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The *bat* gene of *Halobacterium halobium* encodes a trans-acting oxygen inducibility factor

(archaea/bacteriorhodopsin/purple membrane/*bop* gene cluster)

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ABSTRACT **Oxygen and light affect the expression of the bacterioopsin gene (*bop*), which encodes a light-driven proton pump in the purple membrane of *Halobacterium halobium*. This response is thought to be mediated by a set of genes located adjacent to the *bop* gene. DNA fragments containing either the *bop* gene or the entire *bop* gene cluster reversed the phenotype of purple membrane-deficient strains with mutations in the *bop* gene. Purple membrane synthesis was constitutive in one of these strains transformed with the *bop* gene alone. The same strain transformed with the *bop* gene cluster was inducible by low oxygen tension. Moreover, another strain that constitutively expresses purple membrane remained constitutive when transformed with the *bop* gene alone but the phenotype of the strain changed to inducible when transformed with the *bop* gene cluster. Additional experiments have confirmed that one of the genes of the *bop* gene cluster, the *bat* gene, encodes a trans-acting factor that is necessary and sufficient to confer inducibility of purple membrane synthesis by low oxygen tension.**

Bacteriorhodopsin (BR) is a chromophore-associated integral membrane protein found in the halophilic archaeon, *Halobacterium halobium*. BR is a member of the seven-transmembrane-crossing superfamily of proteins, which includes eukaryotic G protein-coupled receptors with a conserved topology and secondary structure. BR is one of the best-characterized representatives of this family and is one of only a handful of integral membrane proteins for which a high resolution three-dimensional model is available (1). BR is the only protein found in the halobacterial purple membrane where it functions as a light-driven proton pump. The subsequent electrochemical gradient is used to generate ATP (2, 3). This one protein proton pump represents, by far, the simplest photosynthetic mechanism known.

Growth conditions of low oxygen tension and high light intensity induce BR synthesis (4) such that the purple membrane constitutes up to 50% of the cell surface area (3). Transcript levels from the gene encoding the apoprotein of BR, bacterioopsin, increase under these conditions (5). Purple membrane-overproducing mutants exist, which possess higher levels of bacterioopsin mRNA than the wild type (6, 7) and constitutively express BR under high oxygen tension (5, 8). The nature of the mutations resulting in constitutivity is undefined. However, work described here provides information concerning the constitutive mechanism in one mutant strain.

The structural gene encoding bacterioopsin (*bop*) is located within a cluster of genes that includes the *bat, brp*, and *blp* genes. The bacterioopsin activator gene (*bat*) has been proposed to encode a soluble protein capable of detecting low oxygen tension and activating bacterioopsin gene expression in trans (5, 9). This proposed role for the *bat* gene was based on observations that (*i*) mutations in the *bat* gene result in a purple membrane-deficient (Pum$^-$) phenotype and such mutants have a negligible amount of *bop* transcripts under aerobic conditions (10); (*ii*) wild-type *bat* transcription is induced under conditions of low oxygen tension (5); (*iii*) the putative Bat protein possesses a region of striking homology (i.e., 34% identity, 59% similarity over ≈100 amino acids with no gaps) to the oxygen sensor, NifL, of *Klebsiella pneumoniae* (5, 11); and (*iv*) the predicted secondary structure of the putative Bat protein is characteristic of a soluble protein (12).

The bacterioopsin-related protein gene (*brp*) has been proposed to be a membrane-bound light sensor that modulates *bat* gene expression, thus affecting *bop* gene expression indirectly (5, 13). This proposed role for the *brp* gene was based on the observations that (*i*) wild-type *brp* transcription is induced by light but not by low oxygen tension (5); (*ii*) mutations in the *brp* gene result in a Pum$^-$ phenotype, although many such mutants make low levels of *bop* transcripts under aerobic conditions (10); (*iii*) *bat* transcription is affected in *brp* mutants (10); and (*iv*) the predicted secondary structure of the putative Brp protein is characteristic of a membrane protein (12). In contrast to the *bat* and *brp* genes, the bacterioopsin-linked protein gene (*blp*) does not regulate *bop* gene expression but is instead coregulated with the *bop* gene by low oxygen tension (7). Shared sequence motifs observed upstream of the *bop* and *blp* genes have been implicated as regulatory factor binding sites (7).

Recent development of a halobacterial transformation system (14) and a limited array of *Escherichia coli*/halobacterial shuttle vectors [e.g., pWL102 (15); pUBP2 (16)] enable detailed genetic studies in halobacteria. Ni *et al.* (17) complemented a Pum$^-$ mutant containing an insertionally inactivated *bop* gene with a shuttle vector containing the *brp* and *bop* genes. Moreover, a shuttle vector with only the *bop* gene and a few hundred base pairs of upstream sequences was sufficient for complementation in a Pum$^-$ mutant derived from a wild-type background strain that had either an insertionally inactivated or deleted *bop* gene (18, 19). A similar result was obtained when the *bop* gene was integrated into the chromosome of a Pum$^-$ strain via homologous recombination (20).

The discovery of the *brp* and the *bat* genes provided the first clues about the molecular mechanisms whereby low oxygen tension and light induce *bop* gene expression. However, regulatory pathways and activation mechanisms have not been fully defined. In the following study, Pum mutants with alterations in the *bop* gene were transformed with either the *bop* gene, the *bat* gene, or the entire *bop* gene cluster and analyzed for inducibility of purple membrane synthesis in response to low oxygen tension.

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Abbreviations: BR, bacteriorhodopsin; Pum, purple membrane; *bop*, bacterioopsin; *bat*, bacterioopsin activator.

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MATERIALS AND METHODS

Materials. RNAzol was purchased from Cinna/Biotecx Laboratories (Friendswood, TX). Nylon Hybond membranes and [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) were from Amersham. Restriction endonucleases and nucleic acid modifying enzymes were obtained from Bethesda Research Laboratories or Boehringer Mannheim.

Bacterial Strains, Medium, and Growth Conditions. Halobacterial strains used in this study are indicated in Table 1. Growth conditions for inducing experiments were as follows. Halobacterial cultures were established by subculturing to an OD_{660} of ≈0.01 into 50 ml of complex peptone medium (5) supplemented with mevinolin (gift of A. W. Alberts; Merck Sharp & Dohme) to a concentration of 10 μM in a 250-ml Erlenmeyer flask. Cultures were grown in a New Brunswick Scientific gyrotory waterbath shaker (model G76D) at 40°C and 220 rpm. Aliquots were taken during the early, middle, and late logarithmic/early stationary stages of growth to assay for inducibility of purple membrane synthesis. During the late logarithmic/early stationary stage of growth, oxygen naturally becomes limiting. Growth stages were determined by optical density at λ_{660} using a Beckman DU50 spectrophotometer. Early logarithmic stage of growth, OD_{660} = 0.1–0.2; mid-logarithmic stage of growth, OD_{660} = 0.4–0.6; late logarithmic/early stationary stage of growth, $OD_{660} > 1.0$.

Plasmid Constructions. A series of restriction fragments were cloned into the *E. coli/H. halobium* shuttle vector pUBP2, which encodes the halobacterial marker mevinolin resistance (16). In the first construction, an ≈9-kbp *Bst*EII fragment containing the *bop* gene cluster (Fig. 1) was isolated from a cosmid containing the *bop* gene and ≈40 kbp of surrounding genomic sequence of *H. halobium* strain R1 (27). The fragment was cloned into the unique *Bst*EII site of *E. coli* plasmid pCas3.1, which is a derivative of the positive selection vector pKGS (28). Subsequently, the fragment was reisolated, filled-in, and cloned into the *Sma* I site of pUBP2. In a second plasmid construction, a 6-kbp *Dde* I/*Bst*EII fragment containing the *bop* gene cluster (Fig. 1) was isolated from the 9-kbp *Bst*EII fragment, filled-in, and subcloned into the *Sma* I site of pUBP2. In a third plasmid construction, a 1.2-kbp fragment (to be described elsewhere) containing the *bop* gene and ≈370 bp of upstream sequences (Fig. 1) was isolated from *H. halobium* strain R1 DNA using PCR and cloned into the *Pst* I/*Bam*HI sites of pUBP2. In a fourth plasmid construction, a 2.2-kbp *Bam*HI fragment containing the *bat* gene (Fig. 1) was isolated from the 9-kbp *Bst*EII fragment and cloned into the *Bam*HI site of pUBP2. This *Bam*HI fragment contains the *bat* gene along with 112 bp of sequences upstream of the translation initiation codon and 30 bp of sequences downstream of the TGA stop codon. The upstream sequences contain the transcription initiation site of the *bat* gene localized at position −18 relative to the *bat* ATG codon by primer extension analyses (data not shown). The downstream sequences contain the putative transcriptional termination structure for the *bat* gene (7, 9).

Table 1. Halobacterial strains

Strain	Parent	Pum phenotype	Ref.
NRL		+	21
ET1001	NRL	+	22
S9	R1	+	23
L33	S9	−	24
ET15	ET1001	−	22
IV-8	II-7	−	25
IV-14	II-7	−	26

+, Pum$^+$; −, Pum$^-$.

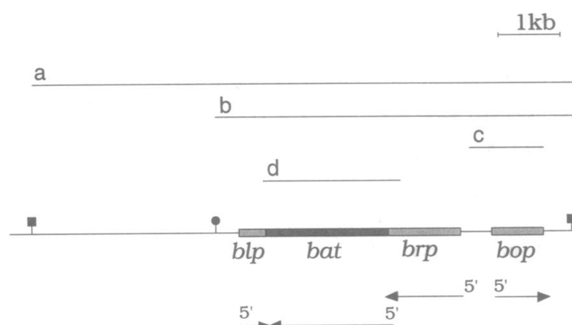

FIG. 1. Map of the *bop* gene cluster. Positions of the genes are indicated by shaded bars. The array of arrows located beneath the map denotes the extent and direction of transcription from the four genes. ■, *Bst*EII restriction sites; ●, *Dde* I restriction site. The size and location of fragments cloned into pUBP2 and used for transformation experiments are indicated by lines above the map: a, 9-kbp *Bst*EII fragment; b, 6-kbp *Dde* I/*Bst*EII fragment; c, 1.2 kbp PCR fragment; d, 2.2-kbp *Bam*HI fragment.

Transformation of Halobacteria. *H. halobium* is prone to cell lysis during transformation procedures (16). Since surfactants are known to promote halobacterial lysis (29) all media and glassware used were soap-free. Transformation was done according to Blaseio and Pfeifer (16) and Cline and Doolittle (30). Transformants were plated on solid complex (YET) medium containing 25 μM mevinolin. Plates were incubated for up to 2 weeks at 42°C prior to scoring of transformants for purple or white color. The presence of the appropriate plasmid construct was verified in transformants by Southern analysis (31).

Inducibility Analyses. Inducibility of purple membrane synthesis was assayed during the growth cycle by examination of the phenotype (i.e., presence or absence of purple membrane) and by determination of *bop* transcript levels. In strains L33 and ET1001, where a Pum phenotype is easily detectable because no other pigments are present, 1-ml culture samples were centrifuged and the pellet was scrutinized for purple color in comparison to wild-type (purple) and mutant (white) strains. To assess the presence of purple membrane in wild-type strain NRL, where the purple color is partially masked by reddish ruberins, bacteriorhodopsin in these samples was bleached with hydroxylamine (32) and difference spectra were recorded from OD_{400} to OD_{800}. For determination of *bop* transcript levels, total RNA was extracted from 4-ml culture samples (early time points) and 2-ml culture samples (mid and late time points) and subjected to Northern analysis as described (7). Probes used were generated by random priming (33) using a PCR fragment encompassing the *bop* gene (7) and a DNA fragment containing the 7S RNA gene (34). Northern blots were visualized by exposure on x-ray films or they were visualized and digitized on a PhosphorImager (Molecular Dynamics).

RESULTS

Inducibility of Pum$^+$ Transformants by Low Oxygen Tension. Pum$^-$ strains L33, ET15, IV-8, and IV-14, all of which contain insertions in the *bop* gene (see Table 1), were transformed to a Pum$^+$ phenotype with the plasmid pUBP2 containing either the *bop* gene cluster (Fig. 1, fragments a and b) or only the *bop* gene with transcriptional initiation and termination signals (Fig. 1, fragment c). Southern analyses showed that the plasmid constructs in the transformants replicated independently and did not integrate into the chromosome (data not shown). Selected Pum$^+$ transformants recovered from the above experiments were examined in comparison to wild-type and Pum overproducing strains to

determine if purple membrane synthesis and *bop* transcript levels were inducible by low oxygen tension.

Wild-type strain NRL showed a lack of purple membrane in the early and mid-logarithmic stages of growth. However, during the late logarithmic stage of growth when oxygen naturally becomes limiting, purple membrane synthesis was induced (Table 2). In contrast, the Pum-overproducing strains ET1001 and S9 showed purple membrane production at all stages of growth, indicative of a constitutive phenotype (Table 2). Northern blot analysis showed that *bop* transcript levels paralleled purple membrane production in these strains (Fig. 2). However, transcript levels in strain ET1001 increased slightly upon entry to the late logarithmic stage of growth.

To determine the consequences of expression of the plasmid encoded *bop* gene cluster in a wild-type background, we transformed the 9-kbp construct into wild-type strain NRL and analyzed inducibility by low oxygen tension. The *bop* gene cluster provided in trans on a multicopy plasmid did not alter the wild-type phenotype of inducibility of strain NRL (data not shown). An increase in *bop* transcript levels was observed but the pattern of transcription throughout the growth cycle more closely resembled that of NRL than ET1001 (Fig. 2). In addition, the *bop*-specific signal was removed and the blots were rehybridized with a probe specific for another RNA, 7S RNA (Fig. 2). This experiment showed relatively constant levels of 7S RNA throughout the growth cycle, indicating that there was no major effect of oxygen availability on transcript levels of the gene encoding 7S RNA.

Transformants chosen for inducibility analysis were those in which plasmid constructs had been transformed into Pum mutant L33. L33 is derived from Pum-overproducing strain S9 (23), which constitutively expresses purple membrane. Strain L33, which does not produce bacterioopsin due to an insertional inactivation of the *bop* gene, produces only truncated *bop* mRNA, which is rapidly degraded (35). As shown in Table 3, the 9-kbp (Fig. 1, fragment a) and 6-kbp fragment (Fig. 1, fragment b) constructs transformed into strain L33 displayed the wild-type pattern of inducibility. In contrast, transformants containing the 1.2-kbp fragment (Fig. 1, fragment c) construct in strain L33 expressed purple membrane constitutively (Table 3) much the same as in the parental strain S9 (Table 2). Transcriptional analysis yielded similar results as the phenotypic inspection. The 1.2-kbp construct in L33 was constitutive, whereas the 9-kbp construct in L33 was inducible (Fig. 3*A*). As a control, the blots were rehybridized with 7S RNA, which was expressed at a relatively constant level throughout the growth cycle (Fig. 3*A*). The above experiments suggest that the 9-kbp and 6-kbp fragments (but not the 1.2-kbp fragment; see Fig. 1 for map) encode one or more factors capable of conferring inducibility of purple membrane synthesis by low oxygen tension in trans.

Reversion of Constitutive Strain ET1001. To test the above hypothesis more directly, all three plasmid constructs (i.e., 9 kbp, 6 kbp, and 1.2 kbp; Fig. 1) were transformed into the constitutively expressing strain ET1001, and transformants

Table 2. Analysis of purple membrane synthesis during the growth cycle in halobacterial strains

Strain	Pum phenotype	Logarithmic stage of growth		
		Early	Middle	Late
ET1001	Constitutive	+	+	+
S9	Constitutive	+	+	+
NRL (wild type)*	Inducible	−	−	+

+, Pum+; −, Pum−.
*In strain NRL, where the purple color is partially masked by ruberins, the presence of purple membrane was determined by bleaching (see text).

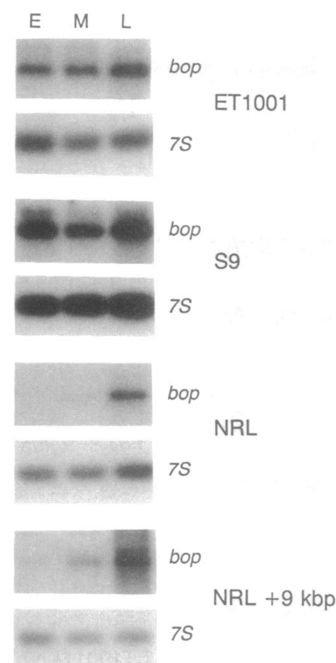

FIG. 2. Northern analysis of inducible and constitutive purple membrane-expressing strains. Samples were taken from the early (E), middle (M), and late (L) logarithmic/early stationary stages of the growth cycle. Strains analyzed are indicated to the far right of the autoradiograms. Probes are indicated in italics to the near right of the autoradiograms. Following hybridization and analysis with *bop* gene-specific probes, blots were stripped and rehybridized with a 7S RNA-specific probe.

were tested for inducibility by low oxygen tension (Table 4). As shown in Table 4, the 9-kbp and 6-kbp fragments resulted in a wild-type pattern of inducibility whereas the 1.2-kbp fragment resulted in a constitutive pattern. These data support the hypothesis that the 9-kbp and 6-kbp fragments encode a trans-acting factor that confers inducibility of purple membrane synthesis by low oxygen tension.

Localization of the Gene Encoding the Oxygen Inducibility Factor. The data described above aid in localization of the gene encoding the oxygen inducibility factor. Since the 1.2-kbp fragment fails to confer inducibility but both the 9-kbp and 6-kbp fragments do, the gene encoding the factor is located in a region encompassing the *brp* gene, the *bat* gene, the *blp* gene, and 208 bp of sequence upstream of the transcription initiation site of the *blp* gene and downstream of the *Dde* I site (see Fig. 1). Since previous work suggested that the *bat* gene may encode an oxygen sensor (5), the *bat* gene seemed a reasonable candidate to encode this "oxygen inducibility factor." To test this hypothesis, we used a plasmid construct containing only the *bat* gene with relevant transcriptional signals (Fig. 1, fragment d) to transform strain ET1001. Indeed, as shown in Table 4 and Fig. 3*B*, the *bat* gene conferred inducibility by low oxygen tension on strain ET1001. Comparison of *bop* mRNA expression patterns unequivocally showed more *bop* RNA in the early and middle stages of growth relative to the late stage of growth in ET1001 or ET1001 transformed with the 1.2-kbp fragment than in

Table 3. Inducibility analysis of Pum+ transformants

Transforming DNA fragment	Recipient strain	Logarithmic stage of growth		
		Early	Middle	Late
1.2 kbp	L33	+	+	+
6 kbp	L33	−	−	+
9 kbp	L33	−	−	+

+, Pum+; −, Pum−.

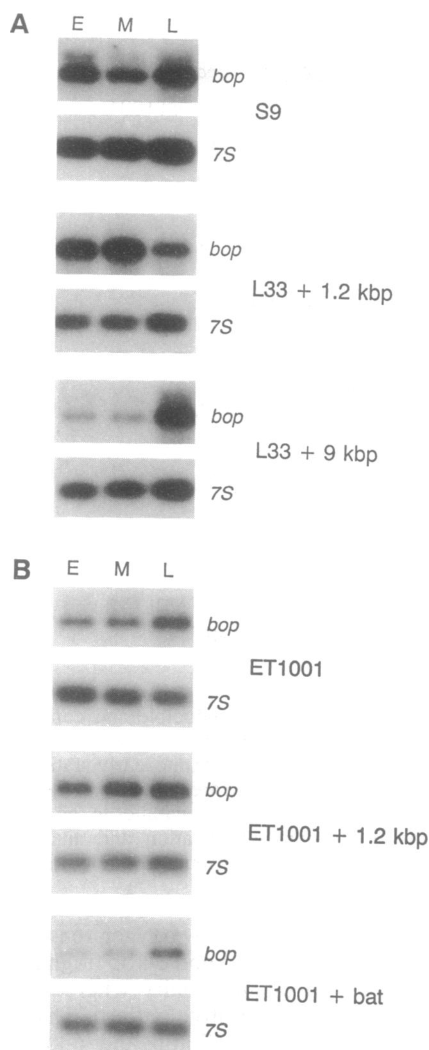

FIG. 3. Northern analyses of purple membrane-deficient mutant L33 (*A*) and constitutively expressing strain ET1001 (*B*) transformed with various DNA fragments. Untransformed strains ET1001 and S9, which is the parental strain of L33, are provided as a reference. Samples were taken from the early (E), middle (M), and late (L) logarithmic/early stationary stages of the growth cycle. Strains analyzed and the DNA fragments they were transformed with are indicated to the far right of the autoradiograms. Probes are indicated in italics to the near right of the autoradiograms. Following hybridization and analysis with *bop* gene-specific probes, blots were stripped and rehybridized with a 7S RNA specific probe.

ET1001 transformed with the *bat* gene (Fig. 3*B*). Moreover, the 7S RNA control showed relatively constant transcript levels in all cases.

Sequence Homologies Between Bat and Other Proteins. Homology searches of the GenBank data base using the BLAST program (36) revealed significant similarities between the putative Bat protein and several proteins involved in oxygen detection and regulation in nitrogen-fixing bacteria, some of which have been noted previously (5, 11, 37). The most striking of these was between a central portion of the Bat protein (i.e., residues 177–300) and the N terminus of the oxygen-sensing regulatory protein, NifL, of *K. pneumoniae* [i.e., 30% identity/56% similarity over 120 amino acids; (11)] and *Azotobacter vinelandii* [i.e., 27% identity/51% similarity over 124 amino acids; (37)]. In addition, we detected homology between the N terminus of the Bat protein (i.e., residues 42–95) and the N terminus of the putative activator, the FixJ protein of *Bradyrhizobium japonicum* (27% identity/48% similarity over 54 amino acids).

Table 4. Inducibility analysis of transformants of constitutive strain ET1001

Transforming DNA fragment	Recipient strain	Logarithmic stage of growth		
		Early	Middle	Late
1.2 kbp	ET1001	+	+	+
6 kbp	ET1001	–	–	+
9 kbp	ET1001	–	–	+
bat gene	ET1001	–	–	+

+, Pum$^+$; –, Pum$^-$.

DISCUSSION

We have analyzed *bop* transcript levels and purple membrane synthesis throughout the growth cycle in various halobacterial strains and transformants. In wild-type strain NRL, induction was observed in the early stationary phase of growth. Similar observations have been made previously for strain NRL (7) and wild-type strains NRC817 (5) and NRC-1 (11). Since low oxygen tension and high light intensity are known to induce purple membrane synthesis (3, 4), it is likely that the oxygen-limiting conditions that naturally occur during stationary phase are the cause of the observed induction in wild-type strains, although other stationary-phase effects cannot be ruled out. In contrast to the wild-type strains, the two Pum-overproducing strains, S9 and ET1001, showed constitutive purple membrane synthesis throughout the growth cycle. However, ET1001 appears to be less deregulated than S9 since *bop* transcript levels in ET1001 showed a slight increase, in contrast to transcript levels in S9, which remained relatively more constant (see Fig. 2). These data suggest that the mutation that affects inducibility by low oxygen tension in these two strains may be different. The constitutive response of Pum-overproducing strains ET1001 and S9 is similar to that seen previously for ET1001 (7) and strain II-7 (5, 8).

Analysis of the inducibility of Pum$^+$ transformants of a Pum$^-$ strain that was derived from the constitutive strain, S9, revealed that transformants that received the *bop* gene cluster were inducible whereas transformants that received only the *bop* gene expressed purple membrane constitutively. These data suggest that one or more of the genes located upstream of the *bop* gene are involved in inducible regulation by oxygen tension. This hypothesis was corroborated by the observation that transformants of the constitutive strain ET1001 that received the same two constructs (i.e., *bop* gene cluster or *bop* gene) showed inducible and constitutive purple membrane synthesis, respectively. Indeed, we demonstrated that one specific upstream gene, the *bat* gene, when provided in trans, was necessary and sufficient to confer inducibility on constitutive strain ET1001. These data support our previous proposal that the *bat* gene product serves as an intermediary in the transmission of the oxygen signal (5).

What is the oxygen-sensing mechanism? A central domain of the putative Bat protein is homologous to the N-terminal domain of the putative oxygen-sensing regulatory protein, NifL, of *K. pneumoniae* and *A. vinelandii* (5, 11, 37). The exact mechanism of oxygen detection by the NifL proteins is unknown. However, the N terminus of NifL from these two organisms is significantly homologous to the N terminus of FixL of *Rhizobium meliloti* (37), which has been shown to be involved in heme binding (38, 39). Based on information available on the NifL and FixL proteins and their homology to the putative Bat protein, it is possible that Bat senses oxygen levels by similar molecular mechanisms.

The Bat protein most likely functions as an activator rather than a repressor since mutations in the *bat* gene totally inactivate *bop* gene expression (10). Indeed, based on these earlier studies it was proposed that the Bat protein is a soluble activator of *bop* gene expression (10, 12). Moreover, we

detected amino acid homology between the N termini of FixJ of *B. japonicum* and the Bat protein. FixJ activates the nitrogen fixation regulatory genes, *nifA* and *fixK*, in *R. meliloti* (40). Alternatively, the Bat protein could be an oxygen-sensing intermediary factor that modifies an activator. Such a regulatory mechanism has been observed for NifL of *K. pneumoniae*, which detects high oxygen levels and inactivates the activator, NifA (41). Interestingly, all of the proteins detected to be homologous with the Bat protein thus far are oxygen sensors or activators that regulate nitrogen fixation genes in response to oxygen levels.

In addition, our experiments provide clues concerning the nature of constitutivity in strain ET1001. Restoration of wild-type inducibility to the constitutively expressing strain, ET1001, via complementation with the *bat* gene from an inducible strain indicates that the Bat protein is missing or altered in ET1001. If the Bat protein is indeed an activator as suggested above, then it may have mutated to a constitutive state in strain ET1001. If such is the case, the constitutive activator in ET1001 might be expected to be dominant, whereas in actuality, it is recessive to the inducible activator provided in trans. One possibility to explain this observation is that the inducible activator of the wild-type binds more tightly to the target site than the constitutive activator. Alternatively, the wild-type form of the activator may possess a dominant phenotype since it is provided in trans on a multicopy plasmid (see ref. 42). The extra gene copies could result in a higher cellular concentration of the wild-type activator that initiates a competition for DNA or protein binding sites favoring the inducible form of the activator over the constitutive form. The nature of the mutation in ET1001 that results in constitutivity can be directly addressed by sequence comparison of the *bat* gene from this strain with that of wild type. Depending upon whether the Bat protein functions as an activator or an intermediary factor, the constitutive mutation(s) could affect the Bat protein by alteration of residues involved in oxygen sensing (e.g., metal binding), DNA binding, or protein–protein interactions with an activator.

We propose that oxygen detection in the Bat protein is linked to an activation mechanism in such a way that *bop* gene expression is triggered by an oxygen concentration-dependent switch in which low oxygen concentration results in induction. For example, when oxygen concentration is high, the switch would remain untriggered and *bop* gene expression would not be activated. Alternatively, when oxygen concentration is low, the switch would be triggered and *bop* gene expression would be activated. Thus, in constitutively expressing strain ET1001, the Bat protein would be incapable of switching off its inherent activation activity or the activation activity of yet another factor with which it interacts.

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