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for

Analysis of Specific Mutants in the Lasalocid Gene Cluster: Evidence for Enzymatic Catalysis of a Disfavoured Polyether Ring Closure

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Cloning and sequencing of the *las* gene cluster

Streptomyces lasaliensis cultures were grown in M79 medium (10 g glucose, 10 g peptone, 2 g yeast extract, 6 g NaCl, 10 g casein hydrolysate, 1 L H_2O) (50 mL) at 30°C in a rotary shaker (200 rpm) for 48 h. The cells were harvested by centrifugation and the pellet was resuspended and washed in 10.3% sucrose (w/v). The genomic DNA was isolated using the salting out procedure[1]. The DNA was partially digested with BamHI to give fragments of average size 30-60 kbp and dephosphorylated using shrimp alkaline phosphatase. The fragments were ligated into Supercos vector as described in the manual (http://www.stratagene.com/manuals/251301.pdf). The cosmid DNA was packaged into XL-1 blue cells following the procedure in the Gigapack III gold manual (http://www.stratagene.com/manuals/200201.pdf). The transformed cells were plated onto LB agar (20 g tryptone, 5 g yeast extract, 10 g NaCl, 20 g Agar, 1 L H_2O) containing 100 µg mL^{-1} carbenicillin and 50 µg mL^{-1} kanamycin. Positive colonies were inoculated into liquid 2TY (16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 L H_2O) containing 100 µg mL^{-1} carbenicillin and 50 µg mL^{-1} kanamycin and incubated at 37°C overnight with shaking (250 rpm). The culture was spun down and used in Southern blotting.

The Southern blotting was carried out following standard procedures[2] using as a probe 1.2 kbp fragment of DNA encoding the ketosynthase domain of the erythromycin PKS kindly provided by Dr Yuliya Demyduk. The colonies showing the strongest hybridisation were sent for end sequencing. End sequencing and restriction analysis identified a number of overlapping cosmids whose ends contained PKS encoding DNA. The cosmid M7G was partially digested with *Sau3AI* to give 2-10 kbp fragments and subcloned into pHSG397. These subclones were then end sequenced and the sequences were assembled using the *phred*/*phrap*/*consed* software package (www.phrap.org) to give the sequence of cosmid M7G. Chromosome walking was used to identify the overlapping cosmids S3F, U12G and 4B12 which were subcloned and sequenced giving the entire lasalocid gene cluster.

Table S1. Deduced function of genes flanking the lasalocid cluster

ORF	Size[a]	Homology and Origin	Identity/ Similarity %	Proposed Function
orf01	270	ref|YP_002207684.1| *Streptomyces sviceus* ATCC 29083	47/61	Hypothetical protein
orf02	302	ref|NP_851542.1| *Streptomyces rochei*	70/82	Ku70/Ku80
orf03	226	ref|NP_862104.1| *Streptomyces violaceoruber*	69/81	Putative secreted protein
orf04	465	ref|YP_001220856.1| *Clavibacter michiganensis subsp. michiganensis* NCPPB 382	72/83	Putative MFS-type efflux protein
orf05	290	gb|AAA97193.1| *Escherichia coli*	100/100	KpLE2 phage like element
orf06	172	gb|AAG50897.1|AC068901_6 *Arabidopsis thaliana*	99/100	Transposase
orf07	157	emb|CAE55859.1| *Escherichia coli Nissle* 1917	99/99	Gene fragment
orf08	157	gb|ABV54432.1| uncultured prokaryote	33/47	Gene fragment
orf09	118	dbj|BAD86806.1| *Streptomyces sp.* KO-3988	54/64	Gene fragment
las1	249	dbj|BAC68117.1| (PteH) *Streptomyces avermitilis* MA-4680	59/71	Type II thioesterase
las2	518	gb|AAO65793.1|AF440781_12 (MonT) *Streptomyces cinnamonensis*	54/72	Resistance protein
las3	161	ref|NP_627826.1| *Streptomyces coelicolor* A3(2)	60/77	Transcription regulator
las4	793	ref|NP_824078.1| *Streptomyces avermitilis* MA-4680	42/54	LuxR-family regulator
las5	454	gb|AAQ84149.1| (PlmT7) *Streptomyces sp.* HK803	79/89	Ethylmalonyl CoA synthase
las6	573	gb|AAQ84148.1| (PlmT8) *Streptomyces sp.* HK803	62/75	3-hydroxybutyryl-CoA dehydrogenase
lasAI	4986	gb|AAZ77693.1| (ChlA1) *Streptomyces antibioticus*	53/63	Polyketide synthase KS, AT,ACP, KS, AT DH, KR, ACP, KS, AT, DH, ER, KR, ACP
lasAII	5469	gb|AAX98191.1| (Orf16) *Streptomyces aizunensis*	52/65	Polyketide synthase KS, AT, DH, KR, ACP, KS, AT, DH, ER, KR, ACP, KS, AT, KR, ACP

ORF	Size[a]	Homology and Origin	Identity/ Similarity %	Proposed Function
lasAIII	1108	gb|AAZ94389.1| *Streptomyces neyagawaensis*	61/71	Polyketide synthase KS, AT, ACP
lasAIV	1647	gb|AAX98190.1| (Orf15) *Streptomyces aizunensis*	56/70	Polyketide synthase KS, AT, KR, ACP
lasAV	3679	gb|AAX98191.1| (Orf16) *Streptomyces aizunensis*	56/68	Polyketide synthase KS, AT, DH, ER, KR, ACP, KS, AT, ACP
lasAVI	1877	gb|AAZ94389.1| *Streptomyces neyagawaensis*	55/67	Polyketide synthase KS, AT, DH, KR, ACP
lasAVII	1311	gb|ABB52545.1| *Streptomyces sp.* KCTC 0041BP	48/59	Polyketide synthase KS, AT, ACP, TE
las7	122	ref|YP_001108251.1| *Saccharopolyspora erythraea* NRRL 2338	38/56	Putative regulator
lasC	472	dbj|BAE93732.1| (TmnC) *Streptomyces sp.* NRRL 11266	49/64	Epoxidase
lasB	282	gb|ABC84468.1| (NigBI) *Streptomyces violaceusniger*	47/62	Epoxide hydrolase
orf10	309	ref|YP_001614508.1| *Sorangium cellulosum* 'So ce 56'	58/71	Gene fragment
orf11	253	ref|YP_002196839.1| *Streptomyces pristinaespiralis* ATCC 25486	34/43	Lipoprotein
orf12	241	ref|YP_001824965.1| *Streptomyces griseus subsp. griseus* NBRC 13350	34/50	Hypothetical protein
orf13	71	gb|AAA27767.1| *Aplysia californica*	47/69	Gene fragment
orf14	262	ref|ZP_02862447.1| *Anaerofustis stercorihominis* DSM 17244	27/50	Hypothetical protein
orf15	201	ref|NP_628475.1| *Streptomyces coelicolor* A3(2)	55/64	TetR family transcription regulator
orf16	248	ref|YP_001848906.1| *Mycobacterium marinum* M	66/78	Short-chain dehydrogenase/reductase
orf17	189	ref|NP_821494.1| *Streptomyces avermitilis* MA-4680	79/88	Hypothetical protein
orf18	152	ref|YP_703377.1| *Rhodococcus sp.* RHA1	67/84	Hypothetical protein
orf19	329	ref|YP_001142355.1| *Aeromonas salmonicida subsp. salmonicida* A449	34/48	Carboxy-peptidase
orf20	76	ref|XP_001275792.1| *Aspergillus clavatus* NRRL 1	31/45	Hypothetical protein
orf21	51	ref|YP_002202646.1| *Streptomyces sviceus* ATCC 29083	86/96	Hypothetical protein
orf22	187	ref|NP_624749.1| *Streptomyces coelicolor* A3(2)	72/81	TetR family transcription regulator
orf23	278	ref|NP_624748.1| *Streptomyces coelicolor* A3(2)	76/88	Hydrolase
orf24	156	ref|ZP_03155039.1| *Cyanothece sp.* PCC 7822	34/49	Hypothetical protein
orf25	224	ref|XP_750009.1| *Aspergillus fumigatus* Af293	50/61	Hypothetical protein
orf26	104	ref|YP_001870139.1| *Nostoc punctiforme* PCC 73102	41/54	Hypothetical protein
orf27	166	ref|YP_002209471.1| *Streptomyces sviceus* ATCC 29083	89/95	Cupin domain protein
orf28	167	ref|YP_638324.1| *Mycobacterium sp.* MCS	53/69	MarR family transcription regulator
orf29	314	ref|YP_002209470.1| *Streptomyces sviceus* ATCC 29083	78//87	Alcohol dehydrogenase

ORF	Size[a]	Homology and Origin	Identity/ Similarity %	Proposed Function
orf30	297	ref|ZP_03169544.1| *Streptomyces sp.* Mg1	68/79	AraC family transcription regulator
orf31	1101	ref|ZP_03190930.1| *Streptomyces pristinaespiralis* ATCC 25486	47/61	Putative hydrolase
orf32	359	ref|YP_002196880.1| *Streptomyces pristinaespiralis* ATCC 25486	81/88	Sugar ABC transporter
orf34	322	ref|YP_002196882.1| *Streptomyces pristinaespiralis* ATCC 25486	84/92	Sugar ABC transport
orf35	341	ref|YP_001828117.1| *Streptomyces griseus subsp. griseus* NBRC 13350	79/88	Sugar ABC transporter
orf36	338	ref|YP_001828118.1| *Streptomyces griseus subsp. griseus* NBRC 13350	77/86	LacI family transcription regulator
[a] Numbers refer to amino acid residues				

Constructs for gene disruption and complementation

To disrupt *lasC* two fragments corresponding to the left and right chromosomal regions flanking *lasC* were PCR amplified from a cosmid clone. Primer C1 TGGACT-CGCTCTCGCATATGTTCGTC which contained an NdeI site and primer C2 AGC-ATCGAGGAGACGAGCATTCCG were used to amplify a 1.5 kbp left flanking fragment including 68 bp of the N terminus of *lasC*. Primer C3 TATTCGGTGTAGTTGG-GAAGCTTGCTGG which contained a HindIII site and primer C4 CGTGGCCCTCA-ACACCCTGTCG were used to amplify a 1.5 kbp right flanking fragment including 160 bp of the C terminus of *lasC*. The phusion polymerase amplified fragments were digested with NdeI and HindIII respectively and blunt end ligated. The fragments were then ligated into NdeI and HindIII digested pYH7 to form the deletion plasmid pLS?C containing a truncated *lasC* gene with a 1191 bp deletion. (Figure S1).

To complement *lasC* a 1843 bp region containing the gene *lasC* and its native promoter was amplified by PCR using primers CComp1 CGCAGATCCATCTCGGTGT-CGG and CComp2 GTCCGGCGCGAACAGCTGGA. The PCR product was blunt-end ligated into PvuII digested pSET152 to form the plasmid pSETlasCNP. The insert was sequenced and the plasmid was introduced in the *?lasC* mutant by conjugation. An apramycin resistant strain incorporating pSETlasCNP was selected and the presence of the plasmid was verified by PCR.

To disrupt *lasB* two fragments corresponding to the left and right chromosomal regions flanking *lasB* were PCR amplified from a cosmid clone. Primer B1 CGGAATG-CTCGTCTCCCATATGCTG which contained an NdeI site and primer B2 GTGCCCA-GCGGGTCCACCAG were used to amplify a 1512 bp left flanking fragment including

124 bp of the N terminus of *lasB*. Primer B3 GCGATGACACTAAGCTTGGTCAAC-GG which contained a HindIII site and primer B4 CATCGAGTACGTGATGGTGATC-GGC were used to amplify a 1491 bp right flanking fragment including 105 bp of the C terminus of *lasB*. The phusion polymerase amplified fragments were digested with NdeI and HindIII respectively and blunt end ligated. The fragments were then ligated into NdeI and HindIII digested pYH7 to form the deletion plasmid pLS?B containing a truncated *lasB* gene with a 618 bp deletion (Figure S2).

To complement *lasB* a 1675 bp region containing the gene *lasC* and its native promoter was amplified by PCR using primers BComp1 CCGCCGGCTGCATTACGACC and BComp2 GGCCGGGAACTTCTCCTCCTTC. The PCR product was blunt-end ligated into PvuII digested pSET152 to form the plasmid pSETlasBNP. The insert was sequenced and the plasmid was introduced in the *?lasB* mutant by conjugation. An apramycin resistant strain incorporating pSETlasBNP was selected and the presence of the plasmid was verified by PCR.

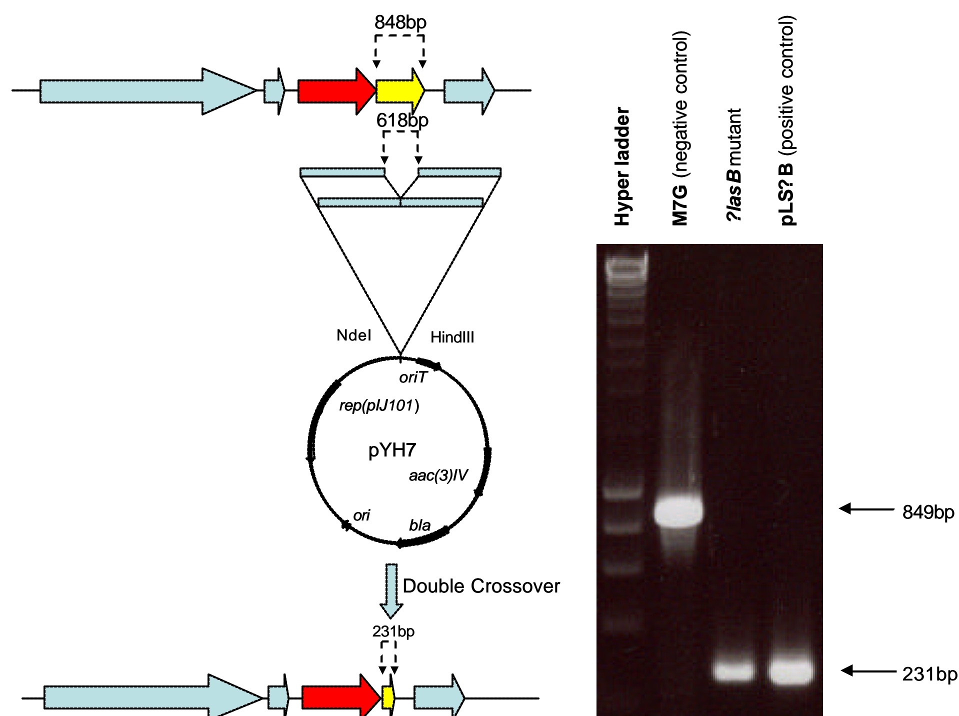

Figure S1. In frame deletion of *lasB*. A) Schematic representation of the in-frame deletion in *lasC* using shuttle vector pYH7. The numbers above the dashed lines represent, respectively the expected size of the PCR product amplified from *S. lasaliensis* wild-type, the size of the internal deletion and the expected size of the PCR product amplified from the *?lasB* mutant. The sizes of the flanking regions used to obtain homologous recombination between construct and chromosome are indicated. B) Confirmation of *?lasB* by PCR screening. Cosmid M7G served as a negative control and plasmid pLS?B served as a positive control.

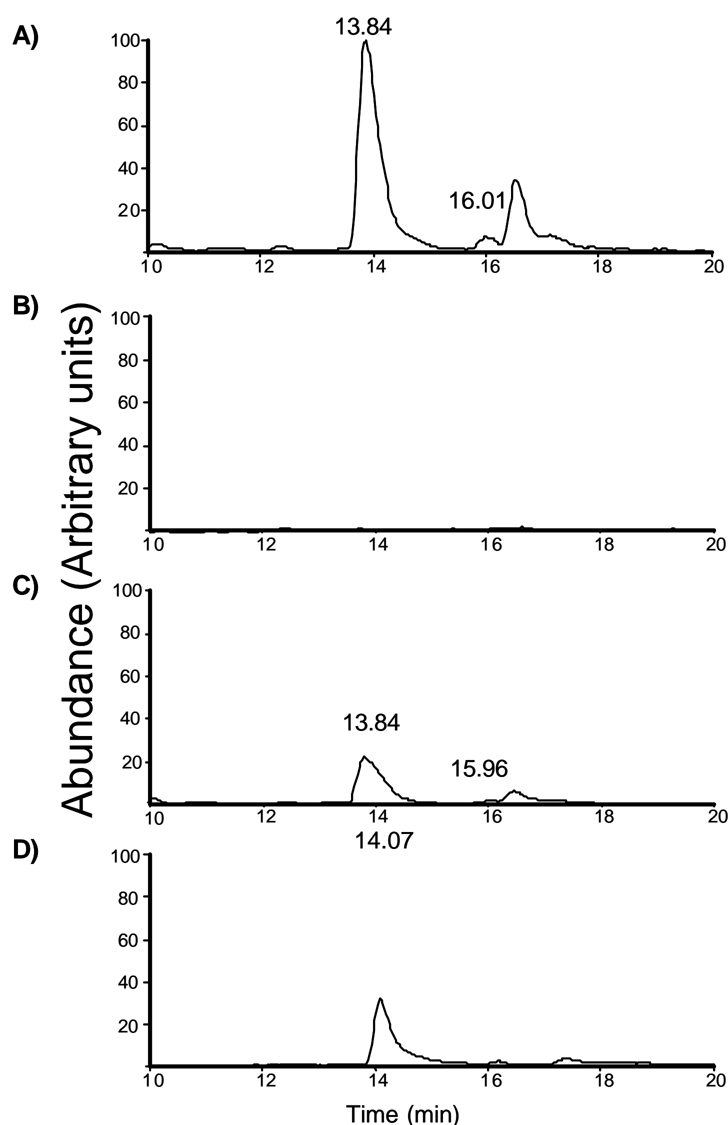

Figure S2. HPLC analysis of lasalocid and iso-lasalocid from *S. lasaliensis* strains. A) *S. lasaliensis* wild-type. B) *S. lasaliensis ?lasC* mutant. C) *S. lasaliensis ?lasC* complemented with expression plasmid pSETlasCNP. D) Lasalocid A standard

Table S2. ^{1}H NMR data for lasalocid A and iso-lasalocid A[a]		
Carbon	^{1}H NMR d (ppm) of lasalocid A	^{1}H NMR d (ppm) of iso-lasalocid A
5	7.14 (d, J = 7.6, 1H)	7.10 (d, J = 7.5, 1H)
6	6.59 (d, J = 7.6, 1H)	6.57 (d, J = 7.6, 1H)
8	3.41, 2.39	3.49, 2.43
9	1.77, 1.40	1.75, 1.41
10	1.70	1.63
11	4.15 (dd, J = 1.4, 9.5)	4.19 (d, J = 9.7, 1H)
12	2.77 (m, 1H)	2.78 (m, 1H)
14	2.52 (m, 1H)	2.59 (m, 1H)
15	3.89 (dd, J = 10.4)	3.81 (d, J = 10.0)

16	2.17	2.24
17	1.87, 1.41	1.92, 1.20
19	3.45	3.86
20	1.99, 1.44	1.77
21	1.62	1.42
23	3.94	3.90
24	1.18 (d, J = 6.9, 3H)	1.13 (d, J = 6.6, 3H)
25	1.66, 1.33	not assigned
26	0.94 (t, J = 7.4, 3H)	0.81-0.89
27	1.70, 1.36	not assigned
28	0.80 (t, J = 7.4, 3H)	0.81-0.89
29	1.02 (d, J = 6.4, 3H)	1.03 (d, J = 6.4, 3H)
30	1.94, 1.28	1.92, 1.31
31	0.81 (t, J = 7.3, 3H)	0.81 (t, J = 7.4, 3H)
32	0.90 (d, J = 7.1, 3H)	0.89 (d, J = 7.1, 3H)
33	0.86 (d, J = 6.3, 3H)	0.85 (d, J = 6.9, 3H)
34	2.16 (s, 3H)	2.19 (s, 3H)

[a] The ^{1}H NMR data were recorded in $CDCl_3$ (500 MHz).

2. The ^{13}C NMR (500 MHz, $CDCl_3$) data for iso-lasalocid A: *d* 216.0, 174.0, 161.3, 143.9, 134.1, 123.8, 121.0, 115.3, 88.8, 86.0, 83.9, 80.9, 71.5, 70.8, 55.2, 49.3, 39.7, 37.1, 34.3, 34.0, 31.1, 29.7, 28.4, 26.3, 22.7, 17.4, 16.4, 15.9, 15.7, 13.1, 12.8, 12.6, 9.7, 7.7.

References

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[2] Boehringer Mannheim, *The DIG system user's guide for filter hybridization*, Boehringer Mannheim GmbH Biochemica, Germany, **1993**