

Inducible-Expression Plasmid for *Rhodobacter sphaeroides* and *Paracoccus denitrificans*^{∇†}

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We have developed a stable isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible-expression plasmid, pIND4, which allows graduated levels of protein expression in the alphaproteobacteria *Rhodobacter sphaeroides* and *Paracoccus denitrificans*. pIND4 confers kanamycin resistance and combines the stable replicon of pMG160 with the *lacI*^q gene from pYanni3 and the *lac* promoter, P_{A1/04/03}, from pJBA24.

Rhodobacter sphaeroides and *Paracoccus denitrificans* are often used for the study of bacterial metabolism, bioenergetics (8), and signal transduction (11). Although inducible-expression plasmids are available for these organisms, e.g., pRKSK1 (5) and pRECTX (9), these plasmids suffer from one or more of the following problems. First, continuous antibiotic selection is essential for maintaining the plasmid in the population, e.g., plasmid pRK415 (7), which is the vector backbone for most of the available expression vectors for these species, is retained by only approximately 10% of the population after 40 generations without antibiotic selection (6) (segregational instability). Second, the inducer affects the expression of many endogenous genes; for example, several *R. sphaeroides* vectors use either light- or oxygen-inducible promoters to deliver high levels of protein expression (5). However, light and oxygen affect the expression of over 35% of the endogenous genes in this organism (2), which limits the use of these vectors in functional studies.

The plasmid developed in this study, pIND4 (Fig. 1), uses the pMG170 vector backbone (6), the *lacI*^q gene from pYanni3 (4), and the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible-expression cassette from pJBA24, which includes the P_{A1/04/03} promoter, a ribosome binding site, a polylinker, and two transcriptional terminators (1). pMG170 confers kanamycin resistance and is an *Escherichia coli* shuttle cloning vector derived from naturally occurring plasmid pMG160 of *Rhodobacter blasticus*, which replicates and is segregationally stable in several other members of the *Rhodobacteraceae* (6). In *E. coli*, pMG170 has a high copy number, replicating using the ColE1 origin, while in *R. sphaeroides*, the pMG160 origin delivers a copy number of 18 to 23 (6).

Vector construction. The construction of pIND4 is described in the supplemental material.

Testing of the expression plasmid. The coding sequence for the *R. sphaeroides cheY6* gene was cloned into pIND4, generating pIND4-Y6. The plasmid was introduced into *R. sphaeroides* strain JPA1336 ($\Delta cheY6$ derivative of WS8N) and *P. denitrificans* strain PD1222 (wild type) via conjugation with the *E. coli* donor strain S17-1 λ pir (10). Cells containing the plasmid were grown from single colonies under aerobic conditions with shaking (225 rpm) in succinate medium containing 25 μ g/ml kanamycin. The effects of different concentrations of IPTG (Fig. 2) and different induction times (Fig. 3) on CheY6 protein accumulation were investigated.

At IPTG concentrations of less than 1 μ M, no expression of CheY6 was detectable in *R. sphaeroides* (the minimum detection limit was \sim 500 molecules per cell). When induced with 1,000 μ M IPTG, expression levels of CheY6 in *R. sphaeroides* exceeded 250,000 molecules per cell (equivalent to \sim 2.3 mg of protein per liter of culture). This represents at least a 500-fold induction of expression between no induction and maximal induction. The expression levels were sensitive to the concentration of IPTG used, with 10 μ M and 100 μ M delivering at least 15-fold and 300-fold inductions, respectively.

For *P. denitrificans*, the maximal induction level achieved with the vector (\sim 140,000 molecules per cell, corresponding to approximately 1.3 mg of the CheY6 protein per liter of culture) was comparable with that for *R. sphaeroides* (\sim 250,000 molecules per cell). However, unlike *R. sphaeroides*, there was leaky expression of CheY6 in *P. denitrificans*, with over 45,000 molecules per cell in the absence of IPTG, which limited the level of induction seen in *P. denitrificans*.

Segregational stability. *R. sphaeroides* and *P. denitrificans* cells were grown in the absence of kanamycin, and the proportion of cells containing the plasmid was estimated by replica plating. After over 85 generations without antibiotic selection, the plasmid was retained in over 97% of bacteria.

Use of pIND4 to analyze an *R. sphaeroides* chemotaxis mutant. *R. sphaeroides* has a complex chemosensory pathway (11). The expression levels of many components of the pathway are

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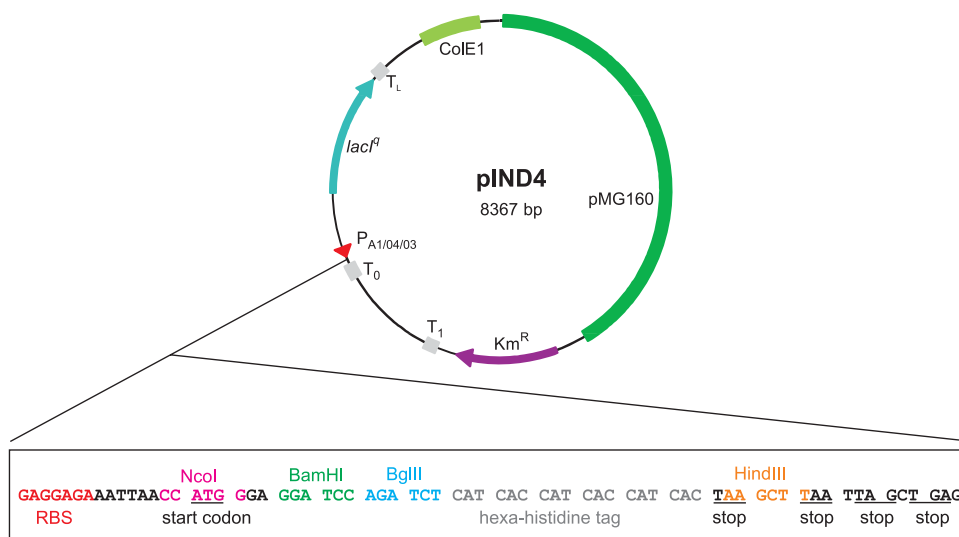


FIG. 1. Plasmid map of the pIND4 expression vector. The key features are labeled: the inducible promoter, $P_{A1/04/03}$ (red); the kanamycin resistance gene, Km^R (purple); the pMG160 sequence (dark green); ColE1 origin of replication, allowing replication of the plasmid in *E. coli* (pale green); transcriptional terminators (gray); and the *lacI* gene (cyan). The vector contains a ribosome binding site (RBS) and provides the option of incorporating a C-terminal hexahistidine tag.

affected by light and oxygen levels (14), making previously used expression plasmids reliant on either light or oxygen as their inducer unsuitable for functional studies of the pathway. The *cheY6(D56N)* mutant of *R. sphaeroides* has a stopped pheno-

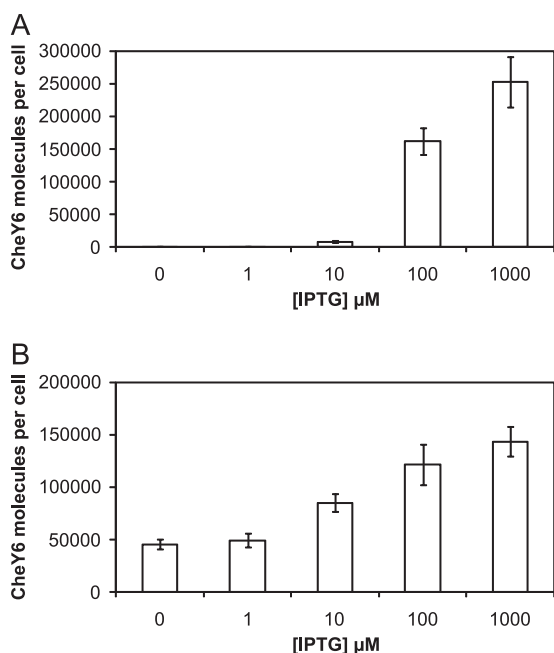


FIG. 2. Effect of IPTG concentration on protein expression levels. Cells were grown in the presence of IPTG from single colonies and harvested when the optical density at 700 nm was 0.6 ($\sim 3.8 \times 10^8$ cells/ml). The protein content of the cells was estimated by quantitative immunoblotting (3). (A) *R. sphaeroides* JPA1336 ($\Delta cheY6$) containing pIND4-Y6. (B) *P. denitrificans* PD1222 containing pIND4-Y6. The zero baseline in each case was cells containing an empty pIND4 vector grown in the presence of 1,000 μ M IPTG. Error bars show the standard errors of the means obtained from nine replicates.

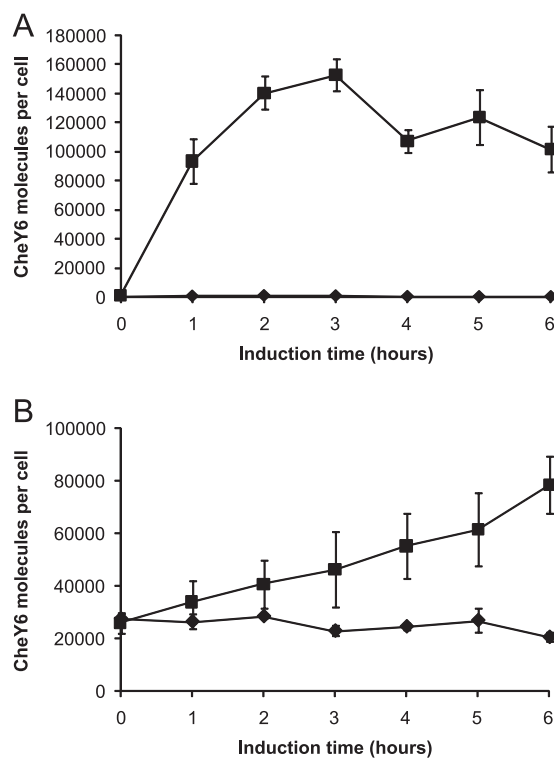


FIG. 3. Induction time course for *cheY6* expression from pIND4-Y6 in *R. sphaeroides* JPA1336 ($\Delta cheY6$) (A) and *P. denitrificans* Pd1222 (B). Cells were grown aerobically in succinate medium without IPTG until the optical density at 700 nm reached 0.3. IPTG (1 mM) was then added, and samples were taken at the intervals indicated. \blacklozenge , uninduced cells; \blacksquare , cells induced with 1 mM IPTG. Error bars show the standard errors of the means obtained from nine replicates.

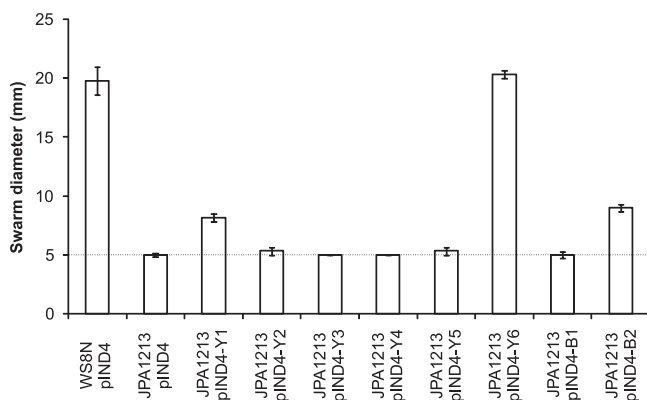


FIG. 4. Swarm plate chemotaxis assay comparing the effect of expression of the chemotaxis response regulators on the chemotactic ability of JPA1213 [*cheY6(D56N)*]. The dotted line shows the swarm diameter produced by a nonmotile strain; swarm diameters significantly larger than this indicate motility. The swarm plates contained 100 μ M propionate and 1 mM IPTG and were incubated for 48 h under aerobic conditions. Error bars show the standard errors of the means obtained from nine replicates.

type, which, due to the stop-start nature of the *R. sphaeroides* flagellar motor, is analogous to the tumbling phenotype of *E. coli* chemotaxis mutants. Previously, we have shown that the phosphorylation site mutant protein, CheY6(D56N), can be phosphorylated by CheA3-P on an alternative residue (S83) and that functional CheA3 is required for the stopped phenotype of the *cheY6(D56N)* mutant, suggesting that phosphorylated CheY6(D56N) causes the stopped phenotype (12). We used pIND4 to test whether we could suppress the stopped phenotype of the *cheY6(D56N)* mutant by overexpressing each of the eight chemotaxis response regulators (RRs). We found that swimming was restored to this strain by the overexpression of the cognate RR of CheA3 (CheY1, CheY6, and CheB2) but not by noncognate RRs (CheY2, CheY3, CheY4, CheY5, and CheB1) (Fig. 4 and see the supplemental material). Presumably, this was because the cognate RRs of CheA3 were able to outcompete CheY6(D56N) for phosphorylation by CheA3-P. These results are consistent with our hypothesis that the phosphorylation of CheY6(D56N) by CheA3 causes the stopped phenotype of the *cheY6(D56N)* mutant.

Anticipated uses of pIND4. The stability of pIND4 combined with its nonendogenous inducible promoter are distinct advantages over previously reported expression plasmids for *R. sphaeroides* and *P. denitrificans*. In *R. sphaeroides*, the plasmid showed minimal levels of leakiness, with greater-than-500-fold induction of CheY6 expression upon the addition of IPTG. In *P. denitrificans*, the plasmid showed some level of leakiness, which may limit its application in this organism. High levels of protein production were obtained for both *R. sphaeroides* and *P. denitrificans* when maximally induced, yielding \sim 2.3 and 1.3

mg of the CheY6 protein per liter of culture. We have also successfully used pIND4 to express CheY6 in *E. coli*, where we obtained a yield of \sim 2.5 mg per liter of culture. These high levels of expression suggest that in addition to functional studies, pIND4 may also be useful for the overexpression and subsequent purification of proteins. The large membrane surface area of *R. sphaeroides* offers the potential for obtaining much higher yields of membrane proteins than with other bacterial hosts (13).

Nucleotide sequence accession number. The pIND4 sequence has been deposited in the EMBL database under accession number FM164773.

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