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ORIGINAL PAPER

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Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria

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Abstract A highly efficient method of transposon mutagenesis was developed for genetic analysis of *Xanthobacter autotrophicus* Py2. The method makes use of a transposon delivery vector that encodes a hyperactive Tn*5* transposase that is 1,000-fold more active than the wild-type transposase. In this construct, the transposase is expressed from the promoter of the *tetA* gene of plasmid RP4, which is functional in a wide variety of organisms. The transposon itself contains a kanamycin resistance gene as a selectable marker and the origin of replication from plasmid R6K to facilitate subsequent cloning of the resulting insertion site. To test the effectiveness of this method, mutants unable to produce the characteristic yellow pigment (zeaxanthin dirhamnoside) of *X. autotrophicus* Py2 were isolated and analyzed. Transposon insertions were obtained at high frequency: approximately 1×10^{-3} per recipient cell. Among these, pigment mutants were observed at a frequency of approximately 10^{-3}. Such mutants were found to have transposon insertions in genes homologous to known carotenoid biosynthetic genes previously characterized in other pigmented bacteria. Mutants were also isolated in *Pseudomonas stutzeri* and in an *Alcaligenes faecalis*, demonstrating the effectiveness of the method in diverse Proteobacteria. Preliminary results from other laboratories have confirmed the effectiveness of this method in additional phylogenetically diverse species.

Keywords Transposon · Tn*5* · Mutagenesis · Carotenoid · *Xanthobacter*

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Abbreviations *Ap* Ampicillin · *Cm* Chloramphenicol · *EDTA* Ethylenediaminetetraacetic acid · *Gm* Gentamycin · *Km* Kanamycin · *MOPS* 3-(*N*-morpholino) propanesulfonic acid · *Tc* Tetracycline · *MIC* Minimal inhibitory concentration

Introduction

Xanthobacter autotrophicus Py2 is classified in the α-subdivision of the Proteobacteria and was originally isolated for its ability to grow on propylene as sole carbon source (van Ginkel and de Bont 1986). This strain was observed to be metabolically quite diverse and has the ability to grow on H_2/CO_2, ketones, alcohols, sugars, carboxylic acids, and aliphatic alkenes (van Ginkel and de Bont 1986). The degradation and/or metabolism of ketones and alkenes, especially halogenated alkenes, are of importance both industrially and environmentally. For this reason, considerable effort has gone into the elucidation of the pathways for propylene and acetone metabolism using a biochemical approach (Ensign et al. 1998; Ensign 2001). Of particular interest is the recent finding that *X. autotrophicus* Py2 catabolizes propylene by a novel pathway involving the cofactor mercaptoethanesulfonic acid (CoM) (Allen et al. 1999). This result was unexpected because previously CoM had been found only in the methanogenic archaea. Although these metabolic traits have been studied extensively via biochemical approaches, relatively few studies involving a genetic approach have been reported. Our interest in *Xanthobacter* metabolism, particularly CoM biosynthesis, led us to attempt genetic analysis of this organism.

A limited number of genetic techniques have been described for *Xanthobacter* species. Plasmid DNA has been introduced by conjugation using a tri-parental mating procedure (Janssen et al. 1989; Swaving et al. 1995; Zhou et al. 1996; Meijer 1997) or from an *Escherichia coli* strain expressing transfer functions (Meijer et al. 1991; Meijer 1997). Broad host range IncP plasmids, including the cloning vector pLAFR5, replicate in *Xanthobacter* allow-

ing the in vivo expression of recombinant genes (Janssen et al. 1989; Zhou et al. 1996). In addition, chemical mutagenesis using MNNG (1-methyl-3-nitro-1-nitrosoguanidine) has been described (Zhou et al. 1996). However, the use of transposon mutagenesis in *Xanthobacter* has not been reported. In our own laboratory, we attempted to mutagenize *X. autotrophicus* Py2 with a number of available transposon mutagenesis systems, but in all cases these experiments were unsuccessful (R.A. Larsen and W.W. Metcalf, unpublished data). For this reason, we set out to develop a method of efficient transposon mutagenesis for *Xanthobacter* that had the potential to be useful in a broad range of other organisms as well. This method employs a vector carrying a mini-Tn*5* transposon and a modified, hyperactive version of the gene encoding Tn*5* transposase.

Wild-type Tn*5* has a broad host specificity and has been shown to transpose in a variety of gram-negative bacteria (Berg and Berg 1983). This feature has made Tn*5* the tool of choice for transposon mutagenesis in this phylogenetic group. Numerous modified Tn*5* derivatives have been developed that carry a variety of selectable markers and various reporter genes (de Lorenzo and Timmis 1994). A particularly useful series of Tn*5* vectors are the "plasposons", a series of Tn*5* vectors designed for mutagenesis and one-step cloning of the transposon mutation (Dennis and Zylstra 1998). The plasposon vectors carry an origin of transfer (*oriT*) allowing them to be moved by conjugation from *E. coli* donor strains expressing the transfer functions of plasmid RP4 into the desired host organism. The transposons carried on these vectors have both an antibiotic resistance gene and a conditional origin of replication such that a transposon insertion along with the adjacent genomic DNA can be maintained as a plasmid in permissive *E. coli* hosts.

Numerous mutations that increase the efficiency of Tn*5* transposition have been isolated. A very useful combination of mutations in both the transposase gene (*tnp*) and the Tn*5* inverted repeats is reported to be 1,000-fold more active than wild-type Tn*5* and allows extremely efficient transposition both in vivo and in vitro (Goryshin and Reznikoff 1998). The hyperactive *tnp* gene contains two mutations, EK54 and LP372, which increase binding to the outside ends of the transposon and increase dimerization, respectively (Zhou and Reznikoff 1997; Goryshin and Reznikoff 1998). An additional mutation (MA56) that prevents expression of the inhibitor protein, which is a truncated derivative of transposase derived from an second translation start site within *tnp*, increases the rate of transposition even further (Wiegand and Reznikoff 1992). In addition, hybrid recognition sequences in the inverted repeats of the transposon optimize binding of the hyperactive transposase to the transposon resulting in additional increases in efficiency (Zhou et al. 1998).

Here, we report the development of a transposon delivery vector that combines the useful features of the plasposon vectors with the new, hyperactive Tn*5* element. It can be introduced by conjugation, transposes at a high frequency, and allows facile cloning of the transposon insertion along with adjacent DNA sequences. The new Tn*5* element was tested in *X. autotrophicus* Py2 by isolating and characterizing pigment mutants. It was also shown to work efficiently in *Pseudomonas stutzeri* and *Alcaligenes faecalis*. Evidence is presented suggesting that this method of transposon mutagenesis is useful in other phylogenetically distant organisms as well.

Materials and methods

Bacterial strains and media

Xanthobacter strains were derivatives of strain Py2 (van Ginkel and de Bont 1986), which was recently identified as *Xanthobacter autotrophicus* (S. Ensign, Utah State University, personal communication). A spontaneous slime-free derivative of *X. autotrophicus* Py2 designated WM1590 was isolated and used for these studies. *Xanthobacter* strains were routinely cultured on MOPS minimal medium (Neidhardt et al. 1974) containing 1% glucose or 1% succinate. Liquid cultures were grown in Luria broth (LB) containing 1% glucose. *Pseudomonas stutzeri* WM567 (smooth, *rpsL*) has been previously reported (Metcalf and Wolfe 1998), *P. stutzeri* WM1967 (smooth, *rpsL*, Δ*ptxA-htxO*) is a derivative of WM567. Details of its construction will be reported elsewhere. *Pseudomonas* strains were grown on tryptone-yeast extract (TYE) medium (Wanner 1986) or 0.4% glucose-MOPS medium. *Alcaligenes faecalis* WM2072 was isolated as a hypophosphite-oxidixing organism using the method described in Metcalf and Wolfe (1998) and was routinely grown on 1% succinate-MOPS medium. Identification of this strain as a strain of *A. faecalis* is based on comparison of its 16S rDNA sequence to sequences compiled by the RDP (Ribosomal Database Project) database (Maidak et al. 1996) (data not shown). *E. coli* strain DH5α/λ*pir* (Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*) (Miller and Mekalanos 1988) was used as the host for cloning the region of transposon insertions as *pir*-dependent plasmids. *E. coli* BW20767 (RP4–2-Tc::Mu-1 kan::Tn*7* integrant *leu-63*::IS10 *recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (Δ*Mlu*I)::*pir*$^+$) (Metcalf et al. 1996) was used as a donor for conjugation experiments. When required, antibiotics were included at the following concentrations for *Xanthobacter*: 30 μg kanamycin (Km)/ml, 12 μg tetracycline (Tc)/ml, 10 μg chloramphenicol (Cm)/ml, and 10 μg gentamycin (Gm)/ml. For *E. coli*, antibiotics were used as follows: 50 μg kanamycin/ml and 50 μg ampicillin (Ap)/ml. For *A. faecalis* WM2072 and *P. stutzeri*, kanamycin was used at 50 μg/ml. Auxotrophic mutants of *P. stutzeri* were grown on MOPS media supplemented with either 0.5 mM cysteine or 0.5 μM nicotinic acid, as appropriate.

To determine the level of antibiotic resistance of *X. autotrophicus* Py2, strain WM1590 was streaked on either MOPS minimal medium with 1% glucose or TYE rich medium containing antibiotics in the following range of concentrations. MOPS glucose medium contained 5–100 μg Cm/ml, 5–100 μg Km/ml, 5–100 μg Gm/ml, 6–24 μg Tc/ml or 20–100 μg Ap/ml. TYE medium contained 5–80 μg Cm/ml, 10–120 μg Km/ml, 5–80 μg Gm/ml, or 6–24 μg Tc/ml. The minimum inhibitory concentration (MIC) for each was defined as the lowest level of antibiotic that inhibited growth completely.

Recombinant DNA techniques

DNA digestion and ligation reactions, and transformation of *E. coli* were performed according to standard protocols (Ausebel et al. 1992). *X. autotrophicus* genomic DNA was prepared using CTAB (cetyltrimethyl ammonium bromide) essentially as described (Ausebel et al. 1992) but with the following preparatory step. After growing overnight in LB with 1% glucose, cells were pelleted by centrifugation, washed in 0.05 M Tris 0.02 M EDTA

Table 1 Plasmids used in the study

Plasmid	Description and/or construction	Source (reference)
pBluescript SK (+)	Ap^R cloning vector	Stratagene
pGRTEMP2	Ap^R, source of hyperactive Tn*5 tnp* gene	W.S. Reznikoff, University of Wisconsin, Madison, Wis., USA
pLAFR5	Tc^R, mobilizable broad host range cosmid vector	(Keen et al. 1988)
p34S-Cm	Ap^R, Cm^R, carries cat gene cassette	(Dennis and Zylstra 1998)
p34S-Tc′	Ap^R, Tc^R, carries *tet* gene cassette	(Dennis and Zylstra 1998)
pTnMod-OCm′	Cm^R, plasposon, pMB1 replicon	(Dennis and Zylstra 1998)
pTnMod-OKm	Km^R, plasposon, pMB1 replicon	(Dennis and Zylstra 1998)
pTnMod-RKm	Km^R, plasposon, *ori*R6Kγ replicon	(Dennis and Zylstra 1998)
pWM265	Tc^R, mobilizable broad host range cloning vector	(Metcalf and Wolfe 1998)
pWM368	Ap^R, used for cloning of hyperactive *tnp* PCR fragment	(Zhang et al. 2000)
pWM91	Ap^R, source of *oriT* PCR fragment	(Metcalf et al. 1996)
pWM403	Ap^R, cloning vector with unique *Pvu*II site: deletion of 448 bp *Pvu*II fragment of pBluescript SK(+)	This study
pWM405	*Pvu*II digested PCR of EZ-TN<KM-1>(primer Tn*5*-IR) inserted into *Pvu*II-digested pWM403	This study
pWM408	*Nde*I, *Bam*HI digested PCR of *tnp* from pGRTEMP2 (primers *tnp*-for and *tnp*-rev) cloned into *Nde*I, *Bam*HI-digested pWM368	This study
pRL1	Cm^R, mobilizable broad host range plasmid: *Sst*I *cat* gene fragment from pTnMod-OCm′ inserted into *Sst*I-digested pWM265	This study
pRL2	Km^R, mobilizable broad host range plasmid: *Sst*I *aph* gene fragment from pTnMod-OKm′ inserted into *Sst*I-digested pWM265	This study
pRL4	*Mbo*I-digested PCR of *oriT* from pWM91 (primers *oriT*-for and *oriT*-rev) inserted into *Bam*HI-digested pWM408	This study
pRL10	*Xba*I, *Nde*I digested PCR of *tetA*p from pLAFR5 (primers *tetA*p-for and *tetA*p-rev) inserted into *Xba*I, *Nde*I-digested pRL4	This study
pRL12	*Kpn*I-*Xba*I fragment of pTnMod-RKm′ containing *aph-ori*R6K inserted into *Kpn*I, *Xba*I digested pWM405	This study
pRL17	Circularized transposon-containing *Pvu*II fragment of pRL12	This study
pRL23	Transposon-containing *Pvu*II fragment of pRL12 inserted into *Sal*I-digested pRL10 treated with T4 DNA polymerase and dNTPs	This study
pRL27	Tn5-RL27 (Km^R-*ori*R6 K) delivery vector: circularized PCR fragment from pRL23 (primers *tetA*p-for and *oriT*-rev)	This study

pH 8.0, 0.05% *N*-lauryl sarcosine, frozen at –20 °C, thawed, and finally resuspended in 500 µl TE (10 mM Tris 1 mM EDTA pH 8.0) (Hayman and Farrand 1990). Plasmids were purified using either QiaPrep Spin Plasmid or Qiagen Plasmid Maxi kits (Qiagen, Valencia, Calif., USA).

Sequencing reactions were carried out using the BigDye sequencing reagent (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's recommendations and analyzed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois (Urbana, Ill., USA). Primers that anneal to transposon sequence and read outwards into the adjacent region are listed below. Additional primers used to complete double-stranded sequencing of *crtE*, *crtYIB* and *ispA* were designed based on sequences obtained using the transposon-specific primers.

Plasmid constructions

Plasmids used in the study and their constructions are described in Table 1. The construction of pRL27, the transposon delivery vector, is shown in Fig. 1

Conjugation

Plasmids were transferred into *X. autotrophicus* Py2, *P. stutzeri* and *A. faecalis* WM2072 recipients by conjugation from *E. coli* strain BW20767 carrying the appropriate plasmids. Donor and recipient strains, which had been grown to mid-exponential phase (OD_{600}≈0.8), were mixed and collected by filtration using a 0.45-µm analytical filter (Nalgene). *E. coli* was conjugated to *X. autotrophicus* at a ratio of 1 (50 µl):10 (500 µl), and to *P. stutzeri* or *A. faecalis* at a ratio of 1 (200 µl):1 (200 µl). The filter on which the cells had been collected was incubated overnight on TYE agar medium at 30 °C (*X. autotrophicus*) or 37 °C (*P. stutzeri* and *A. faecalis*). After incubation, the cells were resuspended in MOPS liquid medium and plated, either directly or after dilution, onto MOPS minimal medium containing an appropriate carbon source and antibiotic (or onto plates without antibiotic to determine total number of recipient cells). For experiments in which auxotrophs of *P. stutzeri* were isolated, selection was carried out on TYE containing 50 µg Km/ml and 200 µg streptomycin/ml.

Screen for mutants

X. autotrophicus Py2 pigment mutants were isolated by screening for colonies with an appearance that differed from the bright yel-

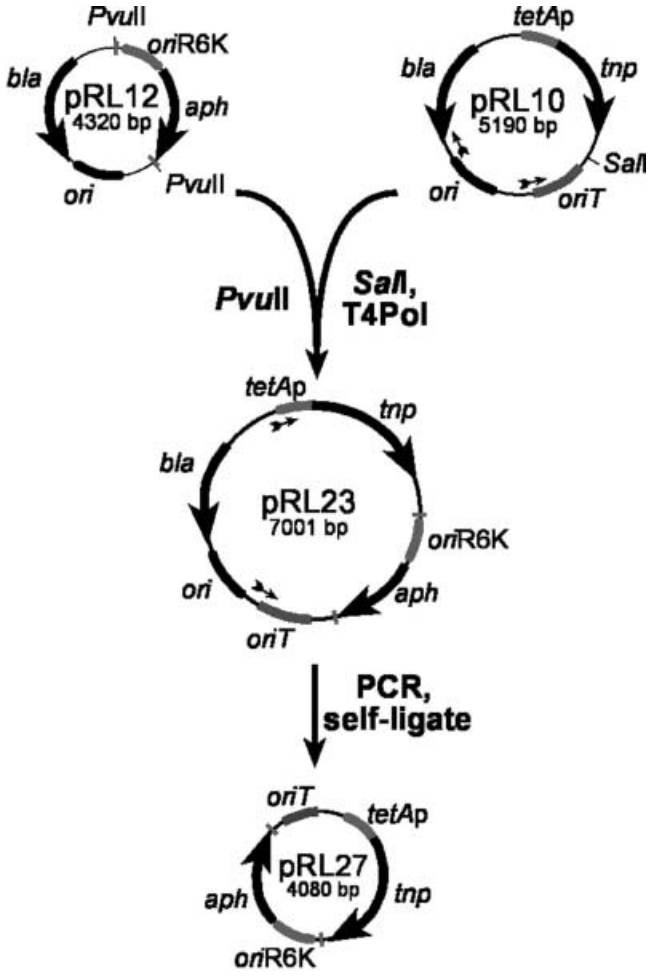

Fig. 1 The construction and physical structure of the transposon delivery vector pRL27. The construction of the plasmid used for transposon mutagenesis is shown. The final vector, pRL27, carries a hyperactive Tn*5* transposase gene (*tnp*) under the control of the *tetA* promoter (*tetA*p) from plasmid RP4. It also carries a mini-Tn*5* element encoding kanamycin resistance (Km^R) and the origin of replication from plasmid R6K (*ori*R6K) to allow cloning of transposon insertion sites. Finally, pRL27 is mobilizable from a transfer-proficient host by inclusion of the origin of transfer (*oriT*) from plasmid RP4. The modified Tn*5* inverted repeats are shown as *gray bars*, the *bla* gene encodes resistance to penicillin and its derivatives, and the *aph* gene encodes resistance to kanamycin

low of the wild-type strain. Potential mutants were restreaked next to the wild-type strain to confirm the phenotype. *P. stutzeri* auxotrophs were obtained by replica plating Km^r mutants on TYE and 1% glucose-MOPS media. Auxotrophs were identified as those isolates that were able to grow on TYE medium but not on glucose-MOPS medium. *A. faecalis* mutants unable to utilize reduced phosphorus sources were identified as described previously (Metcalf and Wolfe 1998).

Cloning of transposon insertions

One-step cloning of Tn*5*-RL27 insertions is similar to cloning of plasposons or mini-Mar elements (Dennis and Zylstra 1998; Zhang et al. 2000). For the studies presented here, cloning of transposon insertions was accomplished by digesting genomic DNA from a transposon-induced mutant with *Bam*HI (which does not cut within the transposon); however, in principle, any enzyme that does not cut within the transposon could be used. Subsequently, the digested DNA was treated with T4 DNA ligase and introduced into *E. coli* DH5α/λ*pir*, where circularized fragments containing the transposon replicate as a plasmids.

Transposon junction plasmids were isolated from selected transformants and subjected to sequencing reactions using the outward-directed transposon-specific primers tpnRL17–1 and tpnRL13–2 (Table 2) which anneal to the *ori*R6K and Km^r ends of the transposon, respectively. Sequences were then compared to the protein sequence database (GenBank) using the BlastX algorithm (Altschul et al. 1990). For each mutant, the join between the transposon sequence (the Tn*5* inverted repeat sequence ending with: GTG TAT AAG AGA CAG) and the genomic DNA sequence as well as the 9-bp target duplication (a characteristic of Tn*5* insertions) were identified.

GenBank accession numbers

The DNA sequences of the *X. autotrophicus* Py2 *ispA*, *crtE* and *crtYIB* loci have been deposited in GenBank under the accession numbers AF408846, AF408847 and AF408848, respectively.

Results and discussion

Development of transposon delivery vectors

In preliminary experiments (described in Materials and methods), the MIC for *X. autotrophicus* Py2 to various antibiotics on TYE medium was determined to be: 10 μg Cm/ml, 10 μg Km/ml, and 6 μg Tc/ml, while on MOPS

Table 2 Primers used in this study

Primer name	Sequence	Restriction sites[a]
tnp-for	GGGGGG**CATATG**ATAACTTCTGCTCTTCATCG	*Nde*I
tnp-rev	GAATTC**GGATCC**TGCAGGTCGACGGATCAG	*Bam*HI
oriT-for	CGGCG**GATC**GATGGCGCGCCGACGTGCTCATAGTCCACGA	*Mbo*I
oriT-rev	CGGCG**GATC**GATGGCGCGCCACAAAACAGCAGGGAAGCAG	*Mbo*I
*tetA*p-for	GAATTC**TCTAGA**ATGATTCTCCGCCAGCAT	*Xba*I
*tetA*p-rev	GGAATTC**CATATG**TGGCCTCCGGAC	*Nde*I
Tn*5*-IR	GAATTC**CAGCTG**TCTCTTATACACATCTCAACG	*Pvu*II
pRL10-for	GAATTC**GGATCC**TTGGTCTGACAGTTACCAATGC	*Bam*HI
pRL10-rev	GAATTC**GGATCC**CGCCAAGCTATTTAGGTGACA	*Bam*HI
Tn*5*IR-2	GAATTC**AGATCT**CAGCTGTCTCTTATACACATCTCAACC	*Bgl*II
tpnRL17–1	AACAAGCCAGGGATGTAACG	
tpnRL13–2	CAGCAACACCTTCTTCACGA	

minimal medium the MIC was determined to be: 10 μg Cm/ml, 5 μg Km/ml, 6 μg Tc/ml and 80 μg Ap/ml. Strains carrying plasmids pRL1or pRL2, which confer resistance to Cm or Km, respectively, were resistant to the corresponding antibiotic at these concentrations. Both plasmids also confer resistance to Tc, which is encoded by the *tetA* gene on each plasmid. These results suggest that each of the three antibiotic resistance genes is expressed in *X. autotrophicus* Py2 and that the gene products are functional. Thus, each of these selectable markers was a potential candidate for use in *X. autotrophicus* Py2. Moreover, the Tc^R phenotype of these strains indicates that the *tetA* gene promoter, *tetA*p, is functional in *X. autotrophicus* Py2. (The *tetA*p promoter in pRL1 and pRL2 is derived from plasmid RP4 and is known to be functional in a wide variety of gram-negative bacteria.)

The transposon delivery vector pRL27 was constructed as described in Table 1 and is depicted in Fig. 1. This plasmid carries an RP4 origin of transfer (*oriT*), which allows transfer of the plasmid into *X. autotrophicus* after conjugation with *E. coli* donors that express the RP4 transfer functions. It also carries a mutant version of the Tn*5* *tnp* gene encoding a hyperactive transposase (Goryshin and Reznikoff 1998) that we placed under the control of *tetA*p. The *tetA*p::*tnp* gene construct is located outside of the transposon so that after transposition the *tnp* gene is lost, resulting in stable transposon insertions. The transposon, designated Tn*5*-RL27, is comprised of optimized Tn*5* inverted repeats (Zhou et al. 1998) flanking the *aph* gene from Tn*903*, encoding Km^r, as a selectable marker and the plasmid R6K DNA replication origin (*ori*R6K) to facilitate subsequent cloning of transposon insertion mutants. Also, because *ori*R6K requires the π protein, encoded by the *pir* gene, these plasmids are incapable of replication in hosts that lack *pir*. Thus, after the plasmids have been introduced into non-*pir* hosts, drug-resistant transconjugants can be obtained only if the transposon inserts into the recipient genome.

Efficient transposon mutagenesis in *X. autotrophicus* Py2 using pRL27

To examine the efficacy of the new transposon delivery vector, we attempted to mutagenize *X. autotrophicus* Py2. In previous experiments we were unable to isolate transposon insertions in *X. autotrophicus* Py2 using a number of available Tn*5* delivery vectors, all of which are introduced by conjugation and utilize the wild-type Tn*5* *tnp* gene under control of its natural promoter (data not shown). In contrast, after conjugation with *E. coli* donors carrying pRL27, transposon-induced *X. autotrophicus* mutants were obtained at a frequency of $1.2\pm0.5\times10^{-3}$ per recipient. An approximately 20-fold lower frequency ($4.5\pm0.5\times10^{-5}$) was observed using pRL23, a precursor to pRL27. This difference is likely due to the presence of the high-copy origin of replication from pBluescript SK (+) on pRL23, which makes this plasmid much less stable in the *E. coli* donor strain (data not shown). Removal of the pBluescript SK (+) sequence from pRL23 (in the construction of pRL27) improved both the stability of the plasmid and its utility as a mutagenic tool.

To confirm that the antibiotic-resistant transconjugants resulted from insertion of the transposon into the host genome we examined non-pigmented *X. autotrophicus* mutants obtained using pRL27. *Xanthobacter* species appear yellow ("xantho"=yellow) due to the presence of zeaxanthin dirhamnoside, an insoluble carotenoid unique to this genus. *X. autotrophicus* mutants unable to synthesize zeaxanthin dirhamnoside were easily observed because they lacked this characteristic bright-yellow colony phenotype. Of the approximately 17,000 Km^r mutants that were screened, 44 putative pigment biosynthetic mutants were obtained. Pigment mutants comprised approximately 10^{-3} of the total Km^R mutants, which is in rough accordance with the number expected based on five target genes in a genome presumed to be approximately 5 Mb.

The genomic region containing the transposon insertion for selected pigment mutants was cloned and sequenced as described in Materials and methods. Comparison of the resulting sequences to those found in GenBank revealed that 22 of the mutants had insertions into genes similar to those encoding known carotenoid biosynthetic enzymes, two mutants had insertions into a gene with similarity to a gene encoding a siderophore receptor, three had insertions into a gene with similarity to heme biosynthesis genes, and two into genes with no similarity to any other protein sequence in the GenBank database (see below for a detailed discussion of individual pigment mutants). The insertion sites of the remaining 15 mutants were not determined. Importantly, none of the 29 transposon junction sequences obtained were related to the delivery vector. Furthermore, analysis of DNA sequence flanking the transposon at either end revealed that each insertion was flanked by the 9-bp duplication that is characteristic of Tn*5* insertions (Berg and Berg 1983). These results clearly indicate that the antibiotic-resistant transconjugants arise by transposition of the Tn*5*-RL27 element from the delivery vector into the host chromosome. In addition, with a single exception, all insertions were at different sites suggesting that transposition was random even within specific target genes, as expected for Tn*5* transposition events.

Zeaxanthin dirhamnoside biosynthesis *in X. autotrophicus* Py2

The zeaxanthin biosynthetic pathway has been well-characterized for many organisms that synthesize various forms of this carotenoid pigment (Armstrong et al. 1990, 1993). Thus, we expected that characterization of pigment mutants in *X. autotrophicus* would lead to the identification of genes with similarity to previously described zeaxanthin biosynthetic genes. Analysis of the DNA sequences from 22 pigment mutants revealed that three separate loci with genes related to known carotenoid biosynthetic genes were mutated (Fig. 2). To examine whether

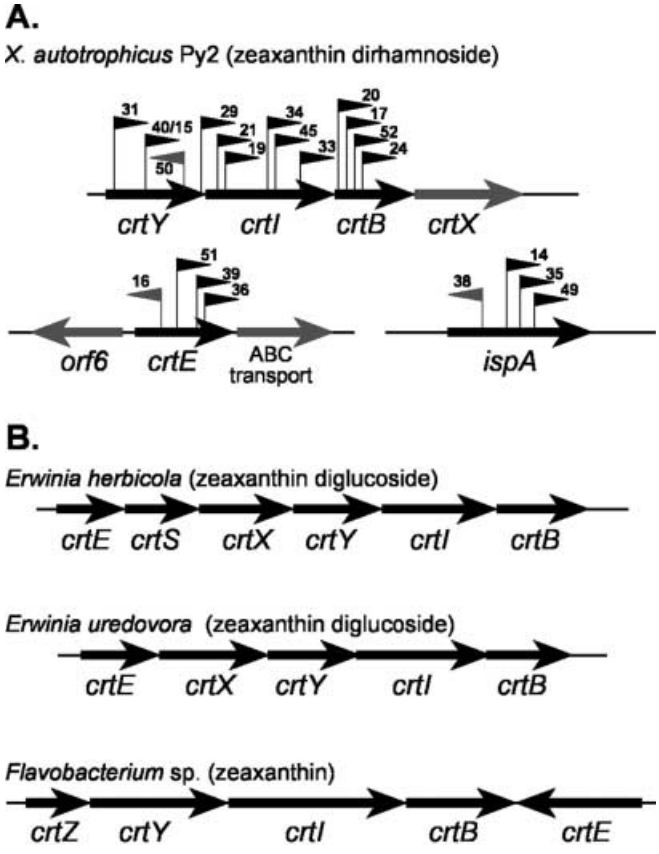

Fig. 2A, B Insertion sites for Tn*5*-RL27-induced pigment mutants of *X. autotrophicus* Py2**. A** The transposon insertion sites for 22 independently isolated pigment-minus mutants were determined as described. Additional ORFs adjacent to the transposon-derived sequences are shown in *gray* and were, for the most part, determined by sequencing only one strand. The transposon insertion sites are indicated by *flags* with the direction of transcription of the Km^R gene indicated by the direction of the *flag*. The allele number of each mutation is shown above the corresponding *triangle*. Genes were named according to that of the most similar sequence in the Genbank database. **B** The arrangement of zeaxanthin biosynthetic genes in other organisms that produce this carotenoid pigment

the three loci could be linked into a larger contiguous fragment we also determined the DNA sequence of regions adjacent to the overlapping transposon junction sequences. Although these sequences did not link the three loci, they did allow identification of additional putative carotenoid genes.

Overlapping sequence from 14 different mutants indicates that *crtY, crtI* and *crtB*, which encode lycopene cyclase, phytoene dehydrogenase and phytoene synthase, respectively, are adjacent genes and probably form an operon in *X. autotrophicus* (Fig. 2A). This arrangement is also found in several other organisms including *Erwinia* sp., *Flavobacterium* sp., *Agrobacterium aurantiacum*, and *Paracoccus marcusii*. The DNA sequence upstream of the *X. autotrophicus crtYIB* operon suggests the presence of a divergently transcribed α-methyl-CoA racemase (not shown), a gene unlikely to be involved in zeaxanthin biosynthesis. Immediately downstream of *crtYIB* and transcribed in the same direction is a gene with similarity to *crtX*, which encodes a glucosyltransferase. In *Erwinia herbicola*, the *crtX* gene product catalyzes the glucosylation of zeaxanthin resulting in zeaxanthin diglucoside (Misawa et al. 1990). The amino acid sequence encoded by *crtX* in *X. autotrophicus* has strong similarity to protein motif PF00201 from the Pfam-A database (Bateman et al. 1999), which is the consensus sequence for UDP-glucoronosyl and UDP-glucosyl transferases. Because zeaxanthin of *X. autotrophicus* is rhamnosylated, we predict that the gene with similarity to *crtX* encodes a rhamnosyl transferase.

Four pigment mutants had insertions in a gene with similarity to those encoding farnesyl pyrophosphate synthase (FPPi synthase), encoded by the *ispA* gene (Fig. 2A). Sequences up- and downstream of this gene suggested that it is adjacent to a peptidoglycan transglycosylase and a protein phosphatase, respectively (not shown). Neither of these genes appears to be coexpressed with *ispA* and neither is likely to have a function related to pigment biosynthesis.

Finally, four pigment mutants had insertions in a gene with high homology to known *crtE* genes, which encode geranyl-geranyl diphosphate synthase. Downstream of *crtE* is an open reading frame (ORF) with similarity to ATP-binding cassette-type transporters (data not shown). The function of this transporter and any relatedness to pigment biosynthesis is unknown. Sequencing upstream of *crtE* identified a gene with similarity to an ORF called Orf6 in *E. herbicola*. Although the function of Orf6 is unknown, the amino acid sequence is similar to that of isopentenyl pyrophosphate isomerases, enzymes that may play a role in the early steps of the pathway in *E. herbicola.*

The gene arrangement of other zeaxanthin-producing organisms is shown in Fig. 2B. The *crtYIB* operon has been maintained in each of the genomes, but the order and direction of transcription of the other required genes varies. Interestingly, Orf6 and *crtX* are adjacent genes in *E. herbicola* but are clearly separated by genes of presumably unrelated function in *X. autotrophicus*. In *Erwina* species, β-carotene is first converted to zeaxanthin and then to zeaxanthin-β-D-diglucoside by the gene products of *crtZ* and *crtX*, respectively. Because both β-carotene and zeaxanthin are yellow and our screen identified only those mutants easily distinguished from wild-type, we were unlikely to isolate mutants blocked at either of these late steps in the pathway. However, reactions similar to these likely occur in *X. autotrophicus*. As described above, we have identified a gene with similarity to *crtX* in the region between *crtB* and *crtE*. We predict that a *crtZ* homolog also exists; however, our sequence information was not extensive enough to confirm the presence of this gene or to determine its proximity to the other pigment genes.

The pigment mutants we isolated fall into four classes based on colony color: white, pale yellow, pale pink, and yellow-brown (Fig. 3). Mutations in *crtE*, *crtB* and *crtI* result in white colonies, presumably due to a block in the

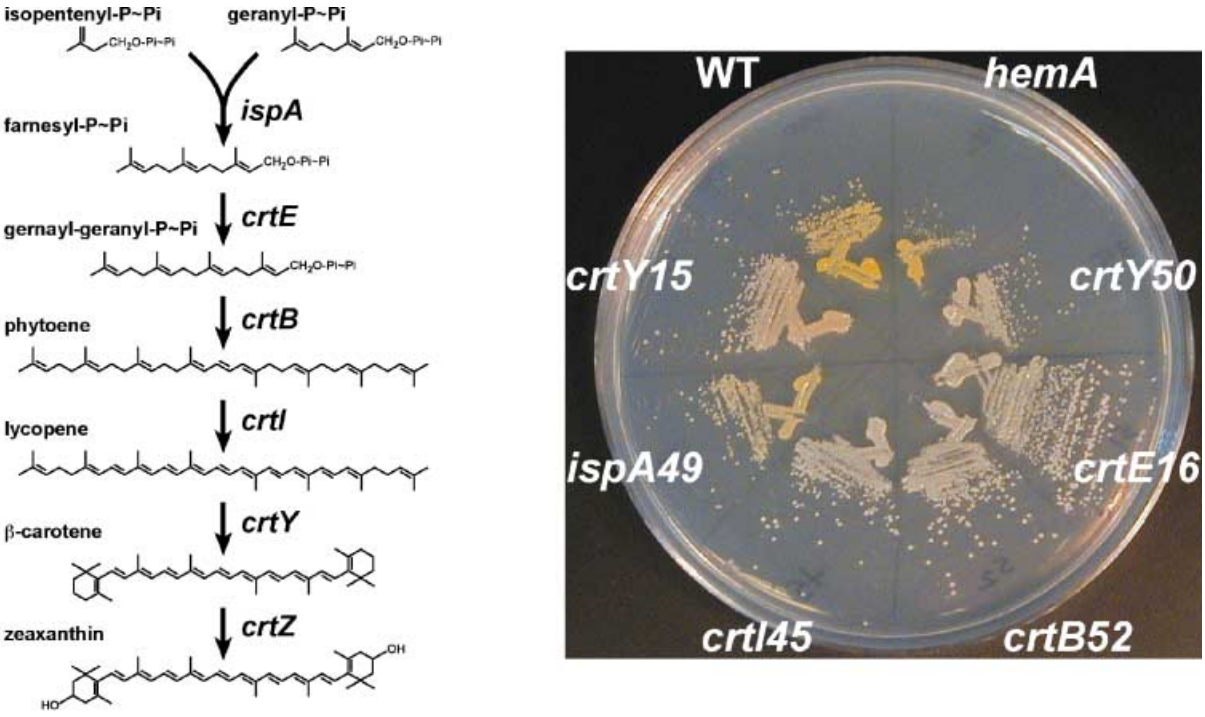

Fig. 3 The proposed pathway of zeaxanthin biosynthesis and the pigment phenotype of selected mutants. The proposed pathway of zeaxanthin biosynthesis is shown on the *left*, a photograph showing the pigment phenotype of selected mutants is shown on the *right*

pathway at the colorless intermediates farnesyl pyrophosphate, geranyl-geranyl pyrophosphate and phytoene, respectively. Interestingly, colonies of the four *ispA* mutants presented a pale yellow phenotype. Thus, there appears to be an alternative pathway in *X. autotrophicus* that allows the continued production of a yellow pigment even in the absence of FPPi synthase. Most of the *crtY* mutants (*crtY31*, *crtY40* and *crtY15*) display a slight pink colony color, which, based solely on the similarity of these genes to known carotenoid pathways, is probably due to an accumulation of the red intermediate lycopene. However, *crtY* appears to be the first gene in an operon with *crtI* and *crtB*, suggesting that transposon insertions in *crtY* should be polar on the downstream genes. If this were true, then the *crtY* mutants should also be unable to produce lycopene due to polarity of *crtI* and *crtB*. We believe expression of the downstream *crtI* and *crtB* genes results from read-through from the Km^R gene in Tn*5*-RL27. In support of this idea, the only *crtY* mutant that is not pink is *crtY50*, which is also the only *crtY* mutant in which the Km^R gene is in the wrong orientation to allow expression of downstream genes (Figs. 2A, 3).

Two additional pigment mutant classes are presumably unrelated to carotenoid biosynthesis. The three yellow-brown mutants all had insertions in *hemA*, which encodes aminolevulinate synthase, an enzyme that catalyzes the first committed step in heme biosynthesis. This was a very subtle phenotype and may be related to changes in cytochrome pigments in the mutants. Two pale yellow mutants had insertions in a gene with similarity to *fhuA*, a gene encoding a siderophore receptor. It seems possible that these mutants accumulate and excrete some brown-pigmented siderophore.

Efficient mutagenesis of other Proteobacteria using pRL27

Our success in *X. autotrophicus* prompted us to examine the utility of the new transposon delivery vector in two other organisms under study in our laboratory, *P. stutzeri* and *A. faecalis*. After mutagenesis using pRL27, antibiotic-resistant transconjugants of these organisms were obtained at frequencies only slightly lower than obtained in *Xanthobacter*: $1.4\pm0.2\times10^{-4}$ for *P. stutzeri* and $3.8\pm0.5\times10^{-4}$ for *A. faecalis*.

We confirmed that the antibiotic-resistant transconjugants obtained in *P. stutzeri* and *A. faecalis* were bona fide transposon insertion mutants by screening for clones with easily identifiable phenotypes. In the case of *P. stutzeri*, Km^r Tn*5*-RL27 mutants were screened for those with auxotrophic phenotypes. Of the 315 mutants that were screened, three were unable to grow on minimal medium. The sequence at the site of insertion for two mutants (*P. stutzeri* WM2406 and *P. stutzeri* WM2408) showed similarities to genes involved in cysteine biosynthesis: *cysD* and *cysN*/*cysC*, respectively. Although neither strain grew on minimal medium without supplementation, both grew with cysteine added indicating that both mutants were, in fact, cysteine auxotrophs. The third mutant, *P. stutzeri* WM2407, contained an insertion in sequence similar to *nadB*, encoding quinolinate synthase (L-aspartate oxidase), an enzyme involved in NAD biosynthesis. On minimal medium supplemented with nicotinic acid,

growth of WM2407 was restored. All three mutants displayed 9-bp duplications flanking the transposon insertions.

A. faecalis mutants were screened for those with defects in the assimilation of alternate P sources. Of two Tn*5*-RL27-induced mutants that were sequenced, one had an insertion in a putative alkaline phosphatase and the other in a hypophosphite transporter. Both insertion mutations were flanked by the expected 9-bp repeat. These mutants will be described in detail elsewhere.

Mutagenesis using pRL27 in other organisms

The data presented above clearly indicate that the transposon delivery vectors are functional and efficient in members of the α-, β- and γ- subgroups of the Proteobacteria; however, additional results suggest that these elements will be useful over a very broad phylogenetic spectrum. Thus far, preliminary data suggest that this method is successful in variety of proteobacteria and also in at least one cyanobacterial species. These include the α-proteobacterium *Ensifer adhaerens* (Mark Martin, Occidential College, personal communication); other γ-Proteobacteria, *E. coli* (Andrei Kuzminov, University of Illinois, personal communication), *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Enteritidis (Stanley Maloy, University of Illinois, personal communication), *Vibrio cholerae* El Tor C6706 (Bonnie Bassler, Princeton University, personal communication), and *Vibrio parahaemolyticus* (Linda McCarter, University of Iowa, personal communication); the δ-proteobacterium *Bdellovibrio* sp. (Mark Martin, Occidential College, personal communication); and a cyanobacterium, *Synechococcus* sp. (Bianca Brahamsha, UCSD, personal communication). Introduction of pRL27 into each of these organisms was by conjugation with the exception of *Salmonella* sp. and *E. coli* in which electroporation was used.

In summary, we have developed an efficient method of transposon mutagenesis that resulted in a high frequency of transposon insertions in *X. autotrophicus* Py2, *Pseudomonas stutzeri* and *Alcaligenes faecalis*. The effectiveness of this method was validated by isolation and characterization of *X. autotrophicus* Py2 mutants unable to synthesize the yellow carotenoid pigment zeaxanthin dirhamnoside. Transposon insertions were found to be random, and the phenotype observed for each of the mutants was consistent with that expected due to a disruption of the gene identified. These data indicate that zeaxanthin dirhamnoside is synthesized in *X. autotrophicus* by a pathway similar to that found in other carotenoid-producing organisms.

Preliminary results from other laboratories indicate that this method of transposon mutagenesis is successful in other bacteria as well, demonstrating its application to a broad range of organisms.

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