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TREX: A Universal Tool for the Transfer and Expression of Biosynthetic Pathways in Bacteria

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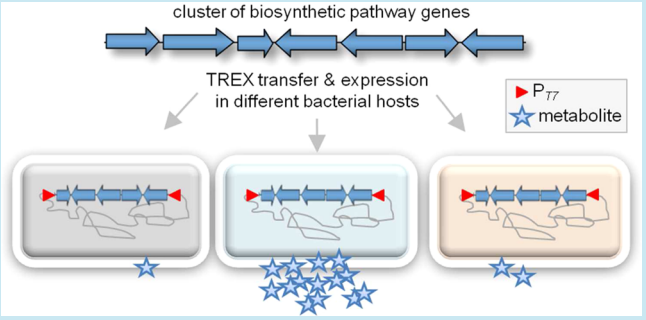
Supporting Information

ABSTRACT: Secondary metabolites represent a virtually inexhaustible source of natural molecules exhibiting a high potential as pharmaceuticals or chemical building blocks. To gain broad access to these compounds, sophisticated expression systems are needed that facilitate the transfer and expression of large chromosomal regions, whose genes encode complex metabolic pathways. Here, we report on the development of the novel system for the transfer and expression of biosynthetic pathways (TREX), which comprises all functional elements necessary for the delivery and concerted expression of clustered pathway genes in different bacteria. TREX employs (i) conjugation for DNA transfer, (ii) randomized transposition for its chromosomal insertion, and (iii) T7 RNA polymerase for unimpeded bidirectional gene expression. The applicability of the TREX system was demonstrated by establishing the biosynthetic pathways of two pigmented secondary metabolites, zeaxanthin and prodigiosin, in bacteria with different metabolic capacities. Thus, TREX represents a valuable tool for accessing natural products by allowing comparative expression studies with clustered genes.



KEYWORDS: *transfer of biosynthetic pathways, functional expression of clustered genes, secondary metabolites discovery, synthetic biology, metabolic engineering*

Microorganisms offer an immense variety of natural compounds with potential pharmacological or biotechnological applications including antibiotic, antiviral, antitumor, or anti-aging activities.[1] However, in most cases, these compounds cannot be isolated because the respective microorganisms cannot be cultured in the laboratory or they produce these compounds in only very small amounts. Therefore, novel approaches to access these substances are of great interest.[2−4] In the past decade, steadily improved DNA sequencing technologies have resulted in uncovering plenty of secondary metabolic pathways that stem from single microbes as well as complex microbial consortia, putting many valuable compounds within reach of scientists. However, their identification and characterization requires the production of sufficient amounts; hence, novel and efficient expression systems preferably in heterologous hosts are urgently needed.[2,5,6]

Unfortunately, the production of natural compounds in heterologous bacterial hosts is hampered by two general limitations. First, their synthesis usually requires the reconstitution of the entire biosynthetic pathways, which are encoded by multiple genes organized within operons and gene clusters.[7] The sizes of such gene clusters as well as their complex genetic structures, reflected by the bidirectional orientation of genes that are arranged in differentially regulated transcriptional units, render their concerted expression difficult. Second, the successful reconstitution of a biosynthetic pathway strongly depends on the host bacterium and its intrinsic or engineered metabolic properties.[8,9]

However, the most appropriate host organisms cannot be identified by a broad screening approach because the concerted expression of clustered genes generally requires an expression host whose RNA polymerases are able to recognize the heterologous promoters.[10] Alternatively, an expression system must allow the coordinated transcription of numerous genes irrespective of their natural promoters and terminators.[11]

We have newly developed the transfer and expression system TREX, which can serve to transfer entire biosynthetic pathways allowing for combinatorial metabolic engineering and thus will help to identify and produce new metabolites. We have demonstrated that TREX can indeed be used as a versatile plug-and-play module that facilitates transfer, randomized integration, and functional expression of large gene clusters in different heterologous bacterial hosts.

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RESULTS AND DISCUSSION

Functional Elements and Implementation of the TREX System. The TREX system was designed to enable comparative expression studies using genomic gene clusters. It consists of two cassettes, designated left and right TREX (L-TREX and R-TREX), which comprise all functional elements required for establishing a novel biosynthetic pathway in a bacterial host (Figure 1A).

The TREX system should generally allow labeling, transfer, as well as bidirectional expression of clustered pathway genes in various bacterial expression hosts by applying the following strategy:

The target gene cluster, which can be located on a standard plasmid, cosmid, or BAC, is labeled by the two TREX cassettes (Figure 1B, step 1). To this end, the tetracycline resistance gene of the L-TREX cassette and the gentamicin resistance gene of the R-TREX cassette (Figure 1A, Tc^R and Gm^R) can be used as corresponding selection markers. The transfer of metabolic pathways often requires the cloning of large DNA fragments rendering restriction endonuclease-based cloning difficult. Recent advances in restriction enzyme-independent cloning[12−14] and recombineering techniques based on λ phage[15−17] or yeast recombinases[18,19] now enable a more easy handling of such large DNA fragments.

In a second step, the TREX-labeled gene cluster can be transferred to any Gram-negative bacterial host (Figure 1B, step 2). Here, the origin of transfer (Figure 1A, oriT, L-TREX) enables the conjugational transfer of large DNA molecules, which allows the efficient delivery of recombinant DNA molecules of almost any size.[20] Hence, a broad range of Gram-negative and some Gram-positive bacterial hosts become accessible.[21,22]

Next, the gene cluster is inserted into the chromosomal DNA of the expression host. In synthetic biology, chromosomal integration is gaining interest as it offers advantages over plasmid-based methods with regard to reduction of metabolic stress and increase of gene stability as well as yield of gene product.[23,24] Integration of heterologous DNA fragments, in turn, can be achieved by homologous recombination or transposition. Transposon-mediated integration of large gene clusters is advantageous as compared to homologous recombination in terms of efficiency.[25] The transposon Tn5[26,27] is functional in a vast variety of bacteria and transposition takes place at random positions.[28] These characteristics secure Tn5 and its derivatives as efficient tools to construct bacterial knockout mutant libraries[29] as well as stable chromosomal insertions of heterologous genes.[30,31]

For TREX, stable maintenance of large DNA fragments in heterologous hosts is ensured by the transposon outer elements (Figure 1A, OE, outside ends, also denoted as inverted repeats (IR) or mosaic ends (ME)) and the transposase-encoding gene (*tnp*) derived from transposon Tn5. The Tn5 elements enable the randomized integration of all pathway genes into a host chromosome (Figure 1B, step 3). During this process, the origin of transfer and the Tc^R marker get lost. Therefore, the TREX-mediated transposition is accompanied by the acquisition of a gentamicin but not a tetracycline resistance. Additionally, loss of oriT prevents subsequent and undesired translocation of the host's chromosomal DNA, which might be important with regard to safety issues.

In the final step, expression of the gene cluster has to be accomplished. For gene clusters with unidirectionally organized genes, insertion of a promoter upstream of the gene cluster has proven very useful.[32] For TREX, two T7 RNA polymerase

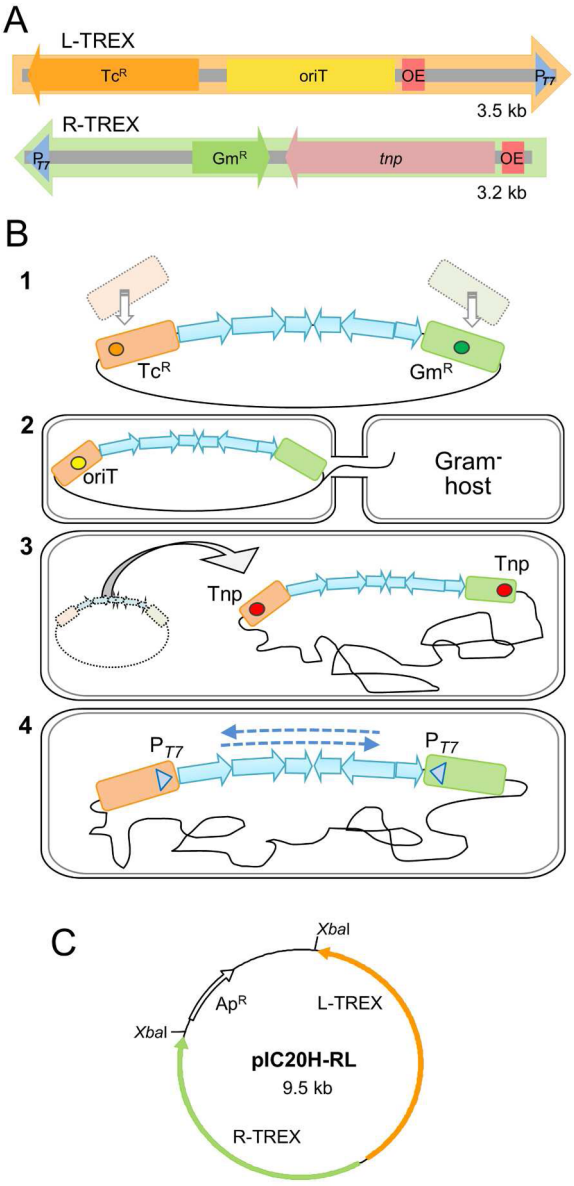

Figure 1. Structure and function of the pathway transfer and expression system TREX. (A) The TREX system consists of two cassettes, the L-TREX (orange) and R-TREX (green) cassette, which comprise all elements for establishing a biosynthetic pathway in a heterologous bacterial host. Tc^R, tetracycline resistance gene (dark orange); oriT, origin of transfer (yellow); OE, outside end of transposon (red); P_{T7}, T7 bacteriophage promoter (blue); Gm^R, gentamicin resistance gene (dark green); *tnp*, transposase gene (light red). (B) The principle of TREX-mediated pathway transfer includes the labeling of a gene cluster of interest with the TREX cassettes (step 1), the conjugational transfer of the TREX-labeled genes to a Gram-negative bacterial host (step 2), the randomized integration of TREX-labeled genes into the bacterial chromosome by transposition (step 3), and finally the bidirectional expression of all clustered genes by T7 RNA polymerase (step 4). (C) For simplified TREX labeling (B, step1), plasmid pIC20H-RL carrying the <L-TREX-R> module was constructed encompassing the L-TREX and R-TREX cassettes in an "inside-out" fashion with the T7 promoters pointing outward (indicated by arrow heads), flanked by *Xba*I restriction sites. Ap^R, ampicillin resistance gene.

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(T7RP)-dependent promoters located within the L- and R-TREX cassettes in opposite directions (Figure 1A, P_{T7}) should allow the bidirectional expression of cluster genes irrespective of their orientation and naturally occurring promoters or regulatory elements (Figure 1B step 4). In contrast to bacterial RNA polymerases, the T7RP was shown to be particularly suited for the expression of clustered genes, since it ignores bacterial termination sites and thus synthesizes notably long transcripts.[11]

To further simplify the TREX labeling (step 1), e.g., for genome-mining approaches, we have constructed a TREX module designated as <L-TREX-R> that encompasses both of the two TREX cassettes where the two T7 promoters point outward the module. This module is provided on plasmid pIC20H-RL (GenBank Accession Number: JX668229) (Figure 1C). This assembly allows the labeling of a plasmid-born target gene cluster in a single step. In this configuration, one T7 promoter necessarily cannot be directly adjoined to the target genes. However, due to its unique properties described above, T7RP is potentially capable of "reading through" the vector backbone. The construction of the TREX cassettes is described in the Supplementary Methods.

TREX-Mediated Functional Expression of a Recombinant Carotenoid Biosynthesis Pathway. The applicability of the TREX system was tested by expression of a 6.9-kb gene cluster derived from *Pantoea ananatis*[33] that encompasses six genes (*crtEXYIBZ*) essential for the biocatalytic conversion of the terpenoid precursor farnesyl pyrophosphate (FPP) into the carotenoids *β*-carotene, zeaxanthin, and zeaxanthin-*β*-D-diglucoside (Figure 2A).

These carotenoids have gained interest for different applications including their use as nutraceuticals or pharmaceuticals.[34] The *crt* cluster contains two opposing transcriptional units with *crtEXYIB* under control of promoter P_{crtE} and the monocistronic *crtZ* operon whose expression is under control of promoter P_{crtZ}. For this study, we chose a commonly used variant of the cluster designated as *crt*Δ*X* in which the *crtX* gene is inactivated by a partial deletion so that the corresponding pathway leads to synthesis of zeaxanthin (Figure 2A). The TREX system serves to express natural gene clusters; we thus used the *crt* cluster encompassing all DNA elements affecting *crt* gene transcription in *P. ananatis* including the original promoters.

In order to comparatively express the clustered carotenoid biosynthesis genes in the non-carotenogenic hosts *P. putida* KT2440 and *E. coli* BL21 (DE3) as well as in the carotenogenic host *R. capsulatus* B10S, we first labeled the zeaxanthin biosynthesis cluster *crt*Δ*X* localized on nonreplicating suicide plasmids (i.e., pUC18 for *P. putida* and *R. capsulatus* or its derivative pUC18ts, containing a temperature sensitive ori, for *E. coli*) by using the <L-TREX-R> module (Figure 1C).

The Gram-negative bacterium *P. putida* has been shown to be a versatile host suitable for the expression of secondary metabolite pathways and thus for the production of various valuable compounds including carotenoids.[6,35,36] Therefore, we initially constructed recombinant *P. putida* strains capable of TREX-mediated expression of clustered *crt* gene. The pUC18-derivative pTREX-crtΔ*X* was transferred to *P. putida via* biparental mating. In this vector, the T7 promoter of the L-TREX cassette is located adjacent to P_{crtE}, whereas the second T7 promoter of the R-TREX cassette is separated from the *crtZ* promoter by a 3.1-kb vector fragment (Figure 2B). As the narrow-host-range ColE1 origin that originates from the vector

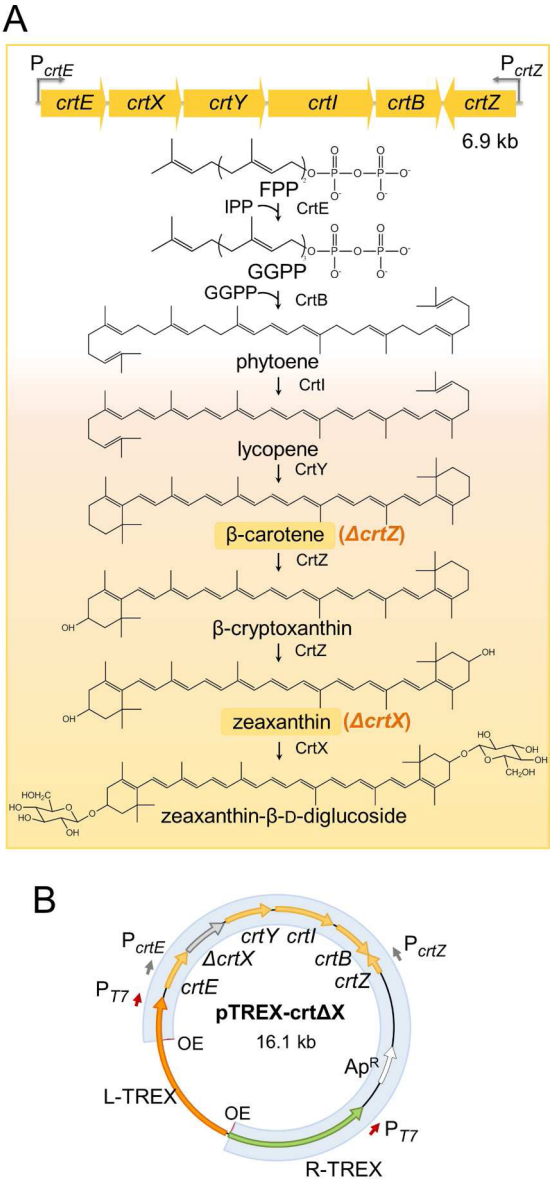

Figure 2. Carotenoid biosynthesis gene cluster from *P. ananatis* used for TREX implementation. (A) The carotenoid biosynthesis gene cluster from *P. ananatis* consists of six genes in two oppositely located transcriptional units. The *crt* genes encode all enzymes necessary for zeaxanthin-*β*-D-diglucoside synthesis.[33] The color shade indicates the individual color of each compound. In this work, two variants were used as indicated: pathway variant crtΔ*Z* (harboring the deletion Δ*crtZ*) leads to *β*-carotene synthesis, whereas in variant crtΔ*X* (with deletion Δ*crtX*) zeaxanthin is formed. FPP, farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate; GGPP, geranyl-geranyl pyrophosphate; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase; CrtY, lycopene cyclase; CrtZ, *β*-carotene hydroxylase; CrtX, zeaxanthin glucosyl transferase. (B) The vector pTREX-crtΔ*X* contains pathway variant crtΔ*X* adjoined by the TREX cassettes. The *crtX* derivative harboring a deletion is marked in gray. Promoters from *crt* cluster (P_{crtE} and P_{crtZ}) as well as T7 promoters (P_{T7}) from TREX cassettes are indicated. The transposing region, determined by the outside ends (OE) of the recombinant TREX transposon is highlighted in blue. Ap^R, ampicillin resistance gene.

pUC18 is not recognized by *P. putida* DNA polymerases, this vector can directly be used as a suicide plasmid. Thus, maintenance of the heterologous gene cluster can only be

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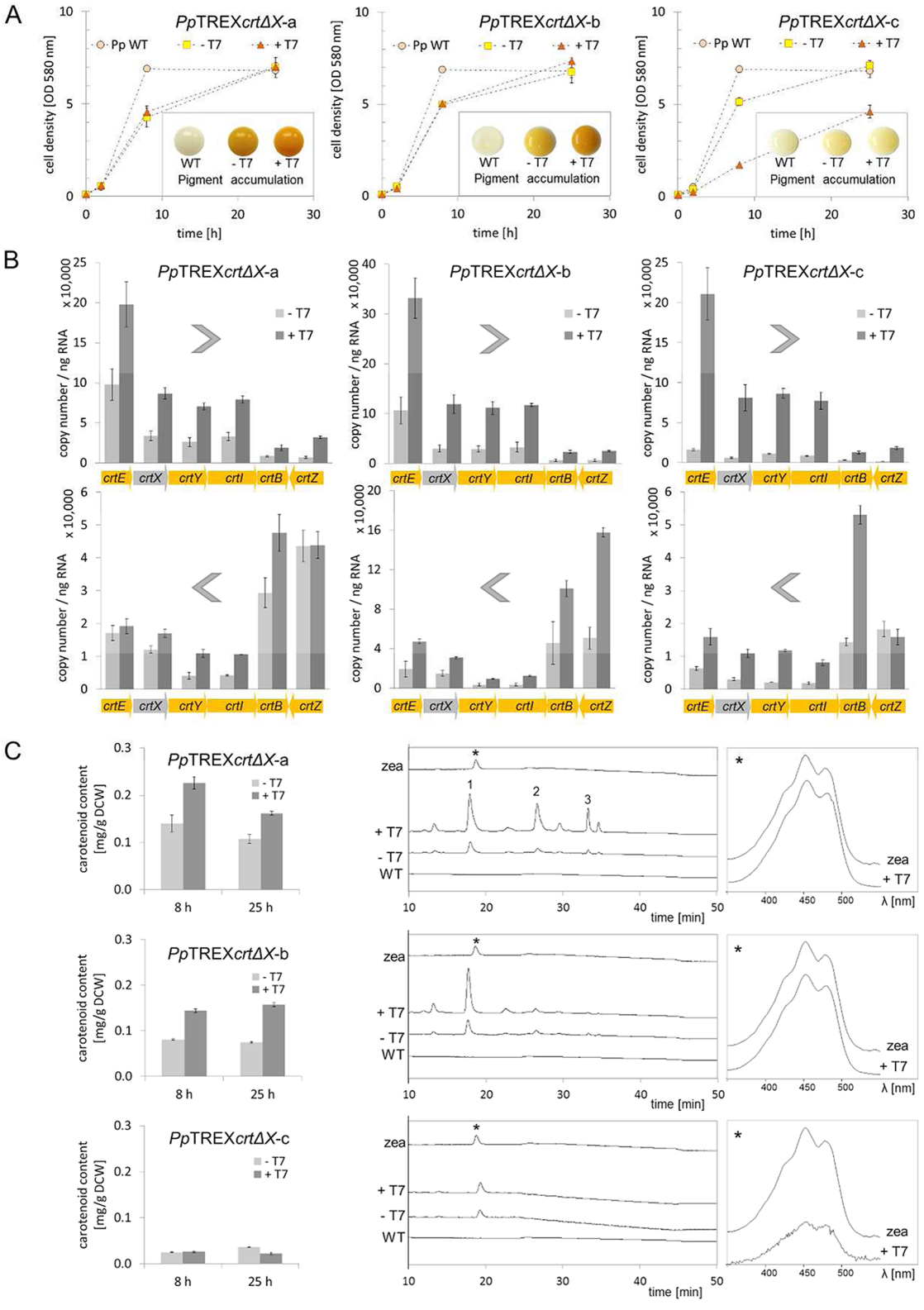

Figure 3. TREX-mediated carotenoid pathway expression in *P. putida*. (A) Random insertion of TREX-crtΔXresulted in phenotypically different *P. putida* strains, designated as *Pp*TREX*crt*Δ*X*-a, -b, and -c, exhibiting distinct growth curves and pigmentation. (B) Quantification of *crt* sense and antisense transcripts in *Pp*TREX*crt*Δ*X*-a, -b, and -c in the absence (−T7) and presence (+T7) of the T7RP *via* RT-qPCR. Arrow heads indicate direction of transcription along the *crt* cluster. Functionally deleted gene *crtX* is marked gray. (C) Analysis of product accumulation within TREX-crtΔX-modified *P. putida* strains in terms of quantity and quality. Extracts from cell cultures were assayed photometrically for overall carotenoid content at 450 nm and analyzed qualitatively using HPLC. HPLC profiles identified: 1, zeaxanthin; 2, *β*-cryptoxanthin; 3, *β*-carotene. Chromatogram and absorption spectrum of the end product zeaxanthin (zea) is included in each diagram. WT, wild type *P. putida* KT2440; * indicates the carotenoid fractions of which absorption spectra are shown (λ = 350−550 nm). Values represent means from three independent measurements. Error bars indicate the respective standard deviations.

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accomplished by random chromosomal integration *via* transposition of the recombinant transposon TREX*crt*Δ*X*.

Conjugational transfer and chromosomal integration of TREX*crt*Δ*X* successfully provided gentamicin-resistant clones with frequencies from 10^{-5} to 10^{-6}. The detected frequencies correspond to the transposition rate of the original Tn*5* transposon (6.5×10^{-5})[37] suggesting that the *crt* gene cluster is not affecting the transposition rate. Presence of *crt* genes in the chromosome of 10 randomly chosen clones was verified by *crt*-specific PCR with genomic DNA as template. Remarkably, after TREX-mediated transposition of *crt* genes, *P. putida* clones showed variable pigmentations with few white to many intensive yellow clones, the latter indicating a basal T7RP-independent *crt* expression in *P. putida*.

The efficiency of bidirectional T7RP-dependent expression of TREX-labeled *crt* genes in *P. putida* was analyzed using three phenotypically different clones with an intensive (*Pp*TREX*crt*Δ*X*-a) or a light yellow pigmentation (*Pp*TREX*crt*Δ*X*-b) as well as a colorless clone (*Pp*TREX*crt*Δ*X*-c). The T7RP-encoding plasmid pML5-T7[11] was transferred into these strains and cell growth, *crt* gene transcription and product formation were subsequently analyzed in the presence and absence of T7RP.

The results of the experiments shown in Figure 3 can be summarized as follows:

(i) *P. putida* strains *Pp*TREX*crt*Δ*X*-a and *Pp*TREX*crt*Δ*X*-b exhibited an intensified yellow pigmentation in the presence of the T7RP (Figure 3A), whereas higher pigment accumulation did not lead to any retardation of cell growth. Surprisingly, the nonpigmented clone *Pp*TREX*crt*Δ*X*-c remained colorless also in the presence of the phage polymerase while clearly showing impaired growth.

(ii) TREX (and thus T7RP)-mediated bidirectional *crt* transcription was quantified by determination of the copy number of all *crt* sense and antisense transcripts including the truncated transcript of the partially deleted *crtX* gene by RT-qPCR. As shown in Figure 3B, weak expression of *crt* genes in the absence of T7RP resulted in the formation of sense and antisense transcripts at low concentrations. The basal T7RP-independent expression might be due to recognition of original *P. ananatis* promoters by *P. putida* RNA polymerases or because of polymerase read-through phenomena potentially occurring at *P. putida* promoters located adjacent to the random TREX*crt*Δ*X* integration sites. However, T7RP-dependent transcription of *crt* genes resulted in an up to 10-fold increase of the corresponding transcript levels throughout the *crt* cluster in both directions. In this context, expression of a reversely orientated *crtZ* gene poses an exception to the described observation since presence of T7RP did not lead to uniformly elevated sense transcripts in all chosen strains (Figure 3B, lower panel).

(iii) Comparison of *crt* transcript copy numbers within the individual *P. putida* clones further revealed variable mRNA levels of single cluster genes and even of a joint transcription unit (i.e., *crtEXYIB*), but the overall expression pattern of the multigene cluster was nevertheless highly conserved irrespective of the presence or absence of T7RP (Figure 3B). In addition, when comparing the net amount of *crt* transcripts within the chosen *P. putida* strains, we found that *crt* transcription efficiency is apparently affected by the insertion locus of the recombinant transposon TREX*crt*Δ*X*, since mRNA concentrations of the target genes are 1.5−4 times higher in *Pp*TREX*crt*Δ*X*-b than in *Pp*TREX*crt*Δ*X*-a and *Pp*TREX*crt*Δ*X*-c. Interestingly, consistencies and differences of *crt* expression patterns and transcript levels within the individual TREX strains did not inevitably lead to the same effects on cell growth as well as product formation (see (i) and (iv)).

(iv) The individual mutations of the three *Pp*TREX*crt*Δ*X* strains that are caused by randomized TREX insertion obviously affected the metabolic rather than the transcriptional level. As shown in Figure 3C, the characterized *P. putida* strains exhibited major differences with respect to carotenoid accumulation and metabolic flux. In detail, T7RP-mediated *crt* expression resulted in 1.5- to 2-fold higher carotenoid formation in *Pp*TREX*crt*Δ*X*-a and *Pp*TREX*crt*Δ*X*-b in comparison to the corresponding strains that did not harbor the phage polymerase gene. In both carotenoid-producing T7RP strains, maximal pigment content was reached in the logarithmic growth phase with product yields of 226 μg/g dry cell weight (DCW) (*Pp*TREX*crt*Δ*X*-a) and 144 μg/g DCW (*Pp*TREX*crt*Δ*X*-b), respectively. In contrast, *Pp*TREX*crt*Δ*X*-c showed almost no carotenoid accumulation, despite its high *crt* transcription levels and its remarkable growth impairment in response to expression. Detailed analysis of the formed products revealed that, beside the expected end product zeaxanthin, the intermediates β-carotene and β-cryptoxanthin could be detected in *Pp*TREX*crt*Δ*X*-a whereas strain *Pp*TREX*crt*Δ*X*-b almost exclusively synthesized zeaxanthin, thereby again underpinning the relevance of individual TREX integration sites on functional expression of the biosynthetic pathway.

In summary, the novel pathway transfer and expression system made it possible to establish the complete biosynthetic pathway for zeaxanthin production in the bacterium *P. putida.* Not surprisingly, our findings furthermore illustrate that distinct transposon insertion sites are of particular significance for cell growth, transcription efficiency, and synthesis of target metabolites. Similar observations have been made in *E. coli* and *Saccharomyces cerevisiae*, where the level of transcripts varied remarkably in dependence on the chromosomal integration site.[38,39] In order to avoid this phenomenon for TREX application and thus minimize the number of clones that have to be screened for product formation, one could potentially employ site-specific transposition, for example, by using transposon Tn*7*.[40] However, in contrast to Tn*5*, this transposon is functional only in bacteria harboring the respective chromosomal integration sites. Since we could demonstrate that the changed mutant physiology as well as the local target gene expression might play an important role in successful establishment of a certain pathway within bacterial screening hosts, only Tn*5*-based integration of pathway genes further opens the opportunity for combined modulation of gene expression and metabolic properties.

Broad Host Range Applicability of the TREX System. Comparative pathway expression in various (also engineered) hosts with different and unique physiological properties offers a new and promising strategy. The applicability of the TREX system in a range of different bacteria allows a selection of hosts exhibiting potentially favorable intrinsic physiological properties. As an example, we chose the carotenogenic bacterium *R. capsulatus* to express the *crt* gene cluster. First, we engineered its natural carotenoid biosynthetic pathway by deleting the neurosporene 1,2-hydratase-encoding *crtC* gene. The corresponding strain *R. capsulatus* Δ*crtC* exclusively accumulated neurosporene instead of its natural carotenoid products

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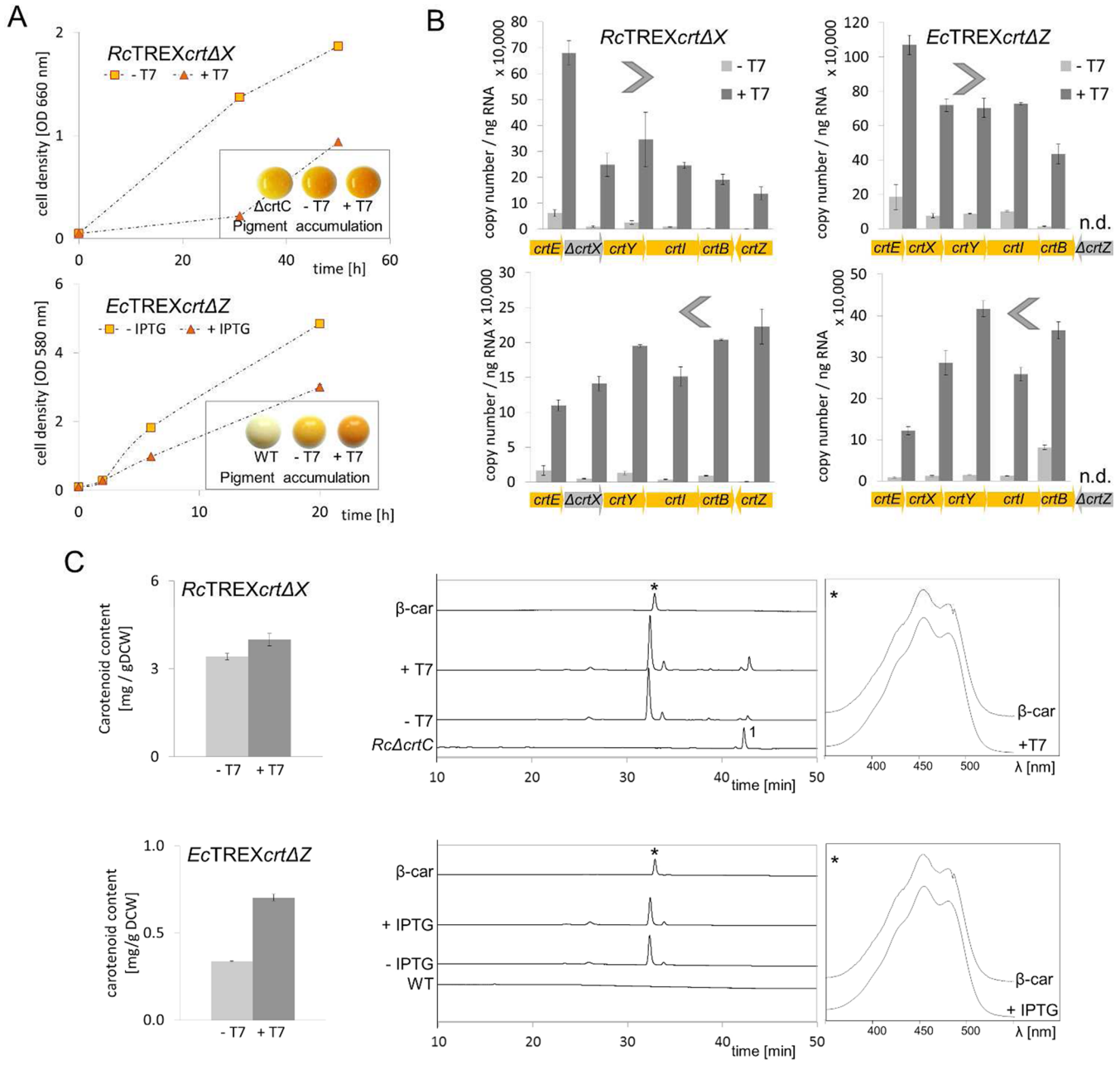

Figure 4. Broad host range application of the TREX system. (A) Growth curves and pigmentation phenotypes of *R. capsulatus* and *E. coli* harboring the TREX-labeled *crt* genes from *P. ananatis*. (B) Analysis of *crt* transcripts in both expression hosts; −T7: absence of T7RP, +T7: presence of T7RP. Genes *crtX* and *crtZ* are marked in gray where deleted. n.d., not determined. Arrow heads indicate direction of transcription along the *crt* cluster. Functionally deleted *crtX* and *crtZ* genes are marked gray. (C) Analysis of carotenoid production and quality in *R. capsulatus* and *E. coli*. Extracts from cell cultures were assayed photometrically for overall carotenoid content at 450 nm and analyzed qualitatively using HPLC. HPLC profiles identify *β*-carotene of the *P. ananatis* pathway. Chromatogram and absorption spectrum of standard *β*-carotene is indicated (*β*-car). 1, intrinsic *R. capsulatus* Δ*crtC* carotenoid neurosporene; *Rc*Δ*crtC*, *R. capsulatus* Δ*crtC*; WT, *E. coli* BL21 (DE3). * indicates the carotenoid fractions of which absorption spectra are shown (λ = 350−550 nm). Values represent means from three independent measurements. Error bars indicate the respective standard deviations.

spheroidene and spheroidenone (Supplementary Figure S1), which in turn may directly be converted to zeaxanthin by the *crt* gene products from *P. ananatis*.

To redirect carotenoid synthesis in *R. capsulatus*, the plasmid pTREX-crtΔ*X* (Figure 2B) was transferred to *R. capsulatus* mutant strain Δ*crtC*, and transposon mutants could be selected with a transposition efficiency of 10^{-6} to 10^{-8}. Surprisingly, in contrast to *P. putida*, only one *R. capsulatus* clone was observed that showed a considerable change in pigmentation, again indicating a basal expression of the heterologous gene cluster. This *R. capsulatus* clone (*Rc*TREX*crt*Δ*X*) was chosen for further investigation. To allow T7RP-dependent expression studies in *Rc*TREX*crt*Δ*X*, vector pML5-P_{fru}T7, which encompasses the T7RP gene controlled by a *Rhodobacter*-specific fructose inducible promoter,[11] was used.

*Rc*TREX*crt*Δ*X* exhibited a strongly impaired growth under T7RP expressing conditions, but the presence of T7RP did not lead to a significant increase of pigmentation (Figure 4A).

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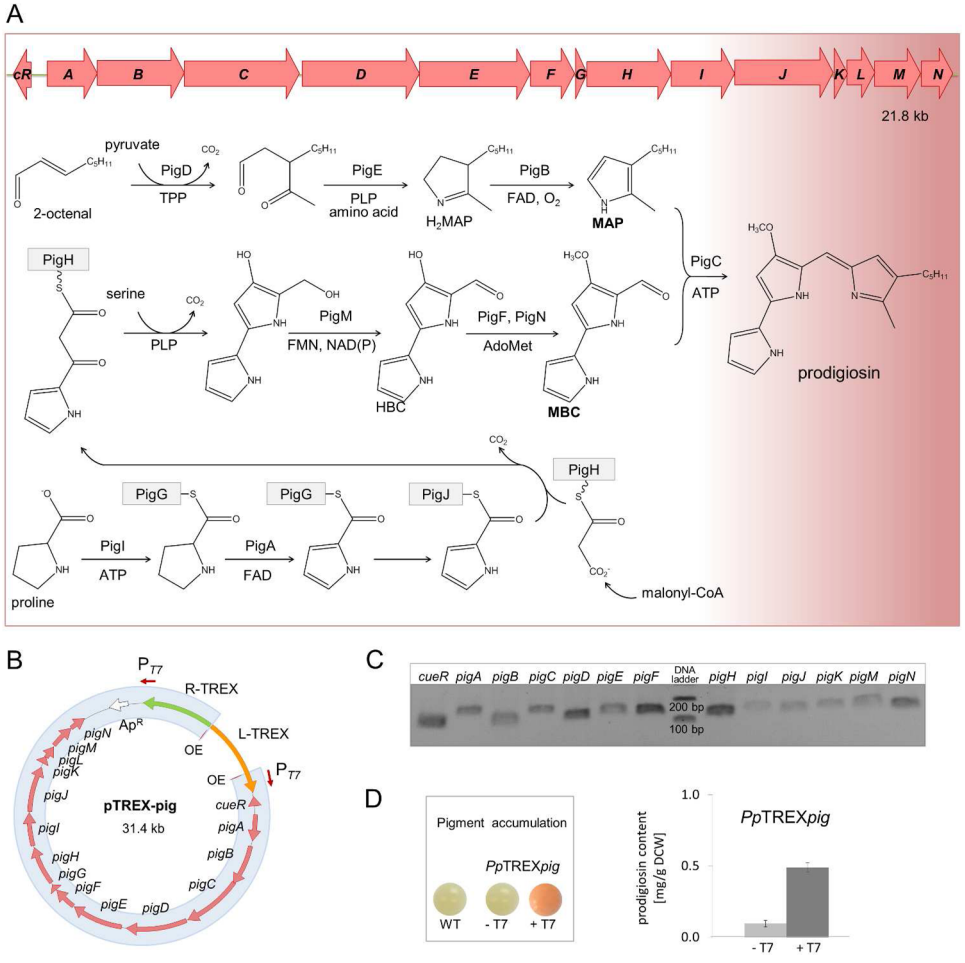

Figure 5. TREX applicability for the expression of large gene clusters. (A) The *pig* cluster from *S. marcescens* consists of 14 uniformly oriented *pig* genes encoding all enzymes essential for the bifurcated prodigiosin synthesis.[47] For this study, the full length *pig* cluster including divergently oriented regulatory *cueR* (cR) was used for TREX implementation. The red shade indicates that only the end-product prodigiosin exhibits a red coloration. TPP, thiamine pyrophosphate; PLP, pyridoxal phosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide; AdoMet, *S*-adenosylmethionine; ATP, adenosine triphosphate; MAP, 2-methyl-3-*n*-amyl-pyrrole; HBC, 4-hydroxy-2,2′-bipyrrole-5-cabaldehyde; MBC, 4-methoxy-2,2′-bipyrrole-5-carbaldehyde. (B) Plasmid pTREX-pig encompasses the entire *pig* gene cluster and the L-TREX (orange) and R-TREX (green) cassettes. T7 promoters from TREX cassettes are indicated (P_{T7}). The transposing region, marked by the outside ends (OE) of the recombinant TREX transposon, is labeled in blue. Ap^R, ampicillin resistance gene. (C) TREX-mediated *pig* delivery in *P. putida* generated strain *Pp*TREX*pig* in which sense transcript of all *pig* genes and *cueR* was detectable by reverse transcription-PCR. PCR products are separated using an agarose gel. (D) Pigmentation and prodigiosin production in *Pp*TREX*pig* in the presence (+T7) and absence (−T7) of T7RP. Prodigiosin accumulation was determined photometrically in cell extracts at 535 nm. WT, *P. putida* wild type KT2440. Values represent means from three independent measurements. Error bars indicate the respective standard deviations.

However, transcription levels of *P. ananatis crt* genes were highly upregulated by T7RP expression with an averaged induction factor of about 50 (Figure 4B). Remarkably, despite T7RP-mediated high-level expression of target genes, carotenoid accumulation is only slightly enhanced in the *R. capsulatus* strain harboring the specific polymerase (Figure 4C). Nevertheless, TREX-mediated pathway expression resulted in an 18-fold higher carotenoid synthesis as compared to *P. putida* with a maximum yield of 4.0 mg/g DCW. Detailed analysis of the accumulated pigments showed that carotenoid biosynthesis of *R. capsulatus* was almost quantitatively redirected to *P. ananatis* pigments (Figure 4C). Apparently, in contrast to *P. putida*, basal expression of heterologous *crt* genes in *R. capsulatus* already produced sufficient amounts of enzymes capable of fully converting the provided precursor neurosporene. Unexpectedly, only *β*-carotene instead of zeaxanthin could be detected within *Rc*TREX*crt*Δ*X*, although *crtZ*, whose gene product catalyzes the 2-fold hydroxylation of *β*-carotene to zeaxanthin, was sufficiently transcribed. We therefore examined the *crtZ* sequence of TREX*crt*Δ*X* in the *R. capsulatus* genome. Sequence analysis of *crtZ* revealed a single point mutation leading to a substitution of Thr13 to Ala within the corresponding gene product, which is probably responsible for the inactivation of the enzyme. Thus, the low yield of pigmented *Rhodobacter* clones after TREX transposition as well as the appearance of a spontaneous *crtZ* mutation suggests that formation of zeaxanthin might be deleterious to the photosynthetic bacterium.

To further analyze TREX broad-host-range applicability, we transferred the *P. ananatis* carotenoid biosynthetic pathway to the standard expression host *E. coli*. Here, the *crt* gene cluster with a deleted *crtZ* gene (*crt*Δ*Z*) instead of *crt*Δ*X* was used, thereby allowing direct comparison of *β*-carotene yields in both bacteria. *E. coli* BL21 (DE3) was transformed with plasmid ptsTREX-crtΔZ, which is a temperature-sensitive analogue of the usual pTREX derivatives. Here, temperature-sensitive

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replication of ptsTREX-crtΔZallowed direct selection of corresponding *E. coli* transposon mutants at 37 °C incubation temperature. One of the pigmented clones (*Ec*TREX*crt*Δ*Z*) obtained was subsequently subjected to comparative expression studies.

In contrast to the findings with *R. capsulatus*, growth of *E. coli* cells was only moderately impaired under conditions where T7RP-dependent expression of *crt* genes was induced (Figure 4A). Furthermore, yellow coloration of *Ec*TREX*crt*Δ*Z* cultures significantly increased upon induction of *crt* gene expression. Quantitative analysis of *crt* sense and antisense transcripts again revealed a concerted bidirectional expression of all pathway genes, thereby demonstrating the robustness of the TREX expression module in different expression hosts. In *E. coli*, T7RP expression led to about 15-fold higher transcription of the *crt* genes in both sense and antisense direction, compared to the noninduced control culture (Figure 4B). Notably, maximal *β*-carotene accumulation in *E. coli* (net yield = 0.7 mg/g DCW) was eight times lower than in *R. capsulatus*, although overall transcription of the corresponding genes was much higher than in *R. capsulatus* (Figure 4C).

Apparently, TREX-dependent reconstitution of metabolic pathways inevitably results in different cellular levels of the targeted metabolites, mainly due to individual inherent metabolic constrains of the host organisms. This observation was further substantiated by transferring TREX*crt*Δ*Z* to *P. putida*. As expected, TREX-dependent establishment of the *β*-carotene pathway resulted in yields of *β*-carotene in *P. putida* (123 *μ*g/g DCW) comparable to those obtained for zeaxanthin (Supplementary Figure S2). In conclusion, comparative expression of the *P. ananatis crt* gene cluster in different bacterial hosts clearly demonstrated the broad host range applicability of the TREX system.

It is accepted that expression of a complex biosynthetic pathway challenges the individual protein expression machinery and the biosynthetic background of a respective host strain.[2,8,41] Here, we demonstrated that different physiological properties of *P. putida*, *E. coli*, and *R. capsulatus* had a clear impact on the formation of the heterologous metabolites. Our data thus corroborate that efficient genome mining should also involve the screening for appropriate natural or engineered host strains whose physiology is suitable for the synthesis of specific classes of natural compounds. In this context, it should be mentioned that the host range of TREX is limited by (i) the conjugational transfer, (ii) the transposition event, as well as (iii) the ability of a host organism to express the T7RP. Functionality of the respective elements that define the applicability and host range of the TREX system in terms of mobilization (i.e., oriT) and transposition (i.e., transposase gene and OE) of the pathway genes have been demonstrated in numerous Gram-negative bacteria.[20,27,42] Furthermore, successful expression of the T7RP in different hosts can easily be achieved by using an appropriate broad host range vector such as pML5-based plasmids,[43,44] which enable P_{lac}- or P_{fru}-mediated expression of T7RP in all host strains tested.[43,45]

TREX-Mediated Expression of the Prodigiosin Gene Cluster. The applicability of TREX for expression also of larger gene clusters was analyzed using the 22-kb prodigiosin (*pig*) gene cluster from *Serratia marcescens* ATCC274.[46] Prodigiosin is a red-pigmented tripyrrolic compound that has gained renewed interest as it exhibits numerous biological activities including antifungal, antiprotozoal, immunosuppressive, and anticancer effects.[47] The *pig* cluster used here consists of 14 *pig* genes (*pigA*−*pigN*) and *cueR*, whose gene products are involved in the regulation and the bifurcated multistep synthesis of this secondary metabolite (Figure 5A).

After conjugational transfer of pTREX-*pig* (Figure 5B) to *P. putida*, the resulting transposon mutants appeared with the same efficiency (10^{-5} to 10^{-6}) as already observed for the *crt* gene cluster. Thus, the transposition rate is obviously not affected by the size of the targeted gene cluster within the tested range (the recombinant TREX-*pig* transposon encompassing the *pig* cluster as well as the TREX elements had a size of almost 30 kb). Analysis of sense transcripts corroborated the simultaneous T7RP-dependent expression of all genes flanked by the respective promoters of the TREX cassettes (Figure 5C). In contrast, no mRNA was detectable in the absence of the T7RP (data not shown). According to these findings, *P. putida* strain *Pp*PATEX*pig* displayed red pigmentation only in the presence but not in the absence of the phage polymerase (Figure 5D). Spectroscopic as well as mass spectrometric analyses (Supplementary Figure S3) revealed that prodigiosin was synthesized in *P. putida* harboring the T7RP with a net yield of 490 *μ*g/g DCW. Apparently, T7RP is able to simultaneously synthesize complementary transcripts and thus functionally express unmodified gene clusters of at least 22 kb size. Furthermore, the convergent expression mode of the TREX system abolishes laborious reassembly of complex gene clusters including promoter insertion that was applied, e.g., for expression of validoxylamine A, zeaxanthin, and erythromycin A encoding gene clusters in *Streptomyces lividans* and *E. coli*.[48−50] Simultaneous synthesis of sense and antisense transcripts might hamper transcription, due to collision of convergently transcribing polymerases as well as posttranscriptional processes including mRNA processing or translation inhibition by complementary mRNAs.[51] Although more detailed analyses remain to be executed, the TREX-driven formation of carotenoids and prodigiosin in considerable amounts clearly demonstrated the functionality and robustness of bidirectional expression. The broader applicability of TREX remains to be examined using more complex gene clusters of larger size.

In summary, TREX is the first toolkit that enables the transfer, randomized genome integration, and bidirectional expression of complex gene clusters in parallel in a broad range of bacterial hosts. Consequently, TREX allows the transplantation of pathways to versatile screening hosts in a plug-and-play fashion and thus will offer new perspectives in the fields of genome mining, metagenome screening, and synthetic biology.

METHODS

Bacterial Strains and Culture Conditions. *Escherichia coli* strains DH5*α*,[52] S17-1,[53] and recombinant TREX-strains were grown at 37 °C under constant shaking (120 rpm) in Luria−Bertani (LB) medium.[54] *Pseudomonas putida* wild type KT2440[36] and mutant strains were grown at 30 °C under constant shaking (120 rpm) in LB medium. *Rhodobacter capsulatus* B10S and Δ*crtC* (see Supplementary Methods) as well as TREX-modified strains were grown microaerobically in the dark at 30 °C using RCV medium. Plasmids were introduced into *R. capsulatus* and *P. putida* by conjugational transfer as described before[55] using *E. coli* S17-1 as a donor strain or into *E. coli* using transformation, respectively.

Antibiotics were added to the culture medium to the following final concentrations [*μ*g/mL]: *E. coli*: 100 (ampicillin), 50 (kanamycin), 10 (gentamicin, tetracycline); *P. putida*:

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25 (gentamicin), 50 (tetracycline); *R. capsulatus*: 10 (spectinomycin), 4 (gentamicin), 0.25 (tetracycline).

General DNA Manipulations. Recombinant DNA techniques were performed essentially as described by Sambrook et al.[54] All plasmids and PCR primers used in this study are listed in Supplementary Table S1 and S2, respectively. Detailed descriptions of vector constructions are given in Supplementary Methods.

Implementation of TREX for *crt* Gene Expression in *P. putida*. To create *P. putida crt* expression strains, *E. coli* S17-1 was transformed with plasmid pTREX-crtΔX, which was further transferred to *P. putida* by conjugation. In *P. putida*, pTREX-crtΔ*X* is a suicide vector. Positive selection for transposon mutants was conducted by using LB medium supplemented with 25 μg/mL gentamicin. For T7RP-dependent *crt* gene expression, vector pML5T7 was transferred *via* conjugation to three *P. putida* clones that exhibited different coloration phenotypes. For T7RP-mediated *crt* gene expression, *P. putida* cells were grown within a 50 mL culture. After reaching the exponential growth phase (OD_{580} = 0.5), T7 RNA polymerase dependent *crt* expression was induced by adding 0.5 mM IPTG.

Implementation of TREX for *crt* Gene Expression in *R. capsulatus*. To generate adequate *R. capsulatus* TREX expression strains, plasmid pTREX-crtΔ*X*was introduced into *R. capsulatus* mutant strain Δ*crtC via* conjugation using donor strain *E. coli* S17-1 as described before.[55] In *R. capsulatus*, pTREX-crtΔ*X* is a suicide vector. Selection for gentamicin-resistant clones identified transposon mutant *Rc*TREX*crt*Δ*X*. For T7RP-dependent *crt* gene expression, plasmid pML5-P_{fru}T7 was introduced *via* conjugation. For *crt* expression studies, *R. capsulatus* was grown microaerobically in the dark, starting with a cell density OD_{660} of 0.05. For induction of T7RP-dependent expression, 8 mM fructose was added to the medium.

Implementation of TREX for *crt* Gene Expression in *E. coli*. *E. coli* T7RP expression strain BL21 (DE3) was used for TREX-mediated *crt* expression. Therefore, plasmid ptsTREX-crtΔZ, which comprises temperature-sensitive ori101, was transformed. After a recovery phase at 30 °C, *E. coli* cells were incubated at 37 °C to allow for selection of transposon mutants *Ec*TREX*crt*Δ*Z* that showed gentamicin resistance and loss of tetracycline resistance. For *crt* gene expression T7 RNA polymerase dependent *crt* expression was induced by adding 0.5 mM IPTG when cultures entered exponential phase (OD_{580} = 0.5).

Transcript Analysis of *crt* Genes Using qPCR. Cells were harvested from expression cultures during late exponential growth phase at cell densities of OD_{580} = 3−4 for *E. coli* and *P. putida* and OD_{660} = 1−1.5 for *R. capsulatus*. For slow growing cultures *Pp*TREX*crt*Δ*X*-c + pML5T7 and *Rc*TREX*crt*Δ*X* + pML5-P_{fru}T7 samples were taken at lower cell densities. Total RNA was extracted from biological triplicates using RNeasy Protect Bacteria Mini Kit (Qiagen). DNA contaminations were removed by treatment with DNase (Promega). To specifically quantify the sense and antisense transcript of each *crt* gene, the corresponding single stranded cDNA was synthesized in a first reaction. Therefore, 900 ng (*P. putida*), 300 ng (*R. capsulatus*), or 1,000 ng (*E. coli*) total RNA was subjected to reverse transcription using the High Capacity cDNA RT Kit (Applied Biosystems) with 20 pmol of a strand-specific *crt* primers in single 20 μL reactions. Quantitative real time PCR (qPCR) was performed on ABI-Applied Biosystems 7900 HT Thermal Cycler using power SYBR Green PCR Mastermix (Applied Biosystems) with standard cycling protocols in 20 μL reactions. qPCR was performed using 2 μL of each strand specific cDNA template and 200 nM of respective primers. In all cases, the size of the resulting PCR products was about 75 bp. All primers used for qPCR are listed in Supplementary Table S2. For data evaluation we proceeded according to MIQE guidelines[56] considering assay precision, specificity, and PCR efficiency. Precision determination revealed an error of <0.21 cycles, using templates in triplicate reactions. Furthermore, we used negative controls without reverse transcriptase to exclude DNA contamination. To demonstrate primer specificity, we routinely analyzed the melting curves of all PCR products as well as their product sizes on agarose gels. To take individual PCR primer performances into account, PCR efficiencies were determined by means of calibration curves as described before.[56] Cq values of each qPCR reaction were used to calculate transcript copy numbers by means of plasmid DNA-based standard calibration curves.

Carotenoid Analysis. Cell material corresponding to OD_{580} = 4 (*P. putida* and *E. coli*) or OD_{660} = 0.2 (*R. capsulatus*) was harvested by centrifugation. Before extraction with 1 mL ethanol, cells were resuspended in 50 μL H_2O. Pigment extracts were cleared by centrifugation and absorption at 450 nm was determined spectrophotometrically. Total carotenoid yield was assessed using molar extinction coefficients (β-carotene: ε = 140,500 (l mol^{-1} cm^{-1}); zeaxanthin: ε = 144,500 (l mol^{-1} cm^{-1})) based on published specific extinction coefficients.[57]

For HPLC chromatography, cell material was harvested by centrifugation. Afterward, carotenoids were extracted with 500 μL acetone at 50 °C for 5 min from cells corresponding to an optical density (OD_{580} for *E. coli* and *P. putida*, OD_{660} for *R. capsulatus*) of 15. Cell debris was removed by centrifugation, and supernatant was mixed with 500 μL H_2O. The mixture was extracted with 2 mL petrolether. The pigmented ether phase was collected and dried under a soft flow of nitrogen. Pigments were resolved with 15 μL hexane and 200 μL acetonitrile and subjected to HPLC analysis. All extraction steps were performed in darkness or under low light. The chromatographic separation method was developed based on work by de Azevedo-Meleiro and Rodriguez-Amaya.[58] Analysis was performed on LC-10Ai series (Shimadzu) equipped with SPD-M10Avp photodiode array detector and installed with LabSolution/LCSolution program version 1.22SP1. Here, 20 μL samples were injected onto a C30-reverse-phase HPLC column (250 mm × 4.6 mm ID, S-5 μm, YMC-Europa GmbH) attached to a guard column filled with the same material (20 mm × 4.0 mm i.d.). The column oven temperature was maintained at 25 °C. Solvent flow rate was 1 mL/min. The mobile phase consisted of methanol, acetonitrile (containing 0.05% of triethylamine) and ethyl acetate. A gradient was applied from 15:70:15 (v/v/v) to 20:60:20 (v/v/v) in 20 min, proceeding to 5:15:80 (v/v/v) in additional 20 min and maintaining this proportion for 10 min. The column was re-equilibrated after each run applying starting conditions of 15:70:15 (v/v/v). Chromatograms were run on 450 nm. The diode array detector was used to scan wavelengths from 350 to 550 nm. Carotenoids were identified by comparison of retention times and absorbance spectra with authentic standards. Lycopene, β-carotene, β-cryptoxanthin, and zeaxanthin were purchased from DHI, and neurosporene was from CaroteNature GmbH.

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TREX-Dependent *pig* Gene Expression in *P. putida*. TREX-mediated delivery of prodigiosin genes to *P. putida* was basically performed as already described for *crt* genes. For prodigiosin synthesis, *P. putida* strain *Pp*TREX*pig* as well as the control strains were incubated at 25 °C. When cultures entered the exponential phase (OD_{580} = 0.5), T7 RNA polymerase dependent *pig* expression was induced by adding 0.5 mM IPTG. After cultivating the *P. putida* strains to an optical density of 0.9−1 at 580 nm, cells were harvested, and RNA was extracted using RNeasy Protect Bacteria Mini Kit (Qiagen). DNA contaminations were removed by treatment with DNase (Promega). Specific cDNA of *pig* sense transcripts was obtained applying RevertAid Synthesis Kit (Fermentas) with 150 ng isolated total RNA as template. For the genes *pigG* and *pigL* no cDNA could be generated as no appropriate primers were found. Obtained cDNA was used as template in a standard PCR reaction. The primer sequences utilized for transcript detection are listed in Supplementary Table S2. Samples were applied to agarose gel electrophoresis to visualize PCR products. Prodigiosin formation was determined photospectrometrically at λ = 535 nm. Cells according to a cell density of 1 at 580 nm were harvested by centrifugation and prodigiosin was extracted with 1 mL acidified ethanol (4% of 1 M HCl). Extracts were cleared by centrifugation and pigment accumulation was quantified based on molar extinction coefficient ε = 110,000 at 535 nm.[59]

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures present the phenotype of *R. capsulatus* Δ*crtC*, verification of β-carotene accumulation in *Pp*TREX*crt*Δ*Z* and of prodigiosin accumulation in *Pp*TREX*pig*, the construction of the TREX cassettes as well as plasmid maps of pUC18crtΔ*X* and pUC18crtΔZ. Supplementary tables list all vectors and oligonucleotides used in this study. Supplementary methods describe the generation and characterization of *R. capsulatus* Δ*crtC*. Detailed sequence information of vector pIC20H-RL is provided in GenBank format. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Author Contributions

F.R., S.W., and T.D. developed the idea for the TREX system. A.L., A.M., and T.D. designed the experiments and analyzed the data. A.L., A.M., and A.W. performed the experiments. A.L., K.-E.J., and T.D. wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): Part of this work was included in patent application number DE 10-2007-048134 A1.

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■ ABBREVIATIONS

TREX, tool for the transfer and expression of biosynthetic pathways in bacteria; T7RP, T7 RNA polymerase; L-TREX, element of the TREX system: left TREX cassette; R-TREX, element of the TREX system: right TREX cassette; <L-TREX-R>, module of the TREX system comprising both L-TREX and R-TREX cassette

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