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Expression of Cellulase Genes in *Rhodobacter capsulatus* by Use of Plasmid Expression Vectors

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Broad-host-range plasmid vectors were constructed for expression of heterologous genes in the photosynthetic bacterium *Rhodobacter capsulatus*. These plasmids utilize an RK2-derived replicon for maintenance and conjugative transfer and the *R. capsulatus rxcA* promoter to obtain transcription of genes within appropriately positioned DNA fragments. The expression vectors were used to obtain synthesis of endoglucanase and exoglucanase in *R. capsulatus* from cellulase genes present on exogenously derived DNA fragments. The cellulase genes were expressed either by use of their native translation initiation signals or by in-frame fusion with the *rxcA* B870β gene translation initiation signals to form a hybrid protein. The level of cellulase gene expression was found to be modulated in response to the extent of aeration of plasmid host cultures.

Cellulose is a major structural component of plant cells and, consequently, is abundant in many natural environments. Some organisms are capable of cellulose degradation to yield soluble oligosaccharides and glucose that can be used to help support cell growth. Cellulosic materials are often characterized by a high ratio of carbon:nitrogen, and cellulose digestion by microorganisms is usually accomplished more efficiently if a supplementary source of nitrogen is available (1, 6).

For these reasons various approaches have been suggested as ways to combine nitrogen fixation and cellulolysis in the development of biological systems for transformation of cellulose to products of wider use. For example, cooperative growth on cellulose and N_2 was obtained during coculture of the cellulolytic fungus *Trichoderma harzianum* with *Clostridium butyricum*, in which *C. butyricum* provided nitrogenase activity (27). The use of bacteria that exist as symbionts of higher organisms, and which utilize N_2 for growth on cellulose, has also been proposed (29). An alternative strategy would be to transfer cellulase genes by molecular cloning into a species that is capable of nitrogen fixation.

Phototrophic purple nonsulfur bacteria (photosynthetic bacteria) are an extremely diverse ensemble of species, nearly all of which are capable of nitrogen fixation in addition to photosynthesis (15, 18). It is also known that photosynthetic growth yields of facultatively photosynthetic bacteria may be more than twofold greater than for heterotrophic growth on the same media in darkness (9). This is because carbon substrate, which would be catabolized to products such as CO_2 for energy generation during growth in the absence of light, can be saved for biosynthetic functions when energy is derived from photosynthesis. Therefore, the photosynthetic bacteria have great potential for development as efficient agents of biological transformations such as cellulolysis.

The photosynthetic bacterium *Rhodobacter capsulatus* (formerly known as *Rhodopseudomonas capsulata* [15]) has been widely used in various biochemical and genetic studies. It is possible to introduce by conjugation some plasmid vectors into *R. capsulatus*, but little is known about the factors that effect gene expression in this species (3, 20, 25).

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We describe here the construction and use of plasmid vectors for regulated expression of heterologous genes in *R. capsulatus*. These vectors were used for production in *R. capsulatus* of cellulases encoded by genes originally derived from the gram-positive bacterium *Cellulomonas fimi* (11). The expression of plasmid-borne cellulase genes in *R. capsulatus* was dependent on the presence in the plasmid of a DNA fragment containing an *R. capsulatus* promoter, appropriately positioned with respect to the cellulase genes.

We report attempts to obtain growth of *R. capsulatus* cultures containing expressed cellulase genes with carboxymethyl cellulose (CMC) as sole carbon source; although significant amounts of cellulase activity (measured as carboxymethyl cellulase [CMCase]) were found in extracts of such cells, the enzymes responsible for that activity did not seem to be secreted sufficiently to allow growth on CMC.

MATERIALS AND METHODS

Bacterial strains and plasmids. The wild-type strain B10 of *R. capsulatus* has been described previously (30). A *hsdR* derivative of *Escherichia coli* C600 (4) and PM191 (21) were used as plasmid hosts.

The plasmids used in this study are listed in Table 1. The plasmid pJAJ103 was constructed by deletion of the *lacZp* region contained on a ca. 200-base-pair fragment of pRK404 (10). This DNA fragment extended from a *Pvu*II site in *lacI* to the unique *Hin*dIII site in the *lacZ* allele carried by pRK404.

The plasmid pJAJ21 was constructed by insertion of the *rxcA* promoter on the 1.2-kilobase (kb) *Xho*II fragment from pJAJ101 (3) into the *Bam*HI site of pUC13, such that a *Bam*HI site was regenerated only at the end of the insert nearest the pUC13 *Eco*RI site (28). This resulted in 20 codons of the B870β gene being fused in frame with the pUC13 *lacZα* gene. (For the deduced sequence at the junction of this fusion, see Fig. 3.)

Media and growth conditions. *E. coli* strains were routinely grown at 37°C in L broth (19), solidified when necessary by the addition of agar (Difco Laboratories, Detroit, Mich.) to 1.5%. Cultures of *E. coli* used in CMCase activity assays were grown on M9 salts medium (19) supplemented with 0.2% glycerol–1 μg of thiamine per ml–0.1% Casamino Acids (Difco). This modified M9 medium was further supplemented with 1.1% agar (Difco) and 0.8% high-viscosity CMC

TABLE 1. Plasmids used in this study

Plasmid	Selective markers and comments	Reference or source
pRK404	Tc^r; derivative of RK2; contains *Hae*II restriction fragment that includes the *lac'IPOZ*α segment of pUC19	10
pJAJ7	Tc^r; expression vector derivative of pJAJ103	This study
pJAJ9	Tc^r; expression vector derivative of pJAJ103	This study
pJAJ17	Tc^r; CMCase 1 gene cloned in pJAJ7	This study
pJAJ21	Ap^r; *rxcAp* and first 20 codons of B870β cloned in pUC13	This study
pJAJ103	Tc^r; derivative of pRK404 with *lacZ* promoter removed	This study
pEC1	Ap^r; CMCase 1 gene cloned in pBR322	11
pEC2.2	Ap^r; CMCase 2 gene cloned in pBR322	23
pEC3	Ap^r; CMCase 3 gene cloned in pBR322	11
pREC1	Tc^r; CMCase 1 gene cloned in pJAJ7	This study
pREC 2.2	Tc^r; B870β-CMCase 2 gene fusion of pRW6 cloned in pJAJ103	This study
pREC3	Tc^r; CMCase 3 gene cloned in pJAJ7	This study
pRKEC2.2	Tc^r; CMCase 2 gene cloned in pRK404	This study
pRW6	Ap^r; CMCase 2 gene cloned in pJAJ21	This study
pRK2013	Km^r; helper plasmid for mobilization of RK2 derivatives	10

for use as growth medium in Congo red screenings. Tetracycline (10 μg/ml) and ampicillin (70 μg/ml) were incorporated as needed.

R. capsulatus was grown at 34°C in a modified version of synthetic medium RCV (2) supplemented with 20 mM phosphate buffer–0.1% yeast extract, designated RC6. For Congo red screening RCV medium containing 20 mM phosphate buffer, 1.1% agar, and 0.8% CMC (high viscosity) was used. Cultures of *R. capsulatus* were grown photosynthetically in screw-cap tubes as described previously (2). Conditions for high and low aeration of *R. capsulatus* cultures have been described previously (3).

Preparation of cell extracts. Forty-milliliter cultures were grown to a density of about 10^9 cells per ml and harvested by centrifugation. Cell pellets were suspended in 1 ml of 50 mM KH_2PO_4-$Na_2H_2O_4$ buffer (pH 7.0) and lysed by sonication. The sonicate was centrifuged at 13,000 × *g* for 1 min, and the resultant supernatant fluid was assayed. When necessary extracts were stored at −20°C without loss of CMCase activity.

Measurements of CMCase activity. The colorimetric assay of the reducing groups released from CMC, as described by Stewart and Leatherwood (24), was used to determine CMCase activity with some modifications. Linear proportionality with respect to both length of time and amount of extract used for assay was established for all samples measured. Assays were performed by mixing two volumes of 4% (wt/vol) low-viscosity CMC in 50 mM KH_2PO_4-$Na_2H_2O_4$ buffer (pH 7.0) with one volume of cell extract and incubating at 37°C. At timed intervals between 0 and 90 min, 750-μl fractions were removed from the reaction tube and mixed with 800 μl of 3,5-dinitrosalicylic acid reagent (DNS) (22) to stop the reaction. A 50-μl volume of glucose (1 mg/ml) was added to each tube, the samples were capped and boiled for 15 min and allowed to cool to room temperature, and their absorbancy at 550 nm was determined. To control for background absorbancy of extracts, equal amounts of extract were added to tubes that already contained both CMC substrate and DNS, and these values were determined and subtracted from the experimental measurements. Absorbancy measurements were converted to glucose equivalents by use of standard curves derived from measurements with known amounts of a glucose standard solution (Sigma Chemical Co., St. Louis, Mo.).

The protein content of cell extracts was estimated by the procedure of Lowry et al. (16), with bovine serum albumin used as the standard.

Zones of CMC hydrolysis around colonies on solid media were detected by Congo red staining (26).

Transformation, conjugation, and DNA cloning procedures. Transformation of competent *E. coli* cells with plasmid DNA was carried out as described previously (19). Conjugations were performed by mixing 0.1-ml portions of early-stationary-phase cultures of donor, recipient, and *E. coli* HB101(pRK2013) (helper plasmid) (10). This mixture was centrifuged for 0.5 min at 13,000 × *g* and suspended in 300 μl of sterile 0.3% yeast extract–0.3% peptone, and 10 μl was spotted onto solid RCV medium. After 4 to 16 h of incubation at 30°C, the cells in the spots were streaked for isolation onto second plates of RCV containing 0.5 μg of tetracycline per ml. The absence of amino acids from RCV minimal medium allowed counterselection against auxotrophic *E. coli* donors. Determination of conjugation efficiency was made by suspending cells from a mating spot in liquid RCV and plating onto solidified RCV (for enumeration of viable cells) and RCV supplemented with 0.5 μg of tetracycline per ml (for enumeration of plasmid recipients).

Analysis of plasmids present in cells was done by a modification of the method of Birnboim and Doly (5), in which incubation times were shortened to 5 to 15 min. Preparative isolation of plasmids and DNA fragments and other recombinant DNA techniques were performed as described by Maniatis et al. (19).

Enzymes and reagents. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). DNA polymerase I (Klenow fragment) and nucleotides were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Low- and high-viscosity CMC, D(+)-cellobiose, and Congo red were obtained from Sigma. All other chemicals used were of reagent or higher grade and were obtained from commercial suppliers.

RESULTS

Comparison of *E. coli lacZ* and *R. capsulatus rxcA* promoters. The possibility that the *lacZ* promoter could be used to initiate transcription of *C. fimi* cellulase genes in *R. capsulatus* was investigated. Because it had been shown that the *lacZ* translation initiation signals function in *R. capsulatus* (3), whereas the efficiency of translation directed by *C. fimi* signals is unknown, a gene fusion was designed that would link the amino terminus of β-galactosidase to a cellulase.

The broad-host-range plasmid pRK404 (10) and derivatives thereof were used as vectors to mobilize cloned *C. fimi* cellulase genes into *R. capsulatus*. A portion of one gene, which encodes an endo-1,4-β-D-glucanase (CMCase 2), has been subcloned in *E. coli* as a 2.4-kb *Bam*HI fragment by insertion into the *Bam*HI site of pBR322 (23). This construct, designated pEC2.2, synthesizes in *E. coli* a hybrid protein with the amino terminus of the pBR322 tetracycline resistance gene product. Although the first 76 codons of the CMCase 2 gene are missing (W. K. R. Wong, B. Gerhard, Z. M. Guo, D. G. Kilburn, R. A. J. Warren, and R. C. Miller, Jr., Gene, in press), the hybrid protein still retains

cellulolytic activity. We used this information, along with the published DNA sequence of the pUC9-derived multiple cloning site of pRK404 (10, 28), to construct a broad-host-range plasmid that expressed a chimeric CMCase 2 gene in *E. coli*. This gene derives its first 12 codons, as well as its translation and transcription initiation sequences, from the *lac* alleles carried by pRK404, and it derives its carboxy terminus from CMCase 2. This plasmid, designated pRKEC2.2, was made by ligating the portion of the CMCase 2 gene present on the 2.4-kb *Bam*HI fragment (see above) into the *Bam*HI site of pRK404 (Fig. 1). When pRKEC2.2 was present in cells of *E. coli*, a functional hybrid CMCase 2 was made, as was evidenced by zones of clearing around colonies on CMC-containing solid media after staining with Congo red. Isolates containing plasmids with the 2.4-kb *Bam*HI fragment in the opposite orientation did not produce zones of clearing in the Congo red assay.

Transfer of pRKEC2.2 from *E. coli* into *R. capsulatus* was done by conjugation (10), and tetracycline-resistant recipients were isolated. However, none of these recipients gave significant clearing in the Congo red assay (Fig. 2). One isolate was shown to contain pRKEC2.2, as evidenced by the presence of the 2.4-kb fragment in the correct orientation, as shown by restriction endonuclease digestion analysis. This isolate was further tested for the presence of CMCase 2 activity by measuring the reducing sugars present after incubation of crude extracts in the presence of CMC. The results of this assay also showed that a very low amount of CMCase 2 activity was present in cells of *R. capsulatus* that contained pRKEC2.2 (<1 U [μg of glucose per min per mg of protein]).

Although one could imagine alternative explanations for our findings, the simplest interpretation of these results is that the *E. coli lacZ* promoter does not function well in *R. capsulatus*. Therefore, another plasmid was engineered to position the CMCase 2 gene for expression as part of a genetic unit derived from *R. capsulatus*. For this purpose a DNA fragment encompassing the promoter and part of the B870β gene of the *rxcA* operon was chosen. The B870β gene encodes a light-harvesting membrane protein and is the first of four genes known to encode pigment-binding peptides, which have been shown to be transcribed as a polycistronic message. This 1.2-kb fragment previously had been isolated and subcloned (3) and was available as an insert in pUC13 (28), designated pJAJ21 (Fig. 3). The insert consisted of *R. capsulatus* DNA sequences extending from an *Xho*II site through the *rxcA* promoter region and ending at the *Sph*I site located about midway through the B870β gene of the *rxcA* operon. The deduced sequence of 26 base pairs of polylinker DNA (28), which were introduced after the resected *Sph*I site in passage of this fragment through intermediate M13 constructs to introduce a *Bam*HI site near the *Sph*I site, is given at the bottom of Fig. 3.

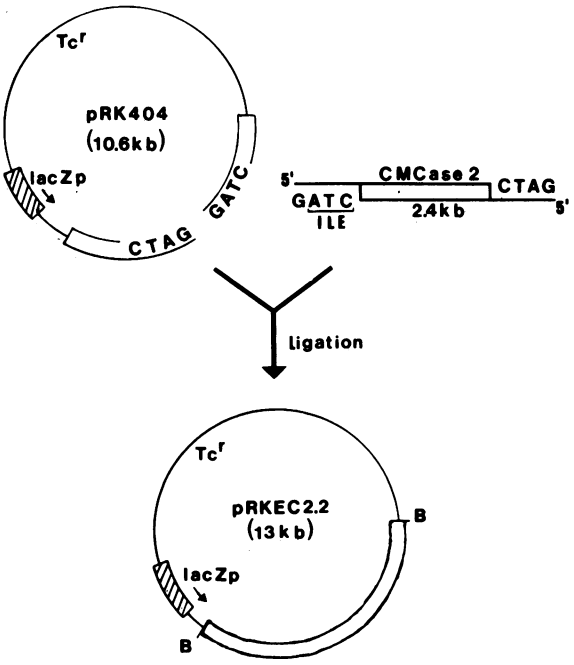


FIG. 1. Fusion of CMCase 2 gene to the *lacZ* promoter to form pRKEC2.2. The hatched box represents the *lacZ* promoter, with the direction of transcription given by the arrow. The boxed segment represents the 2.4-kb *Bam*HI (B) fragment of pEC2.2 that contains the CMCase 2 gene. The single-stranded ends resulting from *Bam*HI digestion are shown, and the codon for isoleucine (ILE) is given to indicate the translational reading frame of CMCase 2 and the *lacZ* allele of pRK404. Sizes (in kilobases) are approximate.

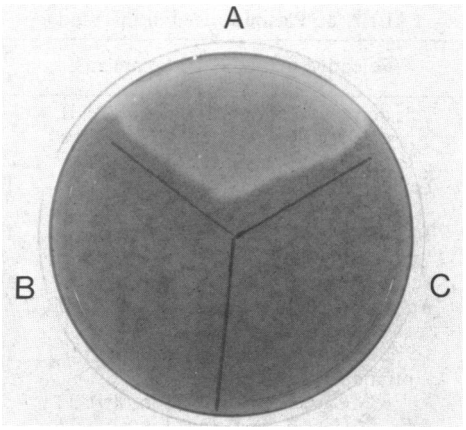
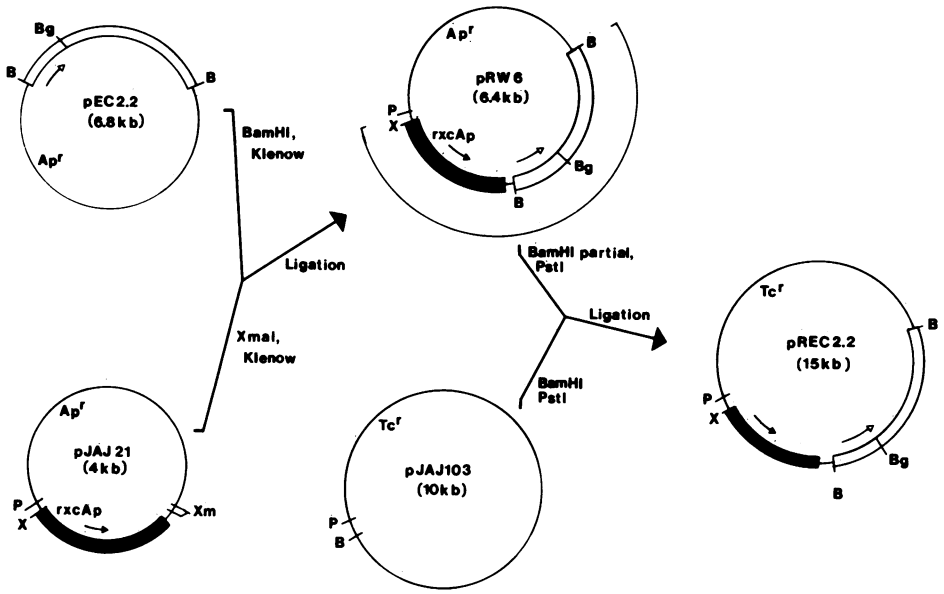


FIG. 2. Congo red stain showing CMC hydrolysis. Solidified RCV medium supplemented with CMC and tetracycline was streaked with plasmid-containing derivatives of *R. capsulatus* to give triangular zones of partially confluent growth. The resultant cells were removed from the surface, and the polysaccharides remaining in the medium were stained with Congo red. The plasmids present in cells were pREC2.2 (A), pRKEC2.2 (B), and pRK404 (C).

Because the DNA sequence of the B870β gene (31) and the polylinker sequence (28) are known, it was possible to design a procedure for obtaining an in-frame gene fusion between B870β and CMCase 2. This procedure (Fig. 3) included *Xma*I digestion of pJAJ21 and filling in of the sticky ends; similarly, the 2.4-kb *Bam*HI fragment containing the CMCase 2 gene was purified, and the ends were filled in. The blunt-ended DNA fragments were mixed, ligated, and then used to transform *E. coli*. The desired ligation product had a *Bam*HI site regenerated at the junction of pJAJ21 and the CMCase 2 gene. Moreover, regeneration of the *Bam*HI site ensured that the translational reading frames of the B870β and CMCase 2 genes were the same (Fig. 3). The appropriate construct was identified by analytical restriction endonuclease digestion of candidate plasmids, and *E. coli* cells containing this plasmid were found to give clearing zones in the Congo red plate assay. This plasmid was designated pRW6.

The *rxcA* promoter region and the B870β-CMCase 2 gene fusion were isolated as a fragment resulting from *Pst*I and *Bam*HI partial digestion of pRW6 (Fig. 3). This fragment was ligated with *Pst*I- and *Bam*HI-cut pJAJ103, a derivative of



5'...GAA CTG GGG AAT TCG AGC TCG CCC GGG ATC CAG...3'

B870β MCS CMCase 2

FIG. 3. Fusion of the CMCase 2 gene to the *rxcA* promoter to form pREC2.2. The boxed portion of pEC2.2 represents the 2.4-kb *Bam*HI fragment that contains the CMCase 2 gene, and the direction of transcription is given by the arrow. The heavy black segment of pJAJ21 represents the *rxcA* promoter and the 5' portion of the B870β gene, with the direction of transcription indicated by the arrow. The intermediate construct (pRW6) contains an in-frame fusion (shown at bottom of the figure) between the 5' end region of the B870β gene and the 3' portion of the CMCase 2 gene. MCS designates the sequence derived from multiple clonings of M13 phage vectors. This fusion was transferred to pJAJ103 as the segment delineated by the arc concentric with pRW6. The restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; X, *Xho*II; Xm, *Xma*I. Sizes (in kilobases) are approximate.

pRK404 from which the *lac* promoter had been removed (see above). The ligation products were transformed into *E. coli*, and transformants were screened for the presence of the correct plasmid. This plasmid was designated pREC2.2, and one such transformant was used as donor in a conjugation with *R. capsulatus*.

It was found that cultures of *R. capsulatus* that contain pREC2.2 produce high levels of CMCase 2 activity. This activity can be detected by the Congo red assay (Fig. 2). High levels of CMCase activity (1,575 U) were also found in crude extracts of *R. capsulatus* cells that contained pREC2.2, when assayed for reducing sugars released after incubation of extracts with CMC.

Construction of expression vectors for use in *R. capsulatus*. The pREC2.2 construct was a special case in which the CMCase 2 gene was fused to a *rxcA* gene, yielding a hybrid protein. We wished to construct vectors of more general applicability that could be used for expression of genes with their own translational control signals. Such plasmids would not present problems associated with obtaining the correct translational reading frame with respect to *rxcA* genes.

Two potentially useful plasmids were constructed by ligation of the *Xho*II-*Bam*HI fragment from pJAJ21 (which contains the *rxcA* promoter) into the *Bam*HI site of broad-host-range plasmid pJAJ103 in both orientations. Because the *Xho*II end of the fragment (although complementary to *Bam*HI ends) does not regenerate a *Bam*HI recognition sequence after ligation to a *Bam*HI end, a unique *Bam*HI site was present downstream of the *rxcA* promoter in the expression vector pJAJ7 (Fig. 4). However in pJAJ9, which contained the insert in the opposite orientation, there was a *Pst*I site, in addition to the *Bam*HI site, that was suitable for potential expression of genes present on inserted DNA fragments. Both plasmids also contained a single *Sst*I site (GAGCTC; Fig. 3). These plasmids were used either for expression of cloned genes with their native translational sequences or for construction of gene fusions in a fashion analogous to that used for expression of CMCase 2 (see above).

The ability of these vectors to express genes present on DNA fragments was tested by subcloning the *lacZ* structural gene from pMC903 as part of a 7.6-kb *Bam*HI-*Bgl*II fragment (7) into the *Bam*HI site of pJAJ7 and pJAJ9. This DNA fragment contained a portion of the *trp* operon preceding the *lacZ* gene. It was found that *lacZ* is expressed in *R. capsulatus* when present in the correct orientation in either of the two vectors, but not when present in pJAJ103 (which does not contain the *rxcA* promoter).

Expression of cellulase genes subcloned into pJAJ7. At present three distinct genes from *C. fimi* that encode cellulases have been identified and cloned in *E. coli* (11). The gene we designated CMCase 2, lacking its first 76 codons, was expressed in *R. capsulatus* as described above. A second cloned gene, encoding an exo-1,4-β-D-glucanase (CMCase 1), retains its native translational initiation sequences, as does the third gene, which encodes an

endoglucanase (CMCase 3) that is expressed at lower levels in *E. coli* than CMCase 2 (11).

The CMCase 1 and CMCase 3 genes were available as *Bam*HI fragments of 6.6- and 5.6-kb, respectively, that were present on recombinant plasmids pEC1 and pEC3 (11). The subcloning procedures that were followed are outlined below. Essentially, these fragments were independently inserted into the *Bam*HI site of pJAJ7 and transformed into *E. coli*, and their orientation was determined by analytical restriction endonuclease digestion. Those constructs in the correct orientation were designated pREC1 (containing CMCase 1) and pREC3 (CMCase 3) and were transformed into *R. capsulatus* by conjugation. After purification of recipients cultures were grown, and crude extracts were made for assay of CMCase activity. The results of these assays showed that extracts of *R. capsulatus* cultures that contained pREC1 or pREC3 had 11 and 3 U of activity, respectively. In contrast, cultures containing pJAJ7 without an insert, or with the CMCase 1 gene in the opposite orientation (pJAJ17), did not produce significant CMCase activity. Other experiments (data not shown) with extracts of cells that contained pREC1 or pREC3 showed proportionality between CMCase activity and amount of extract used or length of time of assay, whereas extracts of cultures bearing pJAJ7 or pJAJ17 did not.

The results of these experiments show that several different heterologous genes can be expressed in *R. capsulatus* by use of the vectors described above and that gene expression is dependent on transcription initiated by the *rxcA* promoter.

All three CMCase activities in cultures of *R. capsulatus* were associated with the cell pellet after centrifugation. However, small amounts of CMCase 2 must leak from cells to give clearing of CMC plates after Congo red staining (Fig. 2). Accordingly, an experiment was performed to evaluate the extent of growth of *R. capsulatus* cultures that contained pREC1 and pREC2.2 with 0.1% CMC as the sole source of carbon. Inocula were grown photosynthetically in the RCV mineral salts base with 5 mM cellobiose as the carbon source, supplemented with tetracycline. Three different inocula were grown that contained pREC2.2, pREC1, or pRK404. These cultures were used to inoculate duplicate tubes of cellobiose or CMC medium, and tubes were also inoculated with a mixture of equal amounts of cells containing pREC2.2 or pREC1. All cultures that were placed into the medium that contained cellobiose grew well. In contrast, the cultures that were transferred into the CMC-containing medium grew for about one doubling period (evidently because of carry-over of cellobiose with the inoculum), after which there was no further increase in culture turbidity. A medium that contained both cellobiose and CMC supported growth. It was concluded that insufficient CMCase is released from cells of *R. capsulatus* to produce enough cellobiose or glucose for growth.

Other properties of expression vectors. It has been reported that pRK404, from which pJAJ7 and pJAJ9 were derived, is quite unstable in the absence of selection (10). We estimated the frequency of loss of pJAJ7 and pJAJ9 in *R. capsulatus* as described below. RC6 liquid medium was inoculated with a suspension of cells that had been grown in the presence of tetracycline. After the culture had gone through eight mass doublings, dilutions were made and spread in duplicate on solid media of RC6 and RC6 supplemented with tetracycline. Approximately 70 to 80% of the number of cells that grew on RC6 were found to grow in the presence of tetracycline. In practice we routinely include tetracycline in growth media for plasmid-containing strains and have found that growth rate and yield are not measurably affected by the presence of the antibiotic.

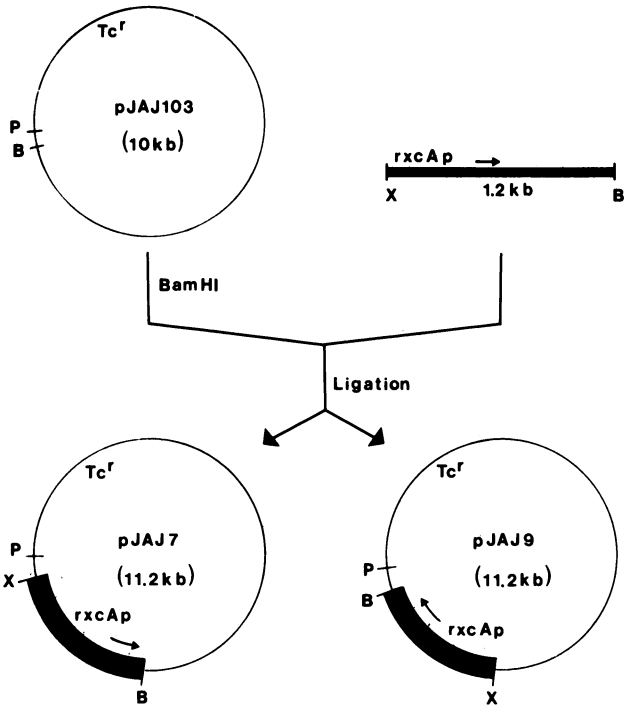


FIG. 4. Construction of expression vectors. The heavy black segment represents the 1.2-kb *Xho*II-*Bam*HI fragment that contains the *rxcA* promoter and the 5′ end of the B870β gene, with the direction of transcription shown by the arrow. Restriction endonuclease sites are abbreviated as follows: X, *Xho*II; B, *Bam*HI; P, *Pst*I. Sizes (in kilobases) are approximate.

Transcription of the *rxcA* operon is stimulated by oxygen starvation of *R. capsulatus* cultures (3). When the levels of CMCase activity present in extracts of cells containing pREC1 grown with high aeration were compared with the levels found with cells grown under conditions of low aeration, about a fourfold increase in CMCase specific activity with oxygen-limited cells was found relative to that obtained with more highly aerated cultures. Thus, expression of heterologous genes can be modulated by controlling the oxygen that is available to cultures containing these plasmids.

Although endogenous plasmids have been reported to be present in *R. capsulatus* BH9 (14), we were able to purify the plasmids described in this report from strain B10 by use of standard techniques (5, 19) with no discernible contamination by other plasmids.

The vectors described above can be mobilized by conjugation from *R. capsulatus* into *E. coli* and, presumably, other species that make up the host range of RK2 (10). The efficiency of plasmid transfer from *E. coli* to *R. capsulatus* in conjugation of these vectors is so high (frequency of about 10^{-1} per recipient) that it would be possible to screen recipients for the presence of minor components of a mixed population, such as in shotgun cloning experiments.

DISCUSSION

With the application of recombinant DNA techniques to an expanding circle of organisms, it has become clear that

there are often obstacles to heterologous gene expression. The *R. capsulatus* photopigment biosynthesis genes have been transferred into *E. coli* and *Pseudomonas fluorescens*, but no pigment formation was observed in cells of either species (20). We have evidence that this absence of expression is due, at least in part, to a lack of transcription of *R. capsulatus* photosynthesis genes when resident in *E. coli* (J. T. Beatty, C. W. Adams, and S. N. Cohen, manuscript in preparation). Results of other unpublished experiments indicate that plasmid-borne *lac* genes, expressed at high levels in *E. coli*, are not expressed on transfer to *R. capsulatus*. Therefore, it was not surprising that the *lacZ*-CMCase 2 gene fusion of pRKEC2.2 was not expressed in *R. capsulatus*, whereas the B870β-CMCase 2 fusion was expressed at high levels. Although it might appear that the *lacZ* translational signals function poorly in *R. capsulatus* and result in inefficient translation of the *lacZ*-CMCase 2 fusion mRNA, this is unlikely because it was possible to obtain translation of *lacZ* mRNA in *R. capsulatus* when *lacZ* was subcloned into pJAJ7 or pJAJ9 (see above). However, it is conceivable that the β-galactosidase-CMCase 2 hybrid protein, or its mRNA, is extremely unstable in *R. capsulata*. This lack of reciprocity in expression of genes transferred between *E. coli* and *R. capsulatus* does not extend to all genes, because some plasmid replication and antibiotic resistance genes function in both species. Species-dependent transcriptional or translational specificity, or both, has been shown in vitro with extracts of *Rhodopseudomonas sphaeroides* (8). We are currently engaged in experiments that may give a better understanding of mechanisms of gene expression in *R. capsulatus*.

The *C. fimi* cellulase genes have been expressed in *E. coli* when present as fragments inserted into the *Bam*HI site of plasmid pBR322, and the specific activities of CMCase 1 and CMCase 3 were reported as 3.6 and 1.2 units, respectively (11). When these genes were transcribed from the *rxcA* promoter of pJAJ7 in *R. capsulatus*, the specific activities of CMCase 1 and CMCase 3 were found to be 11 and 3 U, respectively, which is about a threefold increase over the values reported for *E. coli*. However, the level of CMCase 2 specific activity in *R. capsulatus* containing pREC2.2 was about 40 times the amount reported for *E. coli* cells that contain the analogous pBR322-derived construct (1,575 compared with 39 U) (11). This discrepancy in relative amounts of enzyme specific activities could be explained by posttranscriptional genetic regulatory processes, such as differential stability or translation efficiency of the mRNAs transcribed from the two different constructs. Alternatively, the hybrid B870β-CMCase 2 protein may be a more active cellulase than the fusion resulting from the pBR322 construct. Additional experiments would be necessary to distinguish between these possibilities.

The cellular location of CMCase enzymes in *R. capsulatus* is not yet known, but it seems likely that they are located in the periplasm, as has been shown for *E. coli* (11). The dramatic clearing around areas of cell growth on CMC-supplemented solid medium, seen after staining with Congo red (Fig. 2), probably reflects the great sensitivity of this assay more than it indicates the excretion of significant amounts of CMCase. This interpretation is substantiated by the lack of growth on CMC of *R. capsulatus* containing CMCases. It may be that the clearing observed by the Congo red assay reflects cell lysis as well as limited excretion. However, it may be possible to isolate mutants of *R. capsulatus* that leak greater amounts of CMCase, as has been described for *E. coli* (12). Moreover, the cloned cellulase genes could be used for the study of basic aspects of protein secretion in *R. capsulatus*.

Photosynthetic bacteria have been shown to be capable of almost stoichiometric conversion of carbon substrates to H_2 and CO_2 under (anaerobic) photosynthetic conditions of growth in the absence of NH_4^+ or N_2 (13, 17). If it is possible to increase the level of CMCase excretion by *R. capsulatus*, it may be feasible to use such strains for the biosynthesis of H_2 from readily available cellulosic wastes.

Although we only tested a few genes for expression by use of pJAJ7 or pJAJ9, it seems likely that a wide variety of procaryotic genes would be expressed in *R. capsulatus* when cloned into these plasmids. Therefore, these vectors could be useful to other researchers with interests in expressing heterologous genes in photosynthetic bacteria.

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