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# The *Rhodobacter sphaeroides* 2.4.1 *rho* Gene: Expression and Genetic Analysis of Structure and Function

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**The gene which encodes transcription termination factor Rho from *Rhodobacter sphaeroides* 2.4.1, the gram-negative facultative photosynthetic bacterium, has been cloned and sequenced. The deduced protein shows a high level of sequence similarity to other bacterial Rho factors, especially those from proteobacteria. However, several amino acid substitutions in the conserved ATP-binding site have been identified. When expressed in *Escherichia* coli, the *R. sphaeroides rho* gene relieves Rho-dependent polarity of the *trp* operon, indicating interference with the transcription termination machinery of *E. coli*. A truncated version of *R. sphaeroides* Rho (Rho′) is toxic to a bacterium related to *R. sphaeroides*, *Paracoccus denitrificans*, and is lethal to *R. sphaeroides*. We suggest that toxicity is due to the ability of Rho′ to form inactive heteromers with the chromosomally encoded intact Rho. We localized a minimal amino acid sequence within Rho which appears to be critical for its toxic effect and which we believe may be involved in protein-protein interactions. This region was previously reported to be highly conserved and unique among various Rho proteins. The lethality of *rho′* in *R. sphaeroides* together with our inability to obtain a null mutation in *rho* suggests that Rho-dependent transcription termination is essential in *R. sphaeroides*. This is analogous to what is observed for gram-negative *E. coli* and contrasts with what is observed for gram-positive *Bacillus subtilis*. The genetic region surrounding the *R. sphaeroides rho* gene has been determined and found to be different compared with those of other bacterial species. *rho* is preceded by *orf1*, which encodes a putative integral membrane protein possibly involved in cytochrome formation or functioning. The gene downstream of *rho* is homologous to *thdF*, whose product is involved in thiophene and furan oxidation.**

*Rhodobacter sphaeroides* 2.4.1 is a facultative photosynthetic (PS) bacterium belonging to the α subdivision of class *Proteobacteria*. To identify the transcriptional regulators of PS gene expression, we developed a genetic screen in a closely related but non-PS organism, *Paracoccus denitrificans*, which is capable of expressing *R. sphaeroides* genetic information (18, 32). We reasoned that activators and repressors of *puf* operon (23) expression, encoded by cosmids from an *R. sphaeroides* 2.4.1 genomic library, would increase and decrease, respectively, *puf*::*lacZ* expression in *P. denitrificans*. In an application of the above-described screen, we were able to identify cosmids carrying known activators of *puf* transcription (14, 15), as well as an uncharacterized cosmid, pUI8074. Unexpectedly, we found that the effect of pUI8074 was due to the presence of a truncated form of the *R. sphaeroides rho* gene.

The bacterial *rho* gene encodes a factor responsible for protein-dependent transcription termination (reviewed in references 35 and 37). Most studies of the Rho factor have been performed on this factor from *Escherichia coli* (39). *E. coli* Rho is a hexameric protein composed of identical subunits of 419 amino acids. The hexamer binds to the nascent RNA and by using the energy of ATP hydrolysis dissociates RNA from the transcription complex at certain sites. Rho possesses an RNA-binding, RNA-dependent ATPase and RNA-DNA helicase activities.

Recently, homologs of *E. coli rho* were cloned from several diverse bacterial species (26, 31, 36, 42); however, none of these were from members of the α subdivision of class *Proteobacteria*. Sequencing of these *rho* genes revealed the structural similarities of their derived gene products to *E. coli* Rho, but very few functional studies have been undertaken with the *E. coli* Rho homologs. In addition, little is understood about the essentiality of Rho-dependent transcription termination in bacteria, e.g., Rho factor is vital for gram-negative *E. coli* (6) but is not vital for gram-positive *Bacillus subtilis* (36).

To investigate the importance of *rho* in *R. sphaeroides* 2.4.1 and specifically to be able to address the role of Rho-dependent transcription termination in PS gene expression, we undertook an initial characterization of the *R. sphaeroides rho* gene. Because of the unexpected phenotype associated with expression of truncated versions of *rho* in both heterologous and homologous hosts, we were prompted to consider the basis for this phenotype.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used are listed in Table 1.

**Growth conditions.** *E. coli* strains were routinely grown at 37°C on Luria-Bertani medium (25) supplemented, when required, with the following antibiotics: tetracycline, 10 μg/ml; ampicillin, 100 μg/ml; and kanamycin, 25 μg/ml. Strain CY15621 containing pUC19 or pUC19-derived plasmids was grown at 30°C on M63 minimal medium (25) containing 0.2% glycerol, 0.1% Casamino Acids (Difco), 100 μg of ampicillin per ml, and 25 μg of IPTG (isopropyl-β-D-thiogalactopyranoside) per ml and supplemented with either 20 μg of L-tryptophan per ml or 10 μg of indole per ml.

*R. sphaeroides* and *P. denitrificans* were grown chemoheterotrophically at 31°C on Sistrom's medium A (5), with succinate as the carbon source. Antibiotics were used, when appropriate, at the following concentrations: tetracycline, 1 μg/ml; and kanamycin, 40 μg/ml.

**Conjugation techniques.** Conjugation was performed essentially as described elsewhere (7) with *E. coli* S17-1 as a donor.

**β-Galactosidase assays.** The activities of β-galactosidase on colonies were estimated by overlaying plates with top agar containing X-Gal (5-bromo-4-

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) and/or description | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α*phe* | Host for plasmid maintenance | 14 |
| S17-1 | Donor for conjugations, C600::RP4-2 (Tc::Mu) (Km::Tn7) $Tra^+$ | 41 |
| CY15621 | Double nonsense mutant, *trpE9851-trpA38* | 21 |
| *R. sphaeroides* 2.4.1 | Wild type | W. R. Sistrom |
| *P. denitrificans* ATCC 17741 | Wild type | American Type Culture Collection |
| Plasmids | | |
| pLX1 | $Sm^r/Sp^r$ IncQ, promoterless *lacZYA* | This work |
| pUI1830Δ | $Sm^r/Sp^r$ IncQ: *pufB*::*lacZYA′* | 18 |
| pLA2917 | $Tc^r$ $Km^r$ IncP; cosmid | 2 |
| pRK415 | $Tc^r$ *lacZ*α IncP | 22 |
| pUC19 | $Ap^r$ *lacZ*α IncColE1 | 44 |
| p74BsHp | pUC19::*rho′*; encodes 374 N-terminal residues of Rho[a] under *lacZ*α promoter | This work |
| p278BsBs | pUC19::*rho*; encodes full-length Rho under *lacZ*α promoter | This work |
| pUI8074 | pLA2917 derivative containing truncated *R. sphaeroides rho* gene | 12 |
| pUI8278 | pLA2917 derivative containing the full-length *rho* gene | 12 |
| p74-B | pUI8074 derivative; deletion of all internal *Bam*HI fragments of the insert DNA of pUI8074 | This work |
| p74-Bt | pUI8074 derivative; deletion of all internal *Bst*EII fragments of the insert DNA of pUI8074 | This work |
| p74-H | pUI8074 derivative; deletion of all internal *Hin*dIII fragments of the insert DNA of pUI8074 | This work |
| p74-HP | pRK415::*orf1* | |
| p74-BsHp | pRK415::*rho′*; encodes 374 N-terminal residues of Rho[a] | This work |
| p74-BsR | pRK415::*rho′*; encodes 339 N-terminal residues of Rho[a] | This work |
| p74-BsNr6 | pRK415::*rho′*; encodes 320 N-terminal residues of Rho[a] | This work |
| p74-BsNr5 | pRK415::*rho′*; encodes 200 N-terminal residues of Rho[a] | This work |
| p278-BsBs | pRK415::*rho*; encodes full-length Rho | This work |
| p74HR::Km::mob | pUC19 derivative containing *rho′* disrupted by the $Km^r$ cartridge as well as *mob* and $Tc^r$ loci from pSUP202 | This work |
| pSUP202 | $Ap^r$ $Cm^r$ $Tc^r$ IncColE1 Mob | 41 |

[a] The truncated Rho proteins contain at their carboxy termini several residues encoded by vector DNA.

chloro-3-indolyl-β-D-galactopyranoside), as described elsewhere (19). β-Galactosidase assays of liquid-grown cultures were performed as previously described (18) at least twice, with standard deviations not exceeding 15%.

**DNA manipulations and sequence analysis.** Standard recombinant DNA techniques (25) and molecular biological enzymes and reagents were used according to the specifications of manufacturers.

Isolation of chromosomal DNA and Southern blotting and hybridization were performed essentially as described elsewhere (25). The DNA probes for hybridization were prepared as previously described (40). These consisted of the 0.23-kb *Eco*RI fragment of p278BsBs as a probe specific to the 3′ end of *R. sphaeroides rho* and plasmid p74-BsR as a probe to vector pUC19 and the 5′ end of *R. sphaeroides rho*.

DNA sequencing (ABI 373A automatic DNA sequencer; Applied Biosystems) and oligonucleotide synthesis were performed at the DNA Core Facility of the Department of Microbiology and Molecular Genetics. Templates for DNA sequencing were pUC19-based subclones of the DNA regions of interest sequenced with primers to *lacZ*. The sequence from cosmid pUI8278 was obtained by using the synthetic 20-mer 5′ CACTCGGGGTGATTGGTCGC 3′. For sequence analysis, the Genetics Computer Group software package (43) was used.

**RNA manipulations.** Isolation of total RNA and Northern (RNA) blotting and hybridization were performed, as described previously, with a *puf*-specific probe (probe f [20]).

**Nucleotide sequence accession number.** The sequence reported here has been deposited in the GenBank database under accession number L76097.

## RESULTS

**Identification of genes responsible for increased expression of *puf*::*lacZ* in *P. denitrificans* ATCC 17741(pUI1830Δ).** Three of ~800 cosmids representing a genomic library of *R. sphaeroides* 2.4.1 resulted in substantially increased *puf*::*lacZ* expression in colonies of *P. denitrificans* ATCC 17741(pUI1830Δ) compared with control levels. Two of these cosmids, pUI8533 and pUI8382, were known to contain the *prr* genes (14, 19) encoding a two-component regulatory system involved in activation of *puf* operon expression. Identification of these known activators of *puf* expression lent further credibility to the choice of *P. denitrificans* as a heterologous host for this genetic screen.

A third cosmid, pUI8074, increased *puf*::*lacZ* expression in ATCC 17741(pUI1830Δ) ~sixfold (Table 2). It was also observed that this cosmid resulted in moderate growth inhibition of ATCC 17741, which was especially pronounced at the lag and stationary phases. pUI8074 has been mapped to the same, 410-kb *Ase*I fragment of *R. sphaeroides* 2.4.1 chromosome I (4) as the *prr*-containing cosmids. However, more precise mapping revealed that pUI8074 does not overlap either pUI8533 or pUI8382 and that it is separated from the *prr* genes by ~120 to 150 kb (data not shown).

Subcloning of cosmid pUI8074 revealed that an ~3.0-kb *Bam*HI-*Hpa*I fragment is solely responsible for growth inhibition (Fig. 1A) and increased the levels of *puf*::*lacZ* mRNA in

TABLE 2. Effect of *R. sphaeroides rho′* on LacZ expression in *P. denitrificans* ATCC 17741

| Cosmid or plasmid[a] | Presence of *R. sphaeroides rho* | LacZ expression from plasmid[b] | |
|---|---|---|---|
| | | pUI1830Δ (*puf*::*lacZ*) | pLX1 (*lacZ*) |
| pLA2917 | –[c] | 41 | NA[d] |
| pUI8074 | *rho′* | 236 | NA |
| pRK415 | – | 47 | 15 |
| p74-BsHp | *rho′* | 113 | 51 |
| p278-BsBs | *rho* | 52 | 16 |

[a] In addition to the plasmid for monitoring LacZ expression, a second cosmid or plasmid (shown in this column) was present.
[b] Data are expressed in Miller units of β-galactosidase.
[c] –, vector control.
[d] NA, not applicable.

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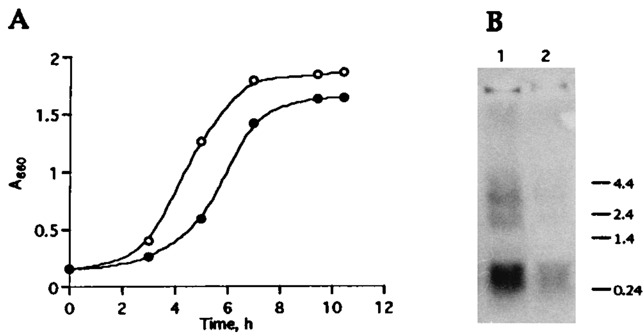


FIG. 1. Effects of *R. sphaeroides rho′* on growth and the level of *puf*::*lacZ* mRNA in *P. denitrificans* ATCC 17741. (A) Typical growth curves of *P. denitrificans* ATCC 17741 carrying vector pLA2917 (open circles) or *R. sphaeroides rho′*-containing cosmid p74-B (closed circles [Fig. 2]). Overnight cultures were diluted to the same optical density, and growth was monitored by measuring the $A_{660}$. The results of one representative experiment are shown; however, similar inhibition of growth was observed for ATCC 17741(p74-B) and ATCC 17741 (pLA2917) in several independent experiments. (B) Northern blot of RNAs from strains ATCC 17741(pUI1830Δ, p74-B) (lane 1) and ATCC 17741(pUI1830Δ, pLA2917) (lane 2), hybridized with the *puf*-specific probe (see Materials and Methods). Lane 2 contains twice as much total RNA (20 μg) as lane 1 (10 μg). Total RNA was isolated from exponentially growing cultures at an $A_{660}$ of 0.5. The sizes of RNA markers (in kilobases) are shown on the right. The size of *pufB*::*lacZYA′* mRNA is expected to be ~4.0 kb. Smaller bands presumably represent prematurely terminated transcripts or degradation products of larger transcripts.

ATCC 17741(pUI1830Δ) (Fig. 1B). This fragment contains ~1.8 kb of insert DNA and ~1.2 kb of vector pLA2917 DNA (plasmid p74-B; Fig. 2). The DNA sequence of this insert revealed the presence of two open reading frames (ORFs) characteristic of *R. sphaeroides* codon usage (Fig. 3). The second ORF was truncated and extended 32 bp into vector pLA2917 DNA until a TGA stop codon was found (data not shown). To determine the role of each ORF, we subcloned each one separately into vector pRK415. *orf1* (plasmid p74-HP; Fig. 2) affected neither growth nor *puf*::*lacZ* expression (data not shown). In contrast, the second ORF (plasmid p74-BsHp; Fig. 2), although lacking its own promoter (see below), resulted in both growth inhibition and increased *puf*::*lacZ* expression in ATCC 17741(pUI1830Δ) (Table 2). This second ORF was found to encode a protein which is ~69% identical to the corresponding portion of the transcription termination factor Rho from *E. coli* (Fig. 4). Significant levels of similarity were also found between this ORF and Rho proteins from other bacterial species. Therefore, we assume that the second ORF represents a truncated *R. sphaeroides* Rho protein, designated Rho′. The putative Rho′ encoded by pUI8074 is truncated after amino acid 374 and contains 10 residues encoded by the DNA of vector pLA2917.

**Cloning and sequence of the full-length *rho* gene.** To understand more fully why *R. sphaeroides rho′* affected growth and expression of *puf*::*lacZ* in ATCC 17741(pUI1830Δ), we cloned the full-length *R. sphaeroides rho* gene and introduced it into ATCC 17741(pUI1830Δ). Using restriction fragment analysis and Southern hybridization (data not shown), we localized the full-length *rho* gene to a 2.7-kb *Bam*HI fragment from cosmid pUI8278 (Fig. 2), which overlaps cosmid pUI8074 (4). According to the nucleotide sequence, the full-length *rho* gene encodes a protein of 422 amino acids (Fig. 3), assuming that the most 5′ methionine, preceded by a putative ribosome binding site, is chosen as a translation start. In that case, the putative *R. sphaeroides* Rho is similar in length and structure to the Rho factors from members of *Proteobacteria* (~62 to 68% identity).

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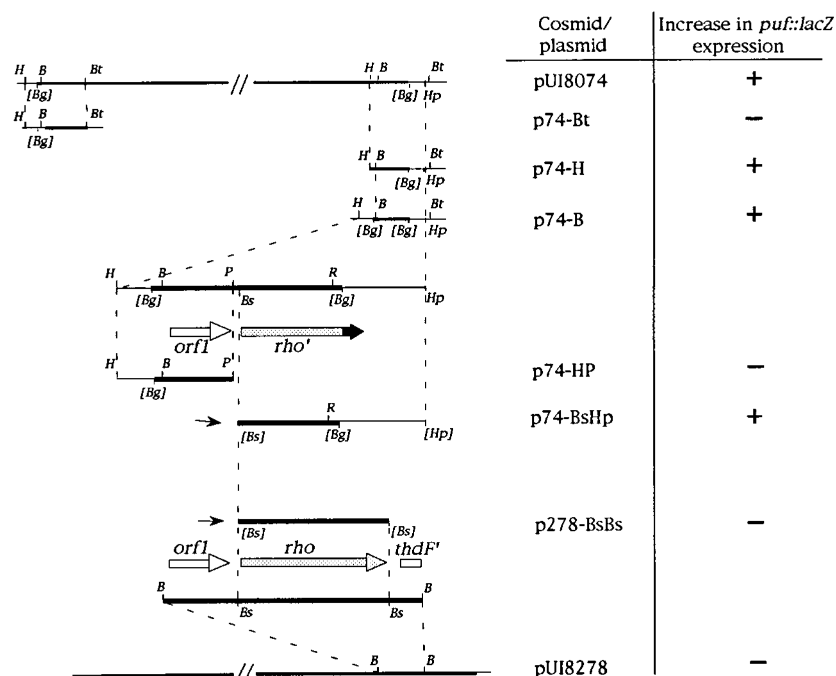


FIG. 2. Cloning of *R. sphaeroides rho′* from cosmid pUI8074 and full-length *rho* from cosmid pUI8278. The restriction sites relevant to cloning are shown as follows: *B*, *Bam*HI; *Bg*, *Bgl*II; *Bs*, *Bsp*EI; *Bt*, *Bst*EII; *H*, *Hin*dIII; *Hp*, *Hpa*I; and *R*, *Eco*RI. Disrupted sites are shown in brackets. Solid arrows in front of plasmids p74-BsHp and p278-BsBs indicate the direction of transcription of promoterless *rho′* and *rho* from vector pRK415 promoters. The tip of the arrow corresponding to *rho′* is in black to stress that the 3′ end of *rho′* is derived from vector pLA2917 DNA. +, an increase in *puf*::*lacZ* expression greater than the standard deviation of measurements (15%).

```
       BamHI
   1 GGATCCTGTGGAAAAGCGCAGGGGTAAGATCGGAAATCCAGTTATCCCTGCGCCCTACTGCATCATTCCCTTCTAATCTTTCTTTTCTTATGAGGATGGCCGACCACTTCGAGGAGACGA
                                                                                                        (M)     (M)
 121 CGATGGGCACGTTCCTGGCCGACTACTACCTGTGGACCAAGTCGCTGCATGTGATTTCGGTGCTGGCCTGGATGGCGGGGCTGTTCTACCTGCCCCGGCTCTTCGTCTATCACGCCGAGG
ORF1   M  G  T  F  L  A  D  Y  Y  L  W  T  K  S  L  H  V  I  S  V  L  A  W  M  A  G  L  F  Y  L  P  R  L  F  V  Y  H  A  E  V

 241 TCGTGAAAGCCGGAACCGAGACGGACGCGCTGTTCCAGACAATGGAGCGGCGGCTTCTGAGGGCGATCATGAACCCGGCCATGATCGCGACCTGGATCTTCGGCCTGCTCCTCGTCTTCA
       V  K  A  G  T  E  T  D  A  L  F  Q  T  M  E  R  R  L  L  R  A  I  M  N  P  A  M  I  A  T  W  I  F  G  L  L  L  V  F  T

 361 CGCCGGGCATCGTCGACTGGTCGATGCTCTGGCCCTGGACGAAGGCCGCCTGCGTTCTGGCGATGACCGGATTTCACATGTGGCTCGCGGCCCGCCGGCGCGACTTCGCGGCGGGCGCCA
       P  G  I  V  D  W  S  M  L  W  P  W  T  K  A  A  C  V  L  A  M  T  G  F  H  M  W  L  A  A  R  R  R  D  F  A  A  G  A  N

 481 ACCGCCACAAGGGGCGCACCTACCGCATGATGAACGAGCTTCCGACGCTCCTCATGCTGGTGATCGTCTTCTCCGCTGTGGCGAAATGGAACTACTGGGGCTTCTGAGCCCCCGGCCCAA
       R  H  K  G  R  T  Y  R  M  M  N  E  L  P  T  L  L  M  L  V  I  V  F  S  A  V  A  K  W  N  Y  W  G  F
                                                                                                             BspEI
 601 GATCATTGACTCGCGGCGTCGCAGCGCCTATGTGCGGCCTTGCAGGGCCGTCCGGTCCATTGCATCTTCCCATCCTATGCTGCAGCGACTCCCCCTTCCGGAGGCGCTCCTGACGAGCGC

 721 AGAGTGCCATGAACGAACGTCTCAACCTCGCTGACCTGAAGGCCAAGACTCCGGCCGATCTACTGGCCATGGCCGAGGAGTGGGAGATCGAGAACGCGCCTTCCATGCGGAAGGGCGAGA
Rho          M  N  E  R  L  N  L  A  D  L  K  A  K  T  P  A  D  L  L  A  M  A  E  E  W  E  I  E  N  A  P  S  M  R  K  G  E  M

 841 TGATGTTCTCGATCCTCAAGGAACATGCGGAGGAAGGCTACGAAGTCGGCGGAGACGGCGTGCTCGAGGTGCTGCAGGACGGTTTCGGCTTCCTGCGCTCGCCCGAGGCCAACTATCTGC
       M  F  S  I  L  K  E  H  A  E  E  G  Y  E  V  G  G  D  G  V  L  E  V  L  Q  D  G  F  G  F  L  R  S  P  E  A  N  Y  L  P

 961 CGGGCCCGGACGACATCTACGTCTCGCCCGAGATCCTGCGTCAGTTCTCGTTGCGCACCGGCGACACGATCGAGGGCGTGATCGTCGCGCCCCGCGAGAACGAACGCTATTTCAGCCTGA
       G  P  D  D  I  Y  V  S  P  E  I  L  R  Q  F  S  L  R  T  G  D  T  I  E  G  V  I  V  A  P  R  E  N  E  R  Y  F  S  L  T

1081 CGCGGGTGACGAAGATCAACTTCGACGATCCCGAACGGGCGCGCCACAAGGTGCACTTCGACAACCTCACGCCGCTCTATCCCGATGAACGGCTGAAGATGGAAGTCGACGATCCCACGA
       R  V  T  K  I  N  F  D  D  P  E  R  A  R  H  K  V  H  F  D  N  L  T  P  L  Y  P  D  E  R  L  K  M  E  V  D  D  P  T  M

1201 TGAAGGACCGTTCCGCGCGCATCATCGATCTGGTGGCGCCGATCGGGAAGGGGCAGCGCGGGCTGATCGTGGCGCCGCCGCGGACCGGCAAGACGGTGCTCCTGCAGAACATCGCCCACT
       K  D  R  S  A  R  I  I  D  L  V  A  P  I  G  K  G  Q  R  G  L  I  V  A  P  P  R  T  G  K  T  V  L  L  Q  N  I  A  H  S
         NruI(5)
1321 CCATCGCGACCAATCACCCCGAGTGCTACCTGATCGTGCTGCTCATCGACGAGCGGCCCGAGGAGGTCACGGACATGCAGCGGTCGGTGAAGGGGGAGGTCGTCTCCTCGACCTTCGACG
       I  A  T  N  H  P  E  C  Y  L  I  V  L  L  I  D  E  R  P  E  E  V  T  D  M  Q  R  S  V  K  G  E  V  V  S  S  T  F  D  E

1441 AGCCCGCCACGCGGCACGTCGCCGTGGCCGAGATGGTCATTGAGAAGGCCAAGCGCCTCGTCGAGCACAAGCGCGACGTGGTGATCCTGCTCGACTCGATCACCCGCCTCGGGCGCGCCT
       P  A  T  R  H  V  A  V  A  E  M  V  I  E  K  A  K  R  L  V  E  H  K  R  D  V  V  I  L  L  D  S  I  T  R  L  G  R  A  F

1561 TCAACACCGTGGTGCCCTCGTCCGGCAAGGTGCTGACCGGCGGCGTCGATGCCAACGCGCTTCAGCGTCCGAAAAGGTTTTTCGGCGCAGCGCGGAACATCGAGGAAGGCGGGTCGCTGA
       N  T  V  V  P  S  S  G  K  V  L  T  G  G  V  D  A  N  A  L  Q  R  P  K  R  F  F  G  A  A  R  N  I  E  E  G  G  S  L  T
            NruI(6)                                                      EcoRI
1681 CGATCATCGCGACCGCCCTGATCGATACCGGCAGCCGGATGGACGAAGTGATCTTCGAAGAATTCAAGGGTACCGGCAACTCCGAGATCGTCCTTGACCGGAAGGTCGCCGACAAGCGCG
       I  I  A  T  A  L  I  D  T  G  S  R  M  D  E  V  I  F  E  E  F  K  G  T  G  N  S  E  I  V  L  D  R  K  V  A  D  K  R  V
                                                   (Sau3A)
1801 TGTTCCCCGCCATGGACATCCTCAAATCTGGCACCCGGAAAGAGGATCTGCTGGTCGACAAGTCCGATCTGCAGAAGACCTATGTCCTGCGCCGCATCCTGAACCCGATGGGCACCACCG
       F  P  A  M  D  I  L  K  S  G  T  R  K  E  D  L  L  V  D  K  S  D  L  Q  K  T  Y  V  L  R  R  I  L  N  P  M  G  T  T  D
                                                   I  K  R  Q  D  E  D  R  F  A
1921 ATGCGATCGAGTTCCTGATCTCCAAGCTGCGGCAGACCAAGAGCAACGCGGAATTCTTCGATTCCATGAACACCTGAGCGACCCGCTCGACAGGACGCCGGAATGGACACGATCTACGCC
       A  I  E  F  L  I  S  K  L  R  Q  T  K  S  N  A  E  F  F  D  S  M  N  T                        ThdF    M  D  T  I  Y  A
                                                 BspEI
2041 TTGGCCAGCGCACGCGGCAAGGCCGGAGTGGCGGTTTTGCGCCTTTCCGGACCGCGGTCGCATGAGGCGGTGCAGGCGTTCGGCGTTCCTTTGCCTTCTCTCCGGCACGCCGCCCTGCGG
     L  A  S  A  R  G  K  A  G  V  A  V  L  R  L  S  G  P  R  S  H  E  A  V  Q  A  F  G  V  P  L  P  S  L  R  H  A  A  L  R

2161 CGTCTGACGTGGAACGGTGAAGTTCTCGACGAAGCGCTGGTGCTGCTCTTCGGCGCTGGCGCCAGTTTCACTGGTGAAACCTCGGCCGAACTGCATCTCCATGGTAGTCCTGCTGCCGTC
     R  L  T  W  N  G  E  V  L  D  E  A  L  V  L  L  F  G  A  G  A  S  F  T  G  E  T  S  A  E  L  H  L  H  G  S  P  A  A  V

2281 TCTTCGGTGCTCCGGGTCCTGTCCGGCCTGCCCGGG
     S  S  V  L  R  V  L  S  G  L  P  G
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *orf1-rho-thdF′* region (GenBank accession number L76097). Restriction sites relevant to plasmid construction are shown above the nucleotide sequence. (Sau3A), the site of fusion of *rho′* to vector pLA2917 DNA in cosmid pUI8074. The protein sequence resulting from this fusion is shown in italics below the sequence of full-length Rho. (M), two alternative start codons for ORF1. Dyad symmetry TGT-$N_{12}$-ACA resembling the PpsR binding site and located within the 3′ end of *rho* is underlined.

It is at least ~50% identical to any other Rho factor whose structure has been reported (26, 31, 36, 42).

Despite a relatively high level of sequence similarity, *R. sphaeroides* Rho differs from its counterparts in several highly conserved residues, including several within the ATP-binding site (see Discussion). Interestingly, some of the residues present in *R. sphaeroides* Rho were found to be identical to the residues in mutant forms of the *E. coli* Rho protein which accumulated when the *E. coli rho* gene was maintained in multicopy plasmids (see Discussion). This prompted us to reexamine the sequence of the corresponding region of *R. sphaeroides rho* from the low-copy-number cosmid pUI8278 template. This sequence did not differ from the sequence previously determined. Therefore, no apparent mutations have been acquired during the maintenance of *R. sphaeroides rho* in multicopy plasmids in *E. coli.*

**Expression of *R. sphaeroides rho* and *rho′* in heterologous hosts.** We tested the effect of full-length *rho* in ATCC 17741 (pUI1830Δ). No differences were found between strains containing cosmid pUI8278 or vector pLA2917. This is consistent with the results of the original genomic library screen, which did not identify pUI8278 as a cosmid resulting in increased LacZ expression. Hence, only the truncated, not full-length, *rho* was responsible for increased *puf*::*lacZ* expression and growth inhibition of *P. denitrificans.*

To permit a quantitative comparison of the effects of *rho* and *rho′*, we cloned the promoterless full-length *rho* into pRK415 to create plasmid p278BsBs (Fig. 2) in a manner similar to that used for *rho′* (plasmid p74-BsHp; Fig. 2). The *Bsp*EI site used for these clonings is located only 31 bp upstream of the putative translation start codon of *rho* (Fig. 3); therefore, *rho* and *rho′* are most likely devoid of the native *rho* promoter and are likely to be transcribed from the vector pRK415 promoter(s) upstream of *tet* and/or *lac*. The effects of both p74-BsHp and p278BsBs on *puf*::*lacZ* expression were compared by using pRK415 as a control. As evident from Table 2, only *rho′*, not *rho*, resulted in increased LacZ levels in ATCC 17741 (pUI1830Δ). The fold increase in *puf*::*lacZ* expression derived from *rho′* contained in p74-BsHp was lower than the increase produced by *rho′* expressed from p74-B apparently under its own promoter (Table 2). Similarly, growth inhibition of ATCC 17741 due to the presence of p74-BsHp was less pronounced than it was in the case of p74-B (data not shown). These results suggest that the magnitude of the *rho′* effect depends upon the level of *rho′* expression and hence the apparent cellular concentration of Rho′.

Using transcriptional fusions of other PS genes to *lacZ* (data not shown) as well as a promoterless *lacZ*, we were able to determine that the effect of *rho′* was not specific. Low-level basal expression of LacZ was observed in ATCC 17741(pLX1)

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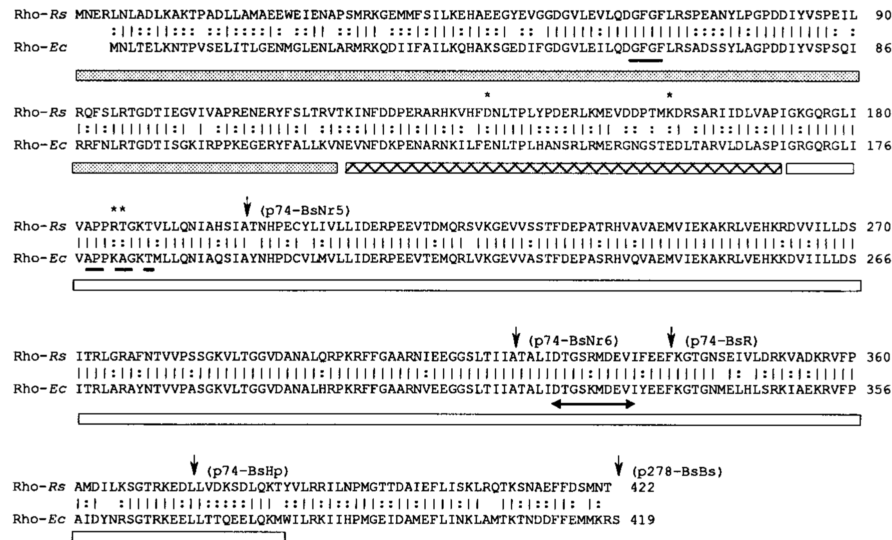


FIG. 4. Alignment of the amino acid sequences of Rho proteins from *E. coli* (Rho-*Ec* [33]) and *R. sphaeroides* (Rho-*Rs*). Vertical lines indicate identical residues; colons indicate homologous residues; residues mentioned in the text are marked by asterisks. Arrows above the *R. sphaeroides* Rho sequence mark points of truncation in Rho′ expressed from the plasmids shown in parentheses. The domain structure of Rho is shown, according to Opperman and Richardson (31), as follows: shaded area, RNA-binding domain; hatched area, connector region; open area, ATP-binding domain. Consensus in the RNA-binding domain is underlined with a plain line; the ATP-binding site is underlined with a dashed line; the Rho-specific sequence (31) is underlined with arrows.

carrying vector pRK415 (Table 2). However, when p74-BsHp, containing *rho′*, was introduced into this strain, the LacZ level increased ~threefold (Table 2). No increase was observed when the full-length *rho* present on p278BsBs was introduced (Table 2). Therefore, the effect of *R. sphaeroides rho′* does not appear to be specific to *puf* expression. However, *rho′* strongly affected the relative abundance of *puf*::*lacZ* mRNA accumulation in ATCC 17741(pUI1830Δ) (Fig. 1B).

Summarizing the observations made above, we conclude that increased LacZ expression and growth inhibition of ATCC 17741(pUI1830Δ) are likely consequences of a more general toxic effect of *R. sphaeroides rho′*. We reason that this effect reflects disturbances in the *P. denitrificans* ATCC 17741 transcription termination machinery caused by *R. sphaeroides* Rho′.

The abilities of *R. sphaeroides* Rho and Rho′ to interfere with the transcription termination machinery of a heterologous host were tested in an *E. coli* CY15621 (*trpE9851-trpA38*) double nonsense mutant (21, 45). The full-length *R. sphaeroides* Rho expressed from multicopy plasmid p278BsBs conferred on strain CY15621 an ability to utilize indole instead of tryptophan apparently because of the relief of polarity in the *trp* operon (plasmid p278BsBs; Fig. 5). Therefore, the *R. sphaeroides* Rho protein specifically interferes with the transcription termination machinery of *E. coli*, perhaps by forming inactive heteromers with the *E. coli* Rho protein (see Discussion).

The effect of Rho′ (plasmid p74BsHp; Fig. 5) on indole utilization by strain CY15621 was much less pronounced.

**Expression of *R. sphaeroides rho* and *rho′* in *R. sphaeroides*.** Because *R. sphaeroides rho′* affects the growth of heterologous hosts, we anticipated that the effect of *rho′* in *R. sphaeroides* could be even more pronounced. Despite several trials, we were unable by conjugation to introduce plasmid p74BsHp, carrying *rho′*, into *R. sphaeroides* 2.4.1.

The apparent frequency of transfer into 2.4.1 for plasmid p74-BsHp was ~$10^2$ to $10^3$ lower than the frequency of transfer for either pRK415 or p278BsBs. However, when plasmids from six randomly chosen transconjugants expected to receive p74-BsHp were isolated and analyzed, all of them were found to have undergone rearrangements and none contained *rho′* (data not shown). The apparent inability of *R. sphaeroides* to maintain both truncated (on plasmid) and intact (on chromosome)

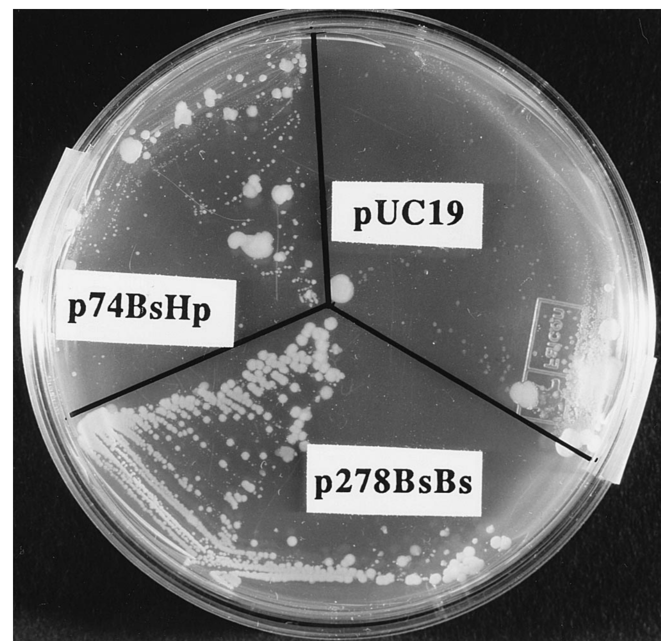


FIG. 5. Relief of polarity in the *trp* operon of *E. coli* CY15621 by *R. sphaeroides rho*. Shown is the growth of strain CY15621 carrying either pUC19 (vector), p278BsBs (*R. sphaeroides rho*), or p74BsHp (*R. sphaeroides rho′*) after 5 days on minimal medium in the presence of indole. Large colonies on the lawns of smaller ones for pUC19 and p74BsHp most likely represent putative pseudorevertants to an indole-utilizing phenotype (independent of the presence of *R. sphaeroides rho*).

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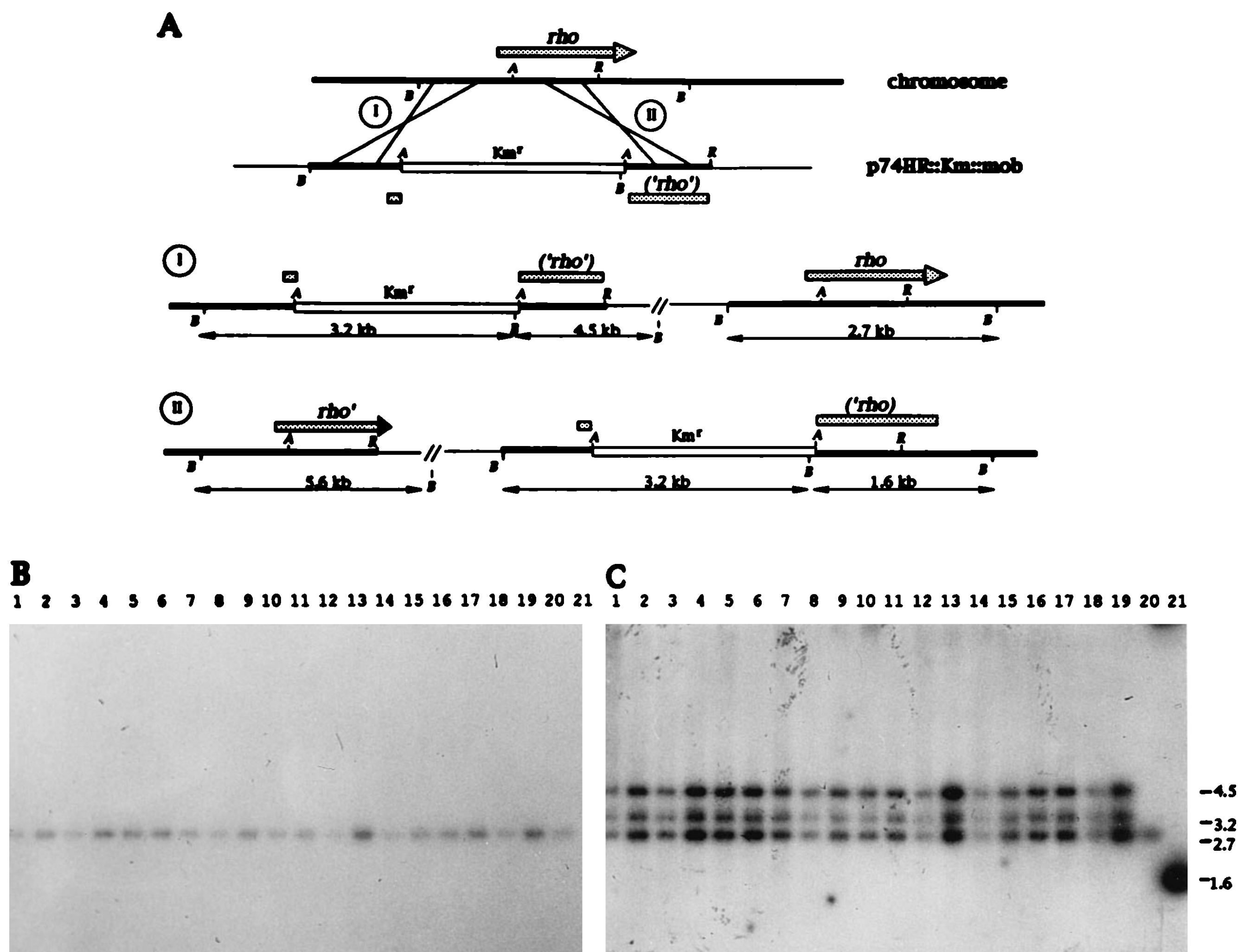


FIG. 6. (A) Possible single crossovers between the chromosomal region containing *rho* and plasmid p74HR::Km::mob. *A*, *Apa*I; *B*, *Bam*HI; *R*, *Eco*RI. The black tip of the *rho′* arrow in scheme II corresponds to the heterologous sequence originated from the vector DNA. (B and C) Southern blot of the *Bam*HI-digested chromosomal DNAs from single-crossover recombinants (lanes 1 through 19) and wild-type strain 2.4.1 (lane 20), as well as a 1-kb ladder (lane 21). The blot was hybridized with the probe specific to the 3′ end of *rho* (B) or the probe specific to vector pUC19 and *rho′* lacking the 3′ end (C). The probes are described in Materials and Methods. The sizes of bands (in kilobases) are shown on the right. The 1.6-kb band of the 1-kb ladder (lane 21) gives a signal with the pUC19-derived probe.

copies of *rho* suggests that interference with Rho-dependent transcription termination is intolerable for *R. sphaeroides*. This further suggests that Rho-dependent transcription termination is essential in this bacterium.

To determine the minimal size of Rho′ still capable of producing a lethal effect in *R. sphaeroides*, we constructed several plasmids containing Rho′ proteins progressively truncated from the carboxy terminus. Plasmid p74-BsR (Fig. 4) encodes a protein containing 339 amino acids of Rho, i.e., 35 residues less than the protein encoded by plasmid p74-BsHp (Fig. 4). We were unable to introduce p74-BsR into *R. sphaeroides* 2.4.1. However, deletion of a further 19 amino acids abolished the toxic effect, as judged by the ability to introduce the corresponding construct (plasmid p74-BsNr6; Fig. 4) into *R. sphaeroides* 2.4.1. Neither Rho′ containing a longer deletion (p74-BsNr5; Fig. 4) nor full-length Rho (plasmid p278-BsBs; Fig. 4) was toxic. Hence, the minimal domain within Rho′ absolutely required for its toxic effect appears to be confined to a small region between residues 320 and 339, although additional sequences downstream of residue 339 may contribute to this effect.

**Essentiality of *rho* in *R. sphaeroides*.** To test the essentiality of Rho-dependent transcription termination in *R. sphaeroides*, we attempted to inactivate the chromosomal *rho* gene. We constructed a suicide plasmid, p74HR::Km::mob, for *R. sphaeroides*, which contains a $Km^r$ cartridge in the *Apa*I site of *rho′* (Fig. 6A). This plasmid was mobilized into *R. sphaeroides* 2.4.1, and ~500 $Km^r$ clones were isolated. All of these were found to be $Tc^r$, which indicated that they originated from single crossovers between the homologous regions of the plasmid and chromosome. Our inability to isolate double-crossover recombinants with a disrupted chromosomal copy of *rho* is in accord with the assumption that the *rho* gene is essential in *R. sphaeroides.*

We further analyzed the DNA structures of 19 randomly picked single-crossover recombinants at the *rho* locus. In all clones, we found that single crossovers took place 5′ of the $Km^r$ cartridge (Fig. 6B and C), which allowed all 19 representatives to preserve an intact copy of *rho* on the chromosome (Fig. 6A, scheme I). Single crossovers 3′ of the $Km^r$ cartridge would result in the absence of an intact *rho* (Fig. 6A, scheme II). The DNA regions available for homologous recombination

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5′ and 3′ of the Km$^r$ cartridge are of the same order, ~960 bp 5′ and ~780 bp 3′ of the cartridge. Previous experience indicates that the number of recombinants originating from single crossovers 5′ and 3′ of the Km$^r$ cartridge should differ only slightly. However, as is evident from Fig. 6B and C, none of the recombinants originated from single crossovers 3′ of the Km$^r$ cartridge. We therefore conclude that *rho* is an essential gene in *R. sphaeroides.*

**Genes surrounding *R. sphaeroides rho*.** We have determined the DNA sequence upstream and downstream of *rho* and found that the genes surrounding *rho* in *R. sphaeroides* differ from those in other bacterial species.

*orf1*, 5′ of *R. sphaeroides rho*, is separated from *rho* by 142 bp (Fig. 3). *orf1* is predicted to encode a protein of either 154, 163, or 165 amino acids, depending on the start of translation. Among the three possible methionine residues, the second and especially the third are preceded by GA-rich sequences resembling Shine-Dalgarno-like sequences. Although the reading frame extends 5′ of the first aforementioned methionine, the codon usage in that region is clearly different from the codon usage of *R. sphaeroides*, which is biased toward G and C in the third position. The *orf1*-encoded protein appears to be hydrophobic, containing several long stretches of hydrophobic amino acids which might represent membrane spanning domains (not shown). Therefore, ORF1 is likely to be an integral membrane protein. No outstanding similarities were found for ORF1 in the databases. The best matches were among electron transfer proteins, i.e., NADH-ubiquinone oxidoreductases from mitochondria of mosquitoes of genus *Anopheles* (~23 to 24% identical residues over the whole length of ORF1) (SwissProtein accession numbers P33511 and P34852) and bacterial and yeast cytochrome *c* oxidases (~16 to 18% identical residues) (GenBank accession number X74341 and SwissProtein accession numbers P31833 and P07657). The following local similarities were also found with another group of proteins, peptidases: ~21% identical residues in a 94-amino-acid overlap with the leader peptidase from *Erwinia carotovora* (SwissProtein accession number P31712) and ~50% identical residues in a 22-amino-acid overlap with the 25-amino-acid human spermatozoon protease acrosin (GenBank accession number M77378). An indirect indication of the possible link of ORF1 with the function or assembly of cytochromes came from an observation that *E. coli* strains overproducing ORF1 turn pink, perhaps because of an excess of accumulated hemes.

Downstream (25 bp) of the *rho* stop codon is an ORF with characteristic *R. sphaeroides* codon usage (Fig. 2 and 3). We sequenced only the 5′ end of this ORF and found that the polypeptide likely encoded by this ORF is similar to the amino termini of the ThdF proteins from *E. coli* (~39% identical residues in the sequenced portion [SwissProtein accession number P255522]), *Pseudomonas putida* (~38% [SwissProtein accession number P25755]), and *B. subtilis* (~31% [SwissProtein accession number P25811]). The exact function of ThdF is unknown. However, *E. coli* ThdF was identified because of its involvement in thiophene and furan oxidation (1). Interestingly, the putative promoter for *R. sphaeroides thdF* most likely lies within the 3′ end of the *rho* gene (data not shown). We also found a region of dyad symmetry, TGT-$N_{12}$-ACA (where N is a nucleotide), inside the 3′ end of the *rho* gene (Fig. 3). This dyad symmetry resembles the proposed binding site (3, 18, 24) for the transcriptional repressor of several PS genes, the PpsR protein (18, 32). If PpsR is capable of binding to this site, it might affect *rho* expression, which could constitute a direct link between the expression of Rho and PS genes. This possibility has not been analyzed.

## DISCUSSION

**Activators of *puf::lacZ* expression identified in *P. denitrificans*.** The genetic screen described here for the identification of transcriptional regulators of the *puf* operon from *R. sphaeroides* 2.4.1 was established in *P. denitrificans*, a non-PS bacterium closely related to *R. sphaeroides*. This screen identified genes encoding known activators of *puf* operon expression, the *prr* genes. This observation, together with our previous report (18), validates the use of *P. denitrificans* as an alternative host for the expression of *Rhodobacter* genes, including PS genes.

**Expression of *rho* and *rho′* in heterologous hosts.** In addition to the *prr* genes, we identified a truncated form of the *R. sphaeroides rho* gene, *rho′*, as being able to activate *puf::lacZ* expression in *P. denitrificans*. We have shown that the effect of *rho′* in *P. denitrificans* is not specific for *puf*. The exact mechanism by which *rho′* affects LacZ expression in *P. denitrificans* has not been addressed, partly because increased LacZ expression was not the sole consequence of the presence of *rho′*. We also observed that *rho′* had what we termed a toxic effect on *P. denitrificans*, expressed as moderate, but reproducible, growth inhibition. A general explanation of these phenomena is that *R. sphaeroides* Rho′ interferes with the normal transcription termination machinery of *P. denitrificans*. We were able to show that *R. sphaeroides* Rho and, to a lesser extent, Rho′ interfere specifically with the transcription termination machinery in the more distant bacterium *E. coli.*

Rho factor from *E. coli* is known to act as a hexamer (34, 37). It seems safe to propose that Rho from *R. sphaeroides* also acts as a multimer. Evidently, *R. sphaeroides* Rho and, to a lesser extent, Rho′ (encoded by pUI8074 and its derivatives) are able to associate with the chromosomally encoded full-length Rho proteins from these heterologous hosts to form less functional heteromers and thus interfere with Rho-dependent transcription termination. In accord with this assumption is the fact that the toxic effect of Rho′ in *P. denitrificans* depends on the level of expression of *rho′*. We reason that full-length *R. sphaeroides* Rho does not cause any deleterious effects when expressed in *P. denitrificans* because of the very high degree of structural and functional similarities between the Rho proteins from these related bacteria. However, when full-length *R. sphaeroides* Rho is expressed in the more distantly related bacterium *E. coli*, it appears to interfere with transcription termination in this bacterium.

**Structural features of *R. sphaeroides* Rho.** Three distinct domains have been proposed to constitute *E. coli* Rho (8, 11, 31), and each of these domains is evident in *R. sphaeroides* Rho (Fig. 4). An amino-terminal domain (~116 residues for *E. coli* Rho) has been shown to function in RNA binding (27). The percentage of identical amino acids between the Rho proteins of *E. coli* and *R. sphaeroides* in this region is ~47%, which is much less than the ~67% identity between the full-length proteins. Lesser homology in the RNA-binding domain may reflect differences in mRNA sequences which function as Rho-dependent transcription termination sites. This would be expected because *R. sphaeroides* has a substantially higher genomic G+C content (~68%) than does *E. coli* (~50%). However, the core of the RNA-binding domain, the GFGF motif (31) (Fig. 4), is conserved.

The connector region between the RNA-binding and ATP-binding domains (Fig. 4) is variable among bacterial Rho proteins; therefore, it is difficult to assess the role of individual amino acid changes within this region. However, there are two noteworthy substitutions. The residue corresponding to E-155 of *E. coli* Rho is identical in all Rho proteins, except for Rho from *Deinococcus radiodurans*, which has a conserved E→D

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substitution (31). Instead of E or D, *R. sphaeroides* Rho contains an oppositely charged K-159 (Fig. 4). The corresponding E-155→K mutation was found in the *E. coli* Rho protein under conditions of *rho* overexpression and was shown to decrease the transcription termination activity of this mutant Rho (28, 38). The E-134→D mutation in *E. coli* Rho also resulted in decreased transcription termination activity (31). We found that *R. sphaeroides* Rho contains D-138 at the corresponding position (Fig. 4).

The ATP-binding domain (Fig. 4) is the most conserved portion of the bacterial Rho proteins, and *R. sphaeroides* Rho is no exception. However, the putative ATP-binding site of *R. sphaeroides* Rho is more divergent than that of any other bacterial Rho protein described so far. A maximum of only one conserved amino acid substitution has been observed so far in the ATP-binding motif, the so-called P loop, from various bacterial Rho proteins (31). However, two substitutions, although conserved, are present in *R. sphaeroides* Rho (Fig. 4). One of these, R-185, corresponds to K-181 in *E. coli* Rho. This residue was shown by cross-linking to be in close proximity to ATP (10, 29), and the K-181→Q mutation resulted in a marked decrease in the efficiency of transcription termination by mutant *E. coli* Rho (9).

The carboxy-terminal domain consists of the last ~40 to 50 residues (31). No function has been assigned to this region, which is variable in both primary sequence and length among the various bacterial Rho factors. The degree of identity between the *R. sphaeroides* and *E. coli* Rho proteins in this region is ~50%.

Thus, despite the high-level overall similarity, a number of structural differences between the *E. coli* and *R. sphaeroides* Rho proteins might contribute to significant functional differences between these molecules. This could explain why the expression of full-length *R. sphaeroides rho* interferes with Rho-dependent transcription termination and thus relieves polarity of the *E. coli trp* operon.

**Regions within Rho important in protein-protein interactions.** *R. sphaeroides* Rho′ expressed from plasmid p74-BsHp (Fig. 4) lacks the 48 carboxy-terminal residues but contains both intact RNA- and ATP-binding domains. We suggest that the most likely reason why it is nonfunctional in *R. sphaeroides* is because of a defect in assembly. In accord with this assumption is our observation that Rho′ is much less efficient, compared with full-length Rho, in relieving polarity of the *trp* operon in *E. coli* (Fig. 5). An indication that the carboxy terminus is involved in Rho assembly was also described for *E. coli* Rho, i.e., the *E. coli* Rho15 temperature-sensitive mutant protein lacking nine carboxy-terminal amino acids (6, 30) forms hexamers less readily than does wild-type *E. coli* Rho (16). However, our data suggest that the region(s) involved in the assembly of a functional transcription termination complex extends beyond this carboxy-terminal sequence.

Because Rho′ lacking as many as 83 residues from the carboxy terminus (plasmid p74-BsR; Fig. 4) is toxic for *P. denitrificans* and apparently lethal for *R. sphaeroides*, we believe it is able to interact with the chromosomally encoded intact Rho and/or other components of the transcription termination machinery to reduce or eliminate the activity of the derived protein complex. However, deletion of an additional 19 residues (plasmid p74-BsNr6; Fig. 4) virtually abolishes the toxicity of this truncated Rho protein. An even more extensive deletion (plasmid p74-BsNr5; Fig. 4) has no deleterious effect. Hence, the data point to the fact that the region between residues 320 and 339 of *R. sphaeroides* Rho may be crucial for protein-protein interactions. We cannot rule out that several amino acid residues derived from vector sequences and attached to the truncated forms of Rho in p74-BsR, p74-BsNr6, and p74-BsNr5 may potentially contribute to the folding of Rho′ and therefore interfere with Rho-Rho′ interactions. However, it is interesting that Opperman and Richardson (31) detected a motif highly conserved among diverse bacteria and unique to the Rho protein (Fig. 4; residues 322-331 of *E. coli* Rho) which lies within the above-mentioned 19-amino-acid deletion. According to the tertiary structure of Rho (11), this region forms a turn located outward of the ATP-binding domain and therefore is potentially capable of participating in protein-protein interactions. Moreover, several assembly mutants were identified in the corresponding region of *E. coli* AtpB, whose secondary structure is believed to be analogous to that of Rho (13).

**Essentiality of *rho* in *R. sphaeroides*.** The observation that *R. sphaeroides* cannot accommodate the presence in *trans* of *rho′* suggests that Rho-dependent transcription termination is essential for this bacterium. Further, our inability to disrupt the chromosomal copy of *rho* indicates its essentiality. Therefore, *R. sphaeroides* is similar to *E. coli* and different from *B. subtilis*. In the latter bacterium, two *rho* null mutations have been reported (36). Although much more data for other bacterial species are required, the existing evidence indicates that *rho* may be an essential gene for gram-negative bacteria but not for gram-positive bacteria.

Because *rho* is essential in *R. sphaeroides*, studying its role in the transcription of PS gene expression in vivo seems problematic. However, the availability of the *R. sphaeroides rho* gene coupled with isolation of the RNA polymerase from this bacterium (17) may permit in vitro studies.

## ACKNOWLEDGMENTS

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