Laboratory Exercise

Laboratory Course on *Streptomyces* Genetics and Secondary Metabolism

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Abstract

The "*Streptomyces* genetics and secondary metabolism" laboratory course gives an introduction to the versatile soil dwelling Gram-positive bacteria *Streptomyces* and their secondary metabolism. The course combines genetic modification of *Streptomyces*; growing of the strain and protoplast preparation, plasmid isolation by alkaline lysis and phenol precipitation, digestions, and ligations prior to protoplast transformation, as well as investigating the secondary metabolites produced by the strains. Thus, the course is a combination of microbiology, molecular biology, and chemistry. After the course the students should understand the relationship between genes, proteins, and the produced metabolites. © 2016 by The International Union of Biochemistry and Molecular Biology, 00:000–000, 2016.

Keywords: *Streptomyces; nogalamycin; rhodomycin; secondary metabolites*

Introduction

Streptomyces are soil dwelling Gram-positive bacteria, which produce secondary metabolites, many of which are used as drugs today [1–3]. The investigation on the secondary metabolites of *Streptomyces* is ongoing in our laboratory to understand better the details and evolution of different antibiotic biosynthesis pathways [4]; we wish to gain insight into the individual proteins [5] and to find and create better antibiotics in the future [6]. The laboratory course "*Streptomyces* genetics and secondary metabolism" is a combination of microbiology, molecular biology, and chemistry and gives the students an overview on *Streptomyces* secondary metabolism.

This course addresses the formation of a particular group of secondary metabolites, the anthracyclines. *S. galilaeus* produces aclacinomycin A, (aclarubicin) [7] used as a drug, for example, acute myeloid leukemia [8]. This aspect showcases the relevance of the course on daily life and it can be used as a transition for highlighting the importance of investigations of secondary metabolites and their use as drugs. For a review see ref. 9. Aclacinomycin A consists of a polyaromatic aklavinone unit with three sugar moieties attached to the backbone at C7 and each other: rhodosamine, 2-deoxyfucose and cinerulose. The last sugar, cinerulose, is attached as rhodinose and is further modified to cinerulose. The aklavinone moiety is called an "aglycone" which is a generic term for natural products devoid of carbohydrate units. Anthracycline aglycones do not harbor biological activities, which emphasize the importance of deoxysugar units for these chemotherapeutic agents. The H075 mutant strain [10] used during the course has a nonsense mutation in the gene *aknP* which codes for dTDP-heksose-3-dehydratase, which makes it impossible for the strain to attach rhodinose, resulting in varying lengths of the sugar chain attached to the aglycone.

One of the incentives of the course is to learn about secondary metabolism and how individual genes affect the produced metabolites. The gene *rdmE* serves as the main example. The product of *rdmE* is an 11-hydroxylase [11–13], which introduces an OH-group at the C11 position of the polyketide backbone (Fig. 1). Due to the attachment the conjugated system is affected and as a result the visible absorbance maximum shifts from yellow to red. The plasmid pMMc (Fig. 2) has genes from two *Streptomyces* species (*S. nogalater* and *S. purpurascens*) responsible for backbone modifications. The fact that the plasmid contains genes from two different species and it is transformed into the third underlines the possibilities provided by synthetic biology for the modification of secondary metabolism of *Streptomyces* and production of novel bioactive compounds.

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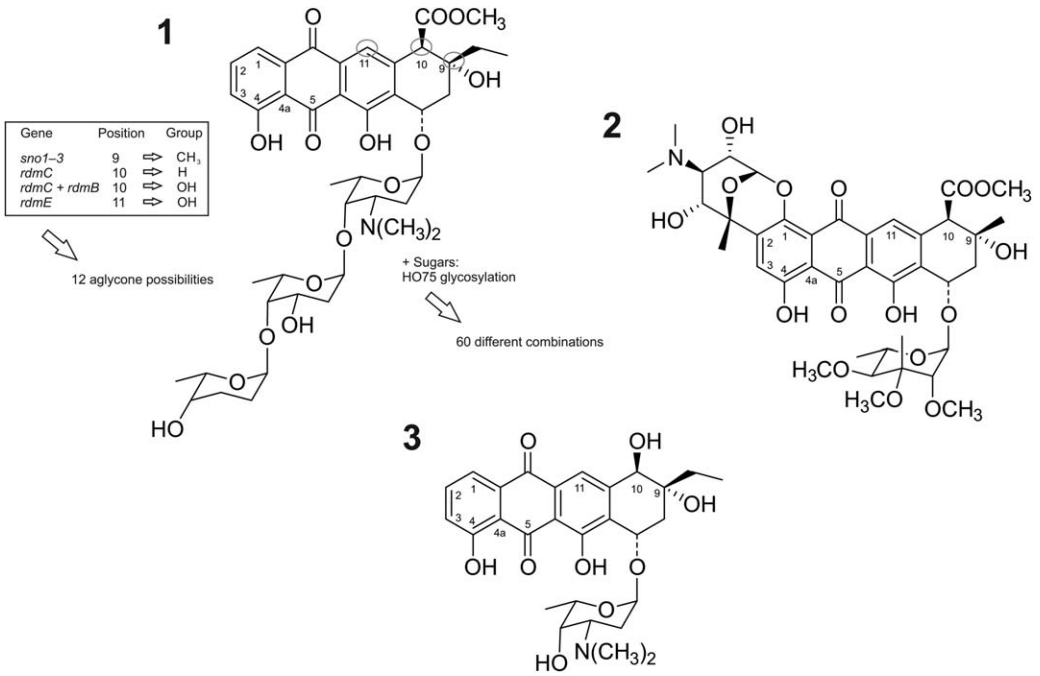

FIG 1 *(1) Structure of a possible hybrid anthracycline produced by the the strain S. galilaeus H075/pMMc. There are in total 12 aglycone possibilities and that combined with the different glycosylation patterns derived from the H075 mutant strain, there are in total 60 different combinations. (2) Nogalamycin, the compound produced by S. nogalater, (3) rhodomycin B, a compound produced by S. purpurascens.*

Nogalamycin and rhodomycin B, anthracyclines produced by the strains *S. nogalater* and *S. purpurascens,* respectively, are shown in Figure 1 (2–3).

During the course the plasmid pMMc is isolated from *S. galilaeus* and the gene for the 11-hydroxylase, *rdmE,* is removed from it. The plasmid is religated and transformed into *S. galilaeus* H075 mutant strain protoplasts, which were prepared by the students. Finally the transformants and controls are grown in a liquid culture to produce secondary metabolites, which are extracted using two different liquid–liquid extraction methods prior to analysis by thin layer chromatography (TLC).

Practical Issues of the Course

The course is intended for third year biochemistry students and is suitable to be done in pairs. The course has been held as a part of a broader course on molecular biology, but it is also suitable to be held independently. The laboratory work takes 2 weeks in total although hands-on time is only approximately 1 week. The complete time is longer than the hands-on time since the strains grow slowly. For example, two groups can be taught overlapping, if needed, by 1-week interval. The outline and timing of the course are presented in Table I for one group while the required equipment, reagents, and facilities are presented in Table II. Media and so forth can be found from the excellent laboratory manuals on *Streptomyces* [14, 15]. The course includes a preliminary assignment (Table III), a starting lecture on the outline and goals of the course, which, optionally, may also cover topics on genetically modified bacteria, hazardous substances, and laboratory safety. An end lecture is provided for reviewing the results and going through possible questions of the students. It is also possible to include a brief introduction to the purification of secondary metabolites and their analysis by high pressure liquid chromatography or mass spectrometry. After the laboratory work is done the students prepare course reports individually.

Overview of the Procedure

Isolation of the *Streptomyces* Plasmid pMMc, Digestion, and Ligation

Prior to the course *S. galilaeus* HO75/pMMc is cultivated for 3–5 days in 12 mL Falcon tubes in agitation at 30°C in 5 mL tryptic soy broth (TSB, Oxoid, UK). The cells are pelleted and stored at −20°C until the course starts. The plasmids are isolated by alkaline lysis and phenol precipitation method adapted from refs. 16–18 since the kits which are nowadays in use are not suitable as such for *Streptomyces* plasmid isolation. Moreover, a traditional isolation procedure helps students to understand the individual steps in the DNA isolation process. The frozen cell pellet is thawed and suspended in 2 mL lysozyme solution and incubated at 37°C for at least 30 minute to lyse the cell walls. After the

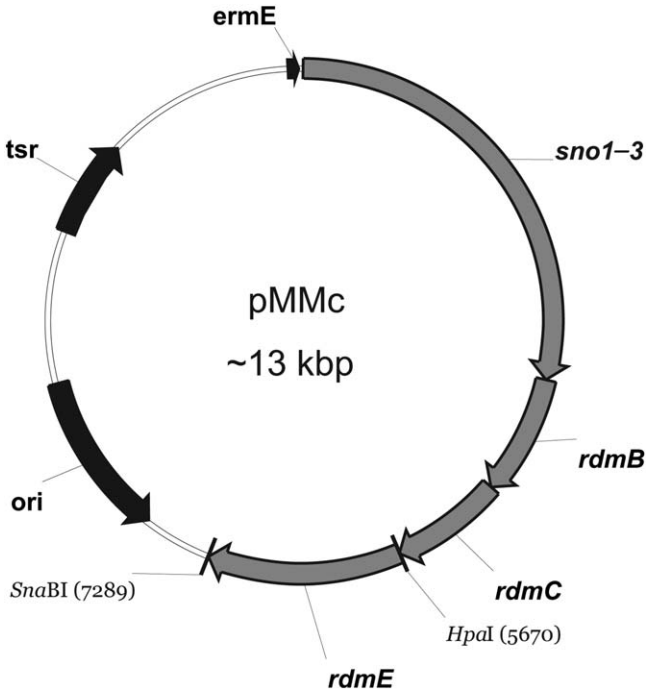

FIG 2 *Schematic picture of the plasmid pMMc. The open reading frames from S. nogalater (sno) or S. purpurascens (rdm). The genes encode the following proteins: sno1–3: minimal polyketide synthase, rdmB: 10-hydroxylase, rdmC: esterase, rdmE: 11-hydroxylase. The plasmid also contains the gene encoding for thiostrepton (tsr) resistance and the origin for replication (ori).*

incubation 1 mL of freshly prepared alkaline SDS solution is added and the suspension is mixed carefully by pipetting up and down. It is important to mix the solution upon addition of the reagent to avoid local high pH changes. The mixture is incubated at 55°C with a loosened cap for 20 minute to denaturate the DNA. From this step on all the additions and omissions to and from the tube are made under a hood. Acidic phenol/chloroform (70–500 μL) is added to the mixture and mixed thoroughly. When adding phenol, one should be careful in pipetting as there is a water layer on top of the actual phenol/chloroform. The mixing should be done carefully in order to avoid shredding of chromosomal DNA. Finally the two phases are separated by centrifugation (1,389 g, 15 min). In this step, the drop in pH results in renaturation of the desired plasmid DNA. The covalently closed circular DNA, RNA, and polysaccharides stay in the aqueous phase and at the same time the proteins, lipids, and the chromosomal DNA go to the interphase. The aqueous phase is moved to a new tube containing 1 mL chloroform for further purification and after mixing the phases are again separated by centrifugation. Finally, the DNA is precipitated from the aqueous phase with an equal volume of isopropanol and 300 mM sodium acetate (unbuffered). After mixing, the tube is left at RT for 5–15 min, followed by centrifugation (3,850 g, 15 min). After discarding the supernatant, the pellet is washed with 3 mL of 70% ethanol and centrifuged again. The pellet is dried well, after which it is dissolved in TE buffer (30–100 μL). The plasmid preparation is then subjected to RNase A treatment (10–33.3 μg/mL) for 30–60 minute at 37°C.

The success of the isolation and the concentration of the plasmid preparation (pMMc) are checked by analytical agarose gel electrophoresis [0.8% (w/v)]. Every pair prepares a sample containing 1/10 of their preparation, loading buffer, and MQ if needed. One pair makes the gels for the whole course. The DNA is visualized by Midori green (NIPPON Genetics EUROPE GmbH, Germany) or ethidium bromide. After the gel run all pairs who have successfully isolated the plasmid will digest their entire preparation. For those who failed, it is good to have a stock ready.

On the second day pMMc is digested with *Eco*105I (*Sna*BI) and *Ksp*AI (*Hpa*I) at 37°C to get pMMc-*rdmE*. If traditional restriction enzymes are used, the best suitable buffer for the digestion is the unique buffer for *Bam*HI (Thermo-Scientific, The United States) making it a good opportunity to demonstrate the star activity of restriction enzymes in practice to the students, by letting one pair make a control reaction with a standard restriction buffer. The start meeting can be held during the time of the digestion. After the meeting an analytical gel [0.8% (w/v)] of the digests is run, so that the students can have a picture of the digestion to include in their report.

In the afternoon a preparative agarose gel electrophoresis [0.6% (w/v)] of pMMc-*rdmE* is initiated and ran overnight. The gel run is done overnight because the low percentage of the agarose in the gel does not allow the use of a high voltage which would cause the gel to melt. After the run the approximately 11 kb pMMc-*rdmE* is extracted from the preparative agarose gel according to the instructions of the manufacturer (GeneJET Gel extraction kit, Thermo Scientific, The United States). It is advisable to offer varying start times for the different pairs, because only one pair can cut the gel at the time and this interlacing will also help in the following step, which is protoplast preparation. The linear pMMc-*rdmE* (~50–100 ng, which can be estimated from the agarose gel) is religated at RT using T4-ligase (Thermo Scientific, The United States) in a final volume of 10 μL.

Preparation of the *S. galilaeus* HO75 Protoplasts

All steps are done under a laminar flow hood to avoid contaminations, which is a challenge to be taken into account if there are many pairs. The preparation of the protoplasts is adapted from ref. 19. Prior to the course, a dense preculture is prepared (100 mL Erlenmeyer containing 10 mL SgYeme grown in agitation for 2–4 days at 30°C). Optionally also glycerol preparations may be used to inoculate the protoplast culture. The students use either the pre-culture or the glycerol preparation to inoculate SgYeme (25 mL,

TABLE I ***The outline and possible timing of the course***

Step		*General*	*Plasmid isolation*	*Protoplasts*	*Transformation*	*Secondary metabolites*
0)	Before the course:	Reserving of needed reagents and instruments, preparing of lectures	• H075/pMMc cultivation in TSB • Pelleting of the cells	• H075 pre-culture • Just in case: readymade protoplasts		• Just in case: readymade cultivations frozen, which can be used for extractions
1)	Mon		• Plasmid isolation • Analytical gel	• Protoplast cultivation start		
2)	Tue	• Start meeting	• Digestions • Analytical gel • o/n preparative gel			
3)	Wed		• Cutting the gel • Gel extraction • Ligation	• Preparation of protoplasts • Checking the protoplasts		
4)	Thu				• Transformation	
5)	Fr				• tsr addition	
6)	Mon					• E1 cultivation start • Plating on ISP4 + tsr plates
7)	Fr	• End meeting				• Looking at the ISP4 + tsr plates • Extraction • TLC

TABLE II ***Needed equipment, reagents, and facilities for the course***

Plasmid isolation, Digestion, and ligation	*Amount*		*Protoplasts*	*Amount*	
TSB (Oxoid)	mL	5	SgYEME	mL	25
tsr in DMSO (50 mg/mL)	μL	5	2.5 M $MgCl_2$	μL	50
Lysozyme solution	mL	2	10% (w/v) glycine	mL	1.25
Alkaline SDS	mL	2	10.3% (w/v) sucrose	mL	10
Acid phenol/chloroform	mL	0.7	Lysozyme solution in P+ buffer	mL	4
Chloroform	mL	1.25	P+ buffer	mL	5
Isopropanol	mL	2.5	R2YE plates	Pieces	1
3M Sodium acetate, unbuffered	mL	0.25	ISP4 plates	Pieces	1
70% Ethanol	mL	3	250 mL sterile baffled Erlenmeyer		
TE buffer	mL	0.1	Falcon centrifuge		
RNase A (10 mg/mL)	μL	1	Sterile syringe with cotton wool		
Analytical DNA gel (0.8%)	Lane	2	Laminar flow hood		
Preparative DNA gel (0.6%)	Lane	1	*Transformation*	*Amount*	
TAE buffer			25% (w/V) PEG4000 in P+ buffer	mL	0.5
Molecular Weight Marker*			R2YE plates	Pieces	1–2
Loading buffer*			tsr in DMSO (50 mg/mL)	μL	4
Midori green, NIPPON genetics EUROPE GmbH			Sterile MQ	mL	1
Eco105I (*Sna*BI)*	μL	1	ISP4 + tsr plates	Pieces	1
KspAI (*Hpa*I)*	μL	1	Laminar flow hood		
*Bam*HI buffer*	μL	2	Eppendorf centrifuge		
B buffer*			*Secondary metabolites*	*Amount*	
Gel extraction kit*	Column	1	E1 medium	mL	10
T4 Ligase*	μL	0.5	tsr in DMSO (50 mg/mL)	μL	10
T4 ligase buffer*	μL	1	Ethyl acetate		
Falcon centrifuge			Methanol		
Eppendorf centrifuge			Formic acid		
Flow hood			1 M HCl	mL	0.5
Incubator and shaker at 30°C			1 M Ammonium acetate, unbuffered	mL	0.5
Incubator or water bath (37°C and 55°C)			Steril MQ		
Equipment for electrophoresis			ISP4 + tsr plates	Pieces	1
Pressurized air			Chloroform	mL	0.7
Centrifuges (for Eppendorf tubes and for Falcon tubes)			Sterile Erlenmeyer (100 mL)	Pieces	1
			Pressurized air		
			TLC plate, TLC chamber		

The needed amounts are presented for each pair, where it is feasible. Standard laboratory equipment (e.g. pipettes) is not listed.

*If not otherwise stated the reagents are from Sigma-Aldrich, *Thermo Scientific.*

Lysozyme solution (in plasmid isolation): 2 mg/mL lysozyme in 0.3 M sucrose, 25 mM Tris (pH 8), 25 mM EDTA (pH 8), lysozyme is added to the solution just before use, alkaline SDS: 0.3 M NaOH, 2% SDS, should be made just before use, Acid phenol/chloroform: solid phenol and chloroform [1:1, (w/v)], 1/1,000 8-hydroxyquinoline (Merck) with a water layer on top, in a brown bottle, TE buffer: 10 mM Tris-Cl (pH 8), 1 mM EDTA (pH 8).

SgYeme: 3 g Yeast extract (Difco, UK), 5 g Bacto peptone (Difco), 3 g Malt extract (Oxoid), 10 g glucose and 110 g sucrose (ultra-pure) in 1 L H_2O, R2YE plates: see refs. 11, 12 for instructions, ISP4 plates: ISP4agar (Difco).

P+ buffer: P buffer that contains additions made after autoclaving, P buffer: 51.5 g sucrose, 0.125 g K_2SO_4, and 1.01 g $MgCl_2 \cdot 6H_2O$ in 400 mL H_2O; dispense in 80 mL aliquots and autoclave. To 80 mL P buffer add: 1 mL KH_2PO_4 (0.5%), 10 mL $CaCl_2 \cdot 2H_2O$ (3.68%), 10 mL TES buffer (5.73%, pH 7.2) and 200 μL Trace element solution to make it to P+ buffer. Lyzosyme solution (in protoplast preparation): just before use lysozyme (2 mg/mL) to P+ buffer and filter sterilize, E1 medium: Glucose [2%, (w/v)], soluble starch [2%, (w/v)], Farmamedia (Trader's Mill Protein Co.) (0.5%), yeast extract (0.25%), K_2HPO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.1%), NaCl (0.3%) and $CaCO_{3\ (0.3\%)}$ in tap water (pH 7.5).

containing $MgCl_2$ [5 mM], and glycin [0.5% (w/v)]) in a 250 mL baffled Erlenmeyer flask. After 3 days of cultivation in agitation at 30°C the protoplasts are prepared. The cells are pelleted in Falcon tubes and washed twice with 5 mL 10.3% (w/v) sucrose after which the cells are left in 4 mL of lysozyme solution for 30 minute at 30°C. The tubes are then mixed and left for another 5–30 minute at 30°C. To the cells, which have their cell walls removed 4 mL of P+ buffer is added and the solution is mixed prior to filtering. The protoplasts are filtered through a sterilized injection syringe containing a cotton wool plug. The filtrate is centrifuged and the pellet is suspended in P+ buffer and divided to 500 μL aliquots for later use. The protoplasts can be used straight away or they may be stored at −20°C (for a short time after which they should be moved to −70°C). To test the efficiency of the protoplast preparation, a dilution of the protoplast suspension in P+ buffer (e.g. 1:100–1:1,000,000) is prepared and the cells are plated on R2YE and ISP4 plates. Protoplasts cannot grow on ISP4 plates, so the students can calculate and understand their success in the protoplast preparation, by comparing the growth on both plates. The cells are cultivated at 30°C and they are examined after 4–5 days.

Transformation of *Streptomyces* Protoplasts

Streptomyces galilaeus H075 protoplasts are transformed with the ligation mixture of the plasmid pMMc-*rdmE* by PEG induced protoplast transformation [20] as follows. The prepared protoplasts can be used fresh or they can be thawed after freezing. One should avoid harsh treatment of the protoplasts, as they are fragile without the cell wall. The protoplasts are centrifuged down (1,389 g, 10 min) and after the supernatant is discarded the ligation mix is added to the protoplasts and mixed by tapping the tube. PEG4000 in P+ buffer [25% (w/v), 500 μL] is added and mixed by pipetting the mixture up and down a few times with a cut pipette tip. The solution is very viscous and one should be careful not to contaminate the pipette. About 200 μL of the mixture is plated on a R2YE plate, the rest can be plated on a second plate or it can be discarded. The plates are incubated at 30°C for 24 hours after which 200 μg of thiostrepton (tsr) (from a 50 mg/mL stock in DMSO diluted with 1 mL MQ) is added. The incubation is continued for about 3 days to see resistant colonies. It should be noted that if there are multiple transformations, one should do one at the time, as PEG is toxic to the protoplasts if they are subjected to it for too long.

Four to five days after the transformation, ISP4 + tsr (50 μg/mL) plates are inoculated with six resistant colonies. Cells are spread to the plate by using sterile toothpicks making a dense grid. The plates are inspected after 4 days incubation at 30°C.

TABLE III *Example of the preliminary assignment*

Read the instructions of the course and the articles [4, 11] and answer the following questions:

1) How does the strain *Streptomyces galilaeus* H075 differ from the wild type? Why are different kinds of mutant strains made?

2) What is the function of RdmE?

3) What hazardous substances are used in this course and how should one work with them?

4) Why are bacteria belonging to *Streptomyces* species especially interesting?

Extraction and Thin-Layer Chromatography (TLC) Analysis of H075/pMMC-*rdmE* Products

Each pair cultivates one to two transformants and some pairs cultivate in addition to this the following controls: the parent strain H075 (without tsr), H075/pMMc (with 10 μg/mL tsr) and H075/pMMc-*rdmE* (with 10 μg/mL tsr). The strains are cultivated in 10 mL E1 [21] in 100 mL Erlenmeyer for 4–5 days. Typically the growth from the secondary ISP4 + tsr plates is used for the inoculation, but because of the time limits of a laboratory course, individual colonies from the transformation plates are used directly for inoculation.

After the cells have grown, two samples (500 μL) are taken from the cultivation with a cut tip into Eppendorf tubes, one for neutral extraction and one for acid hydrolysis. The extraction is done in two ways to show the students that metabolites may react differently in different conditions. The neutral extraction contains the sample, 357 mM ammonium acetate (unbuffered), methanol (1:4), and chloroform (1:4). The tube is mixed for 5–30 minute and centrifuged (4,700 g, 5 min). The chloroform phase is saved and evaporated by the help of pressurized air to a smaller volume. The acid hydrolysis contains the sample and 500 mM hydrochloric acid (HCl). The tube is incubated at 100°C for 20 minute and the mixture is cooled to RT after which 1:1 of chloroform is added. The tube is mixed for 5–30 minute and centrifuged (4,700 g, 5 min). The chloroform phase is saved and evaporated to a smaller volume.

For analysis of the samples by TLC, the mobile phase [toluene:ethylacetate:methanol:formic acid (50:50:15:3)] is prepared and poured to a TLC chamber, where the depth of the solvent mixture should be 3–10 mm. The solvent mix should be left in the chamber for at least 15 minute prior to the run. The extracts are applied in chloroform to the TLC plate (Silica gel 60, Merck, The United States) and the spots are left to dry prior to placing the plate into the TLC chamber. The plate should be straight and it is important that the solution does not touch the samples. The plate is left in the chamber until the solvent front almost reaches the edge of the TLC plate. After the run the plate is taken out, the solvent front is marked and the plate is photographed. If the used

TLC plates are made of glass, the spots can also be investigated under UV light. The TLC plate contains dry anthracyclines and, thus, should be handled with care and as a potential hazard.

Results and Discussion

Plasmid Isolation, Protoplast Preparation, and Transformation

For the evaluation of the success of the plasmid isolation the main points are the purity, the success of the digestion and the yield. The protoplasts can be evaluated by comparing the amount of colonies growing on ISP4 plates to the ones growing on R2YE plates. Only intact cells (not protoplasts) can grow on ISP4 plates, because the vulnerable cell wall free protoplasts are destroyed on these plates by osmosis. The success of the ligation and transformation can be estimated by the amount of transformants on the R2YE plates and further on the ISP4 + tsr plates. In addition, one can investigate the color of the produced metabolites directly on the ISP4 + tsr plate.

Secondary Metabolites and TLC

By looking at the TLC results (Fig. 3) there are two main points to consider: (*a*) what is the difference between the strain which contains RdmE compared with the strain which is without RdmE? The difference is the color due to the change in the conjugated system which is caused by the hydroxylation done by RdmE. (*b*) How does the acidic hydrolysis differ from the neutral chloroform extraction? Why do they differ? Why are there so many different products visible? The acid hydrolysis detaches the carbohydrate moieties and only aglycones are visible whereas in the neutral extraction all different compounds with different carbohydrate moieties are perceptible. The many products are due to the different modifications made to the aglycone and the differently glycosylated products.

Hazardous Substances

The hazardous substances on this course are methanol, phenol, chloroform, toluene, ethyl acetate, HCl, formic acid, tsr in dimethyl sulfoxide, and the compounds produced by *Streptomyces*. Disposal of the different compounds is done according to the local laws and regulations. It is good to point out to the students that even though the safety sheets between different substances do not excessively vary, there are significant differences, for example, between phenol and ethanol.

Conclusion

After the course the students have an idea of the secondary metabolism of *Streptomyces* and they comprehend why *Streptomyces* are studied. From the practical point of view they understand the basics of sterile work and how to deal with slow growing bacteria, they also get an idea how secondary metabolites can be isolated and visualized. In the broader sense they understand the relationship between genes, proteins and the produced metabolites.

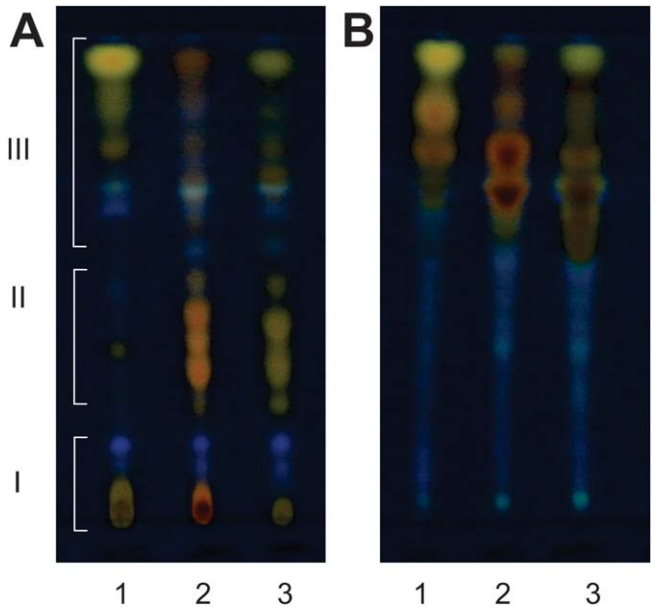

FIG 3

*The TLC plates of the extracted and separated metabolites as seen under UV light. (*A*) neutral extraction (*B*) acid hydrolysis. The products from (*1*) S. galilaeus H075, (*2*) S. galilaeus H075/pMMc, (*3*) S. galilaeus H075/pMMc-rdmE. Under the analysis conditions, glycosylated anthracyclines have low retardation factors (Rf) with aminoglycosides (I: <0.1) and neutral glycosides (II: 0.1–0.5) typically separated from each other. The anthracycline aglycones released by acid hydrolysis have higher Rf-values (III:>0.5). The effect of RdmE in lane 2 can be seen from the appearance of red pigmented anthracyclines lacking from the other samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

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References

[1] Hortobdgyi, G. N. (1997) Anthracyclines in the treatment of cancer. Drugs 54, 1–7.

[2] Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., and Gianni, L. (2004) Anthracyclines: Molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol. Rev. 56, 185–229.

[3] Salvatorelli, E., Menna, P., Cantalupo, E., Chello, M., Covino, E., Wolf, F. I., and Minotti, G. (2015) The concomitant management of

cancer therapy and cardiac therapy. Biochim. Biophys. Acta 1848, 2727–2737.

[4] Kallio, P., Patrikainen, P., Belogurov, G. A., Mäntsälä, P., Yang, K., Niemi, J., and Metsä-Ketelä, M. (2013) Tracing the evolution of angucyclinone monooxygenases: Structural determinants for C-12b hydroxylation and substrate inhibition in PgaE. Biochemistry 52, 4507–4516.

[5] Oja, T., Klika, K. D., Appassamy, L., Sinkkonen, J., Mäntsälä, P., Niemi, J., and Metsä-Ketelä, M. (2012) Biosynthetic pathway toward carbohydrate-like moieties of alnumycins contains unusual steps for C-C bond formation and cleavage. Proc. Natl. Acad. Sci. U.S.A. 194, 2829–2836.

[6] Siitonen, V., Claesson, M., Patrikainen, P., Aromaa, M., Mäntsälä, P., Schneider, G., and Metsä-Ketelä, M. (2012) Identification of late-stage glycosylation steps in the biosynthetic pathway of the anthracycline nogalamycin. Chembiochem 13, 120–128.

[7] Oki, T., Matsuzawa, Y., Yoshimoto, A., Numata, K., Kitamura, I., Hori, S., Takamatsu, A., Umezawa, H., Ishizuka, M., Naganawa, H., Suda, H., Hamada, M., and Takeuchi, T. (1975) New antitumor antibiotics aclacinomycins A and B. J. Antibiot. (Tokyo) 28, 830–834.

[8] Hiddenmann, W. and Mertelsmann, R. (1990) New Findings on Aclarubicin in the Treatment of Acute Myeloid Leukemia, Springer-Verlag, Berlin, New York.

[9] Weber, T., Welzel, K., Pelzer, S., and Wohlleben, A. (2003) Exploiting the genetic potential of polyketide producing streptomycetes. J. Biotechnol. 106, 221–232.

[10] Räty, K., Hautala, A., Torkkell, S., Kantola, J., Mäntsälä, P., Hakala, J., and Ylihonko, K. (2002) Characterization of mutations in aclacinomycin A-non-producing *Streptomyces galilaeus* strains with altered glycosylation patterns. Microbiology 148, 3375–3384.

[11] Lindqvist, Y., Koskiniemi, H., Jansson, A., Sandalova, T., Schnell, R., Liu, Z., Mäntsälä, P., Niemi, J., and Schneider, G. (2009) Structural basis for substrate recognition and specificity in aklavinone-11-hydroxylase from rhodomycin biosynthesis. J. Mol. Biol. 393, 966–977.

[12] Niemi, J., Wang, Y., Airas, K., Ylihonko, K., Hakala, J., and Mäntsälä, P. (1999) Characterization of aklavinone-11-hydroxylase from Streptomyces purpurascens. Biochim. Biophys. Acta 1430, 57–64.

[13] Niemi, J. and Mäntsälä, P. (1995) Nucleotide sequences and expression of genes from Streptomyces purpurascens that cause the production of new anthracyclines in Streptomyces galilaeus. J. Bacteriol. 177, 2942–2945.

[14] Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) Practical -Streptomyces Genetics, The John Innes Foundation, Norwich.

[15] Shepherd, M. D., Kharel, M. K., Bosserman, M. A., and Rohr, J. (2010) Laboratory maintenance of Streptomyces species. Curr. Protoc. Microbiol. Chapter 10:Unit 10E.

[16] Kieser, T. (1984) Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12, 19–36.

[17] Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) Practical -Streptomyces Genetics, The John Innes Foundation, Norwich, pp. 187–188.

[18] Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. (1985) Genetic Manipulations Of Streptomyces: A Laboratory Manual, The John Innes Foundation, Norwich, United Kingdom, pp. 85–92.

[19] Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) Practical -Streptomyces Genetics, The John Innes Foundation, Norwich, pp. 56–58.

[20] Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) Practical -Streptomyces Genetics, The John Innes Foundation, Norwich, pp. 232–235 and 240.

[21] Ylihonko, K., Hakala, J., Niemi, J., Lundell, J., and Mäntsälä, P. (1994) Isolation and characterization of aclacinomycin A-non-producing Streptomyces galilaeus (ATCC 31615) mutants. Microbiology 140, 1359–1365.