reviews

molecular pharmaceutics

Bacterial Hosts for Natural Product Production

Haoran Zhang,[†] Yong Wang,[†] and Blaine A. Pfeifer*

Department of Chemical and Biological Engineering, Tufts University, Medford, Massachusetts 02155

Received October 1, 2007; Revised Manuscript Received December 12, 2007; Accepted December 20, 2007

Abstract: Four bacterial hosts are reviewed in the context of either native or heterologous natural product production. *E. coli*, *B. subtilis*, pseudomonads, and *Streptomyces* bacterial systems are presented with each having either a long-standing or more recent application to the production of therapeutic natural compounds. The four natural product classes focused upon include the polyketides, nonribosomal peptides, terpenoids, and flavonoids. From the perspective of both innate and heterologous production potential, each bacterial host is evaluated according to biological properties that would either hinder or facilitate natural product biosynthesis.

Keywords: Natural products; bacterial hosts; *E. coli*; pseudomonads; *Streptomyces*; *B. subtilis*

1. Introduction

This article will focus on natural products produced from several bacterial systems. In line with the theme of the issue, the natural product classes to be discussed include the polyketides, nonribosomal peptides, terpenoids, and flavonoids—natural product classifications that possess an impressive range of medicinal activities and have been ardently pursued for large-scale production purposes.[1] The bacterial hosts to be profiled have been chosen either because of their innate ability to produce natural products or for their potential as heterologous natural product production hosts.

Driving production efforts are the medicinal properties of natural products.[1–3] However, the production process is complicated by the unique and complex structure of the small molecule natural compounds. Though elegant and impressive strides have been made in synthetic efforts to generate such molecular architecture, a strictly chemical approach will generally not allow an economical production route to the large-scale manufacture of complex natural products. Instead, production has often been left to the original host organisms (bacteria, plants, or fungi) responsible for natural compounds. Further complicating production efforts are intrinsic difficulties in working with native plants or microbes that often fail to grow well outside their native environments and offer little opportunity for genetic manipulation to improve production. This backdrop led to the idea of using heterologous microbes, possessing improved growth properties and molecular biology protocols, to serve as surrogate hosts for complex, medicinal natural products.

Further supporting heterologous production efforts have been relatively recent advances in sequencing information and host-specific molecular biology tools. Numerous natural product clusters have now been sequenced, and such information serves as a starting point for designing gene transfer between host systems. The leap in number of sequenced bacterial genomes has also aided heterologous natural product production. For example, the four primary hosts to be profiled in this article have now been sequenced and such information then provides the genetic blueprint for introducing a new heterologous pathway. In addition, advances in plasmid and promoter options, transcription and translation information, and other general tools to facilitate recombinant gene expression have eased the transition from foreign gene to functional heterologous protein/natural product.

* To whom correspondence should be addressed. Mailing address: 4 Colby Street, Department of Chemical and Biological Engineering, Medford, MA 02155. Tel: 617-627-2582. Fax: 617-627-3991. E-mail: blaine.pfeifer@tufts.edu.

[†] These authors contributed equally to this work.

(1) Baker, D. D.; Chu, M.; Oza, U.; Rajgarhia, V. The value of natural products to future pharmaceutical discovery. *Nat. Prod. Rep.* **2007**, *24*, 1225–1244.

(2) Newman, D. J.; Cragg, G. M.; Snader, K. M. Natural products as sources of new drugs over the period 1981–2002. *J. Nat. Prod.* **2003**, *66*, 1022–1037.

(3) Cragg, G. M.; Newman, D. J.; Snader, K. M. Natural products in drug discovery and development. *J. Nat. Prod.* **1997**, *60*, 52–60.

10.1021/mp7001329 CCC: $40.75
Published on Web 01/31/2008

Table 1. Examples of Native Natural Products Produced in *Streptomyces*

type		compound	host	ref
polyketides	macrolides	erythromycin A	*S. erythraea*	4
		tacrolimus (FK506)	*S. tsukubaensis*	8
		picromycin	*S. venezuelae*	5
	polyene antibiotics	amphotericin B	*S. nodosus*	9
	tetracyclines	tetracycline	*S. aureofaciens*	10
NRPs		vancomycin	*S. orientalis*	6
		bleomycin	*S. verticillus*	11
terpenoids		terpentecin	*S. griseolosporeus*	7
		phenalinolactones	*S.* sp. Tü6071	12

The bacteria to be profiled have either a long-standing or a recently emerging connection to natural product production. Highlighted with each will be the biological characteristics that make them well-suited as either native or heterologous natural product hosts. Following the description of the different bacterial hosts will be a prospective on the future of bacterial-mediated natural product biosynthesis and new directions the field may take.

2. *Streptomyces* and Related Actinomycetes

The actinomycetes are Gram-positive bacteria with a high G+C nucleotide content. A wide range of important secondary metabolites, including antibiotics, herbicides, and growth-promoting substances, are produced by several members of the actinomycetes. *Streptomyces* is the largest antibiotic producing genus among the actinomycetes; more than 60% of the nearly 6000 antibiotics of microbial origin are produced by *Streptomyces* spp. including both antibacterials and antifungals and a wide range of other bioactive compounds such as immunosuppressants and anticancer agents. Specific examples include representative polyketide compounds such as erythromycin from *Saccharopolyspora erythraea*[4] and picromycin from *Streptomyces venezuelae*,[5] nonribosomal peptides such as vancomycin from *Streptomyces orientalis*,[6] and terpenoids such as terpentecin from *Streptomyces griseolosporeus*.[7] Further examples of natively produced *Streptomyces* natural compounds are provided in Table 1.

The *Streptomyces* or relative actinomycetes have also been harnessed as heterologous hosts for the production of various natural products. Several intrinsic characteristics make *Streptomyces* a good candidate for this objective. First, as noted, the genus has traditionally exhibited the ability to produce a wide variety of secondary metabolites. As an example, approximately two-thirds of known bioactive polyketides are produced by the actinomycetes. Therefore, such a capability implies that the bacterial family has the metabolism needed to produce the biosynthetic precursors required for polyketide biosynthesis and the auxiliary enzymes needed to post-translationally modify biosynthetic enzymes, tailor nascent polyketide compounds (to produce the final active natural product), and provide protection through resistance mechanisms available to insulate the bacteria from their own bioactive compounds. Second, several *Streptomyces* species, such as *S. coelicolor*,[13] *Saccharopolyspora erythraea*,[14] and

(4) McGuire, J. M.; Bunch, R. L.; Anderson, R. C.; Boaz, H. E.; Flynn, E. H.; Powell, H. M.; Smith, J. W. "Ilotycin", a new antibiotic. *Antibiot. Chemother.* **1952**, *2*, 281–283.

(5) DeVoe, S. E.; Renfroe, H. B.; Hausmann, W. K. Production of picromycin by cultures deposited as methymycin producers. *Antimicrobial. Agents. Chemother. (Bethesda)* **1963**, *161*, 125–129.

(6) Nishimura, H.; Okamoto, S.; Nakajima, K.; Shimohira, M.; Shimaoka, N. Vancomycin, a new antibiotic substance. I. Physical and chemical properties, and antibacterial studies in vitro and in vivo. *Ann. Rep. Shionogi Res. Lab.* **1957**, *7*, 165–171.

(7) Isshiki, K.; Tamamura, T.; Takahashi, Y.; Sawa, T.; Naganawa, H.; Takeuchi, T.; Umezawa, H. The structure of a new antibiotic, terpentecin. *J. Antibiot.* **1985**, *38*, 1819–1821.

(8) Tanaka, H.; Kuroda, A.; Marusawa, H.; Hashimoto, M.; Hatanaka, H.; Kino, T.; Goto, T.; Okuhara, M. Physicochemical properties of FK - 506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*. *Transplant. Proc.* **1987**, *19* (5 Suppl. 6), 11–16.

(9) Linke, H. A.; Mechlinski, W.; Schaffner, C. P. Production of amphotericin B −14C by *Streptomyces nodosus* fermentation, and preparation of the amphotericin B-14C-methyl-ester. *J. Antibiot.* **1974**, *27*, 155–160.

(10) Jarai, M.; Jozsa, G.; Kollar, J. Biochemical studies on *Streptomyces aureofaciens* IV. Studies on the biosynthesis of chlortetracycline. *Acta. Microbiol. Acad. Sci. Hung.* **19641965**, *11*, 203–210.

(11) Umezawa, H.; Maeda, K.; Okami, Y.; Takeuchi, T. Bleomycin, a novel antibiotic substance. JP 40008117 19650424 Showa1965, 13 pp.

(12) Durr, C.; Schnell, H.; Luzhetskyy, A.; Murillo, R.; Weber, M.; Welzel, K.; Vente, A.; Bechthold, A. Biosynthesis of the terpene phenalinolactone in Streptomyces sp. Tu6071: analysis of the gene cluster and generation of derivatives. *Chem. Biol.* **2006**, *13*, 365–377.

(13) Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A.-M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C.-H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M.-A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A. Complete genome sequence of the model actinomycete *Streptomyces coelicolor*. *Nature* **2002**, *417*, 141–147. A3 (2).

Table 2. Examples of Heterologous Natural Products Produced in *Streptomyces*

type	compounds	host	ref
type II PKS	medermycin	*S. coelicolor* CH999	19
	griseorhodin A	*S. lividans* ZX1	20
	PD 116740	*S. lividans* TK24	21
	aloesaponarinII	*S. lividans* K4–114	22
		S. parvulus	24
	kinamycin	*S. lividans* ZX7	23
iterative bacterial type I PKS	orsellinic acid	*S. lividans* TK24 and *S. coelicolor* CH999	25
	2-hydroxy-5-methyl-1-naphthoic acid	*S. lividans* TK24 and *S. coelicolor* YU105	26
	dechloroneocarzilin A	*S. coelicolor* CH999	27
	aureothin	*S. lividans* ZX1	28
NRPs	daptomycin	*S. lividans* TK23 and TK64	29
	epothilone	*S. coelicolor* CH999	30
modular type I PKS	soraphen A	*S. lividans* ZX7	31
	tylosin	*S. venezuelae*	32
	landomycin A	*S. fradiae* Tü2717	33

S. avermitilis,[15,16] have been sequenced and characterized biologically with protocols developed for the recombinant manipulation of such hosts. These features, together with a nonpathogenic nature and established fermentation technology, have made *Streptomyces* hosts an obvious choice for producing natural products, especially for the polyketide natural product family.

However, when compared to the other host systems presented in this article, the actinomycetes and related strains show a slower and more complex growth profile and are more difficult to manipulate genetically. For example, transforming DNA has traditionally involved PEG-assisted protoplast DNA transfer, which is less efficient than, for example, the simple transformation procedures developed for *Escherichia coli*.[17,18] Moreover, the distinct primary and secondary growth characteristics of actimomycetes can complicate large-scale production efforts because of extended batch runs, expensive and complex media formulations, and the danger of competing microbial contamination.

Table 2 presents numerous examples of polyketide and nonribosomal peptide natural products produced through *Streptomyces* heterologous hosts. *S. coelicolor* emerged as one of the earliest heterologous host systems and, with an expanded knowledge base and the native characteristics outlined above, serves as an ideal host for the heterologous production of many polyketide compounds. However, though capable of supporting natural compounds from a variety of starting precursors, there is a potential "compound bias" associated with *S. coelicolor* (and other heterologous hosts) due to the native production of its own polyketide compounds. For example, *S. coelicolor* naturally produces actinorhodin, a polyketide compound derived from intracellular acetyl- and malonyl-oA groups. Thus, the organism has evolved to readily supply these precursors, but in the case of heterologous compounds that require other precursors, *S. coelicolor* may nonoptimally support biosynthesis. As an example of addressing such a limitation, Lombo et al. introduced a heterologous precursor pathway able to boost additional biosynthetic precursors.[34] The genes *matB* and *matC* from *Rhizobium trifolii*, which encode a malonyl-CoA synthetase and a putative dicarboxylate transport protein, respectively, were coexpressed in *S. coelicolor*. These proteins can directly convert exogenous malonate and methylmalonate into their corresponding CoA thioesters. When the heterologous deoxyerythronolide B pathway, which relies on methylmolonyl-CoA units, was then introduced, the resulting polyketide product showed a 300% improvement in titer.

Additional biosynthetic pathways from related or unrelated microbes have also been heterologously expressed in *S. coelicolor* or other actinomycetes (Table 2). The biosynthetic gene clusters were generally cloned into shuttle cosmids or bacterial artificial chromosome (BAC) plasmids which can be conveniently manipulated in *E. coli* and transferred to a

(14) Oliynyk, M.; Samborskyy, M.; Lester, J. B.; Mironenko, T.; Scott, N.; Dickens, S.; Haydock, S. F.; Leadlay, P. F. Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338. *Nat. Biotechnol.* **2007**, *25*, 447–453.

(15) Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* **2003**, *21*, 526–531.

(16) Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazawa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12215–12220.

(17) Schaffner, W. Direct transfer of cloned genes from bacteria to mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2163–2167.

(18) Hopwood, D.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. In *Genetic manipulation of Streptomyces: A laboratory manual*; The John Innes Foundation: Norwich, U.K., 1985; 356 pp.

Streptomyces host (i.e., *S. lividans*, *S. coelicolor*, etc.). Due to the cloning capacity of a cosmid vector, type II PKS systems that have a small size could be transferred within a single cosmid.[19–24,35] However, a BAC plasmid is capable of accommodating much larger DNA inserts with a typical insert size of 150 kb but with a range from 100 to 300 kb. This characteristic makes the BAC plasmid a good candidate for the transfer of the biosynthesis pathways from the type I PKS systems. Recently, a 128 kb gene cluster from *Streptomyces roseosporus*, responsible for the biosynthesis of daptomycin, was produced in *S. lividans* by heterologous expression; the *S. roseosporus* DNA containing the daptomycin gene cluster was inserted site-specifically in the $\varphi\mu$C31 *attB* site within a BAC shuttle plasmid.[36]

The use of cosmids and BAC vectors to transfer entire gene clusters to *Streptomyces* host systems may also be influenced by regulatory constraints within either the gene cluster or the new host. As an example, the expression of a cosmid containing the biosynthetic genes necessary to produce landomycin A has failed in the past, although different *Streptomyces* strains have been used as hosts (*S. lividans* TK24, *S. coelicolor* CH999, *S. fradiae* Tü2717).[37] It has since been shown that the production of landomycin E in *S. globisporus* 1912 is controlled by the regulatory gene *lndI* located at one end of the biosynthetic gene cluster.[38] The coexpression of a cosmid containing the landomycin A biosynthetic genes from *S. cyanogenus* with *lndI* in a PKS mutant of *S. fradiae* Tü2717 resulted in the production of landomycin A.[33] In this example, a regulatory gene that functioned between different *Streptomyces* hosts was responsible for final natural product production. This is most likely due to similarities between the native and heterologous host systems and common primary and secondary metabolism regulatory elements affecting gene expression and natural product formation. Such dependencies are less likely in hosts that vary from *Streptomyces* or for gene clusters that are re-engineered for inducible expression, situations more common in the remaining bacterial hosts to be profiled.

In 1996, Seto et al. reported that both the mevalonate and nonmevalonate pathways were functional in certain *Streptomyces* strains, thus, providing the precursors (IPP and DMAPP) needed for terpenoid biosynthesis.[39] The gene cluster responsible for the mevalonate pathway from *S. griseolosporeus* strain MF730-N6, which can produce the terpenoid-antibiotic terpentecin, was expressed in the heterologous host *S. lividans*, which naturally possesses only the nonmevalonate pathway for the formation of IPP.[40] However, results showed no activity for heterologous HMG-CoA synthase. In another effort, the same group tried to produce (*R*)-mevalonate in *S. lividans* TK23 by introducing the *aas*, *hmgs*, and *hmgr* genes from the *Streptomyces* sp. strain CL190. After 8 days culture, the final concentration

(19) Ichinose, K.; Ozawa, M.; Itou, K.; Kunieda, K.; Ebizuka, Y. Cloning, sequencing and heterologous expression of the medermycin biosynthetic gene cluster of Streptomyces sp. AM-7161: towards comparative analysis of the benzoisochromanequinone gene clusters. *Microbiology* **2003**, *149*, 1633–1645.

(20) Li, A.; Piel, J. A gene cluster from a marine Streptomyces encoding the biosynthesis of the aromatic spiroketal polyketide griseorhodin A. *Chem. Biol.* **2002**, *9*, 1017–1026.

(21) Hong, S. T.; Carney, J. R.; Gould, S. J. Cloning and heterologous expression of the entire gene clusters for PD 116740 from *Streptomyces* strain WP 4669 and tetrangulol and tetrangomycin from *Streptomyces rimosus* NRRL 3016. *J. Bacteriol.* **1997**, *179*, 470–476.

(22) Kalaitzis, J. A.; Moore, B. S. Heterologous biosynthesis of truncated hexaketides derived from the actinorhodin polyketide synthase. *J. Nat. Prod.* **2004**, *67*, 1419–1422.

(23) Gould, S. J.; Hong, S. T.; Carney, J. R. Cloning and heterologous expression of genes from the kinamycin biosynthetic pathway of *Streptomyces murayamaensis*. *J. Antibiot.* **1998**, *51*, 50–57.

(24) Kim, E. S.; Cramer, K. D.; Shreve, A. L.; Sherman, D. H. Heterologous Expression of an Engineered Biosynthetic Pathway: Functional Dissection of Type II Polyketide Synthase Components in *Streptomyces* Species. *J. Bacteriol.* **1995**, *177*, 1202–1207.

(25) Gaisser, S.; Trefzer, A.; Stockert, S.; Kirschning, A.; Bechthold, A. Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tu57. *J. Bacteriol.* **1997**, *179*, 6271–6278.

(26) Sthapit, B.; Oh, T. J.; Lamichhane, R.; Liou, K.; Lee, H. C.; Kim, C. G.; Sohng, J. K. Neocarzinostatin naphthoate synthase: an unique iterative type I PKS from neocarzinostatin producer *Streptomyces carzinostaticus*. *FEBS Lett.* **2004**, *566*, 201–206.

(27) Otsuka, M.; Ichinose, K.; Fujii, I.; Ebizuka, Y. Cloning, sequencing, and functional analysis of an iterative type I polyketide synthase gene cluster for biosynthesis of the antitumor chlorinated polyenone neocarzilin in "Streptomyces carzinostaticus". *Antimicrob. Agents Chemother.* **2004**, *48*, 3468–3476.

(28) He, J.; Hertweck, C. Iteration as programmed event during polyketide assembly; molecular analysis of the aureothin biosynthesis gene cluster. *Chem. Biol.* **2003**, *10*, 1225–1232.

(29) Penn, J.; Li, X.; Whiting, A.; Latif, M.; Gibson, T.; Silva, C. J.; Brian, P.; Davies, J.; Miao, V.; Wrigley, S. K; Baltz, R. H. Heterologous production of daptomycin in *Streptomyces lividans*. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 121–128.

(30) Tang, L.; Shah, S.; Chung, L.; Carney, J.; Katz, L.; Khosla, C.; Julien, B. Cloning and heterologous expression of the epothilone gene cluster. *Science* **2000**, *287*, 640–642.

(31) Zirkle, R.; Ligon, J. M.; Molnár, I. Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. *Microbiology* **2004**, *150*, 2761–2774.

(32) Jung, W. S.; Lee, S. K.; Hong, J. S.; Park, S. R.; Jeong, S. J.; Han, A. R.; Sohng, J. K.; Kim, B. G.; Choi, C. Y.; Sherman, D. H.; Yoon, Y. j. Heterologous expression of tylosin polyketide synthase and production of a hybrid bioactive macrolide in *Streptomyces venezuelae*. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 763–769.

(33) von Mulert, U.; Luzhetskyy, A.; Hofmann, C.; Mayer, A.; Bechthold, A. Expression of the landomycin biosynthetic gene cluster in a PKS mutant of *Streptomyces fradiae* is dependent on the coexpression of a putative transcriptional activator gene. *FEMS Microbiol. Lett.* **2004**, *230*, 91–97.

(34) Lombo, F.; Pfeifer, B.; Leaf, T.; Ou, S.; Kim, Y. S.; Cane, D. E.; Licari, P.; Khosla, C. Enhancing the atom economy of polyketide biosynthetic processes through metabolic engineering. *Biotechnol. Prog.* **2001**, *17*, 612–617.

(35) Kim, C. Y.; Park, H. J.; Kim, E. S. Heterologous expression of hybrid type II polyketide synthase system in *Streptomyces* species. *J. Microbiol. Biotechnol.* **2003**, *13*, 819–822.

of mevalonate was 0.5 g/L, including an additional copy of the *aas* gene led to a 13% yield increase.[41] Such efforts represent the few examples, especially when compared to polyketide production, attempted for terpenoid biosynthesis through *Streptomyces*; flavonoid biosynthesis remains relatively unexplored in *Streptomyces* hosts.

3. *E. coli*

Nonpathogenic, Gram-negative *E. coli* is a "generally regarded as safe" organism with a rapid growth rate and well-developed molecular biology protocols, ideal characteristics for a heterologous host. In fact, *E. coli* has long been used as a model microorganism for genetic and metabolic engineering. The organism's physiology and use in bioreactor schemes for biological product overproduction (primarily recombinant proteins) are also well documented. However, compared with some of the other hosts featured in this article, *E. coli* lacks the intracellular machinery (i.e., post-transcriptional modification, intracellular metabolism, or biosynthetic enzymes) needed to natively produce a wide range of natural products and is regarded as a minor native natural product producer due to the limited types and numbers of natural products it synthesizes. Nonetheless, *E. coli* does have the ability to produce certain natural products.

E. coli has been discovered to naturally produce a peptide-polyketide hybrid,[42] and it also produces natural products in the families of terpenoids and nonribosomal peptides. For example, *E. coli* is a native producer of isoprene, the smallest member of the terpenoid family; synthesis is achieved through the intermediate methylerythritol phosphate (MEP) pathway.[43,44] *E. coli* also produces NRPs, such as enterobactin, which is synthesized by a three-protein (EntE, B, F), six-module nonribosomal peptide synthetase.[45,46]

Native producers are certainly good candidates for natural product production; however, compared with many native producers, *E. coli* has instead been widely used for heterologous natural product production due to its rapid growth rate, easy and inexpensive large-scale cultivation, well-studied genetic makeup, and versatile genetic manipulation techniques. Being generally regarded as safe, it is suitable for the heterologous production of natural products whose native producers are pathogens. To engineer *E. coli* to produce heterologous natural products, foreign genes need to be introduced and expressed through either the *E. coli* chromosome or recombinant plasmids. However, genetic transfer is just the first step in heterologous natural product biosynthesis. In addition, heterologous protein folding, foreign codon usage, substrate availability, and heterologous gene expression must also be addressed.

i. Polyketides. The first reported heterologous polyketide produced through *E. coli* was 6-deoxyerythronolide B, 6dEB, the precursor of erythromycin, reported in 2001.[47] The effort required active expression of relevant biosynthetic genes and metabolic engineering to supply needed intracellular substrates. A phosphopantetheine transferase (*sfp*) gene from *Bacillus subtilis* was inserted into the *E. coli* chromosomal *prp* operon responsible for propionate catabolism, deleting the *prpRBCD* genes but leaving the *prpE* gene under an introduced and inducible T7 promoter. PrpE converts propionate into propionyl-CoA, one of the precursors needed for 6dEB. A second heterologous enzyme (composed of two subunits), a propionyl-CoA carboxylase (PCC) from *S. coelicolor*, was also introduced to convert propionyl-CoA to (2*S*)-methylmalonyl-CoA, the second precursor required for 6dEB formation. The PCC and the three deoxyerythronolide B synthase (DEBS) genes were introduced by selectable pET plasmids allowing the successful production of 6dEB in *E. coli* after first adjusting process temperature to

(36) Miao, V.; Coeffet-Legal, M. F.; Brian, P.; Brost, R.; Penn, J.; Whiting, A.; Martin, S.; Ford, R.; Parr, I.; Bouchard, M.; Silva, C. J.; Wrigley, S. K.; Baltz, R. H. Daptomycin biosynthesis in *Streptomyces roseosporus*: cloning and analysis of the gene cluster and revision of peptide stereochemistry. *Microbiology* **2005**, *151*, 1507–1523.

(37) Westrich, L.; Domann, S.; Faust, B.; Bedford, D.; Hopwood, D. A.; Bechthold, A. Cloning and characterization of a gene cluster from Streptomyces cyanogenus S136 probably involved in landomycin biosynthesis. *FEMS Microbiol. Lett.* **1999**, *170*, 381–387.

(38) Rebets, Y.; Ostash, B.; Luzhetskyy, A.; Hoffmeister, D.; Brana, A. F.; Méndez, C.; Salas, J. A.; Bechthold, A.; Fedorenko, V. Production of landomycins in *Streptomyces globisporus* 1912 and *S. cyanogenus* S136 is regulated by genes encoding putative transcriptional activators. *FEMS Microbiol. Lett.* **2003**, *222*, 149–153.

(39) Seto, H.; Watanabe, H.; Furihata, K. Simultaneous operation of the mevalonate and non-mevalonate pathways in the biosynthesis of isopentenyl diphosphate in *Streptomyces aeriouvifer*. *Tetrahedron Lett.* **1996**, *37*, 7979–7982.

(40) Hamano, Y.; Dairi, T.; Yamamoto, M.; Kawasaki, T.; Kaneda, K.; Kuzuyama, T.; Itoh, N.; Seto, H. Cloning of a gene cluster encoding enzymes responsible for the mevalonate pathway from a terpenoid-antibiotic-producing *Streptomyces* strain. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1627–1635.

(41) Kuzuyama, T.; Dairi, T.; Yamashita, H.; Shoji, Y.; Seto, H. Heterologous mevalonate production in *Streptomyces lividans* TK23. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 931–934.

(42) Nougayrede, J. P.; Homburg, S.; Taieb, F.; Boury, M.; Brzuszkiewicz, E.; Gottschalk, G.; Buchrieser, C.; Hacker, J.; Dobrindt, U.; Oswald, E. *Escherichia coli* induces DNA double-strand breaks in eukaryotic cells. *Science* **2006**, *313*, 848–851.

(43) Altincicek, B.; Kollas, A.; Eberl, M.; Wiesner, J.; Sanderbrand, S.; Hintz, M.; Beck, E.; Jomaa, H. LytB, a novel gene of the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. *FEBS Lett.* **2001**, *499*, 37–40.

(44) Rohmer, M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep.* **1999**, *16*, 565–574.

(45) Walsh, C. T.; Liu, J.; Rusnak, F.; Sakaitani, M. Molecular studies on enzymes in chorismate metabolism and the enterobactin biosynthetic pathway. *Chem. Rev.* **1990**, *90*, 1105–1129.

(46) Gehring, A. M.; Bradley, K. A.; Walsh, C. T. Reconstitution and Characterization of the *Escherichia coli* Enterobactin Synthetase from EntB, EntE, and EntF. *Biochemistry* **1998**, *37*, 2648–2659.

(47) Pfeifer, B. A.; Admiraal, S. J.; Gramajo, H.; Cane, D. E.; Khosla, C. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science* **2001**, *291*, 1790–1792.

improve the solubility and activity of the large, multidomain biosynthetic enzymes. The synthesis of erythromycin was achieved later by introducing a set of plasmids containing the remaining genes (that included the native erythromycin resistance gene, *ermE*) needed to convert 6dEB to erythromycin.[48]

ii. Hybrid Nonribosomal Peptide Synthetase/Polyketide Synthase Systems. Biosynthesis of epothilone C and D in *E. coli* has also recently been achieved by Mutka et al.[49] The entire 54-kb epothilone gene cluster from myxobacterium *Sorangium cellulosum* was cloned into three plasmids, which were subsequently introduced into *E. coli* to produce the hybrid polyketide/nonribosomal peptide products epothilone C and D.

Several important genetic manipulations were made to engineer epothilone production from *E. coli*. First, the chromosomal methylmalonyl-CoA decarboxylase gene (*ygfG*) was replaced by the *pcc* genes. To facilitate gene expression by the arabinose-inducible pBAD promoter-,the arabinose utilization genes in the *E. coli* chromosome were replaced by the arabinose transport gene mutant *lacY*, such that the *E. coli* strain was modified for consistent arabinose-inducible gene expression.

Besides engineering of the *E. coli* host, many strategies were also used to enhance heterologous gene expression in *E. coli*. To produce the sizable protein EpoD (765 KDa), the *epoD* gene was expressed as two smaller polypeptides, which contained compatible linker pairs to facilitate the interaction of the two polypeptides. To address the problem of low expression levels and limited solubility of expressed epothilone PKS genes in *E. coli*, low temperature expression, promoter engineering, codon optimization, and chaperone coexpression strategies were used.

In addition to the *de novo* production of epothilone C and D (which also required providing intracellular methylmalonyl-CoA through the action of the PCC), precursor-directed biosynthesis was also achieved by feeding the *N*-acetylcysteamine thioester (SNAC) of the normal EpoD substrate ((*E*)-2-methyl-3-(2-methylthiazol-4-yl)acrylic acid) to cells expressing the terminal seven epothilone modules encoded by *epoD*, *epoE*, and *epoF*. Notably, this precursor-directed biosynthesis strategy could be applied for the production of epothilone analogues by using analogues of the normal precursor fed to EpoD.

Another example of heterologous production of a PKS/NRPS hybrid is the reported synthesis of yersiniabactin (Ybt).[50] The Ybt synthetase genes carried by plasmid vectors were introduced into *E. coli* and expressed in a similar manner to the 6dEB case study mentioned above. Since most of the Ybt substrates (salicylate, malonyl-CoA, cysteine, *S*-adenosylmethionine) are native to *E. coli*, only salicylate was fed exogenously to allow Ybt production. An auxiliary enzyme YbtT, thought to act as a second thioesterase domain capable of "proof-reading" biosynthesis[51] was used, together with fed-batch high-cell density bioreactor schemes, to improve Ybt production levels.

To equip *E. coli* as a heterologous host, a main challenge is the lack of intracellular precursors. Therefore, the genetic manipulation of native *E. coli* metabolic pathways is essential for heterologous production and numerous efforts have been made to this end. Examples have been given for the production of 6-deoxyerythronolide and epothilone C and D.[47,49] In addition, the heterologous biosynthesis of ansamycin polyketide precursors in *E. coli* has been reported.[52] To engineer *E. coli* to produce 3-amino-5-hydroxybenzoic acid (AHBA), the starter unit for ansamycin polyketide biosynthesis, a native side reaction which converts AHBA to *N*-acetyl-AHBA needed to be inhibited. To achieve this, a putative *N*-arylamineacetyltransferase (NAT) in the *E. coli* chromosome was knocked out. Also, media devoid of glucose was used to grow *E. coli*, which also reduced the unwanted conversion of AHBA. Furthermore, the NAT inhibitor glycyrrhizic acid was used to further inhibit the side reaction. The manipulation of *E. coli* metabolic pathways involved in ansamycin polyketide precursor biosynthesis conducted at three levels (gene, protein, and growth medium modifications) demonstrated the availability and versatility of methods that can be applied to satisfy the need for precursors during heterologous production of natural products in *E. coli*. Such strategies have since become common during any heterologous production attempts, as illustrated by the examples above.

iii. NRP. Many nonribosomal peptides (NRPs) have also been produced in *E. coli*. With NRP compounds showing many similarities to polyketides, the heterologous production of NRP compounds also requires similar genetic manipulations of the *E. coli* cell. In one example, a single modular type nonribosomal peptide synthetase (NRPS), encoded by the *bpsA* gene from a D-cycloserine (DCS)-producing *Streptomyces lavendulae*, was transferred to *E. coli*. Natively, the *bpsA* gene product requires post-translational modification to catalyze the synthesis of a blue pigment, indigoidine. To achieve heterologous production of indigoidine, the post-translational modification 4′-phosphopantetheinyl transferase (PPTase) gene from *S. verticillus* was cloned and coexpressed together with *bpsA*.[53] The only substrate needed for biosynthesis is L-Gln, which was exogenously fed to the recom-

(48) Peiru, S.; Menzella, H. G.; Rodriguez, E.; Carney, J.; Gramajo, H. Production of the Potent Antibacterial Polyketide Erythromycin C *Escherichia coli*. *Appl. Environ. Microbiol.* **2005**, *71*, 2539–2547.

(49) Mutka, S.; Carney, J.; Liu, Y.; Kennedy, J. Heterologous Production of Epothilone C and D in *Escherichia coli*. *Biochemistry* **2006**, *45*, 1321–1330.

(50) Pfeifer, B. A.; Wang, C. C. C.; Walsh, C. T.; Khosla, C. Biosynthesis of Yersiniabactin, a Complex Polyketide-Nonribosomal Peptide, Using *Escherichia coli* as a Heterologous Host. *Appl. Environ. Microbiol.* **2003**, *69*, 6698–6702.

(51) Geoffroy, V. A.; Fetherston, J. D.; Perry, R. D. Yersinia pestis YbtU and YbtT are involved in synthesis of the siderophore yersiniabactin but have different effects on regulation. *Infect. Immun.* **2000**, *68*, 4452–4461.

(52) Rude, M. A.; Khosla, C. Production of ansamycin polyketide precursors in *Escherichia coli*. *J. Antibiot.* **2006**, *59*, 464–470.

binant *E. coli* cultures to aid heterologous production. In this case, the single module type NPRS and simple substrate requirements helped to facilitate successful heterologous production in *E. coli*.

Another success in NRP production from *E. coli* was the recent report on the total biosynthesis of the nonribosomal peptide echinomycin.[54] Watanabe et al. achieved the *de novo* synthesis of echinomycin by cloning and expressing the echinomycin gene cluster in *E. coli*. This successful total biosynthesis of a complex NRP from simple carbon and nitrogen sources will no doubt continue to fuel further heterologous production efforts.

iv. Terpenoids/Isoprenoids. Several terpenoid and isoprenoid compounds have also recently been heterologously synthesized in *E. coli*. For example, the heterologous production of a series of lipid pigment carotenoids, which are also classified as isoprenoids, have been achieved in recombinant *E. coli*.[55,56] Although the mevalonate-dependent (MEV) pathway has been engineered in *E. coli* for isoprenoid production, metabolic engineering of the deoxyxylulose 5-phosphate (DXP) pathway in *E. coli* has proven a potent method to produce isoprenoids heterologously.[57–61] This is because the DXP pathway does not appear to limit conversion of acetyl-CoA to the isoprenoid precursor isopentenyl diphosphate (IPP). For example, Kim et al. reported the biosynthesis of lycopene by utilizing the DXP pathway in *E. coli* noting the effect different production strains had on final lycopene levels.[59]

However, the mevalonate-dependent (MEV) pathway has also been used to support terpenoid production in *E. coli*.[62] Martin et al. introduced a heterologous and high-flux MEV pathway from *S. cerevisiae* into *E. coli* and achieved a high-level production of amorphadiene, the precursor of the terpene artemisinin. It was reported that the introduced heterologous MEV pathway was superior to the DXP pathway for delivering high-level isoprenoid precursors to terpene synthases for large-scale production. In their research, codon optimization was also adapted to better express amorphadiene biosynthetic genes.[62] In addition, to enable biosynthesis from acetyl-CoA, instead of exogenously added mevalonate, the entire mevalonate pathway was engineered into *E. coli* using plasmids to allow the complete intracellular production of amorphadiene.

v. Flavonoids. Flavonoids are important plant-specific secondary metabolites which have been found to have anti-inflammatory, antiviral, antimicrobial and anticancer activities.[63] Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which phenylalanine or tyrosine is used to produce 4-coumaroyl-CoA.[64,65] The 4-coumaroyl-CoA and malonyl-CoA substrates are two key building blocks for flavonoid biosynthesis catalyzed by a plant type III polyketide chalcone synthase.

Heterologous production of flavonoids in *E. coli* has been reported recently.[66,67] For example, flavones are an important class of flavonoids, and the flavone biosynthetic genes from different plants were introduced and expressed in *E. coli* with the majority of the flavone products secreted into the culture media.[66] Later, Leonard et al. reported the high-level biosynthesis of flavanones, members of the flavones family and the common precursors of flavonoid products in *E. coli*.[68] In their study, several genetic strategies were used to modify the central metabolism of *E. coli* and achieve robust flavanone biosynthesis. The bottleneck of high-level production in *E. coli* comes from the insufficient supply of intracellular malonyl-CoA. Through the coordinated overexpression of four acetyl-CoA carboxylase (ACC) subunits

(53) Takahashi, H.; Kumagai, T.; Kitani, K.; Mori, M.; Matoba, Y.; Sugiyama, M. Cloning and Characterization of a Streptomyces Single Module Type Nonribosomal Peptide Synthetase Catalyzing a Blue Pigment Synthesis. *J. Biol. Chem.* **2007**, *282*, 9073–9081.

(54) Watanabe, K.; Hotta, K.; Praseuth, A.; Koketsu, K.; Migita, A.; Boddy, C. N.; Wang, C. C.; Oguri, H.; Oikawa, H. Total biosynthesis of antitumor nonribosomal peptides in *Escherichia coli*. *Nat. Chem. Biol.* **2006**, *2*, 423–428.

(55) Tobias, A. V.; Arnold, F. H. Biosynthesis of novel carotenoid families based on unnatural carbon backbones: A model for diversification of natural product pathways. *Biochim. Biophys. Acta* **2006**, *1761*, 235–246.

(56) Umeno, D.; Arnold, F. H. Evolution of a pathway to novel long-chain carotenoids. *J. Bacteriol.* **2004**, *186*, 1531–1536.

(57) Farmer, W. R.; Liao, J. C. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog.* **2001**, *17*, 57–61.

(58) Kajiwara, S.; Fraser, P. D.; Kondo, K.; Misawa, N. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.* **1997**, *324*, 421–426.

(59) Kim, S.-W.; Keasling, J. D. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng.* **2001**, *72*, 408–415.

(60) Alper, H.; Jin, Y. S.; Moxley, J. F.; Stephanopoulos, G. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* **2005**, *7*, 155–164.

(61) Alper, H.; Miyaoku, K.; Stephanopoulos, G. Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. *Nat. Biotechnol.* **2005**, *23*, 612–616.

(62) Martin, V. J. J.; Pitera, D. J.; Withers, S. T.; Newman, J. D.; Keasling, J. D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* **2003**, *21*, 796–802.

(63) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.

(64) Beaudoin-Eagan, L. D.; Thorpe, T. A. Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. *Plant. Physiol.* **1985**, *78*, 438–441.

(65) Rösler, J.; Krekel, F.; Amrhein, N.; Schmid, J. Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. *Plant. Physiol.* **1997**, *113*, 175–179.

(66) Leonard, E.; Chemler, J.; Lim, K. H.; Koffas, M. A. Expression of a soluble flavone synthase allows the biosynthesis of phytoestrogen derivatives in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 85–91.

(67) Leonard, E.; Yan, Y.; Koffas, M. A. Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in *Escherichia coli*. *Metab. Eng.* **2006**, *8*, 172–181.

from *Photorhabdus luminescens* (PlACC), the malonyl-CoA pool in *E. coli* was increased, which led to improved flavanone production. Coexpression of PlACC with a biotin ligase ($BirA_{Pl}$) from the same species contributed to further improvement in production. Also, engineering of the acetate assimilation pathways was explored to elevate intracellular acetyl-CoA levels, which subsequently improved malonyl-CoA pools. When combined with the overexpression of ACC, the enhancement of acetate assimilation pathways greatly increased production levels.[68]

A key technical issue encountered during flavonoid (and other) natural product biosynthesis is the frequent need to use cytochrome P450 hydroxylase enzymes, which have developed a notorious reputation as being difficult to functionally produce in heterologous systems.[69] To this end, several strategies have been developed to improve heterologous activity within *E. coli* including: truncated P450 versions excluding transmembrane sequences, the addition (and often hybrid fusion) of accompanying NADPH reductase partnering enzymes, and the use of leading sequences found to enhance functional heterologous expression.[69–76] Such techniques have been coupled to successful flavonoid production within *E. coli* and provide a renewed hope and optimism for continued strides toward heterologous natural product production dependent upon P450 enzymatic activity.

4. *B. subtilis*

Like *E. coli*, *B. subtilis* is generally considered a safe organism. It is a Gram-positive bacterium and has no significant bias in codon usage. *B. subtilis* has the ability to secrete proteins directly into the growth medium, which greatly facilitates their downstream processing. The genome of *B. subtilis* has also been sequenced and, like *E. coli*, a number of powerful molecular biology tools are available for genetic and metabolic engineering. Such engineering is aided by the rapid growth rate of *Bacillus* and the organism's natural competence which allows foreign DNA to be introduced and natively recombined within the chromosome. The convenient growth properties of *B. subtilis* also facilitate fermentation methods and techniques for biological product overproduction.

B. subtilis is known to produce many natural products, including nonribosomal peptides, polyketides, and terpenoids. *B. subtilis* was found to produce antibiotic polyketides such as bacillaene and difficidin.[77,78] The gene clusters involved in the biosynthesis of these polyketides have been identified and characterized recently.[79]

B. subtilis is also able to produce the nonribosomal peptide mycosubtilin (whose synthetase is a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase[80]) and the lipoheptapeptide surfactin.[81] Besides polyketides and nonribosomal peptides, *B. subtilis* is also a native producer of isoprene.[82,83] However, to our knowledge, there is no report of *B. sublitlis* natively producing flavonoid compounds.

(68) Leonard, E.; Lim, K.; Saw, P.; Koffas, M. A. Engineering Central Metabolic Pathways for High-Level Flavonoid Production in *Escherichia coli*. *Appl. Environ. Microbiol.* **2007**, *73*, 3877–3886.

(69) Duan, H.; Schuler, M. A. Heterologous expression and strategies for encapsulation of membrane-localized plant P450s. *Phytochem. Rev.* **2006**, *5*, 507–523.

(70) Barnes, H. J.; Arlotto, M. P.; Waterman, M. R. Expression and enzymatic activity of recombinant cytochrome P450 17 alphahydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5597–5601.

(71) Leonard, E.; Yan, Y.; Koffas, M. A. Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in *Escherichia coli*. *Metab. Eng.* **2006**, *8*, 172–181.

(72) Halkier, B. A.; Nielsen, H. L.; Koch, B.; Moller, B. L. Purification and characterization of recombinant cytochrome P450TYR expressed at high levels in *Escherichia coli*. *Arch. Biochem. Biophys.* **1995**, *322*, 369–377.

(73) Nielsen, J. S.; Moller, B. L. Cloning and expression of cytochrome P450 enzymes catalyzing the conversion of tyrosine to p-hydroxyphenylacetaldoxime in the biosynthesis of cyanogenic glucosides in Triglochin maritima. *Plant. Physiol.* **2000**, *122*, 1311–1321.

(74) Kranendonk, M.; Fisher, C. W.; Roda, R.; Carreira, F.; Theisen, P.; Laires, A.; Rueff, J.; Vermeulen, N. P.; Estabrook, R. W. *Escherichia coli* MTC, a NADPH cytochrome P450 reductase competent mutagenicity tester strain for the expression of human cytochrome P450: Comparison of three types of expression systems. *Mutat. Res.* **1999**, *439*, 287–300.

(75) Pritchard, M. P.; Glancey, M. J.; Blake, J. A. R.; Gilham, D. E.; Burchell, B.; Wolf, R. C.; Friedberg, T. Functional expression of CYP2D6 and human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Pharmacogenetics* **1998**, *8*, 33–42.

(76) Pritchard, M. P.; Ossetian, R.; Li, D. N.; Henderson, C. J.; Burchell, B.; Wolf, C. R.; Friedberg, T. A general strategy for the expression of recombinant human cytochrome P450s in *Escherichia coli* using bacterial signal peptides: expression of CYP3A4, CYP2A6, and CYP2E1. *Arch. Biochem. Biophys.* **1997**, *345*, 342–354.

(77) Patel, P. S.; Huang, S.; Fisher, S.; Pirnik, D.; Aklonis, C.; Dean, L.; Meyers, E.; Fernandes, P.; Mayerl, F. Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity. *J. Antibiot.* **1995**, *48*, 997–1003.

(78) Wilson, K. E.; Flor, J. E.; Schwartz, R. E.; Joshua, H.; Smith, J. L.; Pelak, B. A.; Liesch, J. M.; Hensens, D. Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis*. II. Isolation and physicochemical characterization. *J. Antibiot.* **1987**, *40*, 1682–1691.

(79) Chen, X. H.; Vater, J.; Piel, J.; Franke, P.; Scholz, R.; Schneider, K.; Koumoutsi, A.; Hitzeroth, G.; Grammel, N.; Strittmatter, A. W.; Gottschalk, G.; Suessmuth, R. D.; Borriss, R. Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *J. Bacteriol.* **2006**, *188*, 4024–4036.

(80) Duitman, E. H.; Hamoen, L. W.; Rembold, M. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13294–13299.

(81) Arima, K.; Kakinuma, A.; Tamura, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* **1968**, *31*, 488–494.

Bacillus subtilis is an important prokaryote candidate for the heterologous production of recombinant natural products. As mentioned, members of the genus *Bacillus* are important natural producers of many bioactive natural products, including polyketides and nonribosomal peptides. Therefore, it is logical to assume that *B. subtilis* already possesses certain essential components needed for the heterlogous production of natural products. For example, the sfp phosphopantetheine transferase (used to establish correct polyketide synthase posttranslational modification in *E. coli*) is naturally found within *B. subtilis*.[84] *B. subtilis* is often favored for heterologous protein production because of its high capacity for secreting recombinant protein products outside the cell. In the context of heterologous natural product production, it is unlikely that the often sizable biosynthetic enzymes will be secreted; however, the secretion properties and the Gram-positive nature of *B. subtilis* may aid the export of heterologously produced natural products.

However, the use of *B. subtilis* for the heterologous production of natural products is limited by other native biological properties of the organism. The most important bottleneck is the lack of stable plasmid vectors which can be used to transfer the often sizable natural product genes and gene clusters. The direct integration of natural product genes into the *B. subtilis* chromosome is a solution to this problem, but besides requiring experimental efforts to prep gene transfer, the copy number of the final integrated genes will obviously be less than that of multicopy plasmids, which will limit the gene dosage of the desired natural product system. Also, extra attention should be paid to the expansion of the chromosome by inserting large foreign gene clusters since such efforts could negatively affect the subsequent phenotype of *B. subtilis*. In addition, compared with *E. coli*, *B. subtilis* lacks engineered production strains which have a lower proteases background. Given the protease activity naturally associated with *Bacillus* strains, such an environment may cause rapid degradation of foreign natural product enzymes.[85]

Regardless, several studies on the heterologous production of natural products have been presented. Eppelmann et al. constructed a *Bacillus subtilis* host capable of producing the peptide antibiotic bacitracin.[86] To address the problem of suitable and stable plasmids for the heterologous transfer and expression of large foreign genes, the entire 49 kb bacitracin biosynthesis gene cluster from *Bacillus licheniformis* was integrated into the *B. subtilis* chromosome, replacing the resident 26 kb surfactin synthetases gene cluster by a stepwise homologous recombination method.

Since *B. subtilis* was able to synthesize the building blocks for the bacitracin product, no exogenous or heterologously engineered substrates were needed. It was shown that the bacitracin synthetase was successfully produced in *B. subtilis* without degradation or folding problems. Furthermore, the bacitracin synthetase was post-transtlationally modified by the native *B. subtilis* sfp phosphopantetheine transferase which has been demonstrated to have a high substrate tolerance toward domains of NRPSs and PKSs. The functional production of the bacitracin synthetase led to a high level heterologous production of bacitracin. In this study, cell growth of the recombinant *B. subtilis* was not affected by the heterologous production of bacitracin (an antibiotic), as the self-resistance gene was coexpressed together with the remaining genes needed for biosynthesis. In fact, compared to the native bacitracin producer, the heterologous *B. subtilis* showed an elevated growth rate and improved final production level. This study provides a successful example of *B. subtilis* as a surrogate host for the heterologous production of natural products.

Following the approach in the research above, Doekel et al.[87] further studied parallel heterologous gene expression in *B. subtilis* and *E. coli*. They developed a set of *B. subtilis*/*E. coli* shuttle vectors which were able to self-replicate in *E. coli* and integrate cloned foreign DNA into the *B. subtilis* chromosome. Using these shuttle vectors, a dimodular hybrid NRPS was successfully expressed in both *E. coli* and *B. subtilis*. It was demonstrated that *B. subtilis* could better synthesize the NRPS by maintaining the protein in a stable, nondegradative, and posttranslationally modified active form. However, it should be noted that this result may be due to the similarities/differences in intracellular background between the native (*Bacillus licheniformis*) and heterologous hosts (*B. subtilis* vs *E. coli*).

Besides the examples presented above for nonribosomal peptide compounds, to our knowledge, there have been no reports on heterologous polyketide, flavonoid, or terpenoid production using *B. subtilis* as host.

5. *Pseudomonas putida*

P. putida and related bacteria (such as *P. stutzeri* and *P. syringae*) are metabolically versatile microorganisms used in various fields of biotechnology and bioproduction.[88,89]

(82) Kuzma, J.; Nemecek-Marshall, M.; Pollock, W. H.; Fall, R. Bacteria produce the volatile hydrocarbon isoprene. *Curr. Microbiol.* **1995**, *30*, 97–103.

(83) Julsing, M. K.; Rijpkema, M.; Woerdenbag, H. J.; Quax, W. J.; Kayser, O. Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 1377–1384.

(84) Mootz, H. D.; Finking, R.; Marahiel, M. A. 4′-phosphopantetheine transfer in primary and secondary metabolism of *Bacillus subtilis*. *J. Biol. Chem.* **2001**, *276*, 37289–37298.

(85) Li, W.; Zhou, X.; Lu, P. Bottlenecks in the expression and secretion of heterologous proteins in *Bacillus subtilis*. *Res. Microbiol.* **2004**, *155*, 605–610.

(86) Eppelmann, K.; Doekel, S.; Marahiel, M. A. Engineered Biosynthesis of the Peptide Antibiotic Bacitracin in the Surrogate Host *Bacillus subtilis*. *J. Biol. Chem.* **2001**, *276*, 34824–34831.

(87) Doekel, S.; Eppelmann, K.; Marahiel, M. A. Heterologous expression of nonribosomal peptide synthetases in *B. subtilis*: construction of a bi-functional *B. subtilis*/*E. coli* shuttle vector system. *FEMS. Microbiol. Lett.* **2002**, *216*, 185–191.

(88) Marques, S.; Ramos, J. L. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Mol. Microbiol.* **1993**, *9*, 923–929.

This group of bacteria can produce some compounds with certain antifungal activities, such as the siderophore pseudobactin 358.[90,91]

As heterologous hosts for natural products, pseudomonads have several advantages compared with other host systems. Pseudomonads grow rapidly and are genetically well established. For example, *P. putida* KT2440 is available as a "safe strain" that has been completely sequenced.[92] Besides, pseudomonads can be easily transformed with foreign DNA. The high GC genomic content and codon preference of pseudomonads make them more suitable than *E. coli* for the expression of genes from actinomycetes and myxobacteria. Furthermore, it was shown that pseudomonads harbor a phosphopantethiene transferase with promiscuous substrate specificity, which is able to activate PKSs and NRPSs from myxobacteria[93] and actinomycetes.[94] Additionally, because of the lack of background natural product production, heterologous production efforts are less likely to face crosstalk, contamination, or competition from native pathways; however, such a situation also highlights heterologous metabolic engineering that may be needed to provide intracellular substrates and other features to support foreign natural product production.

Müller and colleagues describe a straightforward strategy that has led to the expression of large biosynthetic gene clusters in *P. putida*.[95] The group focused its work on myxochromide S, a natural compound produced by the myxobacterium *Stigmatella aurantiaca*. Myxochromide S compounds are cyclic peptides connected to unsaturated polyketide side chains. Genes that are involved in the biosynthesis of these compounds are large in size and together comprise more than 60 kb. Müller's group began their work by isolating a cosmid containing parts of the myxochromide biosynthetic gene cluster. Using Red/ET recombineering techniques, the myxochromide S biosynthetic gene cluster was then rebuilt and engineered in *E. coli* to contain the elements required for expression in pseudomonads. Missing additional regulatory elements were also introduced including the toluic acid-inducible P_m promoter, which was inserted in front of the first gene of the cluster. The engineered construct could then be transferred into *P. putida*, and after induction, the recombinant strain produced approximately 40 mg/L of myxochromide S, five times more than *S. aurantiaca.* Here, all the building blocks for the biosynthesis of myxochromide as well as a broad-spectrum phosphopantetheinyl transferase capable of post-translationally activating the carrier domains in the PKS/NRPS hybrid were provided by *P. putida*. This work demonstrates a strategy to express clusters of unknown function, discovered by genome sequencing in foreign organisms, and also the ability to express large DNA fragments isolated from nonculturable bacteria. In addition, the adept use of in vivo recombineering suggests new ways to combine different gene clusters as one DNA fragment, increasing the chance for the production of novel gene constructs and subsequent natural products.

Many polyketides require methylmalonyl-CoA as an extender unit; however, *P. putida* cannot provide this C3 precursor in detectable levels. To address this issue, an engineered *P. putida* KT2440 was constructed by expressing methylmalonyl-CoA epimerase, methylmalonyl-CoA mutase, and meaB (an enzyme complex-stabilizing factor) from the myxobacterium *Sorangium cellulosum* So ce56. After 80 h of culture, methylmalonyl-CoA content could be detected at approximately 0.66 nmol/OD unit. Afterward, myxothiazol, a methylmalonyl-CoA-dependent myxobacterial secondary metabolite, was heterologous produced in this engineered strain with a final production level of 600 μg/L.[96]

A similiar strategy was used to express a silent type III polyketide synthase, which was identified in the genome sequence of the myxobacterium *S. cellulosum* So ce56. The heterologous expression of this gene in three different pseudomonads (*P. putida*, *P. stutzeri*, and *P. syringae*) allowed the corresponding product flaviolin to be identified

(89) Stephan, S.; Heinzle, E.; Wenzel, S. C.; Krug, D.; Müller, R.; Wittmann, C. Metabolic physiology of *Pseudomonas putida* for heterologous production of myxochromide. *Process. Biochem.* **2006**, *41*, 2146–2152.

(90) Ligon, J. M.; Hill, D. S.; Hammer, P. E.; Torkewitz, N. R.; Hofmann, D.; Kempf, H. J.; vanPee, K. H. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest. Manag. Sci.* **2000**, *56*, 688–695.

(91) Lemanceau, P.; Bakker, P. A.; De Kogel, W. J.; Alabouvette, C.; Schippers, B. Effect of pseudobactin 358 production by *Pseudomonas putida* WCS358 on suppression of fusarium wilt of carnations by nonpathogenic *Fusarium oxysporum* Fo47. *Appl. Environ. Microbiol.* **1992**, *58*, 2978–2982.

(92) Nelson, K. E.; Weinel, C.; Paulsen, I. T.; Dodson, R. J.; Hilbert, H.; Martins dos Santos, V. A.; Fouts, D. E.; Gill, S. R.; Pop, M.; Holmes, M.; Brinkac, L.; Beanan, M.; DeBoy, R. T.; Daugherty, S.; Kolonay, J.; Madupu, R.; Nelson, W.; White, O.; Peterson, J.; Khouri, H.; Hance, I.; Chris Lee, P.; Holtzapple, E.; Scanlan, D.; Tran, K.; Moazzez, A.; Utterback, T.; Rizzo, M.; Lee, K.; Kosack, D.; Moestl, D.; Wedler, H.; Lauber, J.; Stjepandic, D.; Hoheisel, J.; Straetz, M.; Heim, S.; Kiewitz, C.; Eisen, J. A.; Timmis, K. N.; Düsterhöft, A.; Tümmler, B.; Fraser, C. M. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida*. *Environ. Microbiol.* **2002**, *4*, 799–808. KT2440.

(93) Gross, F.; Gottschalk, D.; Müller, R. Posttranslational modification of myxobacterial carrier protein domains in *Pseudomonas* sp. by an intrinsic phosphopantetheinyl transferase. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 66–74.

(94) Finking, R.; Solsbacher, J.; Konz, D.; Schobert, M.; Schäfer, A.; Jahn, D.; Marahiel, M. A. Characterization of a new type of phosphopantetheinyl transferase for fatty acid and siderophore synthesis in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **2002**, *277*, 50293–50302.

(95) Wenzel, S. C.; Gross, F.; Zhang, Y.; Fu, J.; Stewart, A. F.; Müller, R. Heterologous expression of a myxobacterial natural products assembly line in pseudomonads via Red/ET recombineering. *Chem. Biol.* **2005**, *12*, 349–356.

(96) Gross, F.; Ring, M. W.; Perlova, O.; Fu, J.; Schneider, S.; Gerth, K.; Kuhlmann, S.; Stewart, A. F.; Zhang, Y.; Muller, R. Metabolic engineering of *Pseudomonas putida* for methylmalonyl-CoA biosynthesis to enable complex heterologous secondary metabolite formation. *Chem. Biol.* **2006**, *13*, 1253–1264.

after the heterologous expression in *E. coli* failed.[97] The production of flaviolin could be verified by HPLC analysis for recombinant strains of *P. stutzeri*/pFG154 though yields were not as high as for *P. putida*/pFG154 using the same fermentation conditions. The production in *P. syringae*/pFG154 could only be verified by HPLC-MS analysis even after prolonged fermentation times. This is the first example of the activation and functional activity of a silent natural product biosynthetic gene by heterologous expression in pseudomonads. However, as in the case of *Streptomyces* and *Bacillus* host systems, little work has been done to evaluate pseudomonads for the heterologous production of terpenoid and flavonoid compounds.

6. Other Bacteria Hosts Options for Natural Products Biosynthesis

Besides the hosts discussed above, there are other bacterial families, such as myxobacteria and *Mycobacteria,* that are also promising for natural product heterologous production. Julien et al. engineered *Myxococcus xanthus* to produce the epothilone mixed polyketide/nonribosomal peptide.[98] The yields of the epothilones from the native *S. cellulosum* strain are generally in the 10–20 mg/L range coupled to a relatively long doubling time (approximately 16 h). In addition, the lack of proper genetic tools to introduce and manipulate *S. cellulosum* DNA made epothilone production through heterologous hosts an attractive option. *M. xanthus* is the best characterized myxobacterium and possesses more amenable genetic engineering tools. *M. xanthus* also produces hundreds of potentially valuable secondary metabolites, many of which are known to have medicinal properties, such as myxalamid, an antibiotic against yeasts, molds, and enterobacteria.[99] Furthermore, *M. xanthus* exhibits a much shorter doubling time (5 h), making the host a better platform for large-scale culture and production efforts. In the example cited for Julien et al., epothilone production using *M. xanthus* as a heterologous host was capable of production levels similar to those observed for the original *S. cellulosum* host.

Mycobacterium ulcerans produces an unusual lipid toxin called mycolactone[100] that also acts as a highly potent immunosuppressive agent. Genome analysis revealed that a virulence plasmid and three polyketide synthase genes on this plasmid are required for mycolactone biosynthesis. The 12-membered core of mycolactone is produced by two large type I modular PKS enzymes, whereas its side chain is synthesized by a third PKS. Such information suggests that *M. ulcerans* may also possess the intracellular machinery needed to serve as a host for heterologous biosynthetic efforts.

7. Future Prospects for Bacterial Natural Product Production

Bacteria, as either native or heterologous hosts, are responsible for numerous natural products from the polyketide, nonribosomal peptide, terpenoid, and flavonoid classes. Production from native sources has been and will continue to be responsible for natural product large-scale manufacture. More recently, however, production from heterologous bacterial hosts, possessing advantageous properties for production, has been used as an alternative route to various natural products. Thus far, there has been enough success with heterologous natural product biosynthesis that the approach has almost become the first option for the production of a particular natural product, especially when that product possesses complex molecular architecture and derives from a fastidious native biological source.

Perhaps the biggest challenge to natural product production from native hosts is the difficulty in isolating the native microbes. For example, soil-based microbes have been responsible for a great number of therapeutically relevant natural products; however, it has been estimated that over 99% of soil dwelling microbes cannot be readily cultivated outside their immediate natural environments.[101] This obviously poses a problem for efforts to isolate and harness the production capabilities of these microorganisms. Currently, work is being done to develop better isolation techniques, ones that provide a more native-like environment to the microbes being isolated, in order to capture new organisms responsible for new natural compounds.[102] Still other research may explore the symbiotic relationship between bacterial strains and their environments, whether that relationship is between the nonliving environment or other living organisms, with the rationale that the symbiotic relationship is required for isolation of natural product-producing bacteria.

Other work recognizes the limitations of isolating native compounds from the soil or other environs and takes a "nondirected" approach to discovering new natural products. Here, environmental DNA is isolated and then transferred to suitable heterologous hosts for screening of phenotypic

(97) Gross, F.; Luniak, N.; Perlova, O.; Gaitatzis, N.; Jenke-Kodama, H.; Gerth, K.; Gottschalk, D.; Dittmann, E.; Müller, R. Bacterial type III polyketide synthases: phylogenetic analysis and potential for the production of novel secondary metabolites by heterologous expression in pseudomonads. *Arch. Microbiol.* **2006**, *185*, 28–38.

(98) Julien, B.; Shah, S. Heterologous expression of epothilone biosynthetic genes in *Myxococcus xanthus*. *Antimicrob. Agents Chemother.* **2002**, *46*, 2772–2778.

(99) Gerth, K.; Thierbach, G.; Gerth, K.; Jansen, R.; Reifenstahl, G.; Höfle, G.; Irschik, H.; Kunze, B.; Reichenbach, H.; Thierbach, G. The myxalamids, new antibiotics from *Myxococcus xanthus* (myxobacterales). I. Production, physico-chemical and biological properties, and mechanism of action. *J. Antibiot.* **1983**, *36*, 1150–1156.

(100) George, K. M.; Chatterjee, D.; Gunawardana, G.; Welty, D.; Hayman, J.; Lee, R.; Small, P. L. Mycolactone: A polyketide toxin from *Mycobacterium ulcerans* required for virulence. *Science* **1999**, *283*, 854–857.

(101) Handelsman, J.; Rondon, M. R.; Brady, S. F.; Clardy, J.; Goodman, R. M. Molecular biological access to the. chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* **1998**, *5*, R245–249.

(102) Kaeberlein, T.; Lewis, K.; Epstein, S. S. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **2002**, *296*, 1127–1129.

traits (such as antibiotic activity).[103–105] The approach sidesteps the issue of isolating native microbes and instead focuses on using heterologous microbes as hosts for environmental DNA encoding for new natural products. Hence, heterologous hosts are used to discover new natural compounds.

Traditionally, however, the idea of heterologous production stemmed from the desire to generate a known natural product. With the advances in natural product gene cluster and genome sequencing, many new natural product pathways have been readily identified. This information, coupled to the biological or genetic limitations of the native hosts, provides an ideal situation to attempt heterologous reconstituted biosynthesis. As highlighted above, this approach has been successful across numerous natural product classes for several bacterial hosts.

The general approach to heterologous natural product biosynthesis can be classified under "heterologous metabolic engineering", which refers to a particular type of metabolic engineering where a new biochemical pathway is reconstituted in a biological host. Within this type of engineering, there are several areas to be explored. The first is the transfer of the pathway to a new host. A natural product is often the result of a number of enzymes working in coordination. In turn, to transfer the pathway to a new host requires that the genes for the pathway be imported to a new organism and designed for coordinated gene expression. This can be complicated by the number or individual size of the genes to be introduced. For example, it is not uncommon for natural product pathways to consist of 10–30 separate enzymes responsible for final biosynthesis. In addition, biosynthetic enzymes may themselves approach and surpass 300 kDa in size (examples include the biosynthetic enzymes needed to generate the 6dEB and epothilone compounds detailed above). Transferring the genes for such systems must then take into consideration the total amount of DNA to be transferred, which can be significant.

Generally, plasmids have been used to shuttle natural product genetic networks between native and heterologous hosts. If the pathway is small enough (~5–30 kb), general cloning or expression plasmids can be used, often by introducing 1–3 plasmids with distinct origins of replication and selection markers.[47,48,106] For systems greater in DNA length, standard plasmids may be taxed by the amount of DNA to be transferred and it becomes increasingly difficult (and impractical) to employ multiple selection pressures. For large gene clusters, cosmid and BAC plasmids stand as alternatives for heterologous transfer. BAC plasmids were designed to accommodate large DNA segments (100–300 kb) and have been employed to a small extent during natural product gene transfer.[107–109] Perhaps the reason for this lack of use are the molecular biology challenges that arise when trying to move and manipulate large segments of DNA that will, by definition, limit the number of convenient and unique restriction sites available for manipulation. Further, manipulation by PCR is also difficult due to the variation in G+C content from certain native hosts and, as mentioned, the individual size of certain biosynthetic genes. One method more recently developed that can address certain issues related to both cloning and the transfer of natural product pathways is Red/ET recombineering.[110–113] In this method, PCR fragments are generated and directly transferred through in vivo homologous recombination to either plasmid or chromosomal DNA. The technique still requires overcoming the issues associated with natural product gene PCR but has been used successfully (especially by Mueller's group, as highlighted above) to transfer natural product genetic networks between hosts. In turn, hosts such as *Bacillus* possess a natural propensity for in vivo homologous recombination (and a lack of ability to accept and maintain plasmids), and this feature has also been used to transfer natural product systems (see above).

(103) Brady, S. F.; Handelsman, J. Cloning and heterologous expression of a natural product biosynthetic gene cluster from eDNA. *Org. Lett.* **2001**, *3*, 1981–1984.

(104) MacNeil, I. A.; Tiong, C. L.; Minor, C.; August, P. R.; Grossman, T. H.; Loiacono, K. A.; Lynch, B. A.; Phillips, T.; Narula, S.; Sundaramoorthi, R.; Tyler, A.; Aldredge, T.; Long, H.; Gilman, M.; Holt, D.; Osburne, M. S. Expression and isolation of antimicrobial small molecules from soil DNA libraries. *J. Mol. Microbiol. Biotechnol.* **2001**, *3*, 301–308.

(105) Rondon, M. R.; August, P. R.; Bettermann, A. D.; Brady, S. F.; Grossman, T. H.; Liles, M. R.; Loiacono, K. A.; Lynch, B. A.; MacNeil, I. A.; Minor, C.; Tiong, C. L.; Gilman, M.; Osburne, M. S.; Clardy, J.; Handelsman, J.; Goodman, R. M. Cloning the Soil Metagenome: a Strategy for Accessing the Genetic and Functional Diversity of Uncultured Microorganisms. *Appl. Environ. Microbiol.* **2000**, *66*, 2541–2547.

(106) Murli, S.; MacMillan, K. S.; Hu, Z.; Ashley, G. W.; Dong, S. D.; Kealey, J. T.; Reeves, C. D.; Kennedy, J. Chemobiosynthesis of novel 6-deoxyerythronolide B analogues by mutation of the loading module of 6-deoxyerythronolide B synthase 1. *Appl. Environ. Microbiol.* **2005**, *71*, 4503–4509.

(107) Shizuya, H.; Birren, B.; Kim, U.; Mancino, V.; Slepak, T.; Tachiiri, Y.; Simon, M. Cloning and Stable Maintenance of 300-Kilobase-Pair Fragments of Human DNA in *Escherichia coli* Using an F-Factor-Based Vector. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8794–8797.

(108) Rong, R.; Slupska, M. M.; Chiang, J. H.; Miller, J. H. Engineering large fragment insertions into the chromosome of *Escherichia coli*. *Gene* **2004**, *336*, 73–80.

(109) Sosio, M.; Giusino, F.; Cappellano, C.; Bossi, E.; Puglia, A. M.; Donadio, S. Artificial chromosomes for antibiotic-producing actinomycetes. *Nat. Biotechnol.* **2002**, *18*, 343–345.

(110) Yu, D.; Ellis, H. M.; Lee, E. C.; Jenkins, N. A.; Copeland, N. G.; Court, D. L. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5978–5983.

(111) Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640–6645.

(112) Muyrers, J. P.; Zhang, Y.; Stewart, A. F. Techniques: Recombinogenic engineering--new options for cloning and manipulating DNA. *Trends. Biochem. Sci.* **2001**, *26*, 325–331.

(113) Murphy, K. C. Use of bacteriophage lambda recombination functions to promote gene replacement in Escherichia coli. *J. Bacteriol.* **1998**, *180*, 2063–2071.

Once introduced to a heterologous host, there is still an issue of coordinated and successful gene expression. This is another area of heterologous metabolic engineering that will need future research and development. Once transferred to a heterologous bacterial host, a designed expression platform must be implemented to allow the engineered production of a heterologous product. This involves coordinated gene expression and active gene products. Depending on the heterologous host, there exist a number of options for coordinated gene expression. For example, in *E. coli*, multiple promoter and repressor systems exist for controlling and influencing gene expression.[114] However, the same repertoire does not exist in most other heterologous bacterial hosts. Gene expression leading to active protein products is another requirement for eventual natural product formation. In the past, postinduction temperature, recombinant chaperones, and strains compensating for rare codons and required posttranslational modification have been used to aid the DNA to active protein process.[47,49,62] Again, such tools have primarily been developed for *E. coli* with other hosts providing situational advantages typically when the pathway transfer occurs between similar native and heterologous hosts. Many problems related to unsuccessful gene expression derive from codon bias between native and heterologous hosts. The problem has become increasingly tractable with improvements in synthetic gene design, even for the large biosynthetic genes previously mentioned.[115]

After a natural product gene cluster has been introduced to a new host and successful natural product biosynthesis reconstituted, the pathway is now subject to the "traditional metabolic engineering" that has been applied to numerous other compounds produced biologically. Here, the new pathway must be viewed in the context of cellular metabolism, and the interplay between the new and established pathways is key to optimizing natural product production. Before experimental steps are taken, the simplest way to explore the interaction between intracellular metabolism and heterologous biosynthesis is through in silico analysis. Numerous efforts have been made to stoichiometrically model microbial metabolism, a prospect made straightforward due to the complete genome sequences available for the four main host systems outlined above. Native metabolism has been modeled through these approaches, and, more recently, heterologously introduced natural product pathways have been introduced to and studied in the context of native metabolism.[116–118] A goal in this area is to build predictive metabolic models indicating the type of native metabolism manipulations necessary to improve heterologous biosynthesis and, more generally, to computationally define metabolic network reactions between native and heterologously introduced metabolism. In addition to in silico efforts, experimental steps are also used to identify interactions between native and heterologous metabolism. Examples include the use of ^{13}C and ^{14}C tracers and high throughput LC−MS schemes to identify pathway carbon flux and bottlenecks that may limit final natural product conversion.[119–121]

Many of the same molecular biology tools used to transfer heterologous pathways to new bacterial hosts are also available to genetically alter metabolism for the sake of improving natural product biosynthesis. Plasmids and the chromosome serve as locations to introduce auxiliary metabolism designed to increase precursor pools fueling the biosynthetic process. In addition, native pathways may be altered to improve production by overexpressing native genes. A nice example of this was the previously described efforts to use separate supporting pathways for lycopene production in *E. coli*. The mevalonate pathway was nonnative to *E. coli* and was hence transferred from *S. cerevisiae* via plasmids. Alternatively, *E. coli* possesses a native DXP pathway that was manipulated through chromosomal promoter replacement of key natural genes involved with supplying DMAPP and IPP needed for lycopene biosynthesis. The system was further optimized via gene deletions (predicted partially by in silico analysis) using the chromosomal engineering methods introduced previously.[60,122] As noted before, different hosts will have a different set of molecular biology tools available to improve natural product production, but as these tools continue to develop, the experimental options available for traditional metabolic engineering to optimally integrate the new natural product pathway into the existing metabolic network will continue to improve.

Finally, once introduced to a new heterologous host, process engineering offers additional opportunities to im-

(114) Baneyx, F. Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.* **1999**, *10*, 411–421.

(115) Kodumal, S. J.; Patel, K. G.; Reid, R.; Menzella, H. G.; Welch, M.; Santi, D. V. Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15573–15578.

(116) Covert, M. W.; Schilling, C. H.; Famili, I.; Edwards, J. S.; Goryanin, I. I.; Selkov, E.; Palsson, B. O. Metabolic modeling of microbial strains *in silico*. *Trends. Biochem. Sci.* **2001**, *26*, 179–186.

(117) Fischer, E.; Sauer, U. Large-scale in vivo flux analysis shows rigidity and suboptimal performance of *Bacillus subtilis* metabolism. *Nat. Genet.* **2005**, *37*, 636–640.

(118) Gonzalez-Lergier, J.; Broadbelt, L. J.; Hatzimanikatis, J. Analysis of the Maximum Theoretical Yield for the Synthesis of Erythromycin Precursors in *Escherichia coli*. *Biotechnol. Bioeng.* **2006**, *95*, 638–644.

(119) Klapa, M. I.; Aon, J. C.; Stephanopoulos, G. Systematic quantification of complex metabolic flux networks using stable isotopes and mass spectrometry. *Eur. J. Biochem.* **2003**, *270*, 3525–3542.

(120) Suthers, P. F.; Burgard A. P.; Dasika M. S.; Nowroozi, F.; Van Dien, S.; Keasling, J. D.; Maranas, C. D. Metabolic flux elucidation for large-scale models using (13)C labeled isotopes. Metab. Eng. 2007, ahead of print.

(121) Pitera, D. J.; Paddon, C. J.; Newman, J. D.; Keasling, J. D. Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab. Eng.* **2007**, *9*, 193–207.

(122) Alper, H.; Miyaoku, K.; Stephanopoulos, G. Characterization of lycopene-overproducing *E. coli* strains in high cell density fermentations. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 968–974.

prove production. Again, these opportunities are dependent on the particular host system. Hosts such as *B. subtilis* and *E. coli* have a particularly noted history in scale-up of high cell density bioreactors for the production of biological products, typically recombinant proteins. The same process tools are available for the enhanced volumetric production of recombinant molecules generated through heterologous natural product biosynthesis. Several examples have emerged over the last 5−10 years employing such strategies to boost volumetric production by as much as 1000-fold.[122–124] This often overlooked aspect of heterologous production is one more incentive for introducing and reconstituting pathways for relevant natural products.

MP7001329

(123) Pfeifer, B.; Hu, Z.; Licari, P.; Khosla, C. Process and metabolic strategies for improved production of *Escherichia coli*-derived 6-deoxyerythronolide B. *Appl. Environ. Microbiol.* **2002**, *68*, 3287–3292.

(124) Lau, J.; Tran, C.; Licari, P.; Gallazo, J. Development of a high cell-density fed-batch bioprocess for the heterologous production of 6-deoxyerythronolide B in *Escherichia coli*. *J. Biotechnol.* **2004**, *110*, 95–103.