Chemistry & Biology, Vol. 12, 349–356, March, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2004.12.012

Heterologous Expression of a Myxobacterial Natural Products Assembly Line in Pseudomonads via Red/ET Recombineering

Silke C. Wenzel,[1,2] Frank Gross,[1,2] Youming Zhang,[3] Jun Fu,[3] A. Francis Stewart,[4] and Rolf Müller[1,2,*]
[1]Pharmaceutical Biotechnology
Saarland University
P.O. Box 151150
66041 Saarbrücken
Germany
[2]GBF: German Research Centre for Biotechnology
Mascheroderweg 1
38124 Braunscheig
Germany
[3]Gene Bridges GmbH
[4]BioInnovationZentrum
Technical University of Dresden
Am Tatzberg 47
01307 Dresden
Germany

Summary

Natural products of microbial origin are widely used as pharmaceuticals and in agrochemistry. These compounds are often biosynthesized by multifunctional megasynthetases whose genetic engineering and heterologous expression offer considerable promise, especially if the natural hosts are genetically difficult to handle, slow growing, unculturable, or even unknown. We describe a straightforward strategy that combines the power of advanced DNA engineering (recombiogenic cloning) in *Escherichia coli* with the utility of pseudomonads as the heterologous host for the analysis and mutagenesis of known and unknown secondary metabolite pathways. The myxochromide S biosynthetic gene cluster from *Stigmatella aurantiaca* was rebuilt and engineered in *E. coli* to contain the elements required for expression in pseudomonads. The successful production in *Pseudomonas putida*, at unprecedented levels, demonstrates the feasibility of the new approach to the analysis and mutagenesis of these important pathways.

Introduction

Myxobacteria are a rich source of natural products with biological activity. So far, about 100 different core structures and 500 variants have been characterized [1]. Many of these compounds illustrate that myxobacteria specialize in rare mechanisms of action. For example, 20 new electron transport inhibitors and 10 substances that act on the cytoskeleton have been found [1]. Most myxobacterial substances are moderately lipophilic, linear or cyclic polyketides and peptides synthesized by large multifunctional enzymes known as polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) [2–5]. These enzymes are organized in a modular fashion and catalyze the successive condensation of acyl-CoA (PKS) or activated amino acid building blocks (NRPS). Usually, each module is responsible for one discrete chain elongation step and can be subdivided into domains controlling not only the choice of the extender unit but also the further modification of the growing chain. These modular enzyme systems present the possibility that the specificity or sequence of reactions can be manipulated by DNA engineering to produce novel therapeutic compounds [6, 7].

Over the past decade, numerous PKS and NRPS biosynthetic gene clusters have been cloned including several from myxobacterial producers, notably the electron transport inhibitors myxothiazol and melithiazol [8, 9] and the potential anticancer agents, epothilones and tubulysins [10–12]. Increasing biochemical knowledge from studies dealing with the corresponding enzymes has led to the emergence of combinatorial biosynthesis technologies aimed at generating novel compounds through genetic manipulation [6, 7]. In contrast to numerous intensively studied streptomycetes, genetic systems for the modification of the slow growing myxobacterial producers have proven difficult. Nevertheless, expression of the epothilone biosynthetic gene cluster in streptomycetes [13] and in *Myxococcus xanthus* [14] has been achieved. The methodology employed relied on several rounds of cloning and heterologous host modification, which was tedious and time consuming. In addition, the yields were worse than in the natural producer, *Sorangium cellulosum* [15].

We now report the genetic engineering and heterologous expression of the first myxobacterial gene cluster in pseudomonads, which were found to present excellent host properties (for the general procedure of the approach see Figure 1). To facilitate the ease of transfer, the entire cluster was rebuilt on one molecule, and the DNA elements for transfer, stable maintenance, and inducible expression were introduced using homologous recombination in *E. coli* [16–18], also termed recombineering or recombiogenic cloning [19].

The gene cluster used here encodes a PKS/NRPS hybrid that directs the biosynthesis of different forms of myxochromide S, which are cyclic peptides with unsaturated polyketide side chains (Figures 2 and 4A) [20]. These compounds are produced by the myxobacterium *Stigmatella aurantiaca* in yields up to 8 mg/l. The gene cluster exhibits several interesting biosynthetic features (Figure 2). The NRPS part of the gene cluster contains six NRPS modules, although only five amino acids are incorporated into the molecule. By comparison of the deduced substrate specificities of the adenylation domains, it was proposed that module four is skipped during the biosynthetic process [20]. In addition, the gene cluster harbors only one type I PKS module that was assumed to form the complete polyketide side chain and therefore believed to act iteratively. The successful expression of the complete myxochromide S biosynthetic gene cluster in the heterologous host *P. putida* now provides direct evidence for the iterative use of the type I bacterial PKS.

*Correspondence: rom@mx.uni-saarland.de

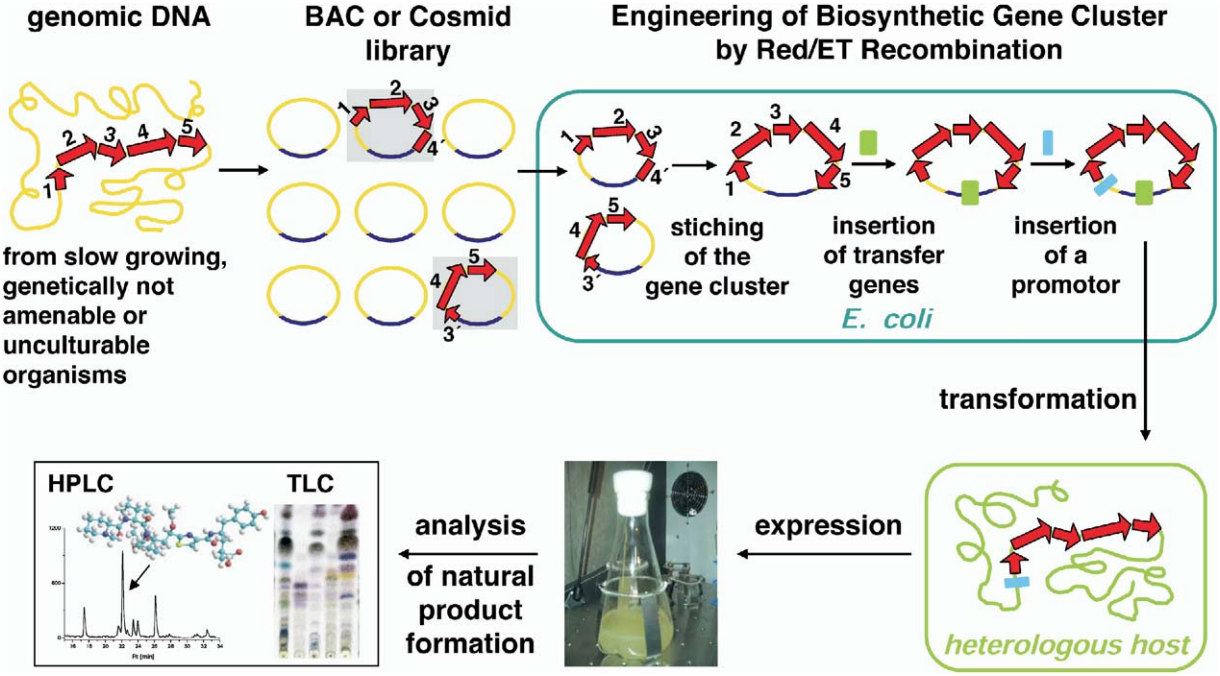

Figure 1. General Strategy for the Cloning and Heterologous Expression of Large Natural Product Assembly Lines

After screening of the library for constructs containing parts of the investigated natural product biosynthetic gene cluster, overlapping fragments can be stitched together in *E. coli* using Red/ET recombineering. The resulting construct harboring the complete natural product assembly line can be further modified in *E. coli* by recombineering, e.g., inserting genes needed for transformation in the heterologous host and/or insertion of promoter region(s). The final construct can be transferred into a suitable heterologous host for expression of the biosynthetic pathway. Natural product formation can finally be analyzed by conventional techniques, e.g., HPLC and/or TLC.

The applied strategy of combining the power of advanced DNA engineering and mutagenesis in *Escherichia coli* with the utility of pseudomonads as the heterologous host provides a new avenue for the analysis and mutagenesis of known and unknown (not only myxobacterial) secondary metabolite pathways in the future (see Figure 1).

Results

Construction of the Complete Myxochromides S Gene Cluster on a Transferable Cosmid

The myxochromide S biosynthetic gene cluster has been cloned and sequenced previously [20]. The original cosmid E196 does not contain the full-length pathway because it is missing the thioesterase (TE) domain of the second NRPS. To complete the myxochromide S biosynthetic gene cluster and add the necessary elements for conjugation, integration, and expression in pseudomonads, the original cosmid E196 was modified sequentially by recombineering using the Red/ET recombination [16–18]. In brief, the backbone of cosmid E196 was modified by single-step insertion of the origin of transfer (*oriT*) for conjugation purposes, the tetracycline-resistance gene for selection in *P. putida*, and a DNA fragment from the chromosome of *P. putida* (*trpE*) to enable the integration of the construct into the genome by homologous recombination, to create the SuperCos derivative CMch34 (Figure 3A). During this procedure, the original ampicillin-resistance gene of SuperCos was deleted. To reconstruct the complete myxochromide S pathway on CMch34, the missing part of the TE domain had to be added. The sequence of the full-length TE domain was available on pMch2, a previously described recovery plasmid from a NRPS mutant strain [20] (see Figure 2). To stitch the missing thioesterase piece of the gene cluster onto CMch34, the zeocin-resistance gene (*zeoR*) was amplified by PCR reaction and inserted into pMch2 by recombineering, resulting in plasmid pMch23. Then, a 3.5 kb StuI/NdeI fragment from pMch23 containing the TE-*zeoR* cassette was recombined with CMch34 to create CMch36. As a final step, the toluic acid inducible P_m promoter was inserted in front of the first gene of the myxochromide S cluster. Together with the chloramphenicol-resistance gene and the *xylS* gene, the P_m promoter was inserted into CMch36 to create CMch37. This insertion was designed to not only place the promoter directly in front of the PKS but also to delete five genes not involved in myxochromide S biosynthesis (Figure 3A). The final construct CMch37 contains only the three genes from the myxochromides S pathway (one PKS and two NRPSs), with the P_m promoter placed in front of the PKS.

Transfer of the Myxochromides S Biosynthetic Gene Cluster into Pseudomonads

The final construct CMch37, containing the complete myxochromide S biosynthetic gene cluster, was trans-

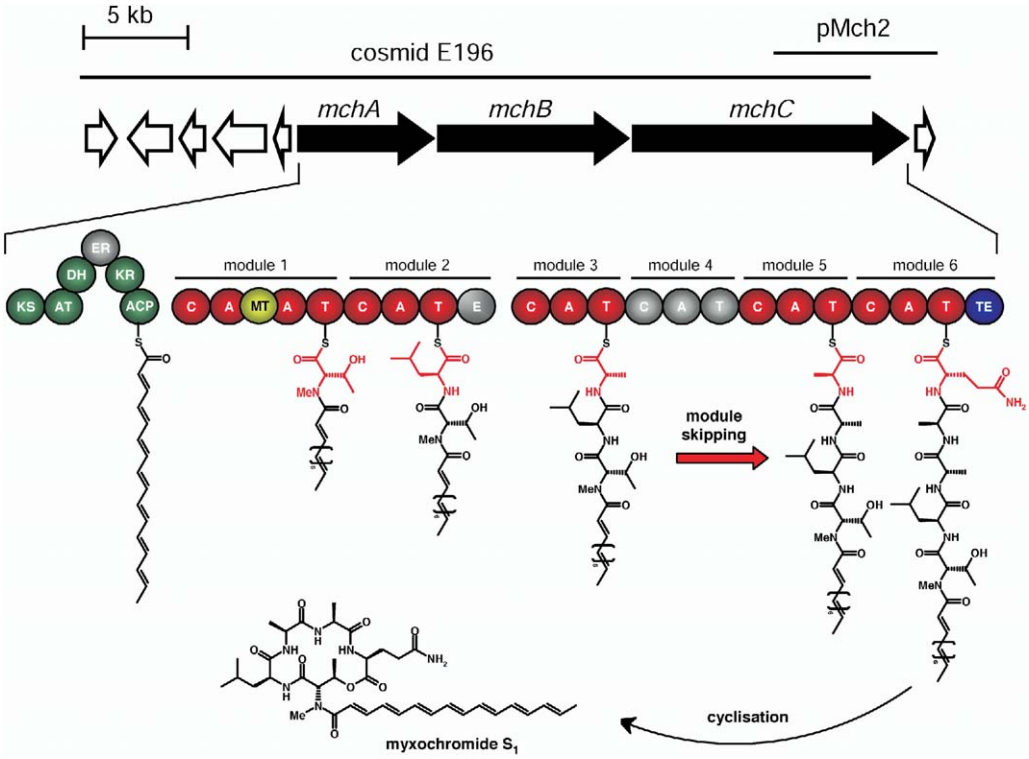

Figure 2. Gene Arrangement and Domain Organization of the *mch* Biosynthetic Gene Cluster from *S. aurantiaca* DW4/3-1 and a Model for the Biosynthesis of Myxochromide S

The inserts of cosmid E196 and additional sequences derived from plasmid pMch2 are indicated by lines. Genes are shown as arrows, which also indicate the direction of transcription. *mchA* encodes a PKS, while *mchB* and *mchC* encode NRPSs. For description of the flanking regions, see [20]. PKS domains are shown in green, and NRPS domains are shown in red. The methyltransferase (MT) domain is shown in yellow, while the thioesterase (TE) domain is marked in blue. Gray domains are presumably inactive. Transfer of the peptide chain from the T (thiolation) domain of module 3 to the T domain of module 5 is indicated. A, adenylation domain; C, condensation domain; KS, β-ketosynthase domain; AT, acyltransferase domain; DH, dehydratase domain; ER, enoylreductase domain; KR, ketoreductase domain; ACP, acyl carrier protein domain. (Modifed from [20].)

ferred into *Pseudomonas putida* by triparental conjugation as previously described [21]. Conjugants were screened on PMM agar containing tetracycline for the selection of *P. putida*::CMch37 mutants. The donor strain was additionally counterselected by ampicillin, which *P. putida* is resistant to. Thousands of exconjugants were obtained per conjugation. Ten randomly chosen colonies were analyzed by PCR, which verified the integration of CMch37 into the *P. putida* chromosome in each case.

Production of Myxochromide S in Pseudomonads

To induce expression from the P_m promoter, toluic acid was added to cultures after 2 hr of fermentation. Compared to 30°C, cultivation at 16°C after the induction resulted in a more than 1000-fold increase of myxochromide S production, reaching a maximum yield of approximately 40 mg/l. This is about five times greater than the maximum found with the natural producing host *S. aurantiaca*. Furthermore, new myxochromide S derivatives could be identified in these extracts by HPLC/MS analysis (Figure 4). In addition to myxochromides S_{1-3} known from *S. aurantiaca* (which were identified by comparison to authentic reference standards), the MS data from extracts indicate the presence of the corresponding compounds lacking the threonine *N*-methyl group and thus representing new myxochromide S derivatives.

Myxochromides were only detected in the cells and not in the fermentation medium, indicating that *P. putida* is not able to export these secondary metabolites out of the cell. A kinetic analysis (data not shown) of myxochromide S production in *P. putida*::CMch37 mutants reveals that the production maximum was reached after 2–3 days, which surpasses the 6 days required for *S. aurantiaca* to reach maximum production.

Discussion

Heterologous expression of complete secondary metabolite pathways is of increasing interest in natural product research and drug discovery. It is a viable alternative to both strain and fermentation process development and molecular biological manipulation of the native producer strain [22]. Several strategies for the heterologous expression of secondary metabolite pathways have been described in the literature so far, ranging from the targeted expression of specific natural pro-

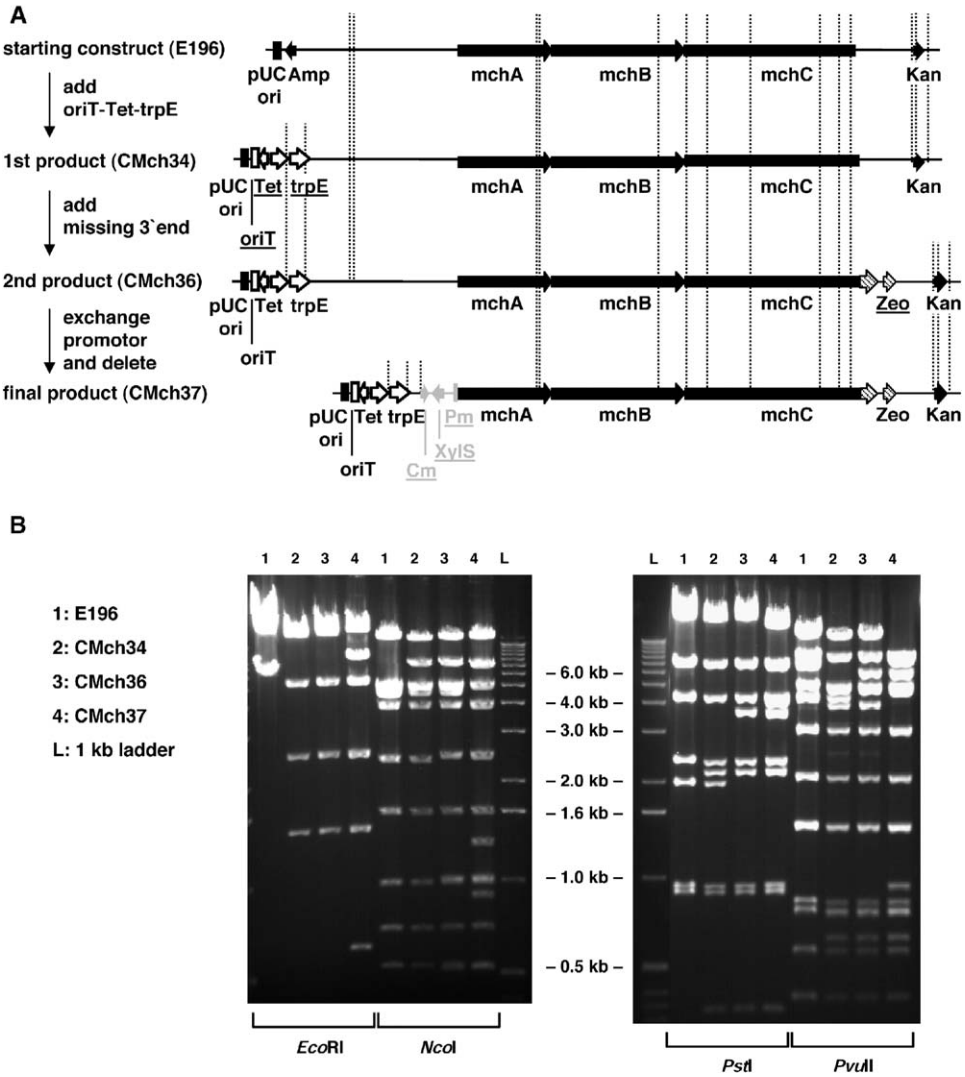

Figure 3. Description of the Cloning Procedure

(A) Maps of cosmid E196 and the Red/ET recombination contructs cosmid CMch34, CMch36, and CMch37 with virtual PvuII restriction, indicated by dotted lines.

(B) Restriction analysis of cosmid E196 and the Red/ET recombination contructs cosmid CMch34, CMch36, and CMch37 with the indicated restriction enzymes including PvuII.

ducts to the expression of large unknown DNA fragments. In the later approach, bacterial artificial chromosome (BAC) shuttle vectors were used for constructing environmental libraries followed by expression in multiple expression hosts (pseudomonads, streptomycetes, and *E. coli*) [23, 24]. However, only products from relatively small biosynthetic gene clusters have been produced with this method so far, and the producing heterologous host has always been from the same genus as the natural producer [23].

For gene transfer into streptomycetes, an elegant method based on the RecA methodology for the assembly of large DNA pieces in *E. coli*-streptomyces artificial chromosomes has been developed [25, 26]. As observed by these authors, cosmid instabilities in the presence of RecA recommend the use of inducible systems, such as used here for regulated expression of the Red proteins. Although more experience with larger complexes is required, we note that undesired DNA deletions and rearrangements were only rarely observed throughout our recombineering.

There are a number of reports on the heterologous production of biosynthetic pathways from streptomycetes in related actinomycetes. Here, mostly relatively small type II PKS systems were used [27–32]. Heterologous expression of large PKS and/or NRPS genes has been achieved by coexpression from several plasmids harboring parts of the biosynthetic pathway [33]. The feasibility of the production of complex natural products (exemplified by the aglycon of erythromycin) after heterologous expression in unrelated bacteria has been shown in a genetically engineered *E. coli* host [22].

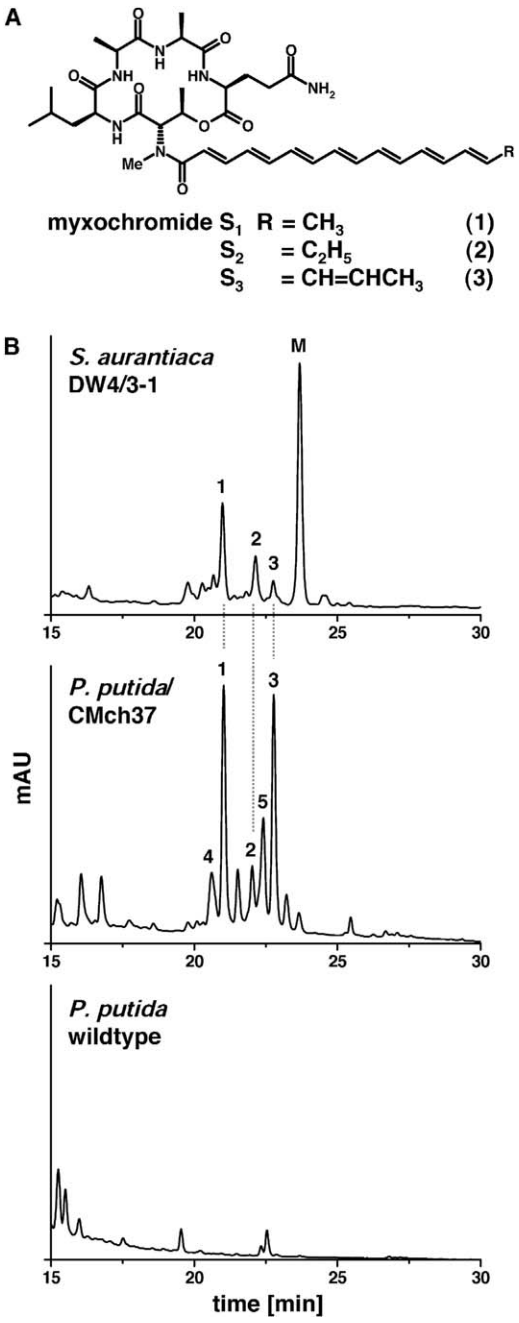

Figure 4. Myxochromide Production in Pseudomonads

(A) Structures of myxochromides S_1–S_3 (1–3) produced by the myxobacterium *S. aurantiaca* DW4/3-1.

(B) HPLC profiles from extracts of the *P. putida*/CMch37 mutant strain in comparison to *P. putida* wild-type and the natural myxochromide S producer *S. aurantiaca* DW4/3-1 (diode array detection at 200–400 nm). Numbers correspond to substances as follows: **1–3**, see (A); **4** and **5** are assumed to be the corresponding Des-*N*-methylderivatives from **1** and **3** (see main text). Some additional novel peaks of currently unknown structure and presumably unrelated to myxochromides occur in *P. putida*/CMch37. M. myxothiazol; mAU, mili absorption units.

Similarly, both epothilone and soraphen derived from the myxobacterium *S. cellulosum* have been produced in streptomycetes [13, 34], and a multistep procedure enabled epothilone production in *M. xanthus* [35]. One could argue that a large BAC library would always enable the cloning of complete biosynthetic gene clusters on one DNA molecule. Nevertheless, this has never been described for a heterologous expression, which is presumably due to the fact that most of the BACs from a library statistically only carry fragments of each gene cluster. In fact, we have not been able to identify a BAC harboring a complete 90 kbp PKS pathway in a library of *S. cellulosum* covering 15 genome equivalents (average insert size ranging from 80 to 180 kbp; O. Perlova and R.M., unpublished data). In addition, it is still a difficult task to prepare BACs of sufficient insert size.

Despite increasing experience with heterologous hosts, typically the levels of production have been much lower than those in the original producer. This may be due to weakness of the natural promoter in the heterologous host. Futhermore, in all of the studies mentioned above, classical and time-consuming cloning procedures were employed, and the expression of large secondary metabolite clusters was only achieved by coexpression from several plasmids. In this study, we describe the application of recombinogenic engineering in *E. coli* for cloning of a large natural product assembly line. This straightforward technique is particularly suitable for large DNA molecules such as BACs and cosmids and is therefore ideal for engineering of PKS and NRPS pathways. We used this method to rebuild the complete assembly line from a myxobacterial PKS/NRPS hybrid gene cluster in one cosmid (final size after deletion of genes not required for myxochromid biosynthesis: 43 kbp) and to introduce the elements required for conjugation, stable integration, and regulated expression. The most important advantage of this method is that the whole secondary metabolite pathway is located on one construct, which can be transferred into the heterologous host strain (see Figure 1). As recombineering is based on homologous recombination, one might have expected difficulties using the described method in the assembly of repetitive sequences. Nevertheless, in related projects employing gene clusters up to 80 kbp in size, we have already demonstrated that even highly repetitive sequences can be recombined. The introduction of several promoters into one pathway can be facilitated by employing counterselectable markers to delete and then reuse the marker genes employed (S.C.W., F.G., Y.Z., A.F.S., and R.M., unpublished data).

To transfer the complete pathway into pseudomonads in one step, the origin of transfer (*oriT*) for conjugation, the tetracycline-resistance gene as selection marker, as well a homologous region (*trpE*) for integration into the pseudomonads genome were inserted into the construct. The *trpE* gene was used for homologous integration into the *P. putida* genome because it is not essential for growth on rich media. The original myxobacterial promoter had also to be changed against a regulatory element working in the heterologous host strain pseudomonads (P_m promoter). We chose the toluic-acid-inducible P_m promoter [36, 37] in order to control the myxochromide S expression. The

use of an inducible promoter is important because the secondary metabolite product of the pathway could be toxic for the host strain. An inducible promoter allows for the control of secondary metabolite expression and thus permits the establishment of the pathway in the host regardless of toxicity. Notably, in the case of an unknown pathway, toxicity after expression will be an interesting first sign that the molecule is antibiotically active.

After conjugation of the final construct (CMch37) into the heterologous host strain *P. putida*, the myxochromide S biosynthetic gene cluster was integrated into the chromosome with high efficiency. All of the recombinants tested were verified as genotypically correct. The resulting *P. putida* mutant strain (*P. putida*/CMch37) was shown to overproduce myxochromide S approximately 5-fold in comparison to *S. aurantiaca*, unambiguously proving that the inserted fragment carries all genes necessary for the biosynthesis.

These data clearly show that only one PKS module is responsible for the formation of the complete polyketide side chain, which is direct evidence for an iteratively working myxobacterial type I polyketide synthase. As for other iterative PKSs, the determination of the polyketide chain length remains a mystery. The functional heterologous expression of the myxochromide pathway now sets the stage for a detailed analysis by mutagenesis of MchA. In addition to the already characterized products from the natural producer *S. aurantiaca* (myxochromides $S_{1\text{-}3}$), new myxochromide S derivatives could be detected in the extracts of the heterologous host by HPLC/MS analysis. The MS data indicate that these compounds correspond to myxochromides S_1 and S_3 lacking the threonine *N*-methyl group. Possibly, S-adenosyl-methionine (SAM)-dependent methylation in *P. putida* catalyzed by the *N*-methyltransferase domain of the first NRPS MchB did not proceed efficiently because SAM may become limiting once production reaches a certain limit. Alternatively, these new minor myxochromide S derivatives may be detectable because of the high yield of myxochromide S produced by the heterologous host. These questions also highlight the need to understand the underlying natural product formation in more detail to enable efficient combinatorial biosynthesis. Due to the flexibility of our approach, these studies are now straightforward. For example, elucidation of the unique module skipping process is underway.

Only two myxobacterial products (epothilone and soraphen) have been expressed in a heterologous host so far, and their production yields in streptomycetes were poor (<0.5 mg/l) [13, 34]. Additionally, epothilone production in the myxobacterium *M. xanthus* has been described [14], but this organism is very difficult to handle in fermentation processes [15]. Thus, efficient heterologous expression of biosynthetic gene clusters from microbial producers other than actinomycetes is still a challenging task, especially for the slow-growing myxobacteria or cyanobacteria. The work presented here demonstrates that *P. putida* provides an important alternative host for the production of myxobacterial secondary metabolites. We chose pseudomonads as the heterologous host for several reasons. First, pseudomonads are genetically well established and grow rapidly, and *Pseudomonas putida* KT2440 is available as a "safety strain" that has been completely sequenced [38]. Pseudomonads and *E. coli* conjugate efficiently, and as opposed to most secondary-metabolite-producing hosts, pseudomonads can be readily transformed with DNA using physical methods. Additionally, several *E. coli* elements, such as certain promoters and certain plasmid replication origins, work in pseudomonads, in contrast to the paucity of such elements in the major secondary-metabolite-producing hosts. Furthermore, pseudomonads have excellent endogenous properties for homologous recombination, which enables efficient integration into the endogenous genome for stable maintenance of introduced DNA molecules. Pseudomonads provide further advantages regarding the expression and activation of myxobacterial secondary metabolite gene clusters. For examples, pseudomonads have a high GC genomic content and a codon preference that is more suitable than *E. coli* for expression of genes from the major secondary metabolite producers, the actinomycetes and myxobacteria. *P. putida* also harbors a phosphopantetheinyl transferase (P-pant transferase) with broad substrate specificity, which is able to activate both the acyl carrier protein domains (ACPs) and the peptidyl carrier protein domains (PCPs) from myxobacteria and streptomycetes [39]. The posttranslational modification of PKS and NRPS systems by attaching the P-pant moiety to a highly conserved serine residue of their ACP and PCP domains (apo-holo-conversion) catalyzed by the P-pant tranferases is essential for the activity of these megasynthases [40]. In accordance with our data, Marahiel and coworkers have recently shown that the P-pant transferase from *P. aeruginosa* in vitro shows relatively low substrate specificity [41]. Unlike most secondary-metabolite-producing hosts such as streptomyces, pseudomonads grow easily and rapidly in culture. With myxochromide S production in *P. putida*, the fermenation time is reduced 3-fold (2 versus 6 days), while the product yield is 5-fold higher (40 versus 8 mg/l) in comparison to the natural producer *S. aurantiaca*. *P. putida* is obviously able to produce all the activated precursors needed for myxochromide S biosynthesis (acetyl-CoA, malonyl-CoA, and propionyl-CoA) and, due to its ability to grow on valine as sole carbon source, it might also be able to produce methylmalonyl-CoA [42], which is important for the production of numerous polyketides. The production of methylmalonyl-CoA in pseudomonads might alternatively be ensured by metabolic engineering of the intracellular CoA pool, which has been described in *E. coli* [43].

Significance

The engineering of a complete polyketide synthase/nonribosomal peptide synthetase biosynthetic gene cluster in *E. coli* and its heterologous expression in pseudomonads is a straightforward strategy that holds promise for discovery and engineering of drugs from nature. This strategy provides not only a useful way to circumvent the slow growth and poor genetics of myxobacterial producers but also a way to scrutinize gene clusters of unknown function, including gene clusters discovered by genome-scale sequenc-

ing projects with little or no supporting information. Our strategy also provides an important step forward in the production of "unnatural natural products" through accurate manipulation of secondary metabolites using advanced DNA engineering and mutagenesis in *E. coli*.

Experimental Procedures

Cloning of the Myxochromide S Cluster

All methods were essentially the same as described previously [16, 18]. For Red/ET recombination, a 50 µl aliquot of Red/ET-competent (ET$^+$) *E. coli* cells harboring the parent molecule was electroporated with 0.3 µg of a linear fragment (either PCR product or fragment obtained from restriction). After electroporation, colonies that grew under selection for the antibiotic resistance gene were examined for the intended Red/ET recombination product by restriction analysis with a set of different enzymes.

All PCRs were carried out using HotStarTaq Polymerase (Qiagen) according to the manufacturer's protocol. For the amplification of the ~600 bp zeocin cassette, Q-Solution (Qiagen) was added (20% final concentration), and pcDNA3.1-Zeo(+) was used as template with oligonucleotides MchET1 and MchET2. The conditions using an Eppendorf mastercycler were as follows: 15 min at 95°C to activate the polymerase, denaturation at 95°C (30 s), annealing at 62°C (30 s) (for cycles 1–4) or at 72°C (for cycles 5–30), and extension at 72°C (40 s); 30 cycles. The PCR product was purified with the NucleoSpin Extract Kit (Macherey-Nagel). The PCR for the amplification of the 2.6 kbp P_m-*xylS*-chloramphenicol cassette was carried out using JB-655-Cm as template with oligonucleotide primers MchET5 and MchET6. DMSO was added to a final concentration of 3%, and 50% glycerol was added to a final concentration of 8%. The conditions using the Eppendorf mastercycler were as follows: 15 min at 95°C denaturation 95°C (30 s), annealing at 53°C (30 s), and extension at 72°C (150 s); 30 cycles. The PCR product was directly used without purification.

Oligonucleotide sequences were as follows. Oligos used for generation of CMch34: left (MchET1), 5′-TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCTCGAATAGGGCGAATTGGGCC-3′; right (MchET2), 5′-AGTGAGGGTTAATTGCGGCCGCGAATTCTTGAAGACGAAAGGGCCTCGTGATATTACTTGGCGGAAGTCTGCTC-3′. Oligos used for generation of pMch23: left (MchET3), 5-CATTCAAGCGCGCGCTGGGGAAGGCAGGCAGGATGGGATCTGATCAGCACGTGTTG-3′; right (MchET4), 5′-TGATAAGCGGTCAAACATGAGAATTCGCGGCCGCATAATACGACTCACTATAGTCAGTCCTGCTCCTCGGCCAC-3′. Oligos used for generation of CMch37: left (MchET5), 5-TGAGCCCCGCGTCCCGCAGGGCCCGGGCGATGCAATACTCACTGGAGATTTAAATCCTGGTGTCCCTGTT-3′; right (MchET6), 5′-TGGACACTACCCCACGGCTAGAAACTTCGGTCAATACAGAATTTCCTATCATGTTCATGACTCCATTATT-3′.

Conjugation into Pseudomonads

The SuperCos derivative CMch37 (size: 43 kbp) was transferred into the pseudomonads strain *P. putida* KT2440 by conjugation. The triparental mating was performed by combining 50 µl of a 300 µl suspension from 1 ml of overnight cultures of *E. coli* XL-1 Blue containing CMch37, *E. coli* HB101 containing the helper plasmid pRK2013, and *P. putida* on a small area of a Luria agar plate [21]. After incubation at 37°C for 4 hr, the plate was transferred to 28°C–30°C and incubated overnight. The cells were scraped from the plate, resuspended in sterile water, and plated out on PMM agar amended with ampicillin (100 µg/ml) and tetracyclin (30 µg/ml). The plates were incubated for 1–2 days at 28°C–30°C. Transconjugants were screened by colony PCR using HotStarTaq Polymerase (Qiagen) according to the manufacturer's protocol. DMSO was added to a final concentration of 3%, and 50% glycerol to a final concentration of 8%. The oligonucleotides Mch35 (5′-CACGAGCTCGTCCTGGATGCCGAGATTG-3′) and Mch36 (5′-GGCTCTAGAAGCGTCCGGCCGTCATAC-3′) were used, yielding an about 700 bp PCR product. The conditions using the Eppendorf mastercycler were as follows: 15 min at 95°C for activation of the polymerase, denaturation for 30 s at 95°C, annealing for 30 s at 53°C, and extension for 40 s at 72°C; 30 cycles.

Expression and Analysis

The *P. putida* strain containing the myxochromide S biosynthetic gene cluster (*P. putida*/CMch37) was incubated in 2 l flasks containing 500 ml Luria broth amended with ampicillin (100 µg/ml) and tetracycline (30 µg/ml). The culture was inoculated with an overnight culture (1:100) and incubated for 1–2 hr at 30°C on a rotary shaker. After induction with toluic acid (5 mM), the culture was transferred to 16°C and incubated for 2–3 days. The cells were harvested by centrifugation and extracted with acetone. The extract was evaporated and redissolved in 500 µl of methanol, and 10 µl of the concentrated extract was analyzed by HPLC-MS (Agilent 1100 LC system). The chromatographic conditions were as follows: column, ET 125 × 2 mm, and precolumn, Nucleosil 120-5-C_{18}; solvent, 5% acetonitrile, 95% water, 5 mM ammonium acetate, 0.003% acetic acid (A), and 95% acetonitrile 5% water, 5 mM ammonium acetate, 0.003% acetic acid (B); solvent gradient from 10% B at 0 min to 100% B within 30 min, followed by 10 min with 100% B; flow rate, 0.3 ml/min. The detection was carried out at 400 nm, and for mass detection a Perkin Elmer Sciex API 2000 mass spectrometer equipped with a TurbolonSpray source was used. Myxochromides S_{1-3} were identified by comparison to the retention times and the MS data of authentic standards (myxochromide S_1: Rt = 21.0 min, [M+H$^+$] = 723; myxochromide S_2: Rt = 22.0 min, [M+H$^+$] = 737; myxochromide S_3: Rt = 22.8 min, [M+H$^+$] = 749). Quantitation of myxochromides was performed by comparison of the peak areas in the HPLC chromatogram at 400 nm with the peak areas in a calibration curve from the myxochromide S_1 standard at 400 nm.

Acknowledgments

The commercial potential of this work is a business prospect of Gene Bridges GmbH. In addition to the coauthors indicated as affiliated with Gene Bridges, A.F.S. and R.M. are consultants and shareholders. The authors would like to acknowledge funding from the German Ministry for Education and Research from a grant in the Biofuture program. S.C.W. and R.M. highly appreciate financial support from the Fonds der Chemischen Industrie (FCI). This paper is dedicated to Prof. Dr. Gerhard Höfle on the occasion of his 65th birthday.

Received: November 24, 2004
Revised: December 20, 2004
Accepted: December 21, 2004
Published: March 25, 2005

References

1. Reichenbach, H. (2001). Myxobacteria, producers of novel bioactive substances. J. Ind. Microbiol. Biotechnol. *27*, 149–156.
2. Hopwood, D. (1997). Genetic contributions to understanding polyketide synthases. Chem. Rev. *97*, 2465–2497.
3. Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. *18*, 380–416.
4. Marahiel, M.A. (1997). Protein templates for the biosynthesis of peptide antibiotics. Chem. Biol. *4*, 561–567.
5. Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat. Prod. Rep. *20*, 275–287.
6. Hutchinson, C. (1999). Combinatorial biosynthesis of antibiotics. In Drug Discovery from Nature, S. Grabley and R. Thiericke, eds. (Berlin: Springer), pp. 233–254.
7. Mendez, C., and Salas, J.A. (2003). On the generation of novel anticancer drugs by recombinant DNA technology: The use of combinatorial biosynthesis to produce novel drugs. Comb. Chem. High Throughput Screen. *6*, 513–526.
8. Silakowski, B., Schairer, H.U., Ehret, H., Kunze, B., Weinig, S., Nordsiek, G., Brandt, P., Blöcker, H., Höfle, G., Beyer, S., and Müller, R. (1999). New lessons for combinatorial biosynthesis

from myxobacteria: The myxothiazol biosynthetic gene cluster of *Stigmatella aurantiaca* DW4/3–1. J. Biol. Chem. *274*, 37391–37399.
9. Weinig, S., Hecht, H.J., Mahmud, T., and Müller, R. (2003). Melithiazol biosynthesis: further insights into myxobacterial PKS/NRPS systems and evidence for a new subclass of methyl transferases. Chem. Biol. *10*, 939–952.
10. Julien, B., Shah, S., Ziermann, R., Goldman, R., Katz, L., and Khosla, C. (2000). Isolation and characterization of the epothilone biosynthetic gene cluster from *Sorangium cellulosum*. Gene *249*, 153–160.
11. Molnar, I., Schupp, T., Ono, M., Zirkle, R., Milnamow, M., Nowak-Thompson, B., Engel, N., Toupet, C., Stratmann, A., Cyr, D.D., et al. (2000). The biosynthetic gene cluster for the microtubule-stabilizing agents epothilones A and B from *Sorangium cellulosum* So ce90. Chem. Biol. *7*, 97–109.
12. Sandmann, A., Sasse, F., and Müller, R. (2004). Identification and analysis of the core biosynthetic machinery of tubulysin, a potent cytotoxin with potential anticancer activity. Chem. Biol. *11*, 1071–1079.
13. Tang, L., Shah, S., Chung, L., Carney, J., Katz, L., Khosla, C., and Julien, B. (2000). Cloning and heterologous expression of the epothilone gene cluster. Science *287*, 640–642.
14. Julien, B., and Shah, S. (2002). Heterologous expression of epothilone biosynthetic genes in *Myxococcus xanthus*. Antimicrob. Agents Chemother. *46*, 2772–2778.
15. Gerth, K., Pradella, S., Perlova, O., Beyer, S., and Müller, R. (2003). Myxobacteria: proficient producers of novel natural products with various biological activities—past and future biotechnological aspects with the focus on the genus *Sorangium*. J. Biotechnol. *106*, 233–253.
16. Zhang, Y., Buchholz, F., Muyrers, J., and Stewart, F. (1998). A new logic for DNA engineering using recombination in *Escherichia coli*. Nat. Genet. *20*, 123–128.
17. Muyrers, J., Zhang, Y., Testa, G., and Stewart, A. (1999). Rapid modification of bacterial artificial chromosomes by ET-recombination. Nucleic Acids Res. *27*, 1555–1557.
18. Muyrers, J., Zhang, Y., and Stewart, A. (2001). Techniques: Recombinogenic engineering—new options for cloning and manipulating DNA. Trends Biochem. Sci. *26*, 325–331.
19. Copeland, N.G., Jenkins, N.A., and Court, D.L. (2001). Recombineering: a powerful new tool for mouse functional genomics. Nat. Rev. Genet. *2*, 769–779.
20. Wenzel, S.C., Kunze, B., Höfle, G., Silakowski, B., Scharfe, M., Blöcker, H.,, and Müller, R. (2005). Structure and biosynthesis of myxochromides S1-3 in Stigmatella aurantiaca: Evidence for an iterative bacterial type I polyktetide synthase and for module skipping in nonribosomal peptide biosynthesis. ChemBioChem *6*, 375–385.
21. Hill, D., Stein, J., Torkewitz, N., Morse, A., Howell, C., Pachlatko, J., Becker, J., and Ligon, J. (1994). Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. Appl. Environ. Microbiol. *60*, 78–85.
22. Pfeifer, B.A., Admiraal, S.J., Gramajo, H., Cane, D.E., and Khosla, C. (2001). Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. Science *291*, 1790–1792.
23. Martinez, A., Kolvek, S.J., Yip, C.L.T., Hopke, J., Brown, K.A., MacNeil, I.A., and Osburne, M.S. (2004). Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. Appl. Environ. Microbiol. *70*, 2452–2463.
24. Daniel, R. (2004). The soil metagenome - a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. *15*, 199–204.
25. Sosio, M., Giusino, F., Cappellano, C., Bossi, E., Puglia, A.M., and Donadio, S. (2000). Artificial chromosomes for antibiotic-producing actinomycetes. Nat. Biotechnol. *18*, 343–345.
26. Sosio, M., Bossi, E., and Donadio, S. (2001). Assembly of large genomic segments in artificial chromosomes by homologous recombination in *Escherichia coli*. Nucleic Acids Res. *29*, E37.
27. Bechthold, A., Sohng, J.K., Smith, T.M., Chu, X., and Floss, H.G. (1995). Identification of *Streptomyces violaceoruber* Tu22 genes involved in the biosynthesis of granaticin. Mol. Gen. Genet. *248*, 610–620.
28. Kim, C.Y., Park, H.J., and Kim, E.S. (2003). Heterologous expression of hybrid type II polyketide synthase system in *Streptomyces* species. J. Microbiol. Biotechnol. *13*, 819–822.
29. Hong, S.T., Carney, J.R., and Gould, S.J. (1997). 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. *179*, 470–476.
30. von Mulert, U., Luzhetskyy, A., Hofmann, C., Mayer, A., and Bechthold, A. (2004). 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. *230*, 91–97.
31. Ichinose, K., Ozawa, M., Itou, K., Kunieda, K., and Ebizuka, Y. (2003). Cloning, sequencing and heterologous expression of the medermycin biosynthetic gene cluster of *Streptomyces* sp. AM-7161: towards comparative analysis of the benzoisochromanequinone gene clusters. Microbiol. *149*, 1633–1645.
32. Kalaitzis, J.A., and Moore, B.S. (2004). Heterologous biosynthesis of truncated hexaketides derived from the actinorhodin polyketide synthase. J. Nat. Prod. *67*, 1419–1422.
33. Kao, C.M., Katz, L., and Khosla, C. (1994). Engineered biosynthesis of a complete macrolactone in a heterologous host. Science *265*, 509–512.
34. Zirkle, R., Ligon, J.M., and Molnar, I. (2004). Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. Microbiol. *150*, 2761–2774.
35. Lau, J., Frykman, S., Regentin, R., Ou, S., Tsuruta, H., and Licari, P. (2002). Optimizing the heterologous production of epothilone D in *Myxococcus xanthus*. Biotechnol. Bioeng. *78*, 280–288.
36. Marques, S., Holtel, A., Timmis, K.N., and Ramos, J.L. (1994). Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. J. Bacteriol. *176*, 2517–2524.
37. Mermod, N., Lehrbach, P.R., Reineke, W., and Timmis, K.N. (1984). Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. EMBO J. *3*, 2461–2466.
38. 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., et al. (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. *4*, 799–808.
39. Gross, F., Gottschalk, D., and Müller, R. (2005). Posttranslational modification of myxobacterial carrier protein domains in *Pseudomonas* sp. by an intrinsic phosphopantethetheinyl transferase. Appl. Microbiol. Biotechnol., in press
40. Lambalot, R.H., Gehring, A.M., Flugel, R.S., Zuber, P., LaCelle, M., Marahiel, M.A., Reid, R., Khosla, C., and Walsh, C.T. (1996). A new enzyme superfamily - the phosphopantetheinyl transferases. Chem. Biol. *3*, 923–936.
41. Finking, R., Solsbacher, J., Konz, D., Schobert, M., Schäfer, A., Jahn, D., and Marahiel, M.A. (2002). Characterization of a new type of phosphopantetheinyl transferase for fatty acid and siderophore synthesis in *Pseudomonas aeruginosa*. J. Biol. Chem. *277*, 50293–50302.
42. Bannerjee, D., Sanders, L.E., and Sokatch, J.R. (1970). Properties of purified methylmalonate semialdehyde dehydrogenase of *Pseudomonas aeruginosa*. J. Biol. Chem. *245*, 1828–1835.
43. Pfeifer, B., Hu, Z.H., Licari, P., and Khosla, C. (2002). Process and metabolic strategies for improved production of Escherichia coli-derived 6-deoxyetythronolide B. Appl. Environ. Microbiol. *68*, 3287–3292.