

Mixed fermentation for natural product drug discovery

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Abstract Natural products continue to play a major role in drug discovery and development. However, chemical redundancy is an ongoing problem. Genomic studies indicate that certain groups of bacteria and fungi have dozens of secondary metabolite pathways that are not expressed under standard laboratory growth conditions. One approach to more fully access the metabolic potential of cultivatable microbes is mixed fermentation, where the presence of neighboring microbes may induce secondary metabolite synthesis. Research to date indicates that mixed fermentation can result in increased antibiotic activity in crude extracts, increased yields of previously described metabolites, increased yields of previously undetected metabolites, analogues of known metabolites resulting from combined pathways and, importantly, induction of previously unexpressed pathways for bioactive constituents.

Keywords Mixed fermentation · Co-culture · Natural product · Secondary metabolite

Introduction

Natural products continue to be an unparalleled resource for pharmaceutical lead discovery. Of all small molecule drugs that were launched between 1981 and 2006, 63% were derived from natural products (Newman and Cragg 2007). Natural products have played a particularly large

role in antitumor, antimicrobial, and antihypertensive areas (Banerjee et al. 2008; Newman and Cragg 2007). Although the vast majority of macro and microorganisms have yet to be described (and almost certainly hold a tremendous reservoir of bioactive constituents), there exists today an unfortunate problem with chemical redundancy in natural products drug discovery.

In response to this, there has been a shift to develop strategies to maximize the chemical diversity obtained from each species, particularly microbial species. Environmental conditions are critical to the synthesis of microbial secondary metabolites (Knight et al. 2003). Maximizing the types of samples collected; diversifying the isolation strategies; optimizing nutrients, temperature, pH, aeration, and incubation time; exploiting talented strains; and genetically modifying strains can greatly influence productivity (Knight et al. 2003). Bode et al. (2002) explored the literature regarding alterations in secondary metabolites with varied cultured conditions. Their group, for example, isolated more than 100 compounds from more than 25 structural classes from six different microbes by altering culture conditions (Bode et al. 2002). The fungus *Sphaeropsidales* sp., which synthesizes the antifungal spirobisnaphthalene cladospirone bisepoxyde, made eight new and six known spironaphthalenes when grown under varied conditions, and new bisnaphthalenes and a rare macrolide when grown in the presence of enzyme inhibitors such as tricyclazole (Bode et al. 2002).

Results of genome analyses reveal why manipulation of microbial culture conditions can be such a fertile avenue for secondary metabolite discovery. Microbial sequencing studies indicate that the biosynthetic potential of many strains is much greater than that observed by fermentation (Bode et al. 2002; Newman and Cragg 2007; Schneider et al. 2008; Udworthy et al. 2007). Because the genomes of

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fungi, actinomycetes, and myxobacteria are much larger than necessary for basic functioning, it was presumed that part of their genomes encoded alternative metabolic pathways (Knight et al. 2003). Indeed, sequencing of *Streptomyces avermitilis*, a known producer of avermectin, revealed at least 25 gene clusters for siderophores, spore pigments, and secondary metabolites of polyketide or nonribosomal peptide origin (Ōmura et al. 2001). Greater than 6% of the *S. avermitilis* genome contains genes for secondary metabolite biosynthesis. More than 200 secondary metabolites have been isolated from strains of *Streptomyces hygroscopicus* (Zhang 2005). *Salinispora tropica*, a marine actinomycete, devotes a very large portion of its genome, approximately 10%, to natural product assembly (Udwarý et al. 2007). The polyketide biosynthetic pathways of *S. tropica* are of greater variety than any other sequenced bacterial genome, and most of the pathways are novel (Udwarý et al. 2007). Aspergilli are another rich source of secondary metabolites (Schneider et al. 2008); they can harbor 30–50 distinct polyketide synthase and nonribosomal peptide synthase gene clusters per species (Payne et al. 2006). *Aspergillus niger* contains 17 nonribosomal peptide synthase and 34 polyketide synthase-encoding genes; most of which are located in clusters (Pel et al. 2007).

Why such a large genomic investment in secondary metabolites? Microbes interact in their natural environments (Slattery et al. 2001; Wiener 1996), presumably even more so in areas with a high microbial burden, for e.g., soil, terrestrial plant surfaces, and marine plant and animal surfaces. Surviving in such competitive environments likely requires strategies such as the production of bioactive secondary metabolites (Slattery et al. 2001; Wiener 1996). It is now apparent that potentially interesting gene clusters, while possibly expressing metabolites that increase competitiveness in natural environments, can remain silent in the unnatural setting of the microbiology laboratory. Varying culture conditions is one method of turning on these pathways.

Another strategy to mimic more natural microbial environments is to grow them in the presence of other microbes. Such mixed fermentations have been used for centuries in food and beverage production and for decades in industrial enzyme and solvent production and wastewater treatment, to name a few applications (Salmon and Bull 1984). Some results of this approach include decreased costs, increased efficiency, and diversified products.

Application of mixed fermentation to natural product drug discovery seems an obvious extension, but the field is in its infancy, probably because of early fears of lack of reproducibility. Results of mixed fermentation include increased antibiotic activity in crude extracts, increased yields of previously described metabolites, increased yields of previously undetected metabolites, analogues of known metabolites resulting from combined pathways, and induc-

tion of previously unexpressed pathways for bioactive constituents.

Mixed fermentation as a strategy for natural product drug discovery

In an early report of mixed fungal fermentation, *Gloeophyllum abietinum* increased its synthesis of six toxins, including oosponol and oospoglycol, when grown in liquid culture with *Heterobasidion annosum* (Sonnenbichler et al. 1994; Table 1). Growth retardation of *G. abietinum* was noted concomitant with enhanced toxin synthesis. Stimulation of toxin synthesis also occurred with cell-free extracts of *H. annosum*, but was not induced by *H. annosum* cell walls, nor by polypeptides or polysaccharides.

In another early report of mixed fungal fermentation, tremendous increases in *Monascus* pigment production and in *Monascus* growth were noted in solid medium when co-cultivated with either *Saccharomyces cerevisiae* or *Aspergillus oryzae* (Shin et al. 1998; Table 1). For these experiments, inducer organisms were added 1 day after *Monascus* was inoculated. Increased pigment production was also noted in liquid culture, but was much less than that on solid medium. No increased pigment production or cell growth occurred upon cultivation of *Monascus* with the bacterium *Bacillus cereus*. Culture filtrates of *S. cerevisiae* were also effective in inducing pigment production in *Monascus*, suggesting that enhanced pigment production was not due to cell-to-cell contact or limitation of components due to consumption by *S. cerevisiae*. Further experiments revealed that hydrolytic enzymes (e.g., chitinase) from a variety of fungi were effective in enhancing *Monascus* pigment production, while those from bacteria were not. The authors suggest that hydrolysis of *Monascus* cell walls stimulates a defense mechanism resulting in overproduction of hydrophobic substances such as pigments, in order to block enzyme attack. Co-cultured *Monascus* exhibited increased numbers and sizes of vacuoles filled with electron dense substances, likely pigments (Suh and Shin 2000a). Increased cell mass and pigment production did not occur in the presence of protein kinase C inhibitors, and protein kinase C activity was only detected in mixed cultures (Suh and Shin 2000b).

Unidentified, surface-associated marine bacteria increased their synthesis of antibiotics when cultured with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Escherichia coli* (Mearns-Spragg et al. 1998). Five-day-old cultures of the terrestrial inducer strains were placed inside semi-permeable dialysis tubing and added to liquid cultures of the marine bacteria. Relative amounts of antibiotics in culture supernatants were determined after

Table 1 Mixed fermentation experiments where secondary metabolites were identified

Microbes co-cultured (B = bacterium, F = fungus)	Secondary metabolites (K = known, N = new)	Microbe responsible for synthesis	Concentration of metabolite in pure culture	Concentration of metabolite after mixed fermentation	Reference
<i>Candida tropicalis</i> (F), <i>Actinomyces levoris</i> (B)	Levorin (K)	<i>Actinomyces levoris</i>		Increased 25–60%	Yakovleva and Sokolova (1978)
<i>Gloeophyllum abietinum</i> (F), <i>Heterobasidion annosum</i> (F)	Oosponol, oospoglycol, fomannoxin, fomannoxinalcohol, fomannosin, melledonal, melledonal C, melleolide D (all K)	<i>Gloeophyllum abietinum</i>	Oosponol ~0.5 mg/L Oospoglycol ~1 mg/L	Oosponol ~46 mg/L Oospoglycol ~14 mg/L	Sonnenbichler et al. (1994)
<i>Monascus</i> sp. (F), <i>Saccharomyces cerevisiae</i> (F) or <i>Monascus</i> sp. (F), <i>Aspergillus oryzae</i> (F) or <i>Monascus</i> sp. (F), <i>Bacillus cereus</i> (B)	Pigments (K)	<i>Monascus</i> sp.		Increased 30– 40 fold Increased 30– 40 fold No increase	Shin et al. (1998)
<i>Pestalotia</i> sp. (F), unidentified marine bacterium	Pestalone (N)	<i>Pestalotia</i> sp.	Not detected	3 mg/L	Cueto et al. (2001)
<i>Streptomyces tenjimariensis</i> (B), unidentified marine bacteria	Istamycins A, B (K)	<i>Streptomyces tenjimariensis</i>	15 mg/L	~30 mg/L	Slattery et al. (2001)
<i>Acremonium</i> sp. (F), <i>Mycogone rosea</i> (F)	Acremostatins A–C (analogues of K)	<i>Acremonium sp.</i>	Not detected	Not reported	Degenkolb et al. (2002)
<i>Rhodococcus fascians</i> (B), <i>Streptomyces padanus</i> (B)	Rhodostreptomycins A, B (N)	<i>Rhodococcus fascians</i>	Not detected	A 2.9 mg/L B 1.5 mg/L	Kurosawa et al. (2008)
<i>Libertella</i> sp. (F), unidentified marine bacterium	Libertellenones A–D (N)	Presumed to be <i>Libertella sp.</i>	Not detected	A 0.5 mg/L B 0.63 mg/L C 158 µg/L D 93 µg/L	Oh et al. (2005)
Marine endophyte (F), marine endophyte (F)	Marinamides A, B (N)	Not reported	Not detected	A 0.33 mg/L B 0.66 mg/L	Zhu and Lin (2006)
Marine endophyte (F), marine endophyte (F)	6-Methylsalicylic acid (K), cyclo-(phe-phe) dipeptide (K)	Not reported	Not detected	A 20 mg/L B 0.83 mg/L	Zhu et al. (2007)
<i>Emericella</i> sp. (F), <i>Salinospora arenicola</i> (B)	Emericellamides A, B (N)	<i>Emericella</i> sp.	A 25 µg/L B 7.6 µg/L	A 2.5 mg/L B 0.76 mg/L	Oh et al. (2007)

24 h. Heat-killed cells of *S. aureus* were also capable of inducing antibiotic production in certain marine strains. Using an even larger collection of surface-associated marine bacteria, these researchers later demonstrated that an unexpectedly large percentage of their collection (35%) synthesized antibiotics in the presence of *P. aeruginosa*, *E. coli*, *Bacillus subtilis*, other surface-associated marine

bacteria, or cell-free culture supernatants (Burgess et al. 1999). Further confirmation that bacterial competition is potentially very common in marine environments was provided by Long and Azam (2001). Eighty-six marine bacteria were examined for their inhibition of 85 other isolates by agar diffusion assays. More than half of the isolates produced inhibitory compounds. Significantly more

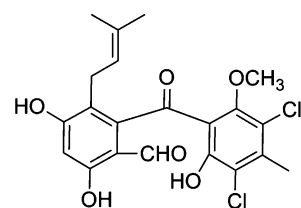
of the attached bacteria than the free-living bacteria produced inhibitory compounds. The attached bacteria also inhibited more isolates than the free-living bacteria. Surface-associated bacteria live in a highly competitive environment. Synthesis of bioactive secondary metabolites may be common in this environment, and these in vitro studies support this idea. Surface-associated marine microbes may be a valuable source of biologically active natural products.

When 76 species of *Streptomyces* were plated in pairs 1 cm apart, 34% of the strains stimulated antibiotic production in 13% of the strains (Ueda et al. 2000). Synthetic A factor, γ -lactone, and butanolides, which are known to stimulate antibiotic production in *Streptomyces*, did not stimulate antibiotic production in these strains.

Fifty-three species of surface-associated marine bacteria were cultured in liquid media with the marine bacterium *Streptomyces tenjimariensis*, a known producer of the antibiotics istamycin A and B (Slattery et al. 2001). The inducing strains were selected based on growth rates similar to *S. tenjimariensis*. Co-culture with 22.6% of the marine isolates resulted in an approximate doubling of the istamycin levels found in *S. tenjimariensis* monocultures (Table 1). The inducing strains included Gram-positive, Gram-negative, and Gram-variable isolates. Pre-establishment of *S. tenjimariensis* (24 h) was critical to enhanced antibiotic synthesis. Co-inoculation or pre-establishment of the inducing bacteria resulted in a significant reduction in istamycin levels compared to monocultures.

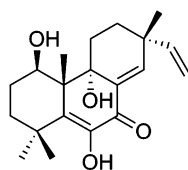
New lipoaminopeptides thought to be the result of biosynthetic pathways from two different fungi were produced in solid co-culture. The fungal parasite *Mycogone rosea* was cultured with the endophytic fungus *Acremonium* sp., and acremostatins A, B, and C were detected (Degenkolb et al. 2002; Table 1). *Acremonium* sp. was established for 7 days prior to inoculation with a 14-day culture of *M. rosea*, and then co-cultured for 14 days. The authors postulate that *M. rosea* contributes 2-methyl-decanoic acid to the terminus of the *Acremonium* products leucinostatins A, B, and K, resulting in acremostatins A, B, and C. The new derivatives were not detected in monocultures of either fungus. Thus, chimeric pathways resulting from co-culture can also lead to new natural products.

New aminoglycoside antibiotics that may be the result of combined biosynthetic pathways were produced by *Rhodococcus fascians* when cultured with *Streptomyces padanus* (Kurosawa et al. 2008; Table 1). Rhodostreptomycins A and B were not detected in pure cultures of either bacterium, and both compounds had broad-spectrum antibacterial activity. There was a correlation between production of rhodostreptomycins and the presence of *S. padanus* DNA in *R. fascians*. The transferred DNA may directly induce aminoglycoside production, or it may contain genes

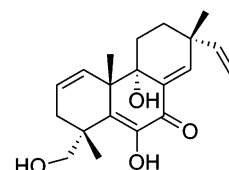


Pestalone

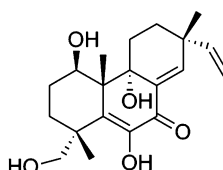
Cueto et al. (2001)



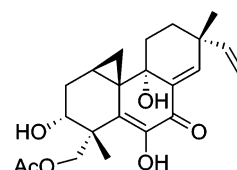
Libertellenone A



Libertellenone B

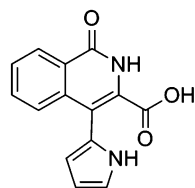


Libertellenone C

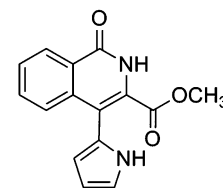


Libertellenone D

Oh et al. (2005)

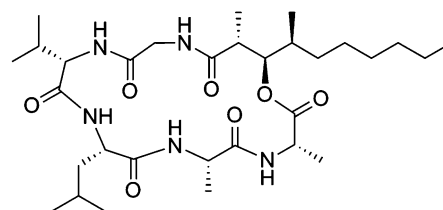


Marinamide

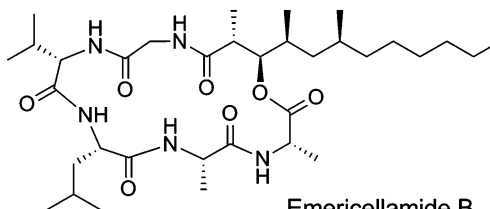


Marinamide methyl ester

Zhu and Lin (2006)



Emericellamide A



Emericellamide B

Oh et al. (2007)

Fig. 1 Structures of new biologically active constituents from mixed fermentations

encoding enzymes for aminoglycoside synthesis. Alternatively, combined pathways from both microbes' genomes could result in rhodostreptomycin synthesis. Hopefully, details of the molecular biology of the transferred DNA are forthcoming.

In 2001, Cueto et al. reported that a surface-associated marine *Pestalotia* synthesized a new antibiotic only when cultured with an unidentified Gram-negative marine bacterium (Table 1). Pestalone (Fig. 1), a new benzophenone antibiotic, was undetectable in pure cultures of the fungus *Pestalotia*. Pestalone had potent activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* and marginal activity against the National Cancer Institute's 60 human tumor cell line screen. In these experiments, the bacterium was added 24 h after *Pestalotia* inoculation, and cultures extracted 6 days later. Neither organic extracts nor cell-free culture supernatants of the marine bacterium induced pestalone synthesis. Interestingly, low yields of pestalone could be induced by ethanol in pure cultures of *Pestalotia*.

A second example of a previously unexpressed pathway induced by microbial competition was provided by Fenical's group (Oh et al. 2005). Four new diterpenoids, libertellenones A–D (Table 1, Fig. 1), were detected when the marine fungus *Libertella* sp. was cultured with an unidentified marine bacterium (the same bacterium used to induce pestalone synthesis). *Libertella* was established in liquid culture for 3 days prior to adding the marine bacterium, and co-culture extractions performed 2 days later. No new metabolites were detected in 49 other marine fungal strains co-cultured with the bacterium. The libertellenones were not detected in pure cultures of the fungus or the bacterium. Heat-killed bacterial cells, cell-free culture supernatants, or ethyl acetate extracts of a viable bacterial culture did not induce diterpenoid synthesis. The authors suggest that diterpenoid induction is controlled by cell–cell interactions and not signaling molecules. Indirect evidence is provided that the libertellenones are *Libertella* products. The diterpenoids had no obvious antibacterial activity (including no activity against the inducing marine bacterium), but had potent activity against a human colon carcinoma cell line.

Further demonstration that mixed fermentation represents a potentially valuable strategy for discovery of novel metabolites was the report of marinamide A and B production in co-culture of two marine fungi (Zhu and Lin 2006; Table 1, Fig. 1). The two unidentified, plant-associated fungi were inoculated simultaneously into liquid media and cultured for 12 days. Marinamide A, a novel 1-isoquinolone alkaloid, and its methyl ester, marinamide B, were not obtained in pure culture of either fungus. Both compounds had activity against *E. coli*, *Pseudomonas pyocyanea*, and *S. aureus*. Additional,

previously undetected metabolites have since been found in co-cultures of these two marine fungi (Zhu et al. 2007; Table 1). The diketopiperazine cyclo-(phe-phe) and 6-methyl-salicylic acid inhibited growth of the plant pest *Heliothis armigera* and the parasitic copepod *Sinergasilus* sp. The metabolites were not obtained in pure culture of either fungus.

Examples of bioactive microbial constituents that were unknown until their production was enhanced 100-fold in mixed fermentation are the cyclic depsipeptides emericellamides A and B (Oh et al. 2007; Table 1, Fig. 1). This report provides another example of a cross-kingdom mixed fermentation. A surface-associated marine fungus, *Emericella* sp., was grown in liquid culture for 3 days prior to inoculation of the marine actinomycete *Salinispora arenicola* and incubated for two more days. Emericellamides A and B had moderate activity against methicillin-resistant *S. aureus*. Once the depsipeptides were isolated from the mixed cultures, the researchers found that the new compounds were indeed produced by *Emericella* sp. in pure culture, but in amounts undetectable by normal LC–MS analysis.

Conclusions and prospects for the future

To date, at least nine new compounds with biological activity have been identified by mixed fermentation, pestalone, libertellenones A–D, marinamides A and B, and emericellamides A and B (Fig. 1). How did these natural products remain undetected for so long? Are the genes not expressed under standard growth conditions? Are external signals required to turn the genes on? Is it simply that chemical detection methods are not powerful enough? Certainly not in every situation, as Fenical's group was able to detect trace quantities of the emericellamides in pure culture (Oh et al. 2007), but not the libertellenones (Oh et al. 2005). In any case, the emericellamides would not have been isolated without the structure determination that resulted from increased yields in mixed culture.

Another encouraging result from early natural product-directed mixed fermentation studies was the isolation of new lipoaminopeptide analogues, where the core structures were provided by *Acremonium*, and the nitrogen termini by *M. rosea* (Degenkolb et al. 2002). While biological activities were not reported, the parent leucinostatin inhibit bacteria and fungi. Thus, co-cultivation of microbes producing related products is another way to increase the chemical diversity of microbial secondary metabolites (Degenkolb et al. 2002).

Competition for limiting natural resources is believed to be the selective force that promotes biosynthesis of biologically active compounds. It is intriguing that in the

majority of reports summarized here, microbes from highly competitive environments, e.g., plant and animal surfaces, were responsible for enhanced or previously undescribed syntheses of bioactive molecules. Other fascinating questions for researchers entering this field to consider include: What is the optimum timing of inoculation of the cultivation partners and of extraction? What, exactly, is responsible for inducing synthesis of the bioactive constituent? Inducing factors varied dramatically in the reports summarized here, from chitinase (Shin et al. 1998) to viable cells (Oh et al. 2005).

Microbes have already played a pivotal role in human medicine. More thorough exploitation of the metabolic potential of microbes via mixed fermentation is proving to be an effective way to augment natural product libraries. With countless possible microbe combinations and increasingly sophisticated chemical isolation and structure determination methods, the potential for mixed fermentation in natural product drug discovery seems quite promising.

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