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Reviews

Sustainable Production of Bioactive Compounds by Sponges—Cell Culture and Gene Cluster Approach: A Review*,†

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Abstract: Sponges (phylum Porifera) are sessile marine filter feeders that have developed efficient defense mechanisms against foreign attackers such as viruses, bacteria, or eukaryotic organisms. Protected by a highly complex immune system, as well as by the capacity to produce efficient antiviral compounds (e.g., nucleoside analogues), antimicrobial compounds (e.g., polyketides), and cytostatic compounds (e.g., avarol), they have not become extinct during the last 600 million years. It can be assumed that during this long period of time, bacteria and microorganisms coevolved with sponges, and thus acquired a complex common metabolism. It is suggested that (at least) some of the bioactive secondary metabolites isolated from sponges are produced by functional enzyme clusters, which originated from the sponges and their associated microorganisms. As a consequence, both the host cells and the microorganisms lost the ability to grow independently from each other. Therefore, it was—until recently—impossible to culture sponge cells in vitro. Also the predominant number of ''symbiotic bacteria'' proved to be nonculturable. In order to exploit the bioactive potential of both the sponge and the ''symbionts,'' a 3D-aggregate primmorph culture system was established; also it was proved that one bioactive compound, avarol/avarone, is produced by the sponge *Dysidea avara*. Another promising way to utilize the bioactive potential of the microorganisms is the cloning and heterologous expression of enzymes involved in secondary metabolism, such as the polyketide synthases.

Key words: sponges, Porifera, bioactive compounds, sustainable production, cell culture, primmorphs.

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INTRODUCTION

Aquatic and especially marine biodiversity is orders of magnitude higher than that of terrestrial life. One reason for this may be the comparably higher viscosity of the surrounding aqueous milieu that allows assembly and

compartmentalization of biotic communities at higher densities. With regard to sponges (Porifera), the phylogenetically oldest phylum grouped with the Metazoa (Müller, 1995), the number of species is approximately 10,000 compared with the 45,000 chordate species known (Hawksworth and Kalin-Arroyo, 1995). While the number of species for chordates is estimated at 50,000 to 55,000, the estimated diversity of the sponge phylum can be considered as a multitude of the species known so far. It cannot be doubted any longer that the Porifera have the characteristics of Metazoa. Data obtained by modern molecular biological techniques group them as the phylogenetically oldest metazoans closest to the hypothetical ancestral animal, the Urmetazoa, from which the other metazoan lineages diverged (Müller, 2001).

Given the high biodiversity of sponge species and the phylogenetic age of these animals of more than 600 million years, one main question arises: Why were these metazoans so successful during evolution and able to avoid extinction? Reasons might be found in the fact that sponges, as sessile filter feeders, do not suffer from nutrient shortage and—in addition—have strong defense systems to defeat foreign invaders. Sponges have developed an amazingly efficient immune system, which is reminiscent of that found in vertebrates (Müller et al., 1999a). In addition, sponges have strategies to defend themselves against foreign organisms, prokaryotic, eukaryotic, or viral attackers, by the production of secondary metabolities that repel them (Sarma et al., 1993; Proksch, 1994). It is known that the most potent antimicrobial compounds, especially the antibiotics, are produced by microorganisms, such as bacteria (e.g., *Streptomyces*) and "Fungi imperfecti" (e.g., *Penicillium*). Consequently, and teleologically speaking, sponges have utilized the capability of these microorganisms as symbionts to enhance their potency to synthesize bioactive compounds. Because of the high abundance of bacteria within sponges, whose concentrations may exceed those in seawater by 2 to 3 orders of magnitude (Friedrich et al., 2001), it has been proposed that sponges may have originated from biofilms (Reitner and Schumann-Kindel, 1999). However, in view of the recent discoveries that sponges contain the key elements of metazoan organization, this proposal should be rejected.

It can be expected that the long term coevolution of sponges with bacteria and microorganisms resulted in a close linkage of the metabolic pathways in both partners, symbionts (microorganisms) and host (sponge). This assumption might be supported by (1) observations that many of the bacteria present in sponges cannot be cultured (Webster and Hill, 2001) and (2) the long lasting difficulties in developing a cell culture from sponges (Rinkevich, 1999). The problem of an in vitro culture of sponge cells could be partially overcome by keeping them in 3-dimensional cultures, as 3D-aggregates (Müller et al., 1999b; Nickel et al., 2001). It was observed that a special form of aggregates, the primmorphs, still contain bacteria (Müller et al., 1999b). As will be outlined below, these primmorphs can be used for the production of bioactive compounds. To utilize the bioactive potential of the bacteria from sponges, the following routes can be taken: optimization of in vitro culture conditions by adding essential nutrients; growing the bacteria that synthesize the bioactive compounds, in primmorphs (Müller and Brümmer, 1998); or cloning the genes that encode enzyme clusters required for the synthesis of low molecular weight bioactive compounds. An example of the latter approach, the isolation of polyketide synthases in sponges (*Suberites domuncula* and *Geodia cydonium*), is given here for the first time.

The Model Sponges

Sponge Collection

Specimens of the marine sponges *Suberites domuncula* (Porifera, Demospongiae) and *Dysidea avara* (Demospongiae) were collected by SCUBA diving from depths between 15 and 35 m in the Northern Adriatic Sea near Rovinj (Croatia). The sponges were brought to Mainz (Germany) and kept there in 10^3-L tanks at 17°C before being used in the experiments. In the studies to determine the bacterial load, either the sponges remained for 6 months in the aquarium prior to use in the experiments (*aquarium animals*), or the animals were kept for only 2 days in the aquarium before use (*field animals*).

Suberites domuncula

This sponge species lives very likely in a symbiotic or commensalic relationship with bacteria (Althoff et al., 1998; Böhm et al., 2001). In *S. domuncula* tissue bacteria accumulate primarily in bacteriocytes, which presumably derived from spherulous cells or archaeocytes. These cells are found either in the cell layer lining the water canals and the lacunae in the epithelium, or in layers that are intimately

associated with it; their diameter is 15 to 20 μm. The bacteriocytes have been analyzed by transmission electron microscopy, by which the bacteria can be visualized in a highly compressed organization. To obtain a first hint about the phylogenetic nature of the bacteria, DNA was extracted from a sponge specimen that had been maintained in an aquarium for 6 months. Only one type of 16S ribosomal DNA sequence could be identified, which corresponded to those found in the genus *Pseudomonas* (Böhm et al., 2001).

Formation of Primmorphs

The procedure to obtain primmorphs from single cells of sponges was applied as described previously (Custodio et al., 1998; Müller et al., 1999b; Müller and Brümmer, 1998). Starting from single cells obtained by dissociation in Ca^{2+}- and Mg^{2+}-free artificial seawater, primmorphs of at least 1 mm in diameter (average, 3 to 7 mm) are formed after 3 to 5 days. For the experiments 5-day-old primmorphs were used. They were cultivated in natural seawater supplemented with 0.2% RPMI1640 medium and silicate to the optimal concentration of 60 μM as described previously (Krasko et al., 2002). Where indicated 30 μM Fe^{3+} (from ferric citrate) was added. The incubation temperature was set to 17°C.

Bacteria

In a recent study bacteria have been isolated from the surface of *S. domuncula* specimens (Thakur et al., 2003). Among those was one strain, termed SB-1, which, on the basis of 16S rDNA analysis has high sequence similarity to *Idiomarina loihiensis* (Alteromonadaceae; 98.8%, a γ-*Proteobacterium*).

Cytostatic Activity

An ethyl acetate extract was prepared from *S. domuncula* tissue as described (Müller et al., 1985a). The cytostatic activity was determined as described (Müller et al., 1985b). Using this crude extract and mouse lymphoma cells as a tumor cell system, a 50% reduction of cell viability or density (ED_{50} value) was obtained at 5.2 ± 0.7 μg/ml after an incubation period of 72 hours. For the determination of the cell density the, methylthiazoletetrazolium (MTT) cell proliferation assay was applied (Holst-Hansen and Brümmer, 1998).

Antimicrobial Activity

The antimicrobial activity of the bacteria, isolated both from *S. domuncula* tissue and from primmorphs, was determined after extraction with n-butanol (Thakur et al., 2003). The human pathogenic strains *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Candida albicans*, along with multi-antibiotic-resistant derivatives, were used as reference organisms for the standard paper disk diffusion assay. The extracts from SB-6 and PB-1 showed only minimal antimicrobial activity (inhibition zone less than 3 mm), while the extract from PB-2 caused strong inhibition against *S. aureus*, *S. epidermidis*, *E. coli*, and *C. albicans* (greater than 15 mm) (Thakur et al., 2003).

Dysidea avara

The demosponge *D. avara* proved to be very rich in the sesquiterpenoid compounds avarol and its derivative avarone (approx. 3 g/kg wet weight). Avarol was discovered by Cimino et al. (1984) and was later reported to display strong bioactivity in vitro and in vivo (Müller et al., 1985b); in particular, cytotoxic, antitumor (in vivo) (Müller et al., 1985b), antibacterial (Seibert et al., 1985), and antiviral (Sarin et al., 1987) activities have been described. The major mode of action of avarol is its effect on the prostaglandin and leukotriene pathways (Schröder et al., 1991).

The enzymatic pathway by which avarol is synthesized in the sponge is not known. It can be postulated that this bioactive compound is produced by a combination of the isopentenyl pyrophosphate–isoprenoid pathway and the shikimate pathway of the host, but a synthesis based on bacterial symbiosis in *D. avara* cannot be excluded.

Production of A Bioactive Compound Using *D. avara*

Considering the results summarized in the previous section, it was challenging to determine if avarol/avarone could be produced in vitro using the primmorph system. This approach was successful using the *D. avara* model (Müller et al., 2000). The 3D aggregates were prepared by applying the basic method, as described above. The dissociated cells were obtained (Figure 1, A-a) and then transferred to natural seawater supplemented with 0.2% RPMI1640 medium. After an incubation of approximately 7 days, round-shaped primmorphs were formed (Figure 1,

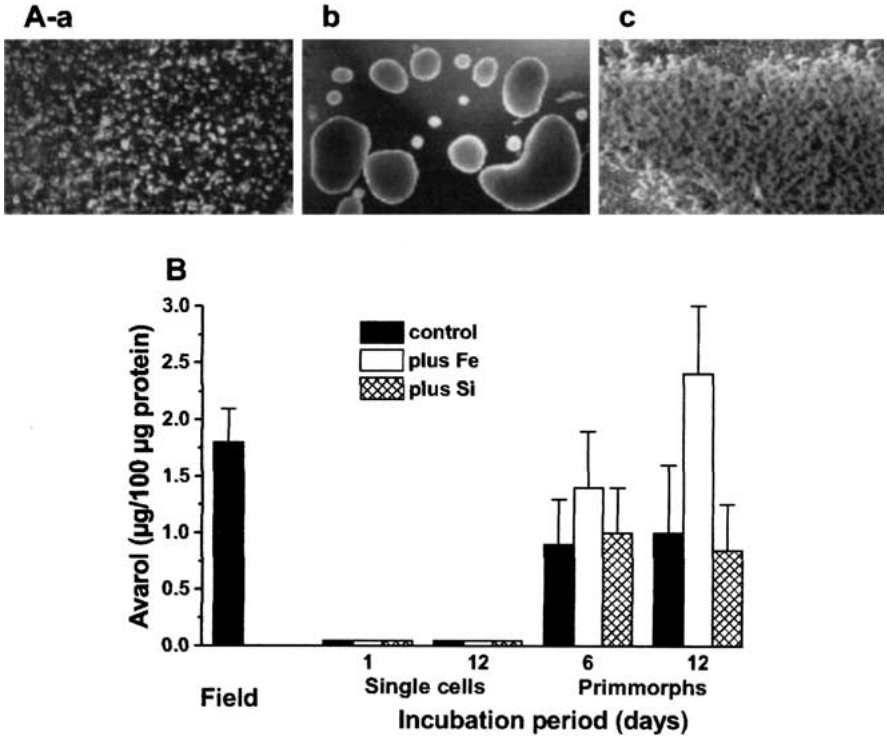

Figure 1. Formation of primmorphs from *D. avara* cells for the production of avarol/avarone. A: Formation of primmorphs. Single cells were prepared (A-a), and allowed to form round-shaped primmorphs (after 7 days) (b), which reorganized after 10 days to adherent mesh primmorphs (c). Magnification: ×50 (a), ×3.5 (b), and ×15 (c); modified according to Müller et al., 2000. B: Production of avarol. The dissociated cells were kept in Ca^{2+}- and Mg^{2+}-free seawater with 0.2% RPMI1640 medium; under these conditions the cells remained in the single state. In parallel, the cells were transferred into natural seawater supplemented with 0.2% RPMI1640 medium and silicate (60 µM) or 30 µM Fe^{3+} (from ferric citrate). The incubation temperature was set to 17°C. After the indicated incubation period, samples were extracted with ethyl acetate, and the extract was separated by HPLC. In the fractions containing avarol, its concentration was determined and the values are given as micrograms of avarol per 100 µg protein. Five experiments were performed each; the means ± SD are given.

A-b), which organized during the following 3 days to adherent mesh primmorphs (Figure 1, A-c).

For the analysis of avarol production, the primmorphs and cells were extracted with ethyl acetate and the extract subjected to high-performance liquid chromatography as described (Müller et al., 2000). The analyses showed that single cells did not contain considerable amounts of this secondary metabolite (Figure 1, B; "single cells"), while avarol could be demonstrated in primmorphs (Figure 1, B; "primmorphs"). In the absence of either Fe^{3+} or silicate, the primmorphs contained approximately 1 µg avarol per 100 µg protein after 6 or 12 days of incubation. In contrast, no avarol could be detected in single cell suspension (Figure 1, B; Müller et al., 2000); we explain this result by the assumption that cells from *D. avara* lose their viability rapidly with the consequence that avarol is released and remained undetectable. This conclusion is in accordance with previous findings which revealed that sponge cells, if kept in the single cell state, become rapidly telomerase negative (Koziol et al., 1998). The level of avarol production could be enhanced by addition of Fe^{3+} to the incubation medium. If the seawater and 0.2% RPMI1640 medium was supplemented with 30 µM Fe^{3+}, the level of avarol in the primmorphs increased to 1.5 (in 6-day-old primmorphs) and 2.5 µg avarol per 100 µg protein (12-day-old primmorphs; significance $P < 0.001$), respectively. In contrast, supplementation of the medium with silicate did not result in a significant change of the avarol level compared with the control. It is interesting to note that the primmorphs cultivated in the presence of Fe^{3+} were able to synthesize as much avarol as cells from field animals (1.8 µg avarol per 100 µg protein); Figure 1, B.

These data demonstrate that the primmorph system is suitable for in vitro production of secondary metabolites from sponges. In addition, the data underscore that single

cells from sponges, at least in our experience thus far, are not able to produce secondary metabolites and—as shown previously (Custodio et al., 1998; Müller et al., 1999b)—are not able to proliferate.

Since avarol is known to be a strong cytostatic secondary metabolite that inhibits growth of mammalian cells at concentrations below 1 μg/ml (Müller et al., 1985b), it was important to know if avarol also inhibits the producer cells in the "bioreactor." Therefore, cells from *D. avara* as well as *S. domuncula* were incubated for 48 hours with avarol. To measure the effect of avarol on cell viability, the MTT assay was applied. The viability of cells from *D. avara* remained almost unaffected by avarol concentrations between 0.1 and 1 μg/ml, while a strong reduction was seen in *S. domuncula* already at a concentration of 0.3 μg/ml (Figure 2, A). This finding strongly indicates that the cells in *D. avara* have developed a resistance mechanism against the toxic effect displayed by avarol, which does not exist in *S. domuncula* cells. For this colorimetric assay it was crucial that the reaction with the MTT reagent was performed at 37°C; at a lower temperature, the onset of the color reaction was—in comparison with mammalian cells—too slow (Figure 2, B).

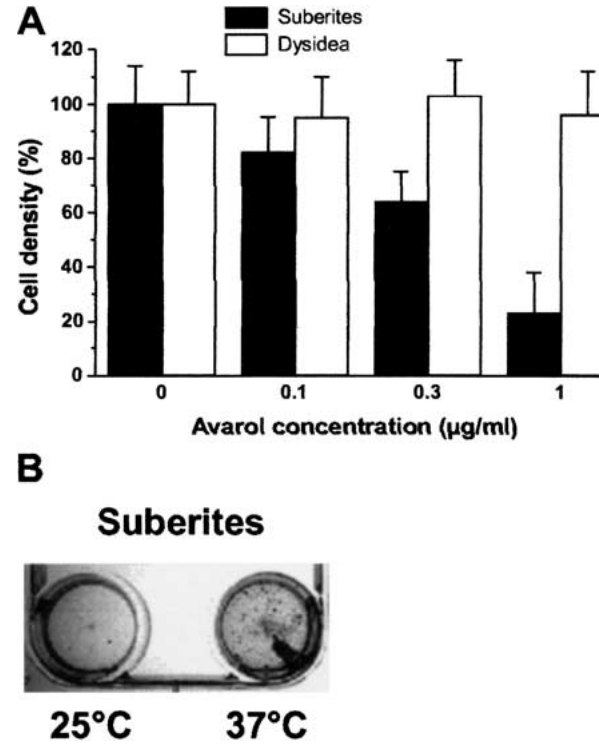

Figure 2. Effect of different avarol concentrations on the viability of cells from *D. avara* and *S. domuncula*. A: Different avarol concentrations were added to the sponge cells (*S. domuncula* or *D. avara*) and incubated in natural seawater, supplemented with 0.2% RPMI1640 medium, silicate (60 μM), and 30 μM Fe^{3+}. After 48 hours the MTT assay was applied to measure the viability. The cell density, which correlates with the MTT color intensity, in the controls was set to 100%. B: Color reaction by the MTT reagent. At a temperature lower than 25°C, the cell suspension remained unstained (left), while after incubation at 37°C for 3 hours, the samples turned yellow and a precipitate was seen (right); the same cell concentrations were used in both assays.

Isolation of Polyketide Synthetase from *S. domuncula* Bacteria

Bacteria in sponges are known to produce polyketides—e.g., swinholide from the lithistid sponge *Theonella swinhoei* (Bewley and Faulkner, 1998)—a class of compounds which display highly potent antibiotic activities. However, most of the bacteria present in sponges are considered to be unculturable. To utilize the bioactive potential of those microorganisms, molecular biotechnological approaches have to be applied. One promising way is to clone the polyketide synthases, which synthesize the core of the bioactive compound, and express them in a heterologous system. In the course of this attempt, two elements of polyketide synthases have been isolated for the first time from a sponge, here from *S. domuncula*.

In most cases it is difficult to isolate DNA from sponge tissue at a high yield. However, it is crucial that both the integrity and the yield are sufficiently high for any kind of quantitative approach. We found that the yield of DNA extraction was strongly dependent on the starting material. If whole tissue was used for the extraction after a standard phenol-chloroform protocol (Sambrook et al., 1989), the yield was comparatively low (Figure 3, lanes d and e). The amount and quality of the DNA in the nucleic acid preparation was checked by agarose gel electrophoresis. The intensity of the band that corresponds to DNA and migrates in the agarose gel at ≥15 kbp is low; primarily the RNA (≤2 kbp) becomes visible, with the dominant bands reflecting the 18S and 28S rRNA. In contrast, if nucleic acid is extracted from cells after dissociation and purification (Müller et al., 1999b), the DNA is resolved at the top of the gel (Figure 3, lanes b and c). Lane a of Figure 3 shows separation of DNA that was obtained after lysis and silica gel fractionation (Seack et al., 1999). Starting from 200 mg of single cells, the yield of DNA was determined to be 250 μg; and from 200 mg of tissue, 55 μg.

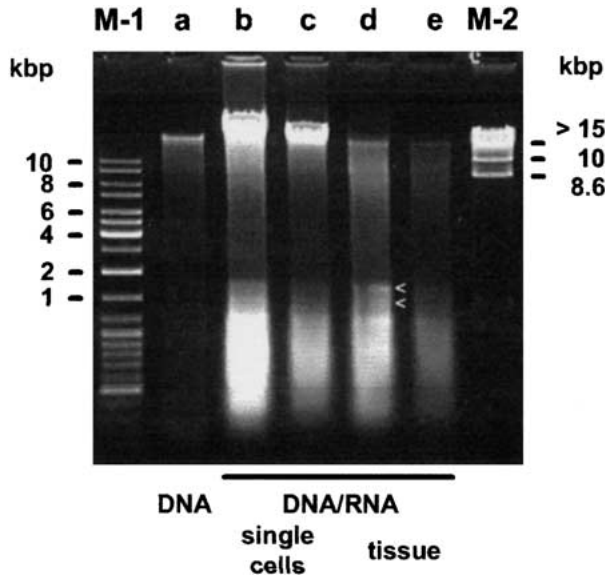

Figure 3. Analysis of DNA, isolated from single cells or from tissue of *S. domuncula*. Nucleic acid was isolated by a standard phenol-chloroform protocol from 200 mg of single cells (lanes b and c) or from 200 mg of tissue (lanes d and e). The resulting fractions were applied onto a gel at the following concentrations: 7.5 µg (lane b), 2.5 µg (lane c), 1.5 µg (lane d), 0.5 µg (lane e). The preparations were size separated on an 1.5% agarose gel and stained with ethidium bromide. The DNA migrates at a size of ≥15 kb, while the RNA is detected in a range ≤ 2 kb. The intactness of the nucleic acid preparation can be checked on the basis of the discrete bands representing the 18S and 28S rRNA species (marked in lane d [<]). In lane a, a DNA purified from RNA was analyzed that had been used for the preparation of the genomic library (Seack et al., 1999). Two sets of size markers were used (M-1 and M-2).

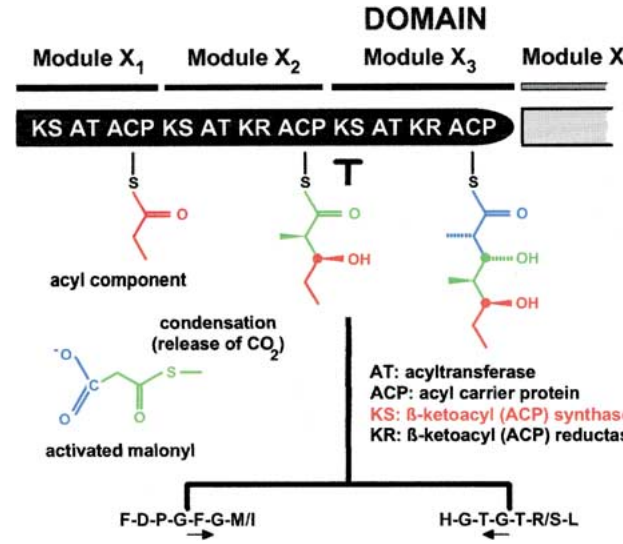

Figure 4. Schematic model of the first domains of the polyketide synthase (based on Khosla et al., 1994). The modular synthetase is composed of at least the following enzymic activities: the β-keto acylthioester synthase (KS) [3-oxoacyl synthase], the acyltransferase (AT), and the acyl carrier protein (ACP) module; in addition, one further activity is included, the ketoacyl-reductase (KR). The first unit comprises the "loading domain." For the isolation of the KS region from *S. domuncula*, primers corresponding to the flanking peptides have been designed.

Polyketide Synthases

Polyketide metabolites are classified as aromatic and non-aromatic polyenes and polyethers, including macrolides (Rawlings, 1997). They are found in most taxa and are especially abundant in actinomycetes; they have been proven to display potent antibiotic, anticancer, and immunosuppressive activities. These complex compounds are synthesized in a metabolic pathway similar to that involved in fatty acid biosynthesis (Kao et al., 1994; Khosla et al., 1994). The polyketide synthases are multienzyme complexes (M_r 10,000 to 100,000 kDa) that use simple organic building blocks, such as acetyl-CoA, and their activated derivatives, such as malonyl-CoA. The polyketide synthases are grouped into *type I* enzymes, which are analogous to the metazoan fatty acid synthases and involved in the synthesis of fungal polyketides such as 6-methylsalicylic acid; they are large multidomain proteins that carry all enzymatically active sites required for the synthesis of polyketides. *Type II* synthases are distributed among several smaller (monofunctional) polypeptides; they are involved in bacterial aromatic natural products such as doxorubicin (Moore and Piel, 2000). Finally, *type III* enzymes, also termed modular polyketide synthases, have an architecture involving multiple copies of active site units reminiscent of those of the fatty acid synthases. Examples of the latter group of modular bacterial polyketides are erythromycin, rapamycin, or rifampicin.

Polyketide Synthase Detection in *S. domuncula*

The polyketide synthases comprise at least the following enzymic activities; ß-keto acylthioester synthase (KS), acyltransferase, and acyl carrier protein (ACP) domain (reviewed in Khosla et al., 1994). In addition, other enzymic activities can be included: e.g., ketoacyl-reductase, dehydratase, enoyl reductase, and thioesterase domains (Figure 4). One enzymic activity that shares similarity to thiolases, a system involved in the fatty acid metabolism, is the KS. The KS mediates the condensation reaction

between the acyl component and the malonyl unit. The acyl component of an activated acyl primer is transferred to a cysteine residue of the enzyme, which in turn is condensed with an activated malonyl; during this reaction carbon dioxide is released (Figure 4). The sequence of the KS around the active site cysteine is well conserved; the signature pattern reads G-x(4)-[LIVMFTAP]-x(2)-[AGC]-C-[STA](2)-[STAG]-x(3)-[LIVMF], where C (cysteine) is the active site residue (PROSITE [PDOC00529], 2002).

In our approach to identifying parts of a polyketide synthase from *S. domuncula*, the KS was isolated by polymerase chain reaction (PCR). Two degenerated primers were designed which are directed against the conserved region of the KS (see Figure 4): the forward primer 325F (5′-TTY GAY CCN GGN TTY GGN AT-3′, corresponding to the amino acids F-D-P-G-F-G-M/I), and the reverse primer R2 (3′-GTR CCI TGI CCI TGI TCI RAI-5′ [where Y= G or C; R = G or A; N = G, A, T, or C, and I = inosine], corresponding to H-G-T-G-T-R/S-L). For the PCR reaction DNA was isolated from tissue and cells and added to the reaction mixture. It contained in 30 µl the 67 mM Tris-HCl buffer (pH 8.8; 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, and 0.01% Tween-20), 200 µM dNTPs, 100 to 200 ng of genomic DNA, 50 pmol of the primer 325F and 20 pmol of R2, and 1.2 U of Thermolase (MB Enzymes). The cycling procedure was as follows: initial denaturation at 95°C, 120 seconds, 5 cycles of denaturation (95°C, 20 seconds), annealing (59°C, 30 seconds), and extension (72°C, 60 seconds), 10 cycles of denaturation (95°C, 20 seconds), annealing (58°C, 30 seconds), and extension (72°C, 60 seconds), and finally 25 cycles of denaturation (95°C, 20 seconds), annealing (56°C, 30 seconds), and extension (72°C, 60 seconds). After purification from the agarose gel, the PCR products were subcloned into the *pGEM-T* vector and sequenced using an automatic DNA sequenator (Li-Cor 4200) (Ausubel et al., 1995). The size of the PCR reaction products obtained from reactions using DNA from *S. domuncula* and *G. cydonium* was approximately 700 bp (Figure 5).

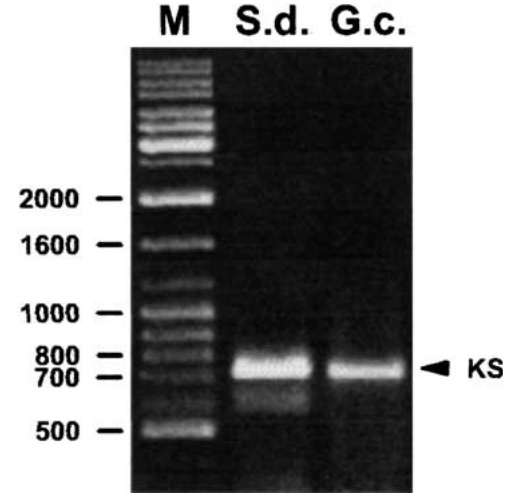

Figure 5. Separation of the PCR reaction products obtained with degenerated primers directed against the KS region and DNA from *S. domuncula* (S.d.) and *G. cydonium* (G.c.). The arrowhead points to the approximately 700-bp large products, which represent fragments of the polyketide synthase. The size markers are indicated.

Polyketide Synthase Sequence Analysis

The PCR products from both assays, using *S. domuncula* as well as *G. cydonium* DNA, resulted in 2 different sequences each, which were termed PKS-3 and PKS-5. Here the sequences from *S. domuncula* are mentioned. The deduced polypeptide sequence comprises the characteristic central part of the KS, with the β-ketoacyl synthase active site (PROSITE [PDOC00529], 2002); the pattern in PKS-3 reads GPAVAVDTACSSSLVAI; in PKS-5, it is GPSVTIDTACSSSLTAI.

The PKS sequences were aligned with two polyketide synthases, the FK506 polyketide synthases from *Streptomyces* sp. and the 6-methylsalicylic acid synthase from *Penicillium griseofulvum* (Figure 6, A). The two *S. domuncula* sequences share 46% identity and 64% similarity with each other; the *S. domuncula* PKS-3 was found to have 52% identical and 68% similar residues with the FK506 polyketide synthase, and 45% identical and 62% similar with 6-methylsalicylic acid synthase. A phylogenetic relationship was established on the basis of tree construction, using these sequences together with the structurally related plant 3-oxoacyl-(acyl-carrier-protein)-synthase I from *Arabidopsis thaliana*, the human fatty acid synthase, and the *S. domuncula* acetyl-CoA:acetyltransferase sequence. The tree was constructed and rooted with the human fatty acid synthase and the sponge acetyltransferase. This calculation revealed that the 2 sponge sequences, PKS-3 and PKS-5, form one branch with the *Streptomyces* sp. FK506 polyketide synthase, which clusters together with the 6-methylsalicylic acid synthase of *P. griseofulvum*. More distantly related are the sequences from *A. thaliana*, the 3-oxoacyl synthase, and the 2 sequences, human fatty acid synthase and *S. domuncula* acetyl-CoA:acetyltransferase (Figure 6, B). These data indicate that the 2 sponge sequences, PKS-3 and PKS-5, are more similar to the bacterial (*Streptomyces*) KS than to the fungal (*Penicillium*) KS.

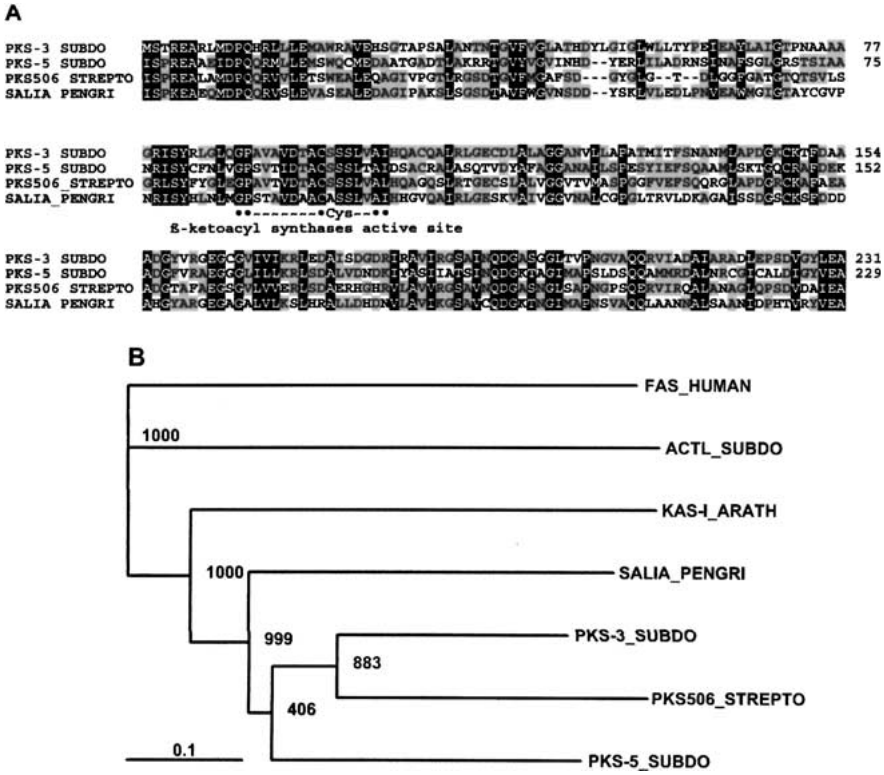

Figure 6. Segments within the polyketide synthases from *S. domuncula*, comprising the KS region, were analyzed. A: As outlined in the text, DNA from *S. domuncula* was used together with 2 degenerated primers to isolate the approximately 700-bp PCR products. They were sequenced, and 2 different deduced amino acids are given (PKS-3_SUBDO; PKS-5_SUBDO). In the alignment, the *S. domuncula* sequences were compared with the corresponding parts from the FK506 polyketide synthase, *Streptomyces* sp., strain MA6548 (PKS506_STREPTO; accession number T17428 [Motamedi and Shafiee, 1998]), and the 6-methylsalicylic acid synthase, *Penicillium griseofulvum* (SALIA_PENGRI; S13178 [Beck et al. 1990]). Residues conserved (similar or related with respect to their similar physico-chemical properties) in all sequences are shown in white on black, and those in at least 3 sequences in black on gray. The characteristic β-ketoacyl synthase active stretch with the active cysteine site is indicated. B: The phylogenetic relationship of the 2 sponge polyketide synthase sequences with the following related sequences is determined: the FK506 polyketide synthase as well as the 6-methylsalicylic acid synthase (see under A) and the plant 3-oxoacyl-(acyl-carrier-protein) synthase I precursor (KAS I) from *Arabidopsis thaliana* (KAS-I_ARATH; NP_199441) and the human fatty acid synthase (FAS_HUMAN; P49327). In addition, the *S. domuncula* acetyl-CoA:acetyltransferase sequence is used for this comparison (ACTL_SUBDO). The tree was computed by neighbor-joining and distance matrix determinations (Felsenstein, 1993; Dayhoff et al., 1978) using the human fatty acid synthase and sponge acetyl-CoA acetyltransferase as outgroups. Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

Polyketide Synthase Bacterial Load

Specific primers were designed against the *S. domuncula* polyketide synthases PKS-3 (PKS3F 5′-CGACCCGCGAGGCCAGGTTG-3′ and PKS3R 5′-TGATTCGTCCTGCTGCTGCCGCAT-3′) and used for an estimation of the bacterial load in *S. domuncula* as well as in *G. cydonium*. Total DNA was isolated both from tissue and from primmorphs. The sponge material either was used directly after collection from the sea (field animals) or was kept first in the aquarium for 6 months and then used for the studies (aquarium animals).

To assess the load of bacteria in a semiquantitative approach, DNA of the different sources was subjected to PCR. The PCR conditions were as described above, using 10 pmol of each primer and 100 ng of genomic DNA. Reactions were run in an "iCycler" (Bio-Rad) under the following conditions: initial denaturation (95°C for 120 seconds), then 30 cycles (denaturation, 95°C for 20 seconds, annealing, 62°C for 30 seconds, extension, 72°C for 35 seconds). Aliquots of 10 µl were resolved in a 1.5% agarose gel and stained with ethidium bromide.

The signals obtained after PCR amplification followed by size fractionation are shown in Figure 7. The PCR

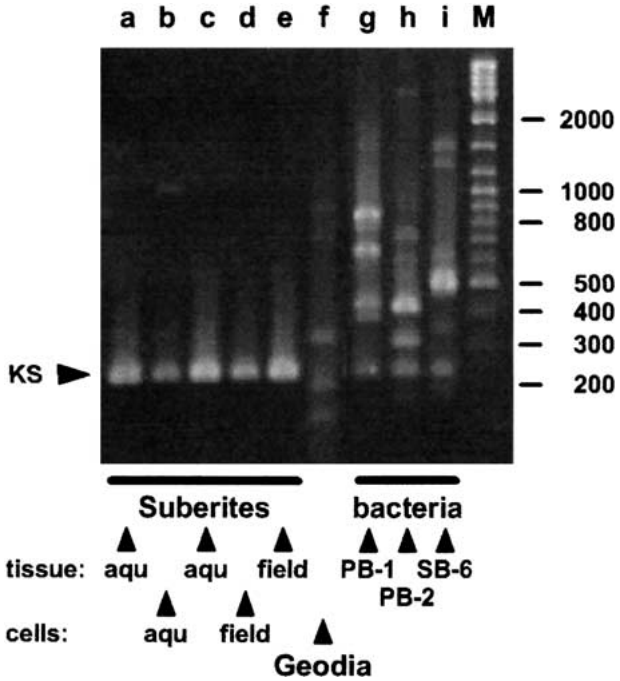

Figure 7. Amplification of the KS gene (PKS-3) obtained from *S. domuncula* in a series of DNA samples. The following DNA samples were used: lanes a–e, samples from *S. domuncula*; more specifically, DNA isolated from tissue of aquarium animals (lane a and c), of field animals (lane e), or from dissociated cells that were obtained from aquarium animals (lane b), or from field animals (lane d). In lane f, DNA from *G. cydonium* was used for the amplification. Finally, DNA was isolated from bacteria which had been isolated from the intact sponge *S. domuncula* (SB-6) or from primmorphs (PB-1 and PB-2); lanes g–i. Lane M: separation of size markers. The PCR conditions are given in the text; the size of the PCR product was 220 bp (arrow indicates KS).

product with the expected size, approximately 220 bp, could be visualized from all *S. domuncula* sponge samples used. However, with this approach the intensities of the bands, reflecting the presence of bacterial DNA, were different. The strongest signals are seen in the assays with DNA from field animals (lane e), in comparison with DNA from aquarium animals (lanes a and c). Surprising was the finding that DNA obtained from *S. domuncula* cells, analyzed immediately after dissociation, also contained the signal (lanes b and d). This finding indicates that these samples also contained the same bacteria. In view of the fact that in *S. domuncula* the bacteria are present intracellularly, this PCR analysis is compatible with the microscopic examination.

In parallel, tissue from *G. cydonium* was analyzed (Figure 7, lane f). The PCR products show no major band. In contrast, at least 6 bands ranging from 100 to 1000 bp became visible. These results are probably due to nonspecific application.

In a final series of experiments, bacteria from *S. domuncula* were analyzed. The bacteria were isolated either from sponge tissue, termed SB-6, or from primmorphs, termed PB-1 and PB-2. After amplification with the specific primers, all 3 DNA samples were found to contain the polyketide synthase gene (Figure 7, lanes g to i).

From this part of the study, it can be concluded that it is possible to identify in *S. domuncula* and in *G. cydonium* polyketide synthase fragments by PCR analysis using degenerated primers. After application of specific primers for the KS gene isolated, this bacterial gene was detected at a higher yield in field animals, as compared with aquarium animals and also in dissociated cells. The different intensities of the signals, especially between DNA from field animals and aquarium animals, indicates that the bacterial load is higher in field animals. This conclusion also confirms findings obtained recently using the ''stress protein'' (2′–5′)oligoadenylate synthetase as a marker (Grebenjuk et al., 2002) or the stress kinases p38 and JNK (Böhm et al., 2001).

In Situ Localization of the Polyketide Synthase Fragment

Recently, we succeeded for the first time with in situ hybridization of sponges (Le Pennec et al., 2003). Here we applied a modified technique to identify the bacterial polyketide synthase in cryosections, which had been prepared as described (Le Pennec et al., 2003). Fresh sponge cubes (about 5 mm^3) were cut, and 8-µm sections were obtained. Then, tissue was digested with proteinase K (1 µg/ml, 15 minutes, room temperature) prior to fixation (30 minutes in 4% paraformaldehyde). After washing 3 times with phosphate-buffered saline (PBS) (1 minute each), pigments were removed by treatment in ascending alcohol solutions (30%, 50%, 70%, 90%, and pure ethanol) and finally in pure acetone. Cuts were then rehydrated and finally washed 3 times in PBS. The DNA probe (double-stranded) used for hybridization was 220 nucleotides long, and obtained by the primer pairs PKS3F/PKS3R (termed PKS3F/PKS3R probe). It was labeled applying the DIG (digoxigenin) DNA labeling kit (Roche). Probes were diluted 50 times in 4× SSC (72 µl) and denatured at 95°C for 1 minute. Solutions were cooled down in an ice bath; 75 µl formamide was added to the probe solution. Hybridization

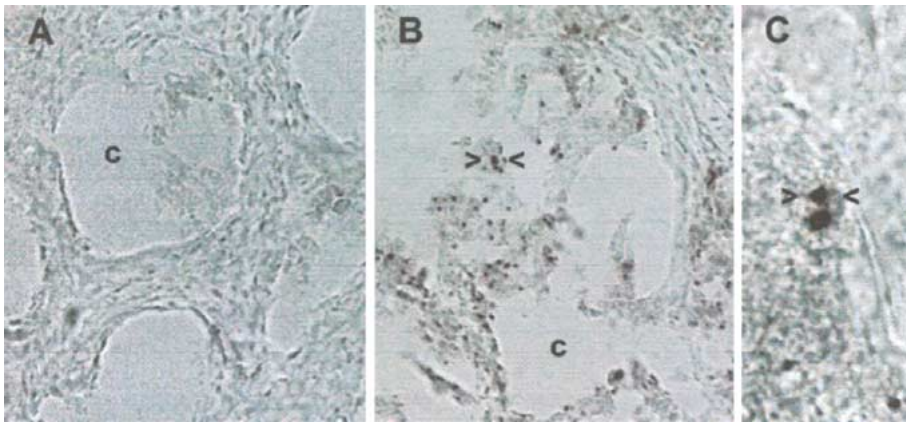

Figure 8. In situ localization of polyketide synthase fragments in sections of *S. domuncula* tissue. Cryosections were prepared and incubated without (**A**) and with the PKS3F/PKS3R DNA probe (**B**, **C**). The positive signals (> <) shown in B and C label cells that are lining the canals (c) within the sponge tissue and probably represent the bacteriocytes. Magnification: ×150 in A and B; and ×600 in C.

was performed with 50 µl of the probe solution at 40°C overnight. Then the slides were washed at 45°C, once with 2× SCC for 2 minutes and 3 times with 0.2× SSC. For the detection of the signals, the slides were transferred into Blocking Solution (Roche) at room temperature for 15 minutes. The antibody anti-DIG was added and the immunocomplexes visualized.

The results shown in Figure 8 indicate that incubation without the PKS3F/PKS3R DNA probe (but with the anti-DIG antibody), no signal in cryosections is seen (A), while with the DNA probe intense signals are visible in those cells which line the canals (B). At a higher magnification it becomes more obvious that the hybridization signal fills almost the complete cell. When this image is compared with the results published earlier (Böhm et al., 2001), it appears to be very likely that the in situ hybridization signals correspond to those in bacteriocytes.

Conclusions

In view of the recent achievements in the field of modern cell biology and molecular biology, and in combined efforts by groups with complementary expertise (e.g., in the German Center of Excellence [BiotecMarin; www.biotecmarin.de]), it can be optimistically projected that sustainable exploitation of the bioactive potential, known to be present in sponges, will become possible.

The recent progress achieved in culturing sponge cells, the establishment of the primmorph system, and the ongoing adaptation of this system for application on a larger scale in bioreactor technology will allow the production of primarily sponge-cell-based bioactive compounds. To improve the growth conditions of sponge cells, factors, in addition to those already identified as inorganic such as silicate (Le Pennec et al., 2003) and ferric iron (Krasko et al., 2002), or organic growth factors such as myotrophin (Schröder et al., 2000), which maintain or stimulate the growth of the sponge cells, have to be identified. Attempts to design the medium have to take into account that the proteinaceous growth factors used in mammalian cell culture cannot be considered useful for sponge cell culture. This must be concluded from the finding that, in spite of a considerable sequence similarity of the growth factors such as dermatopontin (Schütze et al., 2001), the functions of the recombinant proteins are restricted to the given sponge species or perhaps to other members of this phylum. The interaction between the growth factor and the cell surface receptor is also considered to be restricted to a given taxon. A random sequencing of the expressed sponge genome from sponges (http://spongebase.uni-mainz.de/cgi-bin/blast/blastserver.cgi) will help to identify unknown growth factors. Those organic factors which are present in the sponge, but not produced by it, such as lipopolysaccharide (LPS) or okadaic acid, might be of potential use to optimize the medium. The bacterial endotoxin LPS has recently been shown to activate sponge cell metabolism and to stimulate the production of bioactive compounds such as (2′–5′)oligoadenylate (Grebenjuk et al., 2002). Okadaic acid has been proven to stimulate the immune response of sponge cells against bacteria (Wiens et al., 2003). The production of sponge proteins in a recombinant way in bacteria is as feasible as for other metazoan proteins. Successful transfection of mammalian cells (Wiens et al., 2001) as well as of fungal cells (Böhm et al., 2002) with sponge genes has been accomplished.

In focusing on sponge metabolites which are very likely produced by microorganisms, the use of specialized media containing extracts of sponge tissue can be a useful approach for targeting novel bacteria not culturable on

standard media (Webster et al., 2001). The following strategy, however, appears to be promising for utilizing their bioactive potential, too. It is known that considerable natural products produced in the sponge-microbe symbiotic relationship belong to the class of polyketides (Haygood et al., 1999). Since the synthesis of these compounds is under the control of enzyme clusters (Khosla et al., 1999), which have already been analyzed from a series of bacteria and fungi, it becomes feasible to isolate those clusters from bacteria harbored by the sponge. The technique of choice is PCR using degenerated primers. The first successful approach for sponges has been described in this review. It is shown that a fragment of this cluster can be isolated from genomic DNA, isolated from *S. domuncula*. The presence of the sequences in bacteria from the sponge could be verified by in situ hybridization. After having completed the cluster analysis, we propose (and the work in our laboratory is in progress) to express these clusters in a heterologous bacterial-fungal system, which is suitable for continuous growth and efficient production of the bioactive compound.

It is hoped, with the tools and techniques that are available, that these approaches will contribute to successful exploitation of the hitherto inaccessible resources of bioactive compounds from sponges and their associated bacteria in a sustainable and affordable manner.

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