Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Bacterial biosynthesis of a multipotent stilbene

Susan A. Joyce, Alexander O. Brachmann, Itamar Glazer, Lea Lango, Gertrud Schwär, David J. Clarke, and Helge B. Bode**

Stilbene production in *Photorhabdus* strains

In order to investigate the occurrence of stilbenes within the species *Photorhabdus* we analyzed ten different strains by HPLC/MS and could identify stilbenes in each of these strains (Figure S1).

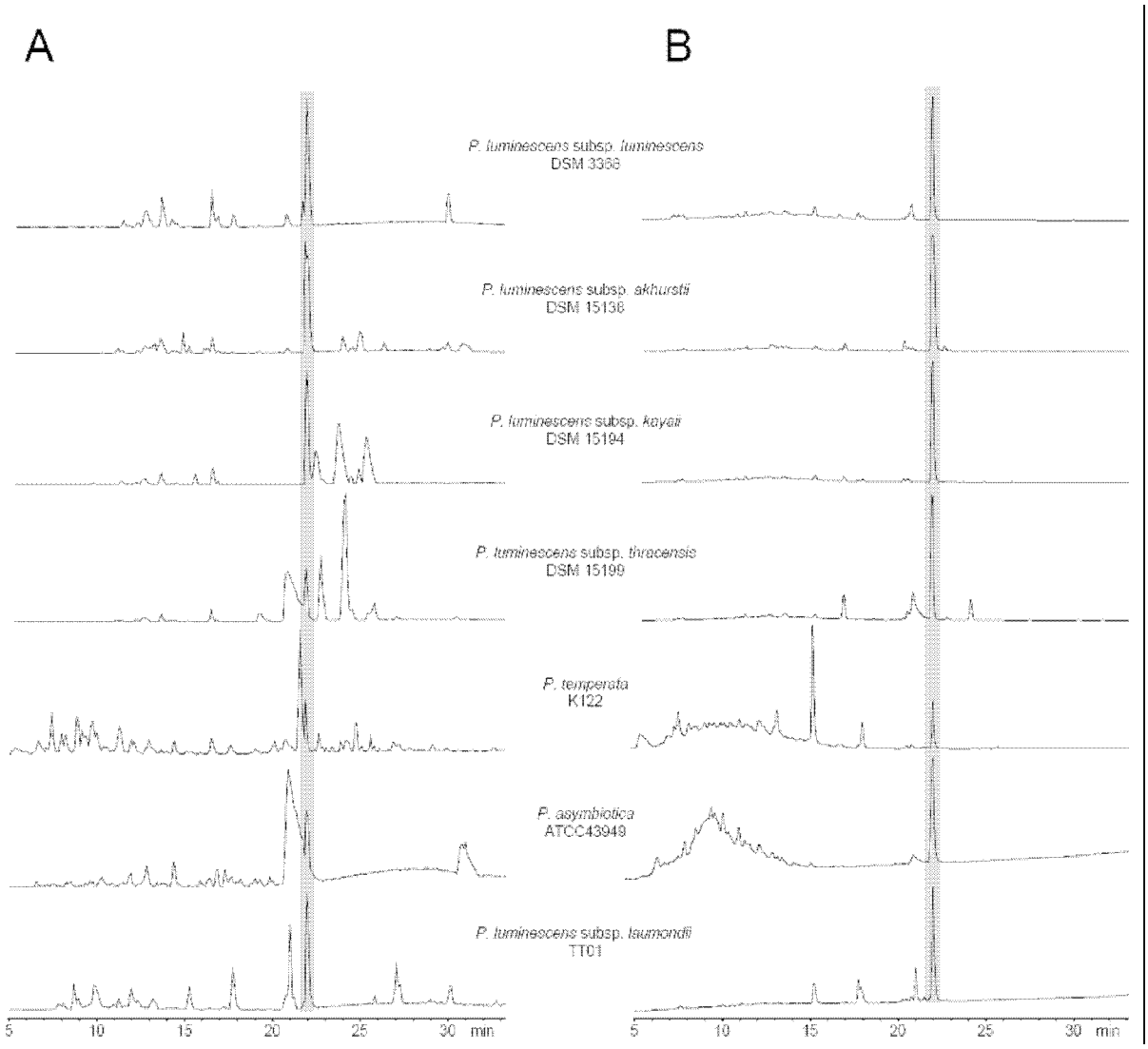

Figure S1. HPLC chromatograms of seven randomly selected *Photorhabdus* strains. MS data (basepeak chromatogram, negative mode; A), UV data (200-600 nm; B). The position of **1** is indicated.

Localisation of the identified genes in the genome of *P. luminescens* TT01

All genes described in the accompanying paper including *ngrA* and *stlA* are located on five loci within 1.5 Mbp of the genome. The distance between *ngrA*, the *bkd* genes, *stlB*, *stlCDE*, and *stlA* is 1.07 Mbp, 268 kbp, 29 kbp, and 75 kbp, respectively. It is clear that *bkdABC* and *stlCDE* are likely to be operons as they are all transcribed in the same direction and are flanked on both sides by divergently transcribed genes and/or large intergenic regions (Figure 2). In addition the spacing between these genes is very small, indicative of polycistronic operons (the numbers in square brackets show the distance between the 3rd position of the stop codon of the upstream gene and the 1st nucleotide of the start codon of the downstream gene (a negative number indicates that the genes overlap)). Interestingly, the intergenic region between *stlD* and *stlE* is relatively large (80 bp) suggesting that there might be addition levels of control of the expression of *stlE*, encoding the ACP. Indeed a transcriptional terminator is predicted to occur in the intergenic region between *stlD* and *stlE* suggesting that *stlE* may be transcribed independently (http://transterm.cbcb.umd.edu/index.php). The *stlB* gene, which is annotated on Photolist (http://genolist.pasteur.fr/PhotoList/) as *fadD*, is immediately upstream from *rnd*, predicted to encode ribonuclease D. However a strong terminator has been predicted in the 96bp region between *stlD* (*fadD*) and *rnd* suggesting that these genes are transcribed independently. Moreover, in *E. coli*, it has been shown that there is a promoter driving *rnd* expression in the intergenic region between *fadD* and *rnd*.

Stilbene biosynthesis in *P. luminescens* TT01

Stilbene biosynthesis was analyzed by feeding of ^{13}C-labelled acetate to strain TT01 followed by NMR analysis. Briefly, [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate (both at 0.5 gL^{-1} with 99% of ^{13}C-enrichment [Sigma-Aldrich]) were added in three equal portions over three days to a LB culture of strain TT01 (1 L) grown in a 5 L-flask with Amberlite XAD-16 (2 %) and **1** was isolated from the XAD-16 crude extract by sequential chromatography as described previously.[12] Labelling was observed for C-1/C-3 and C-4/C-6 and from the different ratio of ^{13}C-enriched doublet to central signal of these carbons (Table S1, Figure S2), it is evident that only one intact acetate unit is incorporated (eg. C-6? C-1) and that the second phenolic carbon (eg. C-3) is not labelled. Therefore, after feeding of [1,2-$^{13}C_2$]acetate, the signal at 156.2 ppm for C-1/C-3 is composed of a non-labelled part (derived from C-3 and non-labelled C-1) and a labelled part (doublet derived from a labelling of C-1/-C-6). Similarly, the same holds true for the signal at 105.1 ppm (C-4/C-6) which is in accordance with the proposed biosynthesis.

In order to identify the precursors of the isopropyl moiety we performed “inverse” feeding experiments in which ^{12}C-labelled putative precursors were added to 1 mL cultures of strain TT01 grown in an [U-^{13}C]medium (^{13}C-ISOGRO [Sigma-Aldrich]) as described.[13] Incorporation was determined by GC/MS analysis after extraction and derivatisation of **1** following standard procedures. The advantage of this approach is twofold in that no expensive ^{13}C or ^{2}H-labelled compounds have to be used and that also compounds can be fed which are not available in labelled form at all. The good production of **1** in strain TT01 and its descendants actually allows to perform these experiments in Eppendorf tubes which additionally minimizes the costs for the [U-^{13}C]medium.

After trimethylsilylation a mass of 398 Da is observed in the GC/MS which shifts to 415 Da in the [U-^{13}C]medium. Incorporation of precursors results in reduction of this mass by the number of carbons that are incorporated. Therefore leucine and valine reduce the observed mass to 410 Da (incorporation of five carbons) and 411 Da (four carbons incorporated), respectively (Table S2). Similarly, incorporation of isovaleric acid into a ? *bkdC* mutant results in a mass shift to 410 Da. The incorporation of valine is unexpected but can be explained by the well-known leucine biosynthesis that uses 2-ketoisovalerate (transaminated valine) as starting compound.[19] This also explains the weaker incorporation of valine compared to leucine (data not shown). Feeding of iso15:0, an abundant fatty acid in *Photorhabdus*, gave similar results compared to the incorporation of isovaleric acid indicating that fatty acid β-oxidation leads to isovaleryl-CoA. Moreover, a higher proportion of lighter isotopomers (409-407) was observed reproducible indicating the incorporation of acetate units obtained by degradation of iso15:0 into additional positions of **1**. Especially the high proportion of the 409 isotopomer might actually support the intact incorporation of a six carbon fragment from iso15:0 which would be in accordance with the proposed biosynthesis mechanism. However, due to its life style that depends mainly on the degradation of insect larvae fat body, *Photorhabdus* seems to have an efficient β-oxidation machinery that hampers such studies.

Interestingly, all *bkd* mutants also showed a complete loss of iso-fatty acid production which could be restored partially by the addition of isovalerate (Table S3). The reason for this inefficient complementation might result from a lack of activation of isovalerate to isovaleryl-CoA due to a missing or less active CoA ligase. Accordingly, a much better complementation of stilbene production was observed when iso15:0 was fed compared to isovalerate because the final end product of the fatty acid β-oxidation is already the required CoA derivative.

Table S1. Structural assignments and ^{13}C enrichments of ST after feeding of ^{13}C-labelled acetate. NMR spectra were recorded at 125 MHz in D_6-DMSO as solvent and internal standard.

carbon atom	δ (ppm)	[1,2-$^{13}C_2$]acetate		[1-^{13}C]acetate
		enrichment[a]/% ^{13}C [b]	*J* (Hz)	enrichment[a]
1/3	156.2	2.6 (70)	67.2	4.4
2	120.1	1.4		1.0
4/6	105.1	8.9 (40)	67.2	1.4
5	134.8	1.2		1.1
7	129.0	1.0		1.0
8	126.8	1.0		1.0
9	137.1	1.3		1.5
10/14	126.4	2.1		1.4
11/13	128.7	1.0		1.8
12	127.4	1.0		2.0
15	23.8	1.2		1.5
16/17	20.7	1.3		1.7

[a] relative enrichment in comparison to natural abundance: 1.0 = no enrichment.
[b] integrals of ^{13}C coupling signals to the integral of the overall signal (central line and ^{13}C couplings); the absolute enrichment was determined for C-4/C-6 via the ^{13}C-satellites in the ^{1}H NMR spectrum to be 7.5%.

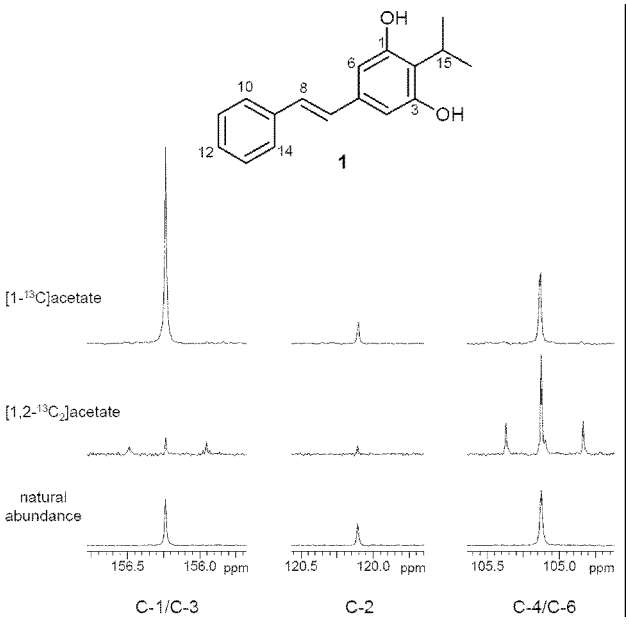

Figure S2. ^{13}C NMR signals of C-1 to C-4 and C-6 after feeding of ^{13}C-labelled acetate.

Table S2. Mass spectrometric data of **1** obtained from different feeding experiments. The most abundant isotopomers are shown in bold.

label	strain	background	*m/z*								
			407	408	409	410	411	412	413	414	415
-	TT01	^{13}C	-	-	-	-	-	1	5	19	**74**
^{12}C-valine	TT01	^{13}C	1	3	6	14	**46**	12	8	5	5
^{12}C-leucine	TT01	^{13}C	2	3	9	**62**	14	6	1	1	2
^{12}C-isovalerate	? *bkdC*	^{13}C	1	5	14	**60**	15	5	-	-	-
^{12}C-iso15:0	? *bkdC*	^{13}C	7	16	26	**40**	9	1			

Table S3. Fatty acid profile of *P. luminescens* strains. TT01, Δ*bkdA*, Δ*bkdB* and Δ*bkdC* with and without added isovaleric acid (IVA; 2 mM) as determined by GC/MS.

Table S4. Oligonucleotides used in this study. Restriction sites are underlined.

	TT01	Δ*bkdA*		Δ*bkdB*		Δ*bkdC*	
		-	+ IVA	-	+ IVA	-	+ IVA
12:0	1.17	2.26	2.10	2.27	2.12	2.28	2.11
iso-13:0	2.08		0.38		0.41		0.35
iso-14:0	0.29	0.07	0.06	0.07	0.08	0.07	0.07
14:0	4.58	8.53	8.69	7.99	8.22	8.58	7.89
iso-15:1w9c	1.03						
iso-15:0	18.24		1.89		2.13		1.93
anteiso-15:0	2.24						
15:0	0.43	0.79	0.90	0.78	0.88	0.80	0.82
iso-16:0	1.21	0.17	0.17	0.17	0.17	0.20	0.15
16:1w7c	12.01	19.12	17.89	19.25	18.97	19.03	18.48
16:0	19.02	35.43	34.59	35.00	35.14	35.02	34.58
iso-17:1	5.77						
iso-17:0	9.52		0.34		0.35		0.37
anteiso-17:0	2.04						0.29
17:0	0.31	0.64	0.73	0.55	0.64	0.65	0.61
18:1 Isomer 2	11.42	24.38	22.42	24.44	21.65	25.23	22.38
18:0	0.44	1.29	1.01	1.08	0.98	1.08	0.98
14:0 3-OH	4.22	5.78	6.84	6.83	6.77	5.59	7.13
iso-14:0 3-OH	0.16	0.02					
iso-15:0 3-OH	1.93		0.20				0.23
16:0 9,10-CH_2	1.90	1.52	1.80	1.56	1.50	1.49	1.63

Oligonucleotide	Sequence (5’-3’)
1883KOL1	TATAATGCATGCGCAGGGCTCAAAGATATAGGTAAGAACC

1883KOL2	CTGTGCCATAAGTTCTCCTTACACTCTCTTTTTCCTCAC
1883KOR1	TTATATGAGCTCCGATTAAGACGCTGCTGATCTCTGGACAG
1883KOR2	GTGAGGAAAAAGAGAGTGTAAGGAGAACTTATGGCACAG
1883PCRF	GTGAGCATAAAGTCTCAAGTTCG
1883PCRR	CCACTCCTGCTGCCCGGTGGTTTCC
1884KOL1	TTATGCATGCGCACAGCCAAAGCTCGGACGG
1884KOL2	GTGGTGCAGATTCCATAAGTTCTCTCCTTAGC
1884KOR1	TTATGAGCTCGGTATCAATTTGCAAACTTGG
1884KOR2	GGAGAACTTATGTAATCTGCACCACATATATC
1884PCRF	GTTGGAGAACGGTGGGAAAATGGCGCTGTCCGG
1884PCRR	CGCCTGTTGGCATTCCCCAGAACGCAATGAATTC
1885KOL1	TATAATGCATGCCTCAGTCAACATTGTTCATTGGTGCGCC
1885KOL2	CCTATCCCGCTTCACTGCTTACATCGTTTCCTCCTTC
1885KOR1	TAATATGAGCTCGTGACTATCATTTCTCTTGGTTAATTG
1885KOR2	GAAGGAGGAAAGCATGTAAGCAGTGAAGCGGGATAGG
1885PCRF	GTACCGAACACGCAGACCATGAGC
1885PCRR	CGACAACAAGGTGTTCACCGTTCC
plu2134-SphI	TGCCACCAGTATATTGCAGA
plu2134-SacI	AAAGTGAGCTCCTGGCTAAAACATTATCCGG
plu2134-3	TCTCAGCTAGCAGCATCAAT
plu2134-4	CCGGAACTTTCGCTTGATAC
plu2163-SphI	ACGTGGCATGCTTCTTGTAGTGTCGTATTCC
plu2163-SacI	AAAAAGAGCTCCAACCAGTACCATCAATATC
plu2163-3	ATCATCTCTTGTTTTCATG
plu2163-4	CATGTCTCTTGCTCATTAGC
plu2164-SphI	ACTGTATTGCATGCCCGCAA
plu2164-SacI	TAGTAGAGCTCAACCAACGCTCTTGTTCAAT
plu2164-3	TGCCCGCTATGATGATAAGG
plu2164-4	TCTAAAGGCAAACGCTACAC
pDS132fw	GATCGATCCTCTAGAGTCGACCT
pDS132rv	ACATGTGGAATTGTGAGCGG

Experimental procedures

General. Feeding experiments with ^{13}C- or ^{12}C-labelled compounds in a ^{12}C- or ^{13}C-labelled background, respectively, have been published [12;13]. Furthermore, fatty acid analysis, GC/MS, and HPLC/MS analysis were performed as described previously [12;13;20].

Bacterial strains. All mutants made in this study including the *stlA* mutants are derived from a spontaneous rifampicin resistant mutant of *P. luminescens* TT01 (TT01 rif).[10] Other *Photorhabdus* species were obtained from the DSMZ strain collection (www.dsmz.de) or Dr. Nick Waterfield (*P. asymbiotica*). Bacteria were routinely cultured in LB broth, on LB agar and, for symbiosis assays, on lipid agar.[6] Where appropriate antibiotics were added at the following concentrations: rifampicin (Rif), 50 µg/ml; ampicillin (Ap), 100 µg/ml; kanamycin (Kn),30 µg/mL; chloramphenicol (Cat), 30 µg/mL.

Transposon mutagenesis. *P. luminescens* TT01 rif was conjugated with *E. coli* S17-1 (λpir) carrying the vector pUT-Kn2, as previously described.[10] Exconjugants were then grown on LB agar supplemented with 33 µg/mL of cinnamic acid and the production of stilbenes was assessed using the *Micrococcus luteus* overlay assay.[10]

Construction of mutants. The genes *bkdA*, *bkdB* and *bkdC* were deleted using a previously described PCR based technique.[12] Briefly, for each gene, the appropriate L1 and L2 primers were used to amplify a region immediately upstream from the gene to be deleted (i.e. left-hand flanking region). At the same time, the appropriate R1 and R2 primers were used to amplify a region immediately downstream from the gene to be deleted (i.e. the right-hand region). Every L1 and R1 primer contains a restriction site, *Sph*1 or *Sac*I, respectively (see Table S4 for list of oligonucleotide primers). On the other hand, the L2 and R2 primers are complementary and can self-prime synthesis of a fused PCR product (left-hand flanking region plus right-hand flanking region) during primerless PCR. The amplified region is then cloned into a suicide vector, pDS132 (*R6K ori, mob RP4, cat, sacB*), using the available *Sph*I-*Sac*I restriction sites. Clones were identified by transforming ligation into *E. coli* EC100 (λpir) and the appropriate construct was then delivered to TT01 rif by conjugation. Deletion mutants were then identified by screening colonies by PCR using the appropriate PCRF and PCRR primers, that lie outside of the flanking regions used to construct the deletion. This method results in the marker-less deletion of the target gene.

The genes *stlB*, *stlC* and *stlD* were disrupted by plasmid integration as described previously.[12] Briefly, an internal fragment (~600 bp) from these genes was amplified by PCR using primers with *Sph*I and *Sac*I restriction sites and the resulting fragment was then cloned into pDS132. The final constructs were then conjugated into TT01 rif as described above and mutants were verified using two PCR primers lying outside the amplified region and two primers specific for the vector backbone as described.[12;20]

Recovery assays. Bacteria to be tested were cultured overnight in LB broth, the OD_{600} was adjusted to 1 with fresh LB and 100 µl were inoculated onto the surface of 0.6 ml of lipid agar (+Rif) in each well of a 12-well microtiter plate. The bacteria were cultured for 2 days at 28ºC. Monoxenic *H. bacteriophora* TT01 IJs (cultured on TT01) were surface-sterilised by washing in 1 x hyamine and 40 sterilised IJs were added to each of the wells. The plates were

incubated at 28ºC for 4 days before recovery was assessed by microscopy. Where appropriate, **1** or **3** were added to the lipid agar at the indicated concentrations.

References

[1] W. H. Richardson, T. M. Schmidt, K. H. Nealson, *Appl. Environ. Microbiol.* **1988**, *54* 1602-1605.

[2] K. J. Hu, J. X. Li, W. J. Wang, H. M. Wu, H. Lin, J. M. Webster, *Can. J. Microbiol*. **1998**, *44* 1072-1077.

[3] K. Hu, J. M. Webster, *FEMS Microbiol. Lett.* **2000**, *189* 219-223.

[4] H. Goodrich-Blair, D. J. Clarke, *Mol. Microbiol.* **2007**, *64* 260-268.

[5] J. Li, G. Chen, H. Wu, J. M. Webster, *Appl. Environ. Microbiol.* **1995**, *61* 4329-4333.

[6] R. J. Watson, S. A. Joyce, G. V. Spencer, D. J. Clarke, *Mol. Microbiol.* **2005**, *56* 763-773.

[7] K. Hu, J. Li, J. M. Webster, *J. Chromatogr. B Biomed. Sci. Appl.* **1997**, *703* 177-183.

[8] I. Eleftherianos, S. Boundy, S. A. Joyce, S. Aslam, J. W. Marshall, R. J. Cox, T. J. Simpson, D. J. Clarke, R. H. ffrench-Constant, S. E. Reynolds, *Proc. Natl. Acad. Sci. USA* **2007**, *104* 2419-2424.

[9] M. B. Austin, J. P. Noel, *Nat. Prod. Rep.* **2003**, *20* 79-110.

[10] J. S. Williams, M. Thomas, D. J. Clarke, *Microbiology* **2005**, *151* 2543-2550.

[11] E. Duchaud, C. Rusniok, L. Frangeul, C. Buchrieser, A. Givaudan, S. Taourit, S. Bocs, C. Boursaux-Eude, M. Chandler, J. F. Charles, E. Dassa, R. Derose, S. Derzelle, G. Freyssinet, S. Gaudriault, C. Medigue, A. Lanois, K. Powell, P. Siguier, R. Vincent, V. Wingate, M. Zouine, P. Glaser, N. Boemare, A. Danchin, F. Kunst, *Nat. Biotechnol.* **2003**, *21* 1307-1313.

[12] A. O. Brachmann, S. A. Joyce, H. Jenke-Kodama, G. Schwär, D. J. Clarke, H. B. Bode, *ChemBioChem* **2007**, *8* 1721-1728.

[13] A. O. Brachmann, S. Forst, G. M. Furgani, A. Fodor, H. B. Bode, *J. Nat. Prod.* **2006**, *69* 1830-1832.

[14] T. A. Ciche, S. B. Bintrim, A. R. Horswill, J. C. Ensign, *J. Bacteriol.* **2001**, *183* 3117-3126.

[15] B. Nowak-Thompson, P. E. Hammer, D. S. Hill, J. Stafford, N. Torkewitz, T. D. Gaffney, S. T. Lam, I. Molnar, J. M. Ligon, *J. Bacteriol.* **2003**, *185* 860-869.

[16] X. Zhu, F. Yu, X. C. Li, L. Du, *J. Am. Chem. Soc.* **2007**, *129* 36-37.

[17] X. Zhu, F. Yu, X. C. Li, L. Du, *J. Am. Chem. Soc.* **2007**, *129* 36-37.

[17] O. Strauch, R. U. Ehlers, *Appl. Microbiol. Biotechnol.* **1998**, *50* 369-374.

[18] The data is nonparametric and, in order to represent the data graphically, the level of IJ recovery (in %) is represented relative to the recovery observed when IJs were added to wild-type TT01 cultured under the same conditions. Therefore, taking the experiment with 1 as an example, 480 IJs (12 plates, each inoculated with 40 IJs) were inoculated onto either TT01 or BMM901 bacteria growing on lipid agar and % IJ recovery was calculated. Non-parametric statistical analysis of this data (Mann-Whitney) shows that the number of IJs recovering on BMM901 growing on lipid agar not supplemented with 1 or 3 is significantly different to the number that recover on either wild-type bacteria or bacteria growing on lipid agar plates supplemented with 1 or 3 (P = 0.01). In addition the number of IJs recovering on BMM901 carrying the plasmid vector is significantly different from both wild-type and BMM901/pBMM901 (P = 0.01).

[19] G. Michal, *Biochemical Pathways*, Spektrum Akad. Verlag, Heidelberg **1999**.

[20] H. B. Bode, M. W. Ring, G. Schwär, R. M. Kroppenstedt, D. Kaiser, R. Müller, *J. Bacteriol.* **2006**, *188* 6524-6528.