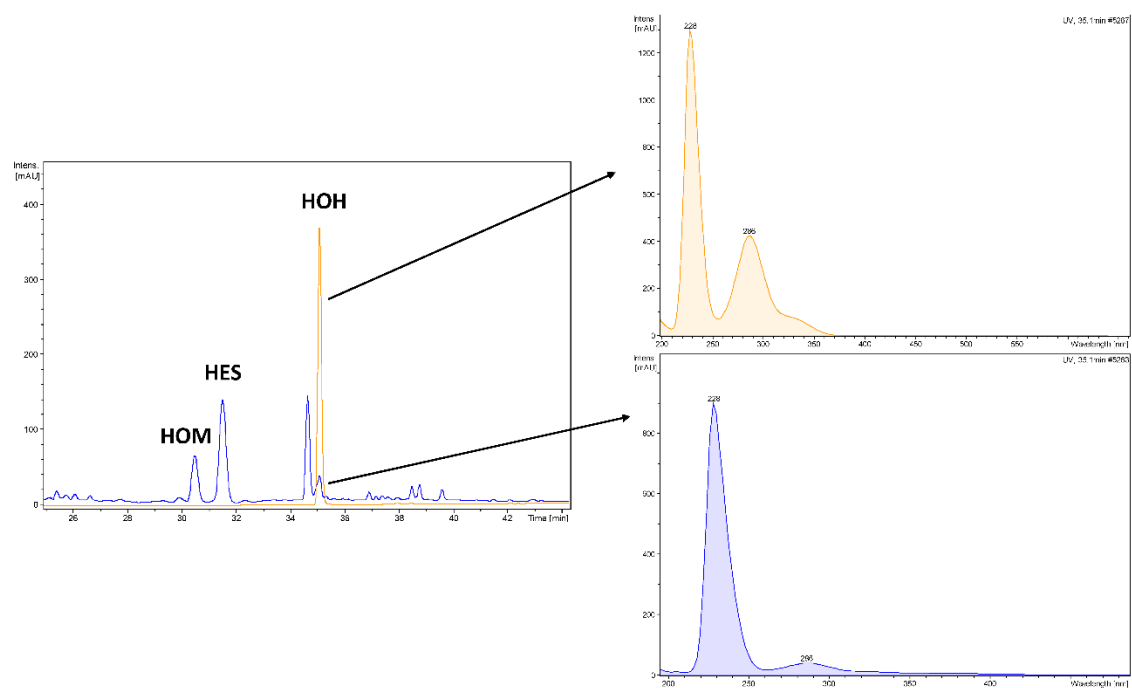


Figure S6: HPLC-DAD chromatograms *S. albidoflavus* UO-FLAV-005- $\Delta hppD$ -HES/HOM (blue) and a pure standard of Homohesperetin (orange). Black arrows indicate the absorption spectra of the tentative dimethyl eriodictyol (blue) and Homohesperetin (orange). Homoeriodictyol (HOM); Hesperetin (HES); Homohesperetin (HOH).

Table S1: List of differentially expressed genes. Abbreviations: WT.Tx: wild-type cultures of *S. albidiflavus* with added L-Tyr, WT-x: control cultures of the wild-type strain.

FeatureID	Regulation	GeneName	log2FC	DE	p-value	p-adjusted	RC Product	WT.T1	WT.T2	WT.T3	WT.S	WT.3	WT.S
ENR_023888	No	2768.86614	-1.3618868	0.1789001	0.1238921	0.1820222	0.886127122 WUS family transcriptional regulator	1261	1811	2318	4074	8482	4464
ENR_023788	Down	14184.821	0.17137628	0.60897728	0.48201818	0.002878897	0.112848227 Paq/Tss family 10 terminal domain-containing protein	411	792	880	847	8028	18028
ENR_023828	Down	10863.87764	-0.18171477	0.17132087	0.01088028	0.2886868	0.148764878 M23 family nucleoside pyrophosphatase	187	461	600	1388	1068	1882
ENR_023820	No	189.717842	1.88808928	0.42277908	0.003817168	0.0628008	0.138127588 R26 family oxidoreductase	288	458	484	117	80	128
ENR_023830	No	232.477748	-0.68613005	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023768	No	2848.90358	-0.20216888	0.28428772	0.01218864	0.3878584	0.028287809 multi-subunit ABC transporter	868	1288	2148	4851	9788	4084
ENR_023832	No	2114.17582	-0.13418178	0.93748484	0.002188828	0.288488872	0.388488811 cytochrome P450	434	782	1184	1078	3840	8711
ENR_023822	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	Up	374.288828	1.47787874	0.1238841	0.287888	0.00208888	0.87888884 type I polyketide synthase	788	861	878	284	171	220
ENR_023830	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	Down	1485.04888	-0.2778888	0.3887888	0.001388388	0.04477728	0.381888848 sugar ABC transporter permease	738	713	1028	2722	2752	2884
ENR_023828	No	880.00888	-0.18787878	0.8413208	0.0848888	0.8427084	0.028177888 hypothetical protein	181	148	188	484	888	1004
ENR_023788	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023828	Down	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023888	No	4088.0218	-0.88888204	0.818281	0.0188881	0.088828	0.028877888 acid shock protein	1878	2488	2721	7388	8888	7182
ENR_023820	Up	887.778182	1.8813218	0.127882	0.0088888	0.2248828	0.48288884 hypothetical repeat-containing protein	1078	1448	1888	422	818	818
ENR_023828	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	No	188.48888	-0.2784882	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023888	No	708.18882	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788	488	841	124	88	128
ENR_023820	No	182.2138	-0.71888811	0.28428772	0.00918778	0.194711	0.288282878 carboxylate ABC transporter permease	428	388	448	1852	1821	1004
ENR_023788	No	278.0882	-0.6881278	0.93820048	0.0140348	0.2881894	0.812788412 hypothetical protein	189	183	177	704	428	471
ENR_023820	Up	1346.76782	2.438178	0.21817888	2.38782810	0.8488288	0.382114384 type I polyketide synthase	4752	4784	4911	884	424	1004
ENR_023828	No	180.08811	1.78887874	0.188787	0.00888428	0.188787	0.412888282 DUF713 family protein	1788					

Table S2: Primers used in this study.

Function	Name	Sequence 5' - 3'
Homology region A BGC20	JSD-BGC20-up1	CAAGACGCTGGCTCTGACATTTCCGCTACTGA ACTACTCGCTGGCGGACAGCAGGGGC
	JSD-BGC20-rp2	CCAGGTGGTTGATGGGTTGATTGCTTTGGTTG AGACGAGGATCAGCGCGTTCACCGGC
Homology region B BGC20	JSD-BGC20-up3	CCTCGTCTCAACCAAAGCAATCAACCCATCA ACCACCTGGTGCCGTGTTCCAGGCTCGTGCC
	JSD-BGC20-rp4	TATGTGACCGTAGAGTATTCTTAGGTGGCAGC GAACGAGACGGTCGAGGGGAGCGTCGC
Recombination checking BGC20	JSD RP-checking-BGC20	TTGGAGTAGAGCCGTTTCGAT
	JSD UP-checkingOK-BGC20	GGTCACCGGTGTCGATAGTC
Deletion checking BGC20	JSD UP-checkingOKBGC20	GGTCACCGGTGTCGATAGTC
	JSD RP-checkingWTBGC20	GTCTACACCACGGCGTTCAT
Protospacer BGC20	JSD-BGC20-PS-up	ACGCCGCGCACCAGGTTGCGGGGG
	JSD-BGC20-PS-rp	AAACCCCCCGCAACCTGGTGCGCG
Homology región A $\Delta hppD$	Primer SBF-UNS7-hppD-regionA-fw	CAAGACGCTGGCTCTGACATTTCCGCTACTGA ACTACTCGGAGCCACTCCTTCTTCAGGA
	Primer SBF-UNS8-hppD-regionA-rev	CCAGGTGGTTGATGGGTTGATTGCTTTGGTTG AGACGAGGACTTCAAGGCCCTCTTCGAG
Homology región B $\Delta hppD$	Primer SBF-UNS8-hppD-regionB-fw	CCTCGTCTCAACCAAAGCAATCAACCCATCA ACCACCTGGGAGTAGGCGACGAGCTTCAT
	Primer SBF-UNS6-hppD-regionB-rev	TATGTGACCGTAGAGTATTCTTAGGTGGCAGC GAACGAGTGCGAGAGGTTCTTGATGC
Recombination checking $\Delta hppD$	Recombination hppD FW	CGCTGCACCTCGACGCCG
	UNS8 REV	CCAGGTGGTTGATGGGTTG
Deletion checking $\Delta hppD$	Deletion hppD fw	GTCGGCTTGTCCTGGACCG
	Deletion hppD rev	GGACCGGAGAACGGCAGC
Protospacer $\Delta hppD$	Protospacer XNR_1801 fw	ACGCGGCCACGTGGCTCTCCAGGA
	Protospacer XNR_1801 rev	AAACTCCTGGAGAGCCACGTGGCC
pSEVA88c1 amplification	Vector FW UNS6	CTCGTTCGCTGCCACCTAAGAATACTCTACGG TCACATACAAGCTTGCGGCCGCGTCG
	Vector REV UNS7	CGAGTAGTTCAGTAGCGGAAATGTCAGAGCC AGCGTCTTGCCTAGGCGGCCTCCTGTG