Recombinant expression, purification, and characterization of the *pufQ* gene product of *Rhodobacter capsulatus*

by

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RECOMBINANT EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE PUFQ GENE PRODUCT OF RHODOBACTER CAPSULATUS

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ABSTRACT

Genetic studies have shown that expression of the *pufQ* gene is required for normal levels of bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus.* Yet, the exact function of the *pufQ* gene is unknown, and a *pufQ* gene product has never been isolated. Recombinant overexpression of the *pufQ* gene in *Escherichia coli* was therefore used to purify and characterize the 74 amino acid gene product, termed the PufQ protein. Site-directed mutagenesis facilitated the cloning of the *pufQ* gene into various expression vector systems of *E. coli* including pKK223-3, pLcII-FX, and pMal-c. Although high levels of *pufQ* transcription were evident from constructs of all three vectors, high levels of protein expression were apparent only in the pMal-c fusion vector. After affinity purification on an amylose column, full length PufQ protein was released from an amino-terminal maltose-binding domain fusion by limited proteolysis with the enzyme Factor X_a. PufQ protein was subsequently purified by high performance liquid chromatography and identified by amino terminal sequence analysis. In further studies, PufQ protein was reconstituted into phospholipid vesicles and its ability to bind to a specific bacteriochlorophyll intermediate (protochlorophyllide) was tested. Additionally, Western blot analysis localized the PufQ protein in a subcellular fraction of *R. capsulatus* where the biosynthesis of chromatophores (photosynthetic membrane compartments) are first thought to occur. Finally, a possible role of PufQ protein in regulating the enzymes of bacteriochlorophyll synthesis was tested by using reconstituted PufQ protein in enzyme assays, and by measuring its effect on the levels of bacteriochlorophyll intermediates. Taken together, these results are consistent with a possible role for PufQ protein in serving as a membrane-bound carrier or activator of intermediates in bacteriochlorophyll biosynthesis.

Dedication

To my beloved wife,

Fatima

Acknowledgements

I would like to extend my sincere gratitude to Dr. W. R. Richards and to Dr. T. Borgford for their invaluable support and guidance during the entire course of the research project. This project was a truly collabrative effort and I would like to also thank Dr. R. Cornell and Dr. J. T. Beatty for their expert advice and the use of their laboratory resources.

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ABBREVIATIONS

ALA	δ-Aminolevulinic acid
ATP	Adenosine triphosphate
Bchl	Bacteriochlorophyll
Bpheo	Bacteriopheophytin
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
CIP	Calf intestinal phosphatase
Copro	Coproporphyrin
Coprogen	Coproporphyrinogen
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	Deionized, distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DPPC	Dipalmitoylphosphatidyl choline
DTT	DL-dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunsorbent assay
EtBr	Ethidium bromide
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
ICM	Intracytoplasmic membrane

IgG	Immunoglobulin G
IPTG	Isopropylthio-β-galactoside
LH-I	Light harvesting I
LH-II	Light harvesting II
MOPS	3-(N-morpholino) propanesulfonic acid
MT	Methyl transferase
MgPME	Mg protoporphyrin monomethyl ester
MgProto	Mg protoporphyrin
M_r	molecular weight
mRNA	Messenger RNA
NMP	N-methyl protoporphyrin
PAGE	Polyacrylamide gel electrophoresis
Pchlide	Protochlorophyllide
PEG	Polyethylene glycol
PMSF	Phenylmethyl sulfonyl fluoride
PBG	Porphobilinogen
RC	Reaction centre
RNase A	Ribonuclease A
RNase T_1	Ribonuclease T_1
SAM	S-Adenosyl-L-methionine
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Tris (hydroxymethyl) methylamine
UPB	Upper pigmented band
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside

1. INTRODUCTION

1.1 Membrane-bound pigment-protein complexes and photosynthesis

Photosynthesis in the purple non-sulfur bacterium *Rhodobacter capsulatus* and the closely related species *Rhodobacter sphaeroides* has been the subject of extensive research over the past number of years. As a result, a clear understanding of the factors and events involved in the process of photosynthesis has emerged.

Rhodobacter species are capable of growth by at least two different modes of metabolism: aerobic respiration and anaerobic photosynthesis (92, 56, 29). Under conditions when oxygen is removed from a culture growing aerobically, a series of events is triggered which results in a marked differentiation of the cytoplasmic membrane (54). When examined by electron microscopy, the differentiated cytoplasmic membrane appears as invaginations which are physically continuous with the cytoplasmic membrane, termed the photosynthetic intracytoplasmic membrane system (ICM) (82, 28). Upon physical disruption of the outer cell membrane (such as by treatment with a French press), these invaginations may be isolated as inside-out ICM vesicles termed chromatophores (80, 35). The functional significance of the ICM lies in the fact that it contains all of the membrane components necessary for the "light reactions" of photosynthesis (section 1.1.3).

The photosynthetic apparatus localized within the ICM of *R. capsulatus* consists of three major pigment-protein complexes. The pigment portion of these complexes consists exclusively of bacteriocholorphyll *a* (Bchl), carotenoids, and bacteriopheophytin (Bpheo), while the various pigment-binding proteins act primarily as structural elements for the proper orientation of Bchl molecules in the membrane. Two of the pigment-protein complexes, B870 and B800-850, designated light-harvesting I (LH-I) and light-harvesting II (LH-II), act as antenna for the absorption

and ultimate transmission of light energy to another pigment-protein complex, the reaction center (RC), where the process of charge separation occurs. Although the role for both light harvesting complexes is similar, they differ in their composition, organization, and proximity to the reaction centre complex.

1.1.1 Light-harvesting complexes

The B800-850 LH-II complex consists of six molecules of Bchl, three molecules of carotenoids, and two each of two small hydrophobic polypeptides designated B800-850-α and B800-850-β. The B800-850 LH-II complex is the most abundant light harvesting species and is present at levels that are variable in relation to the RC complex and inversely proportional to the incident light intensity. Hence, it functions as a peripheral antenna complex, transferring exciton energy to the more centrally located B870 LH-I and RC complex (72).

The B870 LH-I complex consists of two molecules each of Bchl and carotenoids, and two small hydrophobic polypeptides designated B870-α and B870-β in a 1:1 stoichiometry. Unlike the B800-850 LH-II complex, the ratio of the B870 LH-I to the RC complexes is in a fixed stoichiometry of approximately 12-15:1, and energy transfer occurs directly to the RC complex.

The α and β polypeptides from both LH species have been well characterised and found to share common structural features. They are both small (50-60 amino acids) membrane spanning polypeptides which contain polar amino and carboxy-termini, separated by a central hydrophobic stretch of amino acids (96, 97, 104). Various studies (47, 36, 5, 101, 83, 98) using proteolytic mapping, Western blots, and membrane-protein labelling reagents in *R. capsulatus* and *R. sphaeroides* have localized the amino and carboxyl terminals of both polypeptides on the cytoplasmic and periplasmic surfaces respectively. Within the central hydrophobic domain of both the α

and β-subunits, a single conserved histidine residue is thought to interact with Bchl as a fifth magnesium ligand (30, 71). In addition, the β-subunit has a second conserved histidine residue in the amino-terminal domain which is also believed to interact with Bchl. Interestingly, this interaction is present in only the LH-II complex and not in the LH-I complex.

1.1.2 Reaction centre complex

The RC contains three polypeptides referred to as the H (heavy), M (medium), and L (light) subunits, having apparent molecular weights of 28, 24, and 21 kDa respectively, as determined by SDS-PAGE analysis (81). The deduced molecular weights from DNA sequence analysis however have subsequently shown these proteins to have anomalous electrophoretic mobilities. The H subunit is a fairly hydrophilic protein with a deduced molecular weight of 28,534 Da, whereas the L and M subunits are both very hydrophobic proteins having deduced molecular weights of 31,565 and 34,440 Da respectively (110, 111, 112, 116).

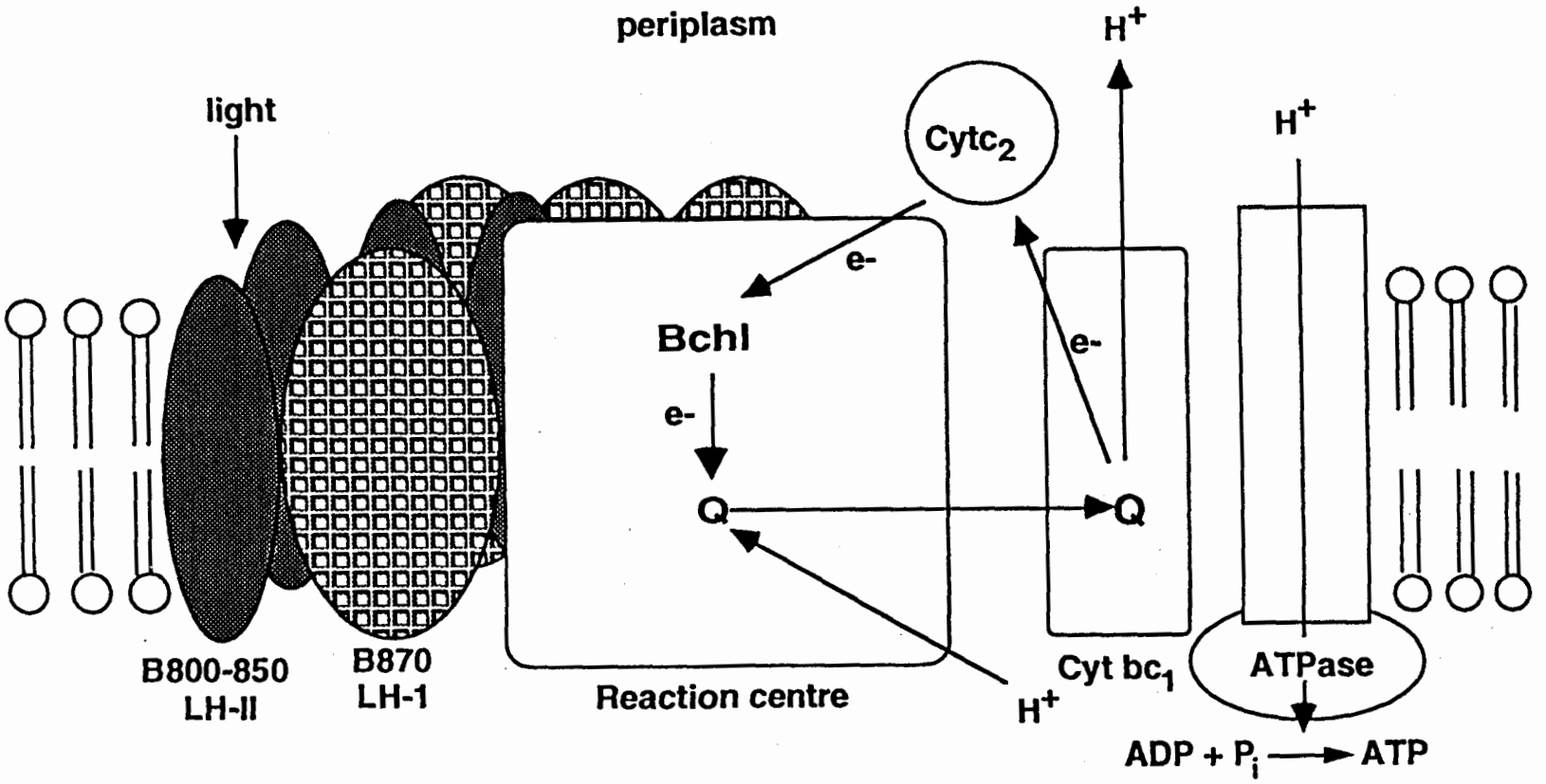
The L and M subunits together bind four molecules of Bchl, two molecules of Bpheo, two molecules of ubiquinone (Q_A and Q_B), a non-heme iron, and a single carotenoid. Whereas the L and M subunits are essential for RC activity, the H subunit binds no pigments and is not required for electron transfer *in vitro* but is required for *in vivo* photosynthetic growth (33). However, the presence of the H subunit greatly accelerates electron transfer from Q_A to Q_B (section 1.1.3) and may also serve as a structural anchor protein for maintaining the proper association of L and M subunits within the membrane (24, 25).

1.1.3 Light reactions of photosynthesis

The light reactions of photosynthesis encompass the following events beginning with the absorption of light energy by Bchl and carotenoid pigment molecules that are bound to polypeptides of the "antenna" or LH complex. The effect is the creation of mobile electronic singlet states, called "excitons"(63). These excitons are then channeled towards the RC via the transfer of resonance energy among neighbouring Bchl molecules.

At the RC, a "special pair" of Bchl molecules (designated P870) donates an electron to a Bpheo molecule (passing a voyeur Bchl molecule in the process), resulting in a positive charge on the special pair. From the Bpheo, the electron transfers to a quinone (Q_A) molecule. The reduced semiquinone $Q_A^{\cdot -}$ then donates the electron to another quinone molecule, Q_B, also located at the RC, while $P870^+$ is reduced by cytochrome c_2 located on the periplasmic side of the membrane. The $Q_B^{\cdot -}$ then becomes protonated, and the process of electron flow is then repeated a second time, resulting in $Q_BH^{\cdot}$ acquiring another electron. The fully reduced Q_BH^- then becomes protonated on the cytoplasmic side of the membrane, and Q_BH_2 diffuses into the membrane-soluble quinone pool. An electron from Q_BH_2 is then returned to the special pair via the cytochrome bc_1 complex and the mobile cytochrome c_2, thereby completing the cyclic flow of electrons (78). During the course of the reduction and subsequent oxidation of Q_B, two protons are removed from the cytoplasmic side and released into the periplasmic side of the membrane for every two electrons that traverses the cyclic electron chain. Hence, the net result is the formation of a proton-motive force across the membrane which provides energy for ATP synthesis via the coupling factor of the ATPase enzyme (fig.1).

fig.1 Schematic diagram, adapted from reference 7, of the photosynthetic components located in the intracytaplasmic membrane. See text (section 1.1.3) for a description of the cyclic flow of electrons.


periplasm
light
H^+
$Cytc_2$
H^+
e-
Bchl
e-
e-
Q
Q
B800-850
LH-II
B870
LH-1
Reaction centre
H^+
Cyt bc_1
ATPase
ADP + P_i → ATP
cytoplasm

5b

1.2 Bchl biosynthesis

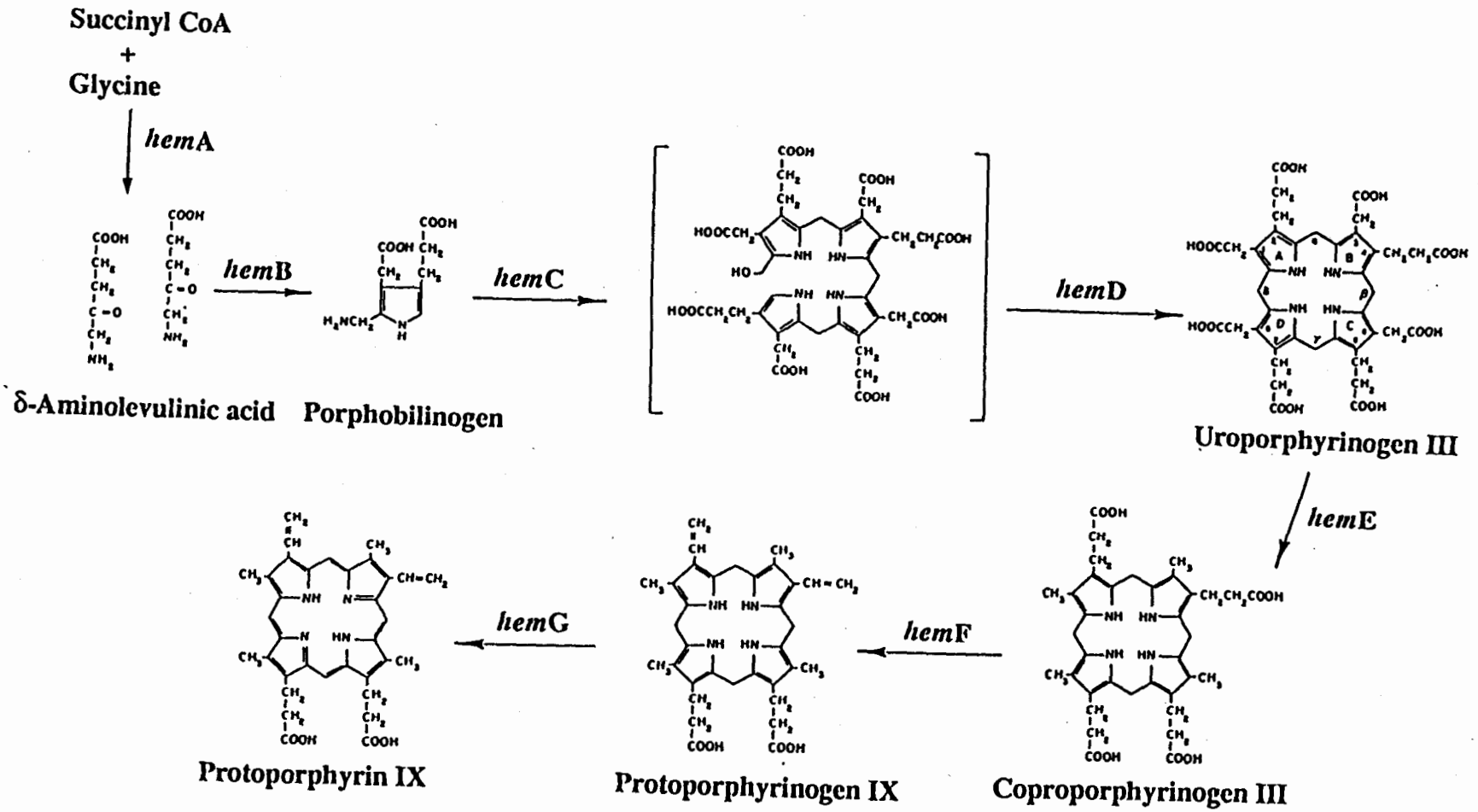
1.2.1 Early stage enzymes and intermediates of the Bchl biosynthetic pathway

Biosynthesis of Bchl can be conveniently represented in two stages (20). The first stage, which is a common pathway shared by heme and Bchl, is shown in fig.2. This early pathway has been well characterized in terms of the structures and order of intermediates, as well as the enzymes involved in catalysis (86). The pathway begins with the condensation of glycine with succinyl-CoA by δ-aminolevulinic acid (ALA) synthase to form ALA. Eight molecules of ALA are then condensed and modified in a series of six enzyme-catalysed reactions to form protoporphyrin IX. The genes encoding enzymes for this part of the pathway are referred to as *hem* genes and encompass genes spanning *hemA* to *hemG*. At this point, the pathway diverges, either by the insertion of Fe^{+2} to form heme by ferrochelatase (*hemH*), or the addition of Mg^{+2} to form magnesium protoporphyrin (MgProto). Enzymes of the magnesium branch catalyse the latter reaction and the subsequent conversion of MgProto to Bchl, and are encoded by genes referred to as *bch* genes (section 1.2.2). Details concerning the enzymes and intermediates of this branch remain fragmented and are discussed below.

1.2.2 *bch* genes and the magnesium branch intermediates of the Bchl biosynthetic pathway

Several *bch* genes were originally identified based on phenotypic mapping studies (13, 103, 114). In these studies, a mutation in a *bch* gene which renders the gene product (an enzyme of the magnesium branch) inactive will block the corresponding

fig.2 Early intermediates of the common pathway for heme and Bchl biosynthesis. The enzymes responsible for each of the steps are encoded by the *hem* genes and shown above the arrows.


Succinyl CoA
+
Glycine
hemA
δ-Aminolevulinic acid
hemB
Porphobilinogen
hemC
hemD
Uroporphyrinogen III
hemE
Coproporphyrinogen III
hemF
Protoporphyrinogen IX
hemG
Protoporphyrin IX

7b

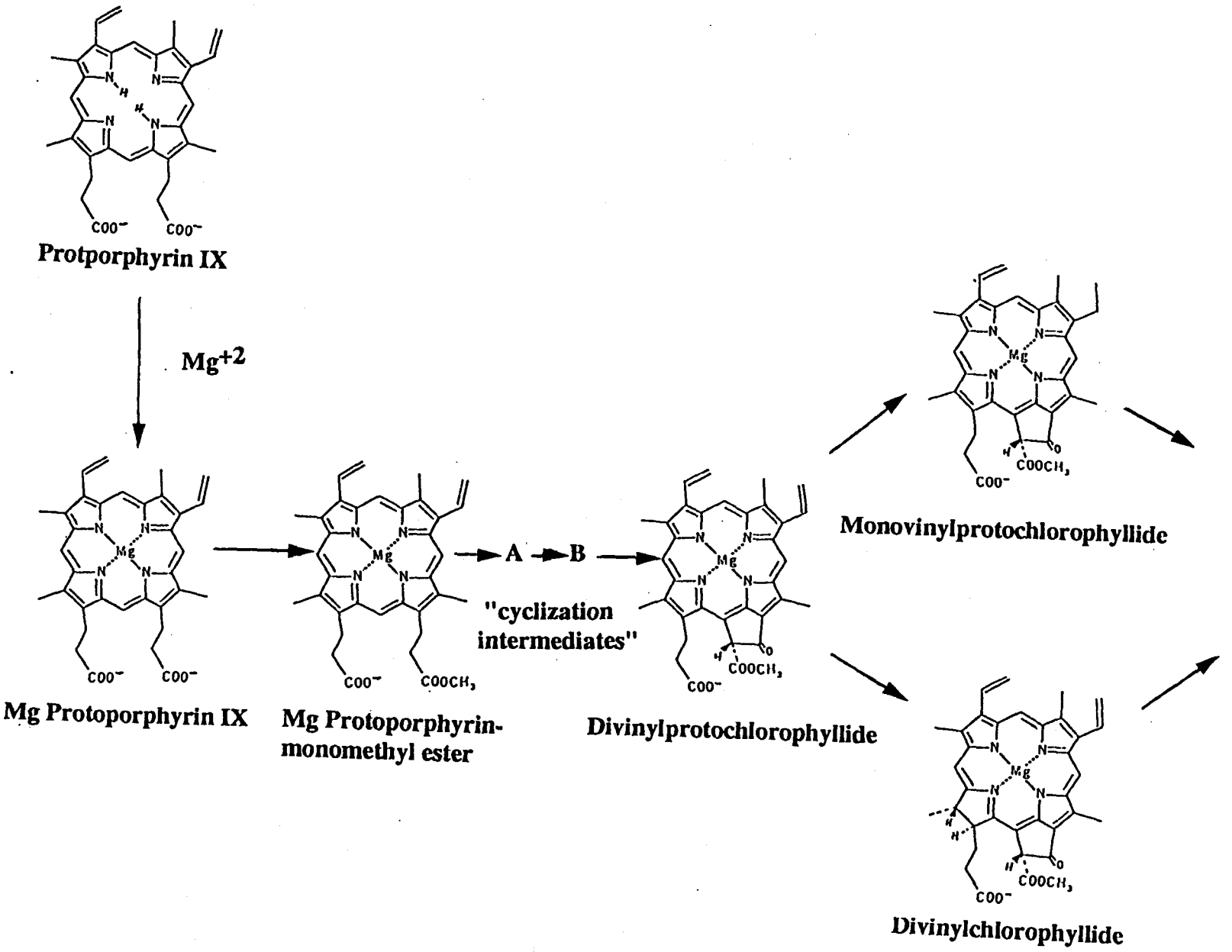
step in the pathway and result in the accumulation of a Bchl intermediate. The intermediate can then be "fingerprinted" on the basis of its absorption and fluorescence emission spectra and correlated with the *bch* mutation. From these studies, *bch* mutants were identified which corresponded to different enzymes of the pathway.

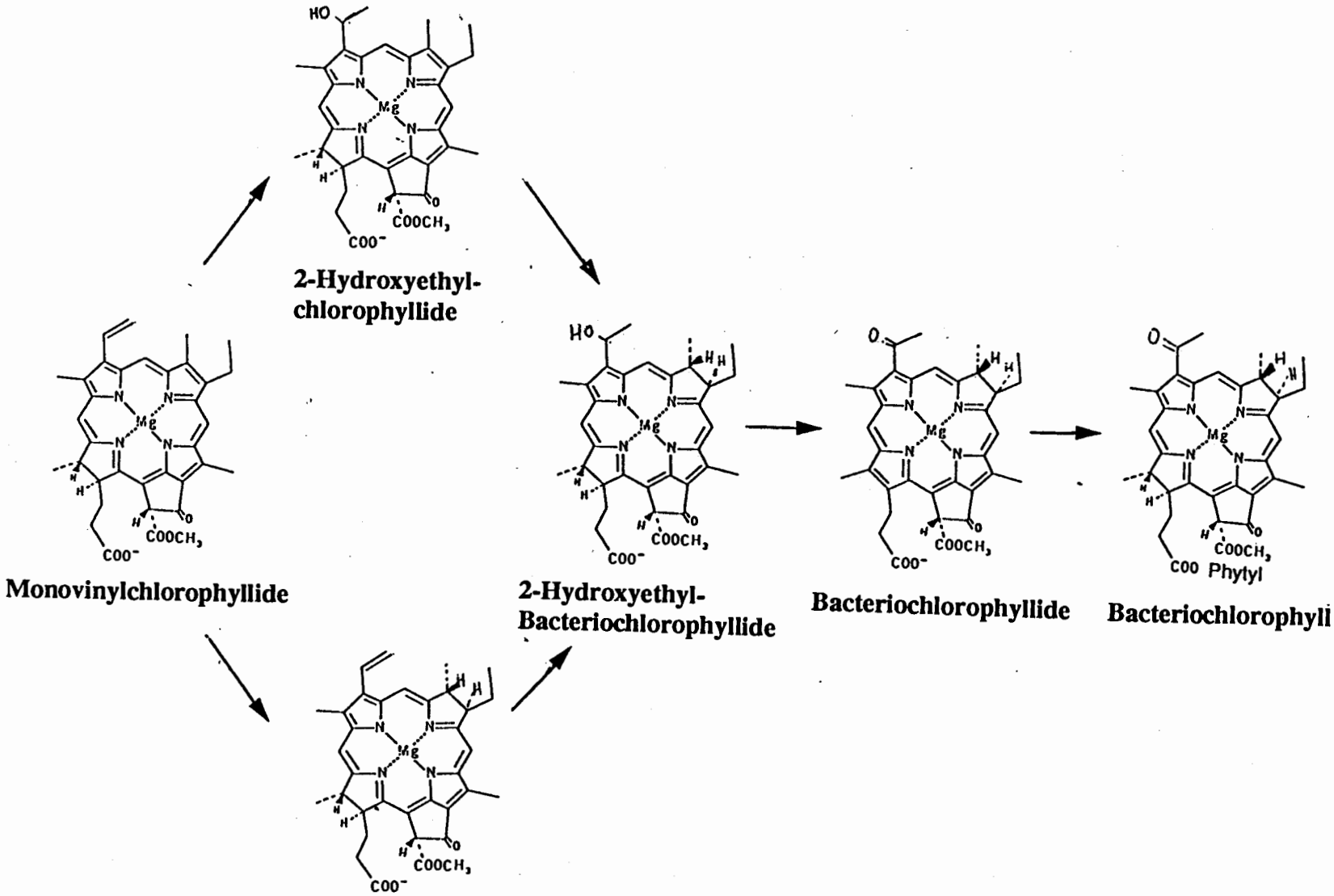
From a careful spectroscopic analysis of pigments excreted by *bch* mutant strains, a biosynthetic pathway was proposed based on the observed Bchl intermediate structures (65, 27, 85). An important feature of the pathway emerged from such analyses. It was observed that certain *bch* mutant strains excreted pigments which could only be accounted for if parallel pathways were employed (84). Hence, a non-linear "metabolic grid" was proposed for ordering the reactions (fig.3). Although this representation of the magnesium branch was proposed over 20 years ago, ambiguities persist in the order and structure of some intermediates, due in part to the lack of available assays for most of the enzymes.

1.2.3 Enzymes of the magnesium branch of the Bchl biosynthetic pathway

In contrast to the first stage of Bchl biosynthesis, the magnesium branch (see fig.3) enzymes have been poorly characterized. In fact, only one enzyme, S-adenosyl-L-methionine (SAM): MgProto methyltransferase (MT), from *R. sphaeroides* has been partially purified and characterized enzymologically (37, 43). This enzyme catalyses the transfer of a methyl group from S-adenosyl-L-methionine to the 6-propionic acid of MgProto to form magnesium protoporphyrin IX monomethyl ester (MgPME). MT was shown to exhibit an equilibrium ordered Bi-Bi reaction mechanism with MgProto as the first substrate (43). In contrast, two other MTs from the alga *Euglena gracilis* and etiolated wheat, display either a random (44) or ping-pong mechanism (45), respectively. In the case of *R. sphaeroides*, enzyme activity was recovered only in the chromatophore fraction of cell extracts, indicating that it was an intrinsic protein.

fig.3 The Mg branch of Bchl biosynthesis. Enzymes of the pathway are encoded by the *bch* genes and discussed in the text (section 1.3.1).


Protporphyrin IX
Mg+2
Mg Protoporphyrin IX
Mg Protoporphyrin-
monomethyl ester
A
B
"cyclization
intermediates"
Divinylprotochlorophyllide
Monovinylprotochlorophyllide
Divinylchlorophyllide


2-Hydroxyethyl-
chlorophyllide
Monovinylchlorophyllide
2-Hydroxyethyl-
Bacteriochlorophyllide
Bacteriochlorophyllide
Bacteriochlorophyll
2-vinyl Bacteriochlorophyllide

The insertion of Mg into protoporphyrin is catalysed by the enzyme magnesium chelatase. While the activity of this enzyme has not been possible to assay in cell free lysates, magnesium chelatase activity was demonstrated in whole cells (39). The substrate in this assay, protoporphyrin, was shown to be converted not to MgProto, but the methylated product, MgPME. Furthermore, in this same assay procedure, if exogenous protoporphyrin is replaced with MgProto, MgPME was shown to form in both whole cells and the cell free lysate (40). These results have been interpreted to mean that the enzymes responsible for Mg insertion and subsequent methylation of protoporphyrin are tightly coupled.

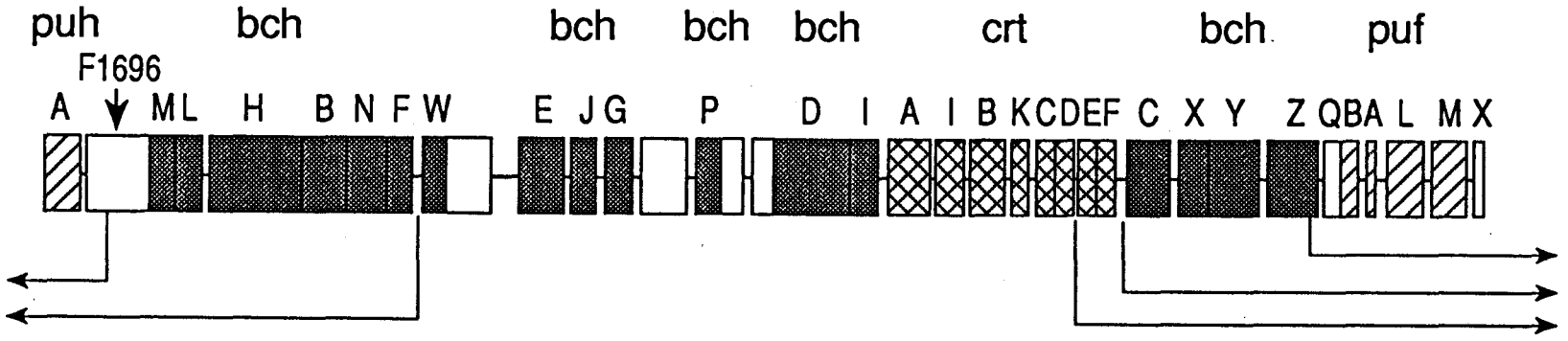
These and other (20, 11, 85) results suggest that insolubility in aqueous solutions may represent a common feature among the magnesium branch enzymes, leading to the difficulties encountered in purification and detecting enzymatic activity. Alternatively, the difficulties in establishing enzyme assays may be due to the formation of one or more tightly associated enzyme complexes.

1.3 Photosynthesis gene cluster

Enzymes of the Bchl and carotenoid biosynthetic pathway of *R. capsulatus* were the first photosynthetic genes to be mapped in a photosynthetic bacteria (114). The results of these and other (103, 117) initial mapping studies indicated that virtually all of the pigment biosynthesis genes as well as the genes encoding RC and B870 LH-I polypeptides were localized within an approximately 46 kb region of the chromosome (representing 1 % of the entire chromosome). These intriguing results focussed attention on, and laid the groundwork for, extensive sequence and genetic analysis on this specific region of the chromosome (reviewed in 7, 58).

It is now clear that the genes encoding most of the structural proteins and many of the enzymes required for the process of photosynthesis in *R. capsulatus* map to a

fig.4 A map of the photosynthetic gene cluster in *R. capsulatus*. The arrows indicate the origin and direction of transcripts. Checkered, hatched, and diagonal boxes represent the *crt*, *bch*, and structural genes respectively. Open boxes indicate loci of undefined function.


puh
bch
bch
bch
bch
crt
bch
puf
F1696
A
M L
H
B N F W
E J G
P
D
I
A
I
B K C D E F
C
X Y
Z Q B A L
M X

11b

region of the chromosome known as the photosynthetic gene cluster (fig.4). The genes encompassing this cluster are organized into operons which transcribe either enzymes required for Bchl and carotenoid biosynthesis, or structural proteins required for the B870 LH-I and RC (*puf* operon) complexes and the H-subunit (*puh A* gene) of the RC complex. An additional *puc* operon, encoding subunits of the B800-850 LH-II complex, is located outside of this gene cluster. As shown in fig.4, the organization of these operons is such that the *crt* and *bch* operons are clustered at the center of this region, and are bounded at either end by the *puf* and *puh* operons.

1.3.1 Organization of *bch* genes

Although the phenotypic mapping studies had generated several *bch* mutant strains, no *bch* genes had been cloned and sequenced until recently. The first gene sequence reported was *bchC*, which was shown to encode 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide *a* dehydrogenase, an enzyme that catalyses the penultimate step in Bchl biosynthesis (107). In these and other studies described below, the assignment of an enzyme to a specific *bch* gene was based on the structure of Bchl intermediates excreted by mutant *R. capsulatus* cells containing an insertional deletion of the chromosomal copy of the cloned *bch* gene.

The results of the *bchC* gene analysis were followed by publication of additional gene sequences for *bchL* and *bchM*, both of which were shown to encode enzymes involved in the early steps of Bchl biosynthesis (113). Recently, the sequence of the entire photosynthetic gene cluster including the *bch* genes was reported by Alberti and Hearst (2). An important result from the sequence analysis was the identification of several additional open reading frames which may encode putative magnesium branch enzymes (see fig.4). It is now clear that in addition to *bchL*, two other genes, *bchB* and *bchN*, are required for the *trans* reduction of ring D of protochlorophyllide (Pchlide) to

form chlorophyllide *a* (18, 6). Based on the high degree of sequence homology to the chloroplast genes of the algae *Marchantia polymorpha* and *Chlamydomonas reinhardtii*, these three genes are thought to encode subunits of the light-independant Pchlide reduction enzyme in *R. capsulatus* (reviewed in 6).

Additional mutational analysis suggests that the *bchM* gene, in conjunction with the *bchE* gene, is responsible for the oxidative cyclization of the cyclopentone ring during conversion of MgPME to divinyl Pchlide (113).

The *bchF* gene encodes an enzyme which is specific for two substrates, chlorophyllide *a* and 2-vinyl-bacteriochlorophyllide *a* (18). It catalyses the hydration reaction of the 2-vinyl group, yielding either 2-α-hydroxyethylchlorophyllide *a* or 2-α-hydroxyethylbacteriochlorophyllide *a*, respectively.

Another gene, *bchA*, has been shown to encode chlorin reductase, an enzyme which is responsible for reducing ring B of chlorophyll *a* to bacteriochlorophyllide (13, 115). Recently however, sequence analysis has revealed that the *bchA* gene in fact consists of three separate coding segments, referred to as the *bchX*, *bchY*, and *bchZ* genes (69).

The accumulation of a common intermediate, protoporphyrin, in mutants of either *bchH*, *bchD*, and *bchI* had suggested that at least these three genes were involved in the Mg insertion of protoporphyrin IX and its subsequent methylation (113, 87, 13). To determine which of these genes was responsible for the two steps, each of the mutants were assayed for Mg chelatase and MT activities in *R. sphaeroides* (41). The results indicated that MT was present only in the *bchD* and *bchI* mutants, while no Mg chelatase activity was detected in any of the three mutants. Based on these results therefore, the *bchH* gene encodes MT, and either one or both of the *bchD* and *bchI* genes appear to encode or regulate the activities of Mg chelatase. Furthermore, the lack of any detectable Mg chelatase activity in the *bchH* mutant suggests an obligatory

presence of MT for Mg chelatase activity, consistent with the idea of a close relationship between both enzymes.

Recently, a comparison search of the newly identified *oli* gene required for chlorophyll biosynthesis in the plant *Antirrhinum majus* (46a) has revealed significant homology with both the *cobN* gene of *Pseudomonas denitrificans* as well as with the *bchH* gene. The homology between *cobN* and *bchH* is particularly interesting in view of the fact that *cobN* is necessary for the insertion of cobalt into the cyclic tetrapyrrole, hydrogenobyrinic acid diamide, which is a precursor of cofactor B_{12} (46a).

1.3.2 Organization of the *puf* operon

The *puf* operon encodes 4 pigment-binding proteins of the B870 LH-I and RC complexes. The β and α subunits of the B870 LH-I are encoded by *pufB* and *pufA* respectively, while the L and M subunits of the RC are encoded by *pufL* and *pufM* respectively. In addition, there are two open reading frames located on either end of the preceeding genes, designated *pufQ* and *pufX*. Though there is significantly more variation in the *pufQ* and *pufX* sequences (48), the gene sequence and organization of the *puf* operon are conserved between *R. capsulatus* and *R. sphaeroides*.

The *pufX* gene encodes a polypeptide of approximately 8000 daltons, and has been shown in *R. capsulatus*, to be essential for the efficient electron flow from the RC to the cytochrome bc_1 complex (67). It has also been demonstrated to be necessary in *R. sphaeroides* for optimal levels of B870 LH-I and B800-850 LH-II complexes (31). The other open reading frame, *pufQ*, encodes a polypeptide of similar molecular weight that is essential for Bchl formation and is the focus of this study.

1.3.3 Superoperonal organization of the photosynthesis gene cluster

Recent studies in *R. capsulatus* have demonstrated the existence of a complex set of overlapping transcripts. Two so-called "superoperons" have been identified in which transcription of the downstream *puf* and *puh* operons is linked to that of upstream pigment biosynthesis genes (see fig.4). A variety of genetic and physical analyses including Northern blotting, the polar effects of mutations, RNA end-mapping, and promoter-probe vectors containing *lacZ* gene fusions were used to elucidate the nature of these overlapping transcripts.

In the case of the first described superoperon (115, 109), transcription is initiated from two weak upstream promoters of the *crtEF* and *bchCA* (or *bchCXYZ*) operons and extends into the third downstream *puf* operon. Although the oxygen regulated *puf*, *crtEF*, and *bchCA* promoters differ in their "strengths" (i.e., ability to initiate transcription), transcription of the *crtEF* operon is a necessary condition for normal levels of *bchCA* and *puf*-encoded polypeptides (115).

In the second superoperon identified (8), transcription is initiated from a promoter located upstream of the *bchFNBHLM-F1696* operon and extends beyond the strong oxygen regulated promoter located at the *F1696* gene and into the downstream *puhA* gene (encoding the H-subunit of the RC).

The physiological consequence of this type of superoperonal gene arrangement has been reviewed (108). The result of constitutive low level expression of both upstream operons is that basal levels of LH and RC polypeptides are synthesised during aerobic growth. This is important during transitions from aerobic to anaerobic (photosynthetic) growth conditions since the presence of pigment-binding polypeptides allows cells to more rapidly form the photosynthetic apparatus. Hence, transcriptional coupling improves the ability of these cells to efficiently adapt to changes in growth conditions.

1.4 Regulation of photosynthesis genes

1.4.1 Post-transcriptional regulation of the *puf* operon

The dramatic effect of oxygen on the levels of pigment-binding polypeptides encoded by the *puf* operon (β and α subunits of B870 LH-I and L and M subunits of the RC) has been well documented (22, 59, 117, 12). In these studies, induction of the ICM in actively growing cells by a shift from high to low oxygen partial pressures resulted in a concomitant increase in the levels of LH and RC polypeptides. However, the stoichiometric amounts of both sets of polypeptides were not the same. The B870 LH-I polypeptides were found to be in a 10-30-fold molar excess over the subunits of the RC (91, 55). The mechanism whereby the cell is able to attenuate the levels of proteins encoded by genes transcribed from the same promoter was shown by Belasco et al. (12) to occur at a post-transcriptional level . Though the *puf* operon is transcribed as a single polycistronic message, Northern blot analysis revealed the presence of two discrete mRNA species having lengths of 0.5 and 2.7 kb. The small transcript encoded only the LH-I β and α polypeptides, whereas the large transcript coded for RC-L, RC-M, and the β and α polypeptides of LH-I. In addition, the 0.5 kb transcript was approximately 9 times as abundant as the 2.7 kb transcript. The presence and relative amounts of both transcripts were attributed to segmental differences in stability of the full-length mRNA. These differences were shown to arise as a result of a large stem-loop structure located between the *pufA* and *pufL* genes which serves to block 3' to 5' exoribonuclease processing of the *puf* operon transcript (21). Subsequent site-directed mutational analysis and high resolution mapping of the 5' end of the *puf* mRNA identified an oxygen regulated promoter region approximately 320 to 370 nucleotides from the initiation codon of *pufQ* (1). Though the length of the primary *puf* transcript is expected to be at least 3.4 kb, the most abundant mRNA

species detected were the 2.7 and 0.5 kb transcripts, both of which had a common 5' end beginning just upstream of *pufB* (but not including *pufQ*). Using a *pufQ*-specific probe however, a minor *pufQBA* transcript of 1.2 kb in length was detected in *R. sphaeroides* (48) and in *R. capsulatus* (1). These results suggest therefore, that the 5' segment of the primary transcript that includes *pufQ* is extremely labile and probably undergoes rapid degradation. Consequently, the relative cellular level of PufQ protein is presumed to be much less than other *puf* encoded polypeptides.

1.4.2 Regulation by oxygen of photosynthesis promoters

Although a large amount of evidence has demonstrated that regulation of the *puc*, *puf*, and *puh* operons occurs at the level of transcription in response to environmental conditions (76, 66, 60, 61), the identification of a gene or a gene product which is responsible for this regulation has remained elusive until recently. The sequence of the weakly regulated *crt* and *bch* promoters has been shown to resemble the recognition sequence for the *E. coli* σ^{70} factor, suggesting that these promoters are recognized by a housekeeping σ factor. Sequence alignment of the oxygen regulated *puf*, *puh*, and *puc* promoters however, has revealed a unique consensus that does not resemble the recognition sequence of the *E. coli* σ^{70} factor (reviewed in 7). A novel σ factor has thus been suggested to interact with these promoters.

Genetic evidence by Sganga and Bauer (93) indicated that expression from these promoters requires both the *regA* and *regB* genes. From this observation, a model for oxygen mediated regulation of these photosynthetic promoters was proposed based on homology of RegA and RegB proteins to the two-component class of bacterial response regulators (95). In this model, a membrane-bound, oxygen sensitive RegB protein activates a cytoplasmic regulator protein, RegA, by a phospho-transfer

mechanism. The phosphorylated RegA is then able to activate transcription of the *puf*, *puh*, and *puc* promoters.

A second model (102, 61) similarly requires the presence of a kinase that phosphorylates a regulator protein (Puf Promoter Binding Protein), thereby leading to activation of transcription.

1.5 Significance of *pufQ* in Bchl biosynthesis

As mentioned previously, environmental factors play a key role in the regulation of ICM formation. However, the extent to which these factors affect the levels of the photosynthetic apparatus assembled in the ICM are not the same. Whereas light intensity simply modulates the levels of pigment-protein complexes in the cell, high oxygen tension may abolish its synthesis completely (28, 3). While the repression by oxygen has a direct effect on *puf* operon transcription, the transcription of *bch* genes in contrast is much less sensitive to environmental oxygen levels (13, 22, 49, 117). Hence, the repression of *bch* genes by light or oxygen, acting alone or in combination to down-regulate transcription, cannot completely account for the down-regulation of Bchl biosynthesis. Though it is clear that some other factor or factors must be influenced by light and oxygen to down-regulate the biosynthesis of Bchl, no post-transcriptional regulatory mechanisms for the *bch* genes have yet been identified.

A possible candidate gene that may encode a regulatory protein of Bchl biosynthesis was first identified as a 74 amino acid open reading frame (fig.5) in *R. capsulatus* (1, 9, 10, 34, 62). This open reading frame, later termed the *pufQ* gene, is also present in *R. sphaeroides* (26, 48), and is located in both at the extreme 5' end of the *puf* operon (fig.4). As such, its expression is regulated by the oxygen sensitive *puf* promoter. The putative *pufQ* gene has a codon usage consistent with other genes of the species (9), and the protein deduced from the DNA sequence (the so-called PufQ

fig.5 The nucleotide and deduced amino acid sequence of the *pufQ* gene. Amino acids that are underlined indicate the putative membane-spanning region of the protein based on its hydropathy plot (1).

```
          10          20          30          40
          |           |           |           |
ATG CAA AGC CAG CGT CTT CGC GCT CAT GGG GTC CAA CAT GTC GAC

MET Gln Ser Gln Arg Leu Arg Ala His Gly Val Gln His Val Asp

    50          60          70          80          90
    |           |           |           |           |
CGC GTG CCG CGT CCC GAG TTC GCG CTT TAC TTT TCG CTG ATC CTG

Arg Val Pro Arg Pro Glu Phe Ala Leu Tyr Phe Ser Leu Ile Leu

         100         110         120         130
          |           |           |           |
ATC GTC GCG GTG CCT TTC GCG CTG GTC GGC TGG GTC ATG GCC CTG

Ile Val Ala Val Pro Phe Ala Leu Val Gly Trp Val Met Ala Leu

   140         150         160         170         180
    |           |           |           |           |
GTG CGC GAG CGC CGC ATC CCC GAG TGC GGG CCC TTC GCC CGC GCC

Val Arg Glu Arg Arg Ile Pro Glu Cys Gly Pro Phe Ala Arg Ala

         190         200         210         220
          |           |           |           |
TGG CGC GAG GCG GGC GAG ATC ACG CCC GAG ATT TTC CGG CCC

Trp Arg Glu Ala Gly Glu Ile Thr Pro Glu Ile Phe Arg Pro
```

protein) contains a centrally located stretch of 25 hydrophobic amino acids (1) long enough to span a membrane (fig.5).

The importance of the *pufQ* gene in regulating the synthesis of Bchl was first demonstrated by genetic studies (9, 62). In strains of *R. capsulatus* in which the entire *puf* operon had been deleted, virtually no Bchl was detected (as determined by the characteristic absorption spectra of the B800-850 LH-II complex in whole cells). However, when a plasmid-borne *pufQ* gene was reintroduced into the strain, wild type levels of Bchl were detected. Bauer and Marrs (9) performed an elegant series of experiments involving the fusion of a *nif* promoter with the *pufQ* and *lacZ* genes. With this promoter, different levels of expression could be obtained by varying the nitrogen sources, and the relative amounts of PufQ protein expressed could be inferred from the measured β-galactosidase activity. When this gene fusion was introduced into a strain of *R. capsulatus* lacking the *puf* operon, it was found that the level of β-galactosidase activity was proportional to Bchl biosynthesis. This effect of *pufQ* on the levels of Bchl was also subsequently demonstrated in *R. sphaeroides* (48).

To test whether the *pufQ* gene is involved in regulating transcription of the *puc* operon, Northern blot analysis of the *puc* operon in the presence and absence of the *pufQ* gene was performed by Forrest *et al* (34). The results indicated there was no effect by *pufQ* on the levels of *puc*-specific transcripts. Furthermore, results from *puc-lacZ* gene fusions demonstrated that *pufQ* was not involved in regulating translation of the *puc* operon. These results confirmed the notion that the PufQ protein may be involved in Bchl biosynthesis, and also suggested its possible role in the assembly of the B800-850 LH-II complex.

If the PufQ protein is involved in regulating the flow of tetrapyrroles in the Bchl pathway, it may exert its effect at some stage in the magnesium branch of the pathway, since the levels of heme are unaffected by the deletion of the *puf* operon. To determine at which step PufQ protein is required, studies were carried out on a series of

R.capsulatus strains which contain functional mutations in specific *bch* genes including *bchA*, *C*, *D*, *E*, *F*, *G*, and *H* (9). These mutants form no Bchl, but instead, accumulate elevated levels of Bchl intermediates ranging from protoporphyrin IX to bacteriochlorophyllide. When deletions of the *puf* operon were then introduced into each of the strains, Bchl intermediates failed to accumulate. Furthermore, the failure to accumulate Bchl intermediates was observed in every combination of *bch* gene mutant/*puf* operon deletion tested by double mutant analysis. This inability to accumulate Bchl intermediates was shown to be due to a specific lack of *pufQ* gene, because its addition *in trans* restored the ability of mutants to accumulate Bchl intermediates. Since PufQ protein is required for all of the steps in the magnesium branch beginning with the insertion of Mg into protoporphyrin IX (catalysed by the enzyme magnesium chelatase), a possible role of the PufQ protein in activating this step of the pathway has been suggested. As this step is the branchpoint in the pathway to heme or Bchl biosynthesis, PufQ protein might be expected to serve as an ideal regulator of magnesium chelatase activity. An alternate view of the role of PufQ protein may be as an activator or carrier protein of Bchl intermediates for bch enzymes to act on. This model of PufQ protein would also account for its requirement for each of the steps in the Bchl pathway tested.

While the chelation of Fe or Mg in protoporphyrin IX has been recognized as the key step in determining the relative levels of heme and Bchl, recent evidence in *R. sphaeroides* suggests that a step prior to the conversion of protoporphyrin may in fact, represent the true branch point (38, 23). The oxidative decarboxylation of coproporphyrinogen III (Coprogen) to form protoporphyrinogen IX had been previously shown to be catalyzed by the enzyme Coprogen oxidase under either anaerobic or aerobic conditions (99, 100). Identification of a *hemF* gene, located outside of the photosynthetic gene cluster, has now been shown to encode the anaerobic version of this enzyme. Thus, two Coprogen oxidases exist in *R.*

sphaeroides, and are activated under either aerobic conditions to form heme, or under anaerobic photosynthetic conditions to form Bchl. The presence of these two forms of the enzyme creates therefore the intriguing possibility of an alternative pathway for heme biosynthesis which utilizes only anaerobically activated enzymes.

The regulation by oxygen on specific enzymes of the protoporphyrin biosynthetic pathway has also been studied in *R. capsulatus* by Biel (14). In this study, the conversion of ALA to porphobilinogen (PBG), which is a step even earlier than Coprogen oxidation, was demonstrated to be tightly regulated by oxygen. The fact that this regulation occurs at a very early stage, and that ALA is a metabolite involved in a variety of other pathways (14), implies that synthesis of PBG may be a committed step in the pathway. It remains unclear however, whether oxygen has a direct inhibitory effect on the enzyme, or if regulation occurs via another oxygen-mediated mechanism.

Despite the clear importance of *pufQ* in Bchl biosynthesis, attempts to isolate a *pufQ* gene product from *Rhodobacter* species have thus far been unsuccessful. Recombinant expression in *E. coli* was therefore used to generate overexpressed levels of PufQ protein. In subsequent studies, the model of PufQ protein acting as a carrier protein of Bchl intermediates was tested by performing binding experiments between PufQ protein reconstituted in phospholipid vesicles and the Bchl intermediate, Pchlide. Further studies localized the PufQ protein in a subcellular fraction of *R.capsulatus* where the first site of ICM formation is thought to occur. To further understand its role in Bchl biosynthesis, reconstituted PufQ protein was used in attempts to develop assays for some of the enzymes of Bchl biosynthesis. Finally, the physiological consequences of *pufQ* on the levels of pigment biosynthesis will be discussed.

2. MATERIALS AND METHODS

2.1 Materials

The following chemicals and enzymes were purchased from the suppliers shown: Dipalmitoylphosphatidyl[*methyl*-^{3}H]choline (50 Ci mol^{-1}), 5-[4-^{14}C]Aminolevulinic acid (47.8 mCi mol^{-1}), *S*-[*methyl*-^{14}C]adenosyl-L-methionine (58 nCi nmol^{-1}), and α-^{32}P dATP (10 mCi mL^{-1}) were supplied by DuPont NEN Research Products and Amersham, respectively. Soybean phospholipids (Asolectin) was supplied by Associated Concentrates. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, mung bean nuclease, fetal calf serum, 5-bromo-4-chloro-3-indoyl-β-D-galactose (X-Gal), isopropylthio-β-galactoside (IPTG), low melting point agarose, acrylamide, and all other electrophoresis reagants were purchased from Bethesda Research Laboratories. The pKK223-3 vector, deoxyribonucleotides, and Mono Q were purchased from Pharmacia Biotech Inc., and T7 DNA polymerase (Sequenase) was purchased from United States Biochemicals Corp. Calf intestinal phosphatase, n-octylglucoside, and ribonuclease T_1 was from Boehringer Mannheim, and goat anti-rabbit serum conjugated to horseradish peroxidase from Mandel Scientific. The Random Priming System I kit and Protein Fusion and Purification kit (containing pMal-c vector, amylose affinity column, rabbit anti-MBP serum, and 24mer *malE* sequencing primer) were purchased from New England Biolabs. Other oligonucleotides were purchased from the Oligonucleotide Synthesis Laboratories of either the University of British Columbia, Vancouver, B.C. (33mer), or the Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, B.C. (16mer and 24mer). Zeta-probe blotting membranes and Bradford assay dye reagant were purchased from Bio-Rad. Immobilon-p filters were purchased from Millipore. Agarose, ampicillin, tetracycline, sodium dodecyl sulphate, sodium cholate, sodium deoxycholate, Triton X-100,

ribonuclease A, dithiothreitol, polyethylene glycol 8000, bovine serum albumin, diaminobenzidine, mixed bed ion exchange resin (Amberlite), S-Adenosyl-L-methionine, ALA, levulinic acid, nicotinamide, and porphobilinogen were purchased from Sigma. Standard cellulose dialysis tubing and HPLC grade acetonitrile were purchased from Fisher Scientific. All other chemicals and reagants were of the highest grade commercially available.

2.2 Buffers and other solutions

Buffer A: 10 mM sodium phosphate, pH 7.0, 30 mM NaCl, 0.25% (v/v) Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethyl sulfonyl fluoride (PMSF).

Buffer B: 10 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 0.25% (v/v) Tween 20, 10 mM β-mercaptoethanol, 1 mM EGTA, and 1 mM sodium azide.

Buffer C: 20 mM Tris-Cl, pH 8.0, and 0.1 M NaCl.

Buffer D: 2.7 mM KCl, 10 mM NaCl, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, pH 7.2.

Buffer E: 50 mM Potassium phosphate, pH 7.5.

Buffer F: 192 mM glycine, 20 % (v/v) methanol, and 25 mM Tris-HCl, pH 8.3.

Buffer G: 20 mM Potassium phosphate, pH 7.5, 10 mM EDTA, and 1 mM PMSF.

10 x DNA Electrophoresis Sample Buffer: 20 % (w/v) Ficoll 400, 0.1 M EDTA, pH 8.0, 1.0 % (w/v) SDS, 0.25 % (w/v) bromophenol blue, and 0.25 % (w/v) xylene cyanol.

Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, and 4 mg mL^{-1} lysozyme.

Solution II: 0.2 N NaOH, and 1.0 % (w/v) sodium dodecyl sulfate (SDS)

Solution III: 3 M potassium acetate, and 2 M glacial acetic acid.

MOPS/EDTA Buffer: 20 mM MOPS [3-(N-morpholino) propanesulfonic acid], 5.0 mM sodium acetate, and 1.0 mM EDTA, pH 7.0.

TBE: 8.9 mM Tris-HCl, 8.9 mM boric acid, and 2 mM EDTA, pH 8.0.

10 x T4 DNA Polymerase Buffer: 500 mM glycine, pH 8.8, 166 mM $(NH_4)_2SO_4$, 60 mM $MgCl_2$, 65 μM EDTA, 100 mM β-mercaptoethanol, and 1.65 mg mL^{-1} bovine serum albumin.

RNA Electrophoresis Sample Buffer: 0.75 mL deionized formamide, 0.15 mL 10 x MOPS/EDTA buffer, 0.24 mL deionized formaldehyde, 0.1 mL diethyl pyrocarbonate (DEPC) treated ddH_2O, 0.1 mL glycerol, and 0.08 mL 10 % (w/v) bromophenol blue.

DNA Sequencing Stop Buffer: 95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue, and 0.05 % (w/v) xylene cyanol.

PBS Buffer: 137 mM NaCl, 2.7 M KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4.

PBS/Tween Buffer: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, and 0.05 % (v/v) Tween-20.

6 x SDS Sample Buffer: 7 mL 0.5 M Tris-HCl containing 0.4 % (w/v) SDS, pH 6.8, 1.0 g SDS, 3.8 g glycerol, 0.93 g DTT, 1.2 mg bromophenol blue, and ddH_2O to a final volume of 10 mL.

Hybridization Buffer: 20 mL deionized formamide, 10 mL of 20 % (w/v) SDS, 10 mL of 2 M NaH_2PO_4 and 4 mM EDTA, pH 7.2, and 1 mg mL^{-1} bovine serum albumin.

Protoplasting Buffer: 15 mM Tris-HCl, pH 8.0, 0.45 M sucrose, and 8.0 mM EDTA.

Gram-negative Lysing Buffer: 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM sodium citrate, and 1.5 % (w/v) SDS.

10 x SSC: 1.5 M NaCl, and 0.15 M Na_3citrate$\cdot 2H_2O$, pH 7.0.

10 x PE 1 Buffer: 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, and 10 mM DTT.

10 x PE 2 Buffer: 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, and 100 mM DTT.

5 x Ligation Buffer: 0.25 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol (DTT), and 25% (w/v) polyethylene glycol-8000.

10 x Polynucleotide Kinase Buffer: 0.5 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine HCl, and 1 mM EDTA, pH 8.0.

10 x CIP Dephosphorylation Buffer: 10 mM $ZnCl_2$, 10 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.3.

10 x T4 DNA Ligase Buffer: 200 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, and 0.5 mg ml^{-1} bovine serum albumin.

STE Buffer: 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM EDTA.

5 x Electrode SDS Buffer: 15g Tris-HCl, 72g glycine, and 5g SDS, pH 8.3, per litre.

Mung Bean Nuclease Buffer: 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$, and 5 % (v/v) glycerol.

TE8 Buffer: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0.

TAE Buffer: 40 mM Tris-acetate, and 1 mM EDTA.

5 x Sequencing Buffer: 200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, and 250 mM NaCl.

TfBI Buffer: 30 mM CH_3COOK, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, and 15 % (v/v) glycerol. Solution adjusted to pH 7.0 and filter sterilized.

TfBII Buffer: 10 mM Na-MOPS, pH 7.5, 75 mM $CaCl_2$, 10 mM KCl, and 15 % (v/v) glycerol. Solution adjusted to pH 7.0 and filter sterilized.

Phenol solution: All DNA extractions were performed using phenol prepared from pure liquified phenol equilibrated to a pH > 7.8 as described in Sambrook (89).

2.3 Bacteriological media, strains, and methods

2.3.1 Media

TY/Mg broth: 20 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, and 10 mM $MgSO_4$ per litre. The $MgSO_4$ was added from a 1 M filter sterilized stock.

2 x TY broth: 16 g tryptone, 10 g yeast extract, and 5.0 g NaCl per litre.

TY broth: 10 g tryptone, 5.0 g yeast extract, and 10 g NaCl per litre.

TY plate: TY broth containing 2.0 % (w/v) agar.

2 x TY/ Amp: 2 x TY broth including ampicillin from a filter sterilized stock to a final concentration of 200 $\mu g\ mL^{-1}$.

TY/Amp plate: TY plate including 200 μg mL ampicillin from a filter sterilized stock.

2 x TY/Tet: 2 x TY broth including tetracycline from a filter sterilized stock to a final concentration of 50 $\mu g\ mL^{-1}$.

TY/Tet: TY media including 50 $\mu g\ ml^{-1}$ tetracycline from a filter sterilized stock.

H- top agar: 10 g tryptone, 8.0 g NaCl, and 8.0 g agar per litre.

M9 minimal media: 1.05 g KH_2PO_4, 0.45 g KH_2PO_4, 0.1 g $(NH_4)_2SO_4$, 0.05 g sodium citrate, 0.2 g glucose, 0.02 g $MgSO_4$, and 0.0005 g vitamin B1 per litre.

M9 plate: M9 media including 2.0 % (w/v) agar.

M9/Tet plate: M9 plate containing 50 $\mu g\ mL^{-1}$ tetracycline from a filter sterilized stock.

RCV media: 0.4 g malic acid, 0.1 g $(NH_4)_2SO_4$, 0.2 g K_3PO_4, 0.012 g $MgSO_4 \cdot 7H_2O$, 0.0075 g $CaCl_2 \cdot 2H_2O$, 0.0020 g Na_2EDTA, 0.0001 g vitamin B1, 0.1 g casamino acids, and 0.3 g H_3BO_3 per litre, adjusted to pH 6.8.

RCV plate: RCV media including 2.0 % (w/v) agar.

RCV/Kn: RCV media containing 10 $\mu g\ mL^{-1}$ kanamycin from a filter sterilized stock.

RCV/Kn plate: RCV/Kn media containing 2.0 % (w/v) agar.

RCV/Kn/Tet: RCV media containing 0.5 $\mu g\ mL^{-1}$ tetracycline and 10 $\mu g\ mL^{-1}$ kanamycin from filter sterilized stocks.

RCV/Kn/Tet plate: RCV/Kn/Tet media containing 2.0 % (w/v) agar.

RCV$^+$: RCV media containing 0.6 % (w/v) glucose, 0.5 % (w/v) pyruvate, and 50 mM dimethyl sulfoxide.

RCV$^+$/Kn: RCV$^+$ media containing 10 μg mL^{-1} kanamycin from a filter sterilized stock.

RCV$^+$/Kn/Tet: RCV$^+$ media containing 10 μg mL^{-1} kanamycin and 0.5 μg mL^{-1} tetracycline from filter sterilized stocks.

2.3.2 Bacterial strains

2.3.2.1 *Escherichia coli* strain DH5α

Genotype: *supE44* Δ*lacU*169 (ϕ*80 lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*

Growth conditions and relevant characteristics: The strain DH5α (89) was maintained and grown on TY plates and 2 x TY broth at 37^{o}C. This strain was used for all routine transformations of plasmids and ligation mixtures unless stated othewise.

2.3.2.2 *Escherichia coli* strain TG2

Genotype: *supE hsd*Δ*5 thi* Δ *(lac-proAB)* Δ *(srl-recA) 306::*Tn *10 (tet^r) F'* [*traD36 proAB$^+$ lacI^q lacZ*Δ*M15*]

Growth conditions and relevant characteristics: The strain TG2 (89) was maintained on M9/Tet plates and grown either in M9/Tet or 2 x TY media at 37^{o}C. *E. coli* strain

TG2 was the host for the plasmid pKK223-3 and its recombinant derivatives. Repression of recombinant gene expression is achieved by a *lacI*^{q} repressor gene provided by the host.

2.3.2.3 *Escherichia coli* strain K12cI

Growth conditions and relevant characteristics: The strain K12cI was maintained and grown on TY plates and and 2 x TY broth at 37^{o}C. These cells constitutively express a lamba cI repressor protein so that transformations of plasmids pLcII-FX and its derivatives that do not subsequently involve induction of recombinant gene expression were performed using this cell line.

2.3.2.4 *Escherichia coli* strain QY13

Genotype: F^{-} lac_{am} trp_{am} *BB'bio-256* N^{+} *c1857* ΔH Sm^{r} *recA*

Growth conditions and relevant characteristics: The strain QY13 (74) was maintained and grown on TY plates and 2 x TY broth at 30^{o}C. Liquid cultures of this strain were grown at either 30^{o}C prior to, or at 37^{o}C following, the induction of recombinant gene expression. A recombinant derivative of pLcII-FX containing the *pufQ* gene, pSF2, was used to transform competent QY13 cells. Regulation of gene expression on plasmid pLcII-FX is achieved by the temperature sensitive repressor cI857.

2.3.2.5 *Escherichia coli* strain TB1

Genotype: *ara* Δ (*lacproAB*) *rpsL* (ϕ80 *lacZ*ΔM15) *hsdR*

Growth conditions and relevant characteristics: The strain TB1 (52) was maintained and grown on TY plates and 2 x TY broth at 37°C. This strain was used for transforming the expression vector pMal-c and the pSF3 construct which contained the *pufQ* gene. Repression of recombinant gene expression by this host strain is achieved by the *lacIq* gene.

2.3.2.6 *Escherichia coli* strain RZ 1032

Growth conditions and relevant characteristics: The strain RZ 1032 (89) was maintained and grown on TY/Tet plates and 2 x TY/Tet broth at 37°C. This strain contains a double mutation of the *dut* and *ung* genes. The *dut* mutation renders the cell incapable of synthesising the enzyme dUTPase (dUTP nucleotidohydrolase) that hydrolyses dUTP into dUMP and diphosphate. An *ung* mutation results in failure to synthesise the enzyme uracil N-glycosylase that hydrolyses uracil from single stranded and double stranded DNA.

2.3.2.7 *Rhodobacter capsulatus* wild type strain B10

Growth conditions and relevant characteristics: The strain B10 (106) was maintained on RCV plates at 30°C. Liquid cultures were grown either semi-aerobically in the light (photosynthetic conditions), or aerobically in the dark in RCV media. Semi-aerobic conditions were achieved by growing cultures in flasks containing media filled to 80% of its nominal volume with gentle stirring.

2.3.2.8 *Rhodobacter capsulatus* strain ΔRC6

Growth conditions and relevant characteristics: The strain ΔRC6 (21) was maintained on RCV/Kn plates and grown as described for wild type *R. capsulatus* in either RCV/Kn or anaerobically in RCV^+/Kn media. This strain is photosynthetically incompetent and contains a deletion of the *puf* operon beginning 40 bp downstream of the *pufQ* gene and extending beyond the *pufB, A, L, M,* genes until 486 bp downstream of the 5' end of the *pufX* gene. A neomycin phosphotransferase structural gene (conferring kanamycin resistance) is interposed between these sites.

2.3.2.9 *Rhodobacter capsulatus* strain ΔRC6(pΔ4)

Growth conditions and relevant characteristics: The strain ΔRC6 (pΔ4) was maintained on RCV/Kn/Tet plates and grown as described for wild type *R. capsulatus* in either RCV/Kn/Tet or anaerobically in RCV^+/Kn/Tet media. This strain is a derivative of ΔRC6 that harbors the plasmid pΔ4 (1).

2.3.2.10 *Rhodobacter capsulatus* CB1200

Growth conditions and relevant characteristics: The strain CB1200 was maintained as described for wild type *R. capsulatus* and grown anaerobically in RCV^+ media. This strain contains a double mutation of the *bchA* and *bchF* genes (Dr. C. Bauer, personal communication).

2.3.3 Preparation of competent *E.coli* cells

Two mL of TY/Mg broth was inoculated with a single colony from a plate grown overnight and incubated at the appropriate temperature (30°C or 37°C) to a cell density at A_{600} of 0.5. This culture was used to inoculate 20 mL of TY/Mg (pre-warmed at 30°C or 37°C) in a 250 mL flask and incubated to a cell density at A_{600} of 0.5. The 20 mL culture was then used to inoculate 200 mL of TY/Mg (pre-warmed at 30°C or 37°C) in a 1 L flask until a cell density at A_{600} of 0.6 was reached. At this point, the culture was chilled on ice and centrifuged at 3500 rpm in a GS3 rotor for 15 min at 4°C. All of the subsequent steps were performed at 4°C. The cell pellet was resuspended in 40 mL of TfBI buffer and re-centrifuged. The cell pellet was then resuspended in 8 mL of TfBII buffer and divided into approximately equal aliquots in microfuge tubes. The competent cells were immediately frozen in dry ice and stored at -70°C.

2.3.4 Transformation of *E. coli* strains

2.3.4.1 Transformation of *E. coli* strains DH5α

Approximately 50 µL of *E. coli* strain DH5α cells which had been previously thawed on ice were used for a transformation experiment. Following the addition of either 1 µL of plasmid or 25 µL of diluted ligation mixture DNA, the cells were incubated on ice for 30 minutes. They were then heat shocked for 2.5 minutes at 37°C, and re-incubated on ice for 1 minute. Nine volumes of 2 x TY were then added and the cells incubated in an environmental shaker for an additional 2 hrs at 37°C. After incubation, 100 µL, 10 µL, and 1 µL of cells diluted to 200 µL with 2 x TY broth were spread-plated on TY/Amp plates and incubated overnight at 37°C.

2.3.4.2 Transformation of *E. coli* strains TG2

A 100 µL aliquot of competent *E. coli* TG2 that had been previously thawed on ice was added to either 1µL of the pKK223-3 derived expression vector pSF1 (fig.8), or 25 µL of diluted ligation mixture. The transformation procedures were the same as described above for strain DH5α.

2.3.4.3 Transformation of *E.coli* strain K12cI

Either 1 µL of the plasmids pLcII-FX and pSF2 (fig.10), or 25 µL of diluted ligation mixture were used for transforming 150 µL of competent *E.coli* strain K12cI cells previously thawed on ice. The transformation procedures were the same as described above for strain DH5α.

2.3.4.4 Transformation of *E. coli* strain QY13

A 100 µL aliquot of competent *E. coli* QY13 that had been previously thawed on ice was added to 2 µL of the pLcII-FX derived expression vector pSF2. The cells were then incubated on ice for 40 minutes and subsequently heat shocked for 5 minutes at 34^{o}C prior to re-incubation for an additional 15 minutes on ice. The cells were then diluted with an equal volume of 2 x TY broth and incubated without shaking for 60 minutes at 30^{o}C. All of the the transformed cells were then spread-plated on TY/Amp plates and incubated at 30^{o}C for 36 hrs. Only freshly plated QY13 cells were used for protein expression tests.

2.3.4.5 Transformation of *E. coli* strain TB1

A 50 μL aliquot of competent *E. coli* TB1 that had been previously thawed on ice was added to 1 μL of the pMal-c derived expression vector pSF3 (fig.12). Alternatively, 25 μL of diluted ligation mixture was added to 100 μL of competent TB1 cells. The transformation procedures were the same as described above for strain DH5α.

2.3.5 Transfection of *E. coli* strain TG2 with bacteriophage M13

Replicative form M13 bacteriophage DNA was added to 50 μL of competent DH5α (previously thawed on ice) and incubated on ice for 30 minutes. The cells were then heat shocked at 37°C for 2.5 minutes and re-incubated on ice for 1 minute. During this re-incubation period, a 0.2 mL aliquot of an overnight TG2 culture was combined with 3 mL of H-top agar and occasionally containing 50 μL of 20 mg mL^{-1} X-gal and 5 μL of 200 mg mL^{-1} IPTG. The cells were then added to the agar mixture and poured gently onto a TY plate. Plaques of M13 were visible after overnight incubation at 37°C.

2.4 Plasmids and bacteriophages

2.4.1 Plasmids used

2.4.1.1 pΔ4

The plasmid pΔ4 (fig.6) was originally constructed by Adams (1) and derived from the following: plasmid pJAJ21 (51) containing the entire *pufQ* gene and part of the

upstream *pufB* gene was cleaved at the *Xho*I and *Sph*I sites to release a 0.9 kb Δ4 fragment. This fragment was then sub-cloned into the *Bam*HI site of plasmid pXCA601 to produce an in frame translational fusion with the *lacZ* gene.

2.4.1.2 pKK223-3

The plasmid pKK223-3 (17) (fig.7) is a transcription expression vector and contains a *tac* promoter which drives expression of recombinant genes.

2.4.1.3 pLcII-FX

The fusion expression vector pLcII-FX (75) (fig.9) is driven by a leftward promoter (P_L) of bacteriophage λ. Recombinant genes are expressed as fusion proteins with a 31 amino acid portion of the λcII protein separated by a tetrapeptide (Ile-Glu-Gly-Arg) Factor X_a recognition sequence.

2.4.1.4 pMal-c

Transcription of the fusion expression vector pMal-c (70) (fig.11) is driven by a *tac* promoter. Recombinant genes are expressed as fusion proteins consisting of maltose binding protein (MBP) and the tetrapeptide (Ile-Glu-Gly-Arg) Factor X_a recognition sequence.

fig.6 Partial restriction map and regulatory sequences of plasmid pΔ4. Plasmid pΔ4 was the source of the *pufQ* gene (indicated by the solid line) in all of the cloning experiments.

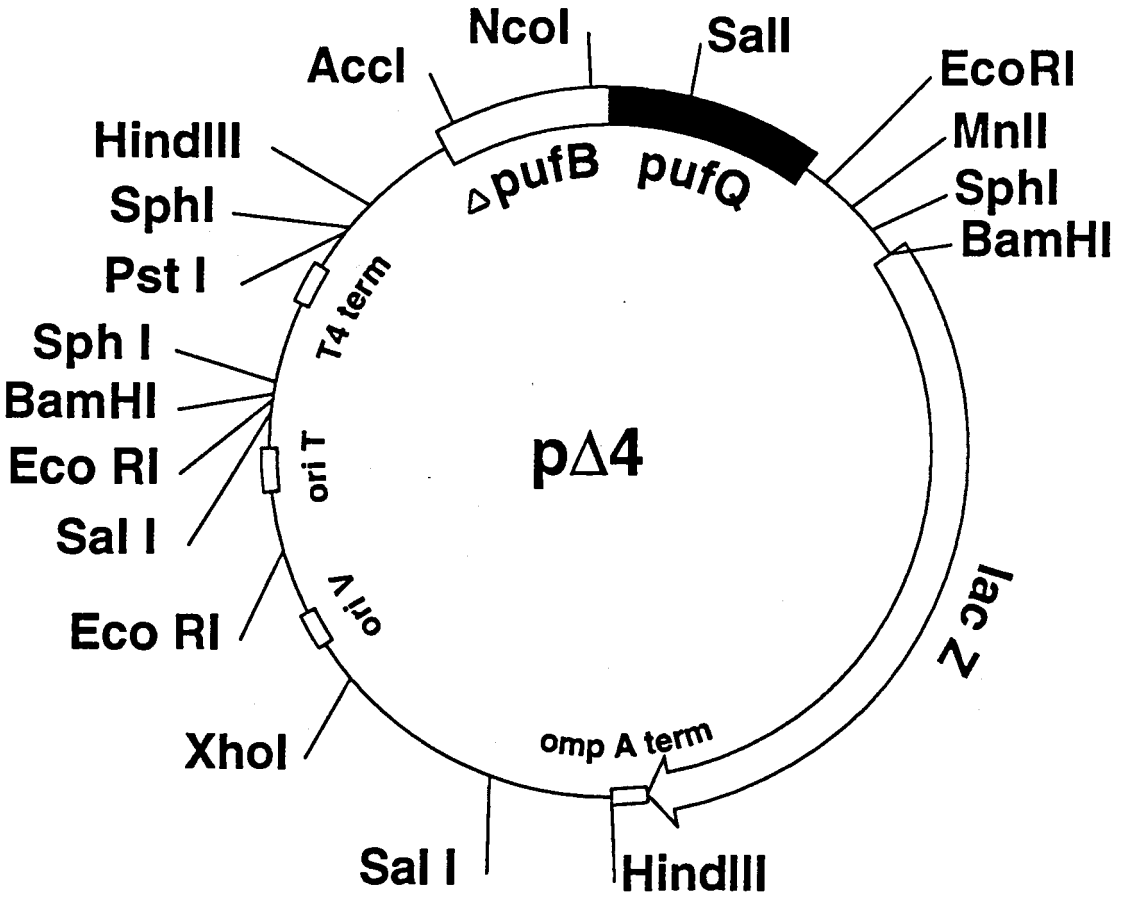

NcoI
SalI
AccI
EcoRI
MnlI
HindIII
SphI
ΔpufB
pufQ
SphI
BamHI
Pst I
T4 term
Sph I
BamHI
pΔ4
Eco RI
ori T
Sal I
ori V
lac Z
Eco RI
XhoI
omp A term
Sal I
HindIII

fig.7 Partial restriction map and regulatory sequences of expression vector pKK223-3. The terms "5S" and "rrnBT_1T_2" refer to the 5s rRNA gene and the transcription terminators T_1 and T_2.

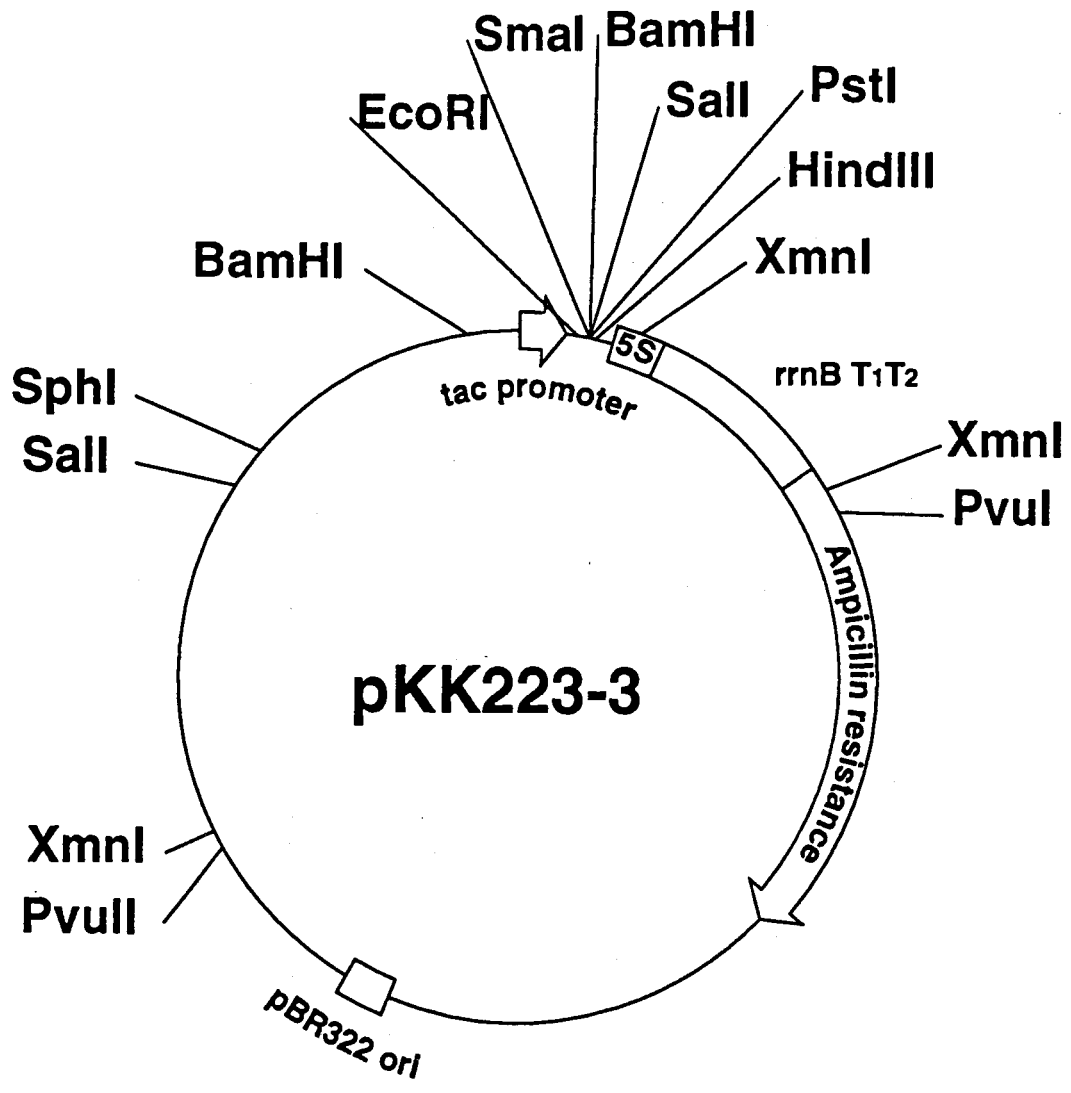

SmaI
BamHI
EcoRI
SalI
PstI
HindIII
BamHI
XmnI
SphI
SalI
5S
tac promoter
rrnB T1T2
XmnI
PvuI
Ampicillin resistance
pKK223-3
XmnI
PvuII
pBR322 ori

fig.8 Partial restriction map and regulatory sequences of expression construct pSF1. Vector pSF1 is a derivative of vector pKK223-3. The hatched arrow indicates the coding region and direction of transcription of the *pufQ* gene.

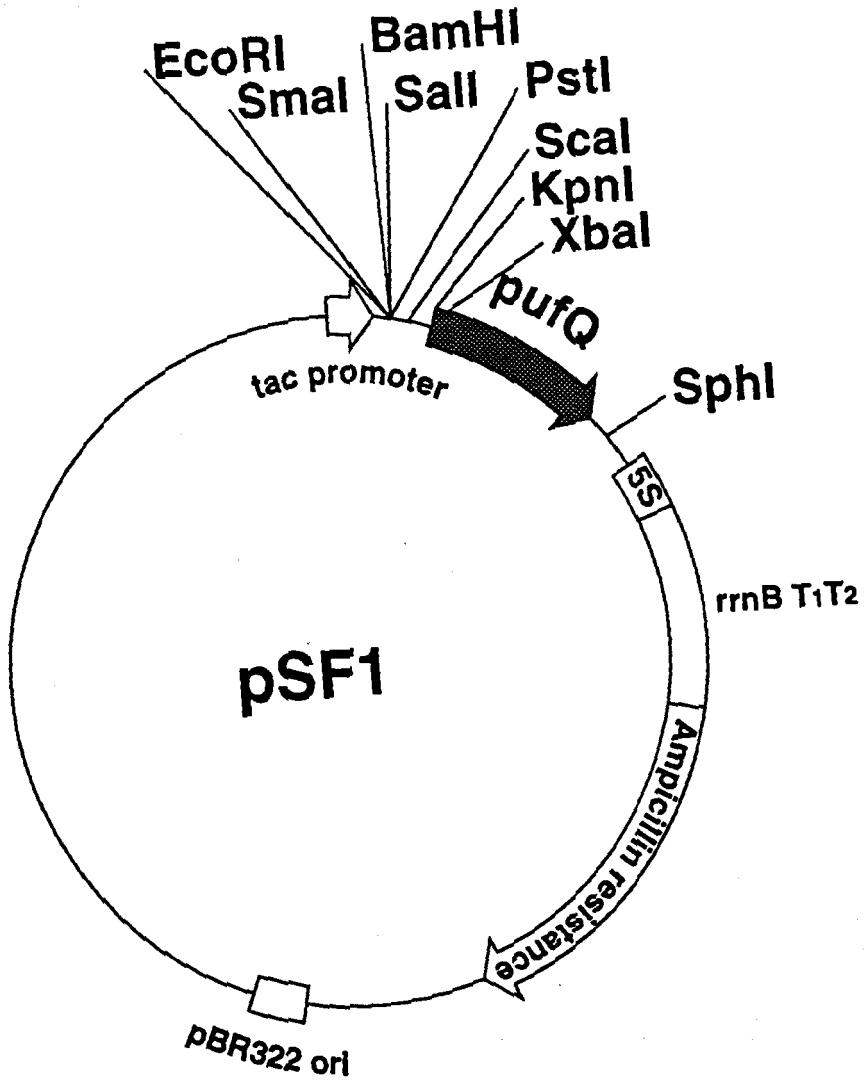

EcoRI
SmaI
BamHI
SalI
PstI
ScaI
KpnI
XbaI
pufQ
SphI
tac promoter
5S
rrnB T1T2
pSF1
Ampicillin resistance
pBR322 ori

fig.9 Partial restriction map and regulatory sequences of expression vector pLcII-FX. The coding region of a portion of the λcII protein (termed cII) and the Factor X_a recognition sequence (termed FX) are denoted by the unshaded arrow. The terms "tR_1" and "P_L" refer to the strong transcription terminator of the *rrnB* operon and the leftward promoter of bacteriophage λ respectively. Sites of anti-termination for transcription initiated at P_L are designated "nutL" and nutR" (88).

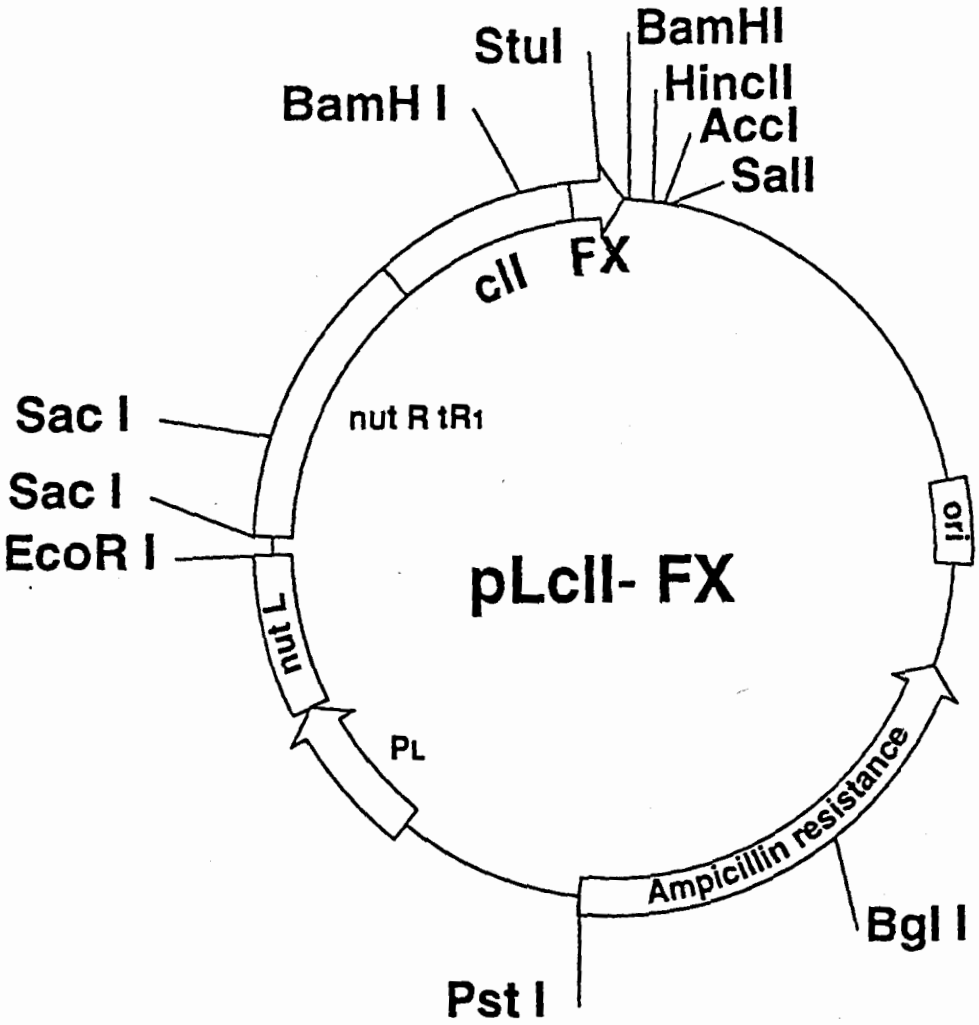

StuI
BamHI
BamH I
HincII
AccI
SalI
cII
FX
Sac I
nut R tR1
Sac I
EcoR I
pLcII- FX
ori
nut L
PL
Ampicillin resistance
Bgl I
Pst I

fig.10 Partial restriction map and regulatory sequences of expression construct pSF2. The hatched arrow indicates the coding region and direction of transcription of the *pufQ* gene.

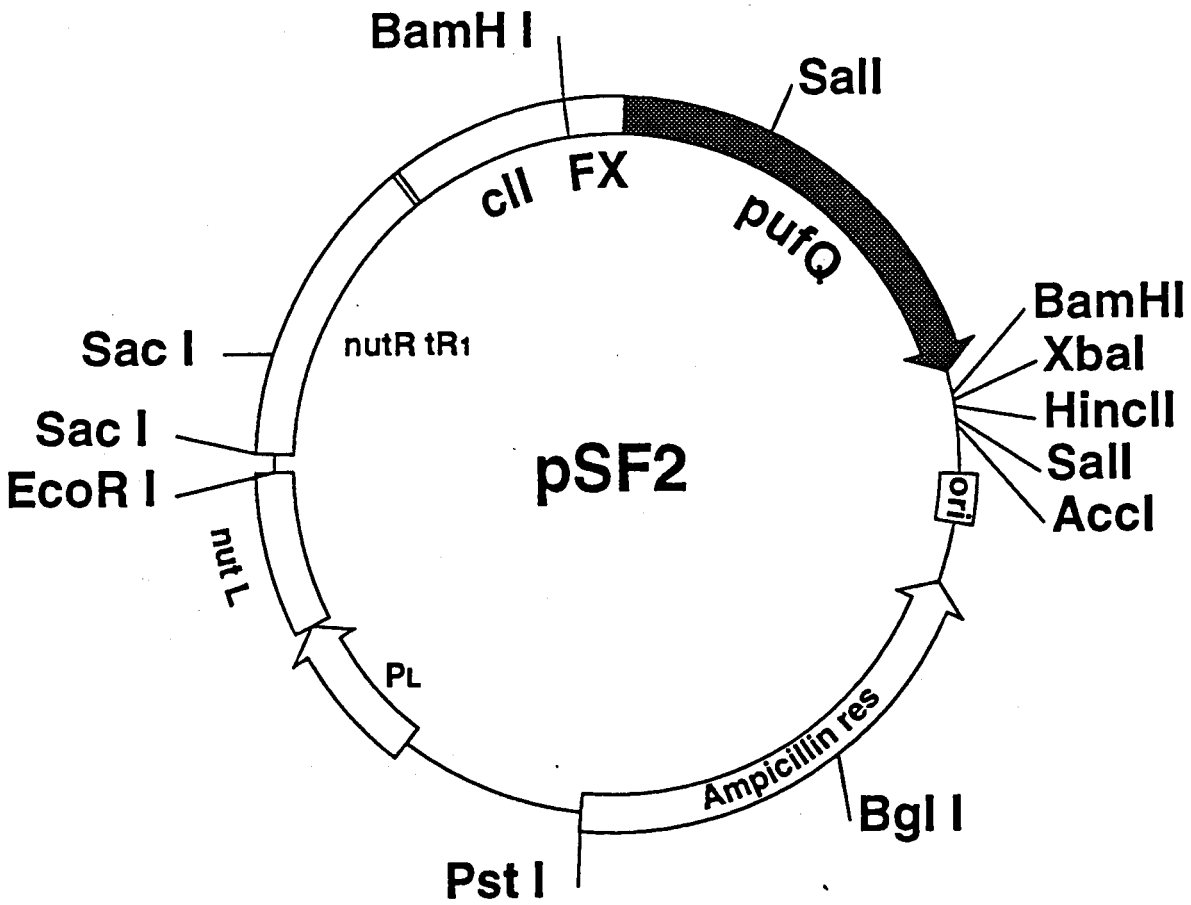

BamH I
SalI
cII
FX
pufQ
BamHI
XbaI
HincII
SalI
AccI
Sac I
nutR tR1
Sac I
EcoR I
pSF2
ori
nut L
PL
Ampicillin res
Bgl I
Pst I

fig.11 Partial restriction map and regulatory sequences of expression vector pMal-c. The coding region of the lacI repressor, MBP (containing a deletion of its signal sequence), and the α subunit of β-galactosidase (termed lacZα) are denoted by the unshaded arrow. The term "P_{tac}" refers to the *tac* promoter.

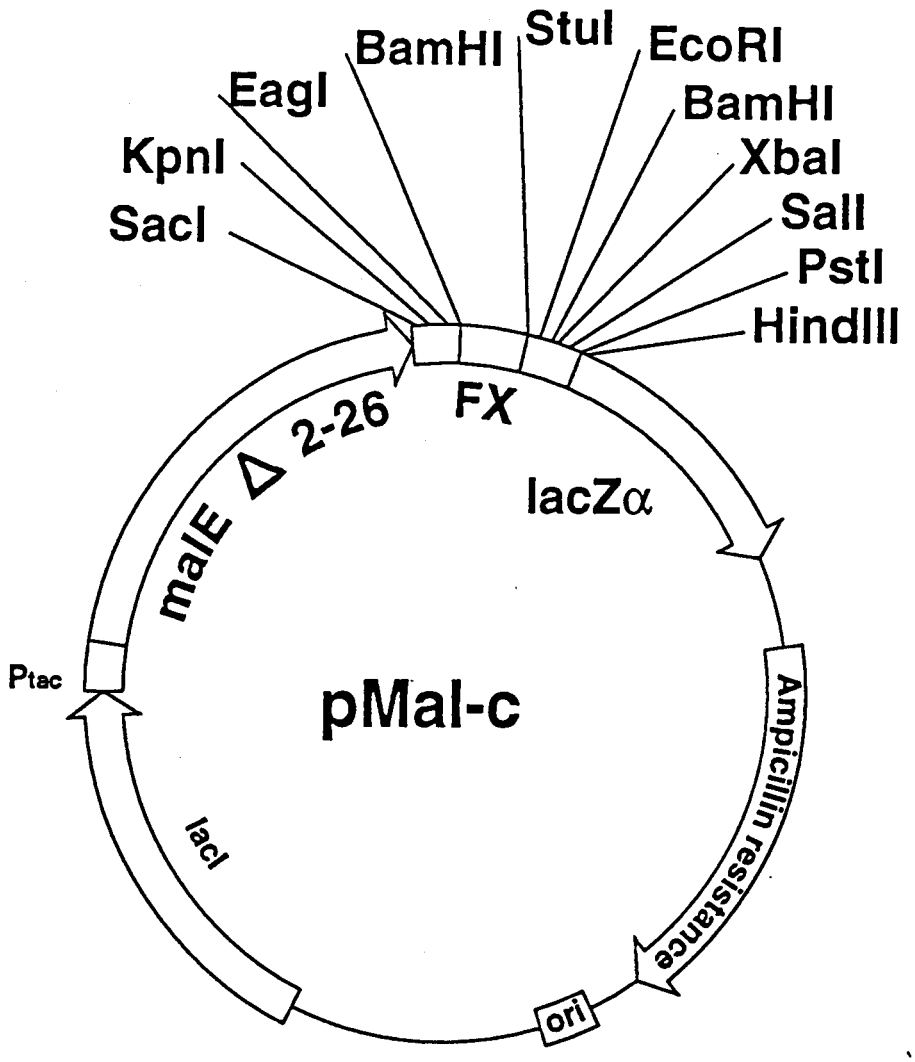
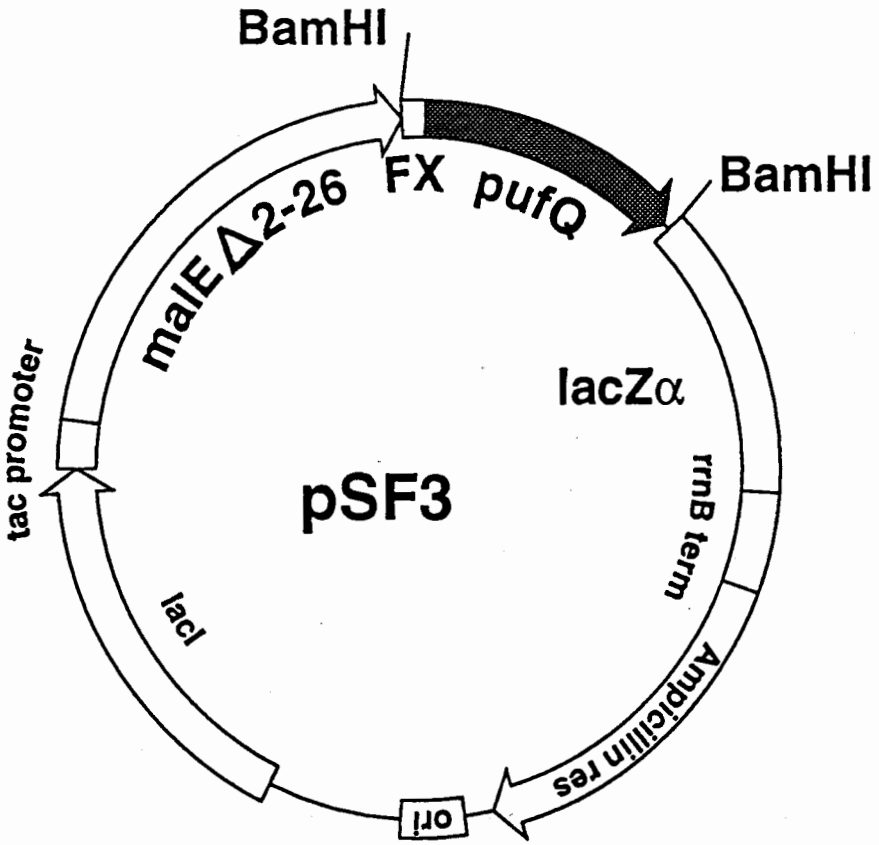

BamHI
StuI
EcoRI
EagI
BamHI
KpnI
XbaI
SacI
SalI
PstI
HindIII
FX
malE Δ 2-26
lacZα
Ptac
pMal-c
Ampicillin resistance
lacI
ori

fig.12 Partial restriction map and regulatory sequences of expression construct pSF3. The hatched arrow indicates the coding region and direction of transcription of the *pufQ* gene.


BamHI
FX
pufQ
BamHI
malE Δ2-26
lacZα
pSF3
tac promoter
lacI
ori
Ampicillin res
rrnB term

2.4.2 Bacteriophages used

The recombinant M13 bacteriophages, M13mp18 and M13mp19 (73), were used as cloning vectors. Transfections by these F-specific phage were performed with the *E. coli* strain TG2.

2.4.3 Preparation of plasmids

2.4.3.1 Small scale plasmid preparation

Plasmids were prepared from a 2 mL culture of bacterial cells grown for either 6-8 hrs or overnight in TY/Amp at 37°C. Cells were pelleted by centrifugation in a microfuge for 5 minutes at 12,000 rpm and resuspended in 100 µL of an ice cold solution of solution I. The cell suspension was then incubated for 5 minutes at room temperature, followed by the addition of 200 µL of solution II. The mixture was then re-incubated until the solution clarified (approximately 5 minutes), at which point 150 µL of solution III was added. After an additional 5 minutes, the solution was centrifuged as before and the supernatant extracted with a mixture of 300 µL phenol:chloroform (2:1) v/v. The aqueous phase was separated by centrifugation and the DNA precipitated with 2 volumes of 95% ethanol. The precipitated DNA was recovered by centrifugation and dried briefly under vacuum. The DNA was finally resuspended in 50 µL TE8 and the RNA removed by digestion with 0.5 µL each of RNase A (0.5 mg mL^{-1}) and RNase T_1 (0.5 mg mL^{-1}) for 1 hr at 37°C. Though this procedure yielded adequately pure plasmid for restriction digest analyses, further plasmid purification was necessary for sequence analysis (section 2.4.5).

2.4.3.2 Large scale plasmid preparation

Two hundred mL of 2 x TY/Amp was inoculated with 2 mL of a mid-log phase *E. coli* culture harboring the plasmid of interest. The culture was grown overnight at 37°C and the cells pelleted by centrifugation at 8,000 rpm for 10 minutes in a GSA rotor. The cells were resuspended in 10 mL of solution I and incubated for 10 minutes. Following successive additions over a 10 minute interval of 20 mL of solution II and 15 mL of solution III, the mixture was re-centrifuged. The supernatant was then extracted with a mixture of 40 mL of phenol:chloroform (5:3) v/v, centrifuged as above, and the aqueous phase extracted again with 25 mL of chloroform. After centrifugation, the aqueous phase was precipitated with 2 volumes of 95% ethanol, incubated for 10 minutes at room temperature, and re-centrifuged. The precipitated DNA was dried under a stream of nitrogen gas, resuspended in 0.5 mL TE8, and incubated with 5 µL each of RNase A (0.5 mg mL^{-1}) and RNase T_1 (0.5 mg mL^{-1}) for 2 hrs at 37°C. The RNA-free plasmid preparation was then extracted with an equal volume mixture of phenol:chloroform (3:2) v/v, and then with one volume of chloroform. DNA was precipitated by combining the solution with 125 µL of 10 M ammonium acetate and 1 mL of 95% ethanol and incubating for 30 minutes at room temperature. The suspension was centrifuged for 15 minutes at 12,000 rpm. The resulting pellet was resuspended in 0.2 mL of TE8, and precipitated once more by incubation at room temperature for 30 minutes with 20 µL of 3 M sodium acetate (pH 5.5) and 450 µL of 95% ethanol. Plasmid DNA was then recovered by centrifugation, dried under vacuum, and resuspended with TE8 to a final volume of 0.2 mL.

2.4.4 Preparation of M13 bacteriophage DNA

Single plaques containing recombinant M13 phage from a lawn of transfected TG2 cells were inoculated into 2 mL of 2 x TY and used to infect 20 µL of *E. coli* TG2 cells grown overnight. The culture was then incubated in an environmental shaker at 37°C for 5 hrs, after which the cells were centrifuged at 12,000 rpm for 5 minutes. The culture supernatant was used to prepare single stranded DNA (section 2.4.4.1), while the pellet was used for preparation of replicative form M13 DNA (see section 2.4.4.2).

2.4.4.1 Single stranded DNA preparation

The soluble bacteriophage particles present in the supernatant were precipitated by the addition of 200 µL of a solution containing 20% polyethyleneglycol-8000 and 3.5 M ammonium acetate for 15 minutes at room temperature. After centrifugation as above, the pellets were resuspended in 100 µL of TE8 and mixed with 50 µL of phenol. The mixture was re-centrifuged and the aqueous phase combined with 100 µL of chloroform. The extraction was repeated, and the viral DNA present in the aqueous phase was precipitated with 10 µL of 3 M sodium acetate (pH 5.5) and 250 µL of 95% ethanol for 15 minutes at room temperature. Following centrifugation for 10 minutes, the pelleted single stranded DNA was washed with 200 µL of cold 70% ethanol, dried under vacuum, and resuspended in TE8 to a final volume of 50 µL.

2.4.4.2 Preparation of replicative form DNA

The procedure for obtaining replicative form M13 DNA from a 2 mL infected culture of TG2 cells (section 2.4.4) was identical to the plasmid preparation (section 2.4.3.1) except for an initial washing of pelleted cells with STE buffer.

2.4.5 Preparation of DNA for sequencing

Plasmid DNA obtained from a 2 mL culture of cells (section 2.4.3.1) was diluted to 120 µL with ddH_20 and further purified for sequencing by two additional DNA precipitations. In the first precipitation, 40 µL of 10 M ammonium acetate was added, followed by addition of 240 µL of 95 % ethanol. The solution was then incubated for 5 minutes and the DNA pelleted by centrifugation in a microfuge at 12,000 rpm for 10 minutes. The resulting DNA was resuspended in 90 µL ddH_2O, and precipitated with 10 µL of 3 M sodium acetate (pH 5.5), and 150 µL of 95 % ethanol for 10 min. Plasmid DNA was then recovered by centrifugation for 20 minutes and resuspended in TE8 to a final volume of 10 µL.

Plasmid and replicative form M13 phage DNA were prepared for sequencing by the following procedure: One µg of double stranded DNA was denatured by the addition of 200 mM NaOH containing 0.2 M EDTA in a final volume of 17 µL. The solution was incubated at room temperature for 3 min and then precipitated at room temperature for 5 min by the addition of 3 µL of 3 M sodium acetate, pH5.5, and 50 µL of 95 % ethanol. Following centrifugation at 12,000 rpm for 5 min, the denatured DNA pellet was dried briefly under vacuum and resuspended in 7 µL with ddH_2O.

One pmol of the appropriate primer in 2 µL of 5 x sequencing buffer was added to the above single stranded template DNA and allowed to anneal by incubation at 37°C for 15 min in a total volume of 10 µL. The annealing mixture was then incubated at room temperature for an additional 10 min and stored on ice prior to performing the sequencing reactions. Sequencing was done by the dideoxy chain termination method (90), using the enzyme Sequenase (version 2.0) and protocols supplied by the United States Biochemical Corporation. Single stranded M13 phage DNA was sequenced identically except for the initial denaturation step.

2.4.6 Separation of nucleotides by electrophoresis

Samples containing each of the dideoxy reactions in stop buffer were heated to 80°C for 3 min prior to electrophoresis on denaturing gels containing 6 % polyacrylamide. The buffer used for preparing the acrylamide gels was 1 x TBE and contained 7 M urea. Electrophoresis was routinely performed in a BRL S_2 sequencing apparatus [30 (w) x 40 (l) cm gel size] at 80 watts in 1 x TBE buffer. Occasionally, smaller gels [19 (w) x 40 (l) cm gel size] were prepared in a custom made sequencing apparatus and electrophoresed at 40 watts. Both gels were pre-run at their respective wattage for 1 hr prior to sample loading. For autoradiography, the gels were dried after electrophoresis using a Bio-Rad gel drier at 80°C for 1 hr and exposed to either Kodak XAR or XK film, depending on the level of radioactive counts. The length of exposure was typically overnight, and the film was developed using either developing tanks (89) or an automated developer.

2.5 Cloning procedures

2.5.1 Size estimations of DNA by electrophoresis

Agarose gels containing 0.5 mg mL^{-1} ethidium bromide (EtBr) were prepared for separation of DNA by electrophoresis. The buffer used for preparing the agarose gels and for electrophoresis was 1 x TAE. Electrophoresis was performed in a horizontal gel apparatus at either 60 volts for small (25 mL) gels or 100 volts for large (75 mL) gels. Gels contained either 1.0 % (w/v) agarose for most electrophoretic separations, or 1.5 % (w/v) agarose for resolution of low molecular weight DNA fragments. DNA lengths were estimated by comparison with a DNA size ladder obtained from digestion

of bacteriophage lambda with the restriction enzyme *Cla*I. The DNA was visualized by monitoring the fluorescence of the EtBr-DNA complex under ultraviolet light.

2.5.2 Purification of DNA

2.5.2.1 Electroelution

Bands on agarose gels corresponding to separated DNA fragments (section 2.5.1) were excised from the gel (without exposure to ultraviolet light) using a scalpel and placed in dialysis tubing containing 200 µL of TE8. The tubing was then sealed and immersed in a mid-size horizontal gel apparatus containing 0.5 x TAE. Electroelution of the DNA from the agarose gel was performed at 200 volts for 15 minutes, followed by reversal of the current for 20 seconds to remove any DNA present on the sides of the tubing. The DNA in solution was then withdrawn and the tubing rinsed with 100 µL of TE8. DNA was recovered by precipitation with 0.1 volume of 3 M sodium acetate (pH 5.5), 1 µL of polyacrylamide carrier, and 1 mL of 95 % ethanol for 2 hrs or overnight at 4°C. Following centrifugation at 12,000 rpm for 30 minutes at 4°C, the pelleted DNA was resuspended in 10 µL of TE8.

2.5.2.2 Low melting temperature agarose

Low melting temperature agarose gels of appropriate percentages were prepared in 1 x TAE and stored at 4°C for approximately 30 minutes prior to electrophoresis (section 2.5.1). Following electrophoresis and gel excision (section 2.5.2.1), the gel slice was placed in a 1.5 mL microfuge tube containing 500 µL of TE8 and heated to 65-68°C for 10 minutes. Following the addition of an equal volume of phenol at the same temperature, the solution was mixed, and centrifuged for 5 minutes at 12,000

rpm. The aqueous phase was then extracted as above with successive additions of phenol/chloroform (1:1) v/v and chloroform. The DNA was precipitated with the addition of 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of 95 % ethanol for 30 minutes at room temperature. Following centrifugation at 12,000 rpm for 15 minutes, the pelleted DNA was dried and resuspended in 10 µL of TE8.

2.5.3 DNA modifications

2.5.3.1 Restriction digests

All restriction digests were performed in the buffers and reaction conditions either supplied or recommended by the manufacturers of the particular enzyme. For the purposes of subsequent gel purifications or enzyme incubations, the DNA from the digests was purified by a combination of phenol/chloroform (1:1) v/v and chloroform extractions, followed by ethanol precipitation using sodium acetate as described in section 2.4.5.

2.5.3.2 Blunt-ending DNA fragments by T4 DNA polymerase

The ends of DNA fragments made blunt by treatment with T4 DNA polymerase were done either by a) filling in the protruding ends using the 5' to 3' polymerase activity or b) hydrolysing the ends using the 3' to 5' exonuclease activity of T4 DNA polymerase. Unless stated otherwise, blunt-ending using T4 DNA polymerase was achieved by the former method.

For filling-in reactions, DNA which had previously been digested with the appropriate restriction enzyme was purified and resuspended in 88 µL dd$H_2$0. Ten µL of T4 DNA polymerase buffer was then added on ice and combined with 2 µL of a 50

mM dNTP stock. The solution was mixed and 10 units of T4 DNA polymerase per μg of DNA was added. The polymerase reaction was incubated for 10 min at 37°C.

For blunt-ending using the exonuclease activity, dNTPs were omitted from the above reaction mixture and 2 units of T4 DNA polymerase per μg of DNA was added. The incubation conditions were the same as described above. In both cases following incubation, the DNA was purified as described in section 2.4.5.

2.5.3.3 Blunt-ending DNA fragments by mung bean nuclease

Blunt-ending of the 5' overhang resulting from digestion with the restriction enzyme *Xba*I was performed by limited single strand hydrolysis using mung bean nuclease. Two sets of reaction conditions and a variety of enzyme concentrations were used to optimize the exonuclease activity while limiting any possible double strand hydrolysis. Incubations were done either at 30°C for 30 min or at 37°C for 10 min using the following units of enzyme for each reaction condition: 0.05, 0.1, 0.5, 1.0, 1.5, and 5.0. Efficient ligations were observed under conditions of 30°C incubations using 1.0 unit of enzyme.

2.5.3.4 Dephosphorylation of plasmids

The 5' phosphate of linearized plasmid DNA was removed by treatment with calf intestinal alkaline phosphatase (CIP). Plasmid DNA digested with the appropriate restriction enzyme was ethanol precipitated as described in section 2.4.5. The resulting DNA pellet was resuspended in 43 μL of ddH_20 and combined with 5.0 μL of 10 x phophatase buffer. Two units of CIP was then added and the reaction incubated at 50°C for 1 hr, followed by incubation for an additional 1 hr at 65°C to inactivate the enzyme. Linearized plasmid DNA was subsequently precipitated (section 2.4.5),

separated by electrophoresis (section 2.5.1), and purified by either gel purification (section 2.5.2.2) or electroelution (section 2.5.2.1). The extent of the dephosphorylation reaction was assessed by comparing transformation efficiencies of control ligation mixtures containing dephosphorylated plasmids incubated in the presence or absence of T4 DNA ligase (section 2.5.3.5).

2.5.3.5 Ligation of DNA fragments into plasmids

DNA fragments were ligated to linearized and dephosphorylated plasmids using the enzyme T4 DNA ligase. The concentration of insert and vector was estimated by visualizing small aliquots of each on agarose gels. For most cases, a 2:1 molar ratio of insert to vector was used in a ligation reaction. The ligation mixture consisted of 1 μL of 5 x ligation buffer and 1 unit of the enzyme diluted to a final volume of 5 μL. Incubations were done at room temperature for either 2 hr in the case of ligations involving cohesive ends, or left overnight for blunt-ended ligations. Immediately prior to transformations, the ligation mixtures were diluted with 20 μL of dd$H_2$0 in order to dilute the concentration of PEG present in the ligation buffer.

2.5.4 Cloning of expression vector constructs

2.5.4.1 Assembly of expression vector pSF1

The first expression system tested for recombinant expression of *pufQ* was derived from vector pKK223-3 (section 2.4.1.2). The coding region of *pufQ*, plus 26 bp of upstream and 10 bp of downstream DNA, was excised by digestion of plasmid pΔ4 with the restriction enzymes *Nco*I and *Eco*RI. The vector pKK223-3 is designed such that a gene can either be cloned at the *Sma*I site of the polylinker at an optimal

distance from the ribosome binding site (RBS) on the vector, or at a downstream *Hind*III site of the polylinker if a RBS is already present on the cloned gene fragment. The *pufQ* gene fragment was blunt-ended by treatment with T4 DNA polymerase and ligated into either the *Sma*I site or a similarly blunt-ended *Hind*III site on the polylinker of pKK223-3. The resulting constructs were termed pKK223-3(*Sma*I) and pKK223-3(*Hind*III) respectively.

For the purposes of mutagenesis, a 250 bp *pufQ* gene fragment from the pKK223-3 (*Sma*I) construct was excised by cleavage at the *Nco*I and *Bam*HI sites of the polylinker, the ends made blunt by treatment with T4 DNA polymerase, and the fragment subcloned into the *Sma*I site of pUC-18. This construct, termed pUC-18(*Sma*I), was subsequently digested with *Eco*RI and *Hind*III and the *pufQ* gene cloned into the replicative form of phage M13mp19 previously cut with *Eco*RI and *Hind*III. The phage construct, with the appropriate orientation for mutagenesis as confirmed by sequencing (section 2.4.5), was designated M13mp19(*250*).

The *pufQ* gene was altered by site-directed mutagenesis to produce a sequence, 5' of the coding region, which is a consensus RBS of genes that are well expressed in *E.coli*. In addition, an *Xba*I restriction site was introduced at the extreme 5' end of the initiation codon to allow facile subcloning of the *pufQ* gene in the fusion vectors. A uracil-containing single-stranded DNA template was prepared from the M13mp19(*250*) construct, and site-specific mutagenesis was performed as described (section 2.6) using a 33mer oligonucleotide (section 2.6.3). Single-stranded phage containing the mutation were identified by sequencing (section 2.4.5), and the mutant phage were designated M13mp19(*250-Xba*I).

Following site-directed mutagenesis, a spontaneous deletion of a thymidine nucleotide 100 bp downstream of the initiation codon in the recombinant phage M13mp19(*250-Xba*I) was detected in all variant clones. The mutation was corrected using another recombinant phage, M13mp8(Δ*pufQ*) (kindly supplied by Dr. T. Beatty,

U.B.C), that contained part of the *pufQ* gene extending from an internal *Sal*I site to beyond the 3' end of the coding region. Sequence analysis of this clone confirmed the presence of a thymidine nucleotide which had been deleted in the M13mp19(*250-Xba*I) construct. For repairing the deletion, the M13mp8(Δ*pufQ*) construct was first cleaved by digestion at an *Eco*RI site located 3' to the end of the *pufQ* coding region. The linearized phage was then blunt-ended by treatment with T4 DNA polymerase and digested again with *Sal*I. The resulting 0.2 kb *pufQ* gene fragment was gel purified by electroelution and ligated to the plasmid described below. The M13mp19(*250-Xba*I) construct was digested with *Eco*RI and *Hind*III and the *pufQ* gene cloned into plasmid pUC-19 that had been digested similarly. The resulting subclone, termed pUC-19 (*250-Xba*I), was digested with *Pst*I and the ends made blunt by using the 3' to 5' exonuclease activity of T4 DNA polymerase. The plasmid was then re-digested with *Sal*I, and the gel purified plasmid was ligated to the above described 0.2 kb *pufQ* gene fragment. Sequence analysis of the resulting construct, termed pUC-19 (*Xba*I), confirmed the presence of the native *pufQ* gene. The pUC-19(*Xba*I) construct was subsequently excised by an *Eco*RI and *Hind*III digest and cloned into the *Hind*III site of pKK223-3 using T4 DNA polymerase to fill in the protruding ends, resulting in the expression vector pSF1.

2.5.4.2 Assembly of expression vector pSF2

The fusion expression vector pLcII-FX (section 2.4.1.3) was used for the construction of pSF2. pLcII-FX was obtained from a recombinant derivative of the vector termed pLcII-FX(*TnC*). In order to regenerate the parent vector, an approximately 1200 bp gene fragment was first cleaved by digestion with *Bam*HI. The plasmid was then dephosphorylated using CIP (section 2.5.3.5) and gel purified prior to ligation with a DNA fragment encoding a Factor X_a recognition sequence separated by

*Bam*HI sites. The DNA fragment was a 30mer oligonucleotide (section 2.6.3) which had been self-annealed by heating to 80°C for 5 min and then allowed to cool slowly to room temperature. The double stranded fragment was then enzymatically phosphorylated as described in section 2.6.4.

The *pufQ* gene was excised from plasmid pUC-19(*Xba*I) by an *Xba*I-*Hind*III digest, blunt-ended with mung bean nuclease (section 2.5.3.3), and cloned into the *Stu*I site of vector pLcII-FX. Sequence analysis of the resulting construct, termed pLcII-FX(*Stu*I), again revealed a spontaneous nucleotide deletion which had occurred during cloning. The deletion of an adenosine in the ATG initiation codon of the *pufQ* coding region was repaired by site-directed mutagenesis as follows: a 250 bp *pufQ* coding region was removed from pLcII-FX(*Stu*I) by digesting at its *Bam*HI sites, and inserted into the *Bam*HI site in the polylinker of the replicative form of phage M13mp18. The phage construct with the appropriate orientation for mutagenesis, as confirmed by dideoxynucleotide sequencing, was designated M13mp18(*250ΔA*). A 24mer oligonucleotide (section 2.6.3) homologous to the M13mp18(*250ΔA*) template DNA with the exception of an additional adenosine residue at the ATG initiation codon of *pufQ* was used to direct the mutagenesis. Site-directed mutagenesis was performed as described (section 2.6) and the mutant phage, designated M13mp18(*250*), was recloned into vector pLcII-FX after digestion of both with *Bam*HI. The resulting construct, designated pSF2, was confirmed by sequencing using a 16mer oligonucleotide homologous to nucleotides encoding amino acids 16-21 of the λcII gene.

2.5.4.3 Assembly of expression vector pSF3

Vector pSF3 was constructed by digestion of vector pSF2 with *Bam*HI and inserting the *pufQ* gene into vector pMal-c similarly cut with *Bam*HI. The pSF3 construct was confirmed by sequencing using a 24mer *malE* primer.

2.6 Site-directed mutagenesis

Site-directed mutagenesis was performed by the method of Kunkel (89). In this procedure, site specific DNA mutations are directed by oligonucleotides which have a specific base pair mutation(s). The mutagenic oligonucleotide is hybridized to single stranded M13 template DNA containing a number of uracil residues in place of thymine (U-DNA). Following *in vitro* synthesis by T4 DNA polymerase of the complimentary strand, the double-stranded circular DNA is transformed in *E. coli*. Mutant M13 phage can then be subsequently isolated due to selection against the wild type U-DNA.

Assembly of the *pufQ* containing replicative form M13 phage constructs, M13mp19 (*250*) and M13mp18(*250ΔA*), and the preparation of their single-stranded versions used for mutagenesis are described in sections 2.5.4.1, 2.5.4.2, and 2.4.4.1 respectively.

2.6.1 Determination of the multiplicity of viral infection

The multiplicity of viral infection is defined as the number of plaque forming units (pfu) per bacterial cell (pfu/cell). This value is calculated based on the number of pfu/mL of M13 bacteriophage suspension divided by the number of bacterial cells/mL in the inoculum of *E. coli* strain RZ1032.

The number of pfu/mL in a bacteriophage suspension was obtained by the following: Ten μL each of the bacteriophage suspension diluted by a factor of 10^{-4}, 10^{-6}, and 10^{-7} were combined with 100 μL of a log phase *E. coli* TG2 culture containing 3 mL of H-top agar. The mixture was then poured over TY plates and the plates incubated at 37°C overnight. The resulting bacteriophage containing plaques were counted and the number of plaque forming units per volume (pfu/mL) of phage suspension determined.

The concentration of cells in the inoculum was obtained by the following: One hundred μL each of the bacterial suspension diluted by a factor of 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6} were spread plated on TY/Tet plates and incubated at 37°C overnight. The colonies were subsequently counted and the number of RZ1032 cells /mL of inoculum determined.

2.6.2 Preparation of U-DNA

Uracil containing single stranded template DNA (U-DNA) that were used for mutagenesis was obtained by transfecting the *E.coli* strain RZ1032 (*ung*$^-$*dut*$^-$) with a M13mp19(*250*) and M13mp18(*250*ΔA) phage suspension. The phage suspension was obtained by inoculating a single M13 plaque with 2 mL of 2 x TY containing 0.2 mL of an overnight culture of *E. coli* TG2. The culture was incubated at 37°C for 2 hrs, and the cells then transferred to a microfuge tube and heated at 60°C for 5 min to destroy the bacterial cells. Following centrifugation at 12,000 rpm for 5 min, the soluble bacteriophage particles were stored at 4°C.

One hundred μL of the phage suspension was inoculated into 100 mL of 2 x TY supplemented with 0.25 μg mL^{-1} uridine and containing 5 mL of an overnight culture of RZ1032. The culture was incubated at 37°C with high aeration (300 rpm) for 6 hrs. The cells were pelleted by centrifugation at 8,000 rpm for 30 min in a GSA rotor at

4°C. The multiplicity of infection (section 2.6.1) was 0.02-0.2 pfu/cell. The phage containing supernatant was precipitated and purified as described in section 2.4.4.1. The concentration of DNA was determined spectrophotometrically at 260 nm (89).

2.6.3 Synthesis of oligonucleotides

The sequence of the oligonucleotides used to direct mutagenesis of the recombinant constructs M13mp19(250) (33mer) and M13mp18(250ΔA) (24mer) and for cloning in vector pSF2 (30mer) are shown below. Bold type indicates altered nucleotides used to direct mutagenesis; amino acids encoded by some of the codons are shown above the sequence; restriction sites and the ribosome binding site (RBS) are underlined.

```
                                                    Met  Glu  Ser ...
33mer 5'-GGATCGGAAGGAGGTGAATCTAG ATG CAA AGC C-3'
                          RBS          XbaI

24mer 5'-CTGGCTTTGCATCCTACCCTCGAT-3'

                Ser  Ile  Glu  Gly  Arg
30mer 5'-GA TCC ATC GAG GGT AGG CCT ACC CTC GAT G-3'
          BamHI                    StuI
```

Oligonucleotides were synthesized on an Applied Biosystems 329 DNA/RNA synthesizer at the Institute of Molecular Biology and Biochemistry (S.F.U.), or at the Oligonucleotide Synthesis Laboratories (U.B.C.) and purified on Sep-Pak C_{18} cartridges (4) as follows. The Sep-Pak apparatus was first equilibrated using a syringe with 10 mL each of HPLC grade acetonitrile followed by ddH_2O. The crude oligonucleotide was next resuspended in 1.5 mL of 0.5 M ammonium acetate and the

solution applied on the cartridge. The Sep-Pak cartridge was then washed with 10 mL of ddH_2O and the oligonucleotide eluted with three 1 mL washes of 20 % (v/v) acetonitrile. The acetonitrile was then removed under vacuum and the oligonucleotide concentrated to a final volume of 100 µL with ddH_2O. The concentration of purified oligonucleotide was determined spectrophotometrically at 260 nm (89).

2.6.4 Phosphorylation of oligonucleotides

The 5' ends of oligonucleotide primers were phosphorylated using the enzyme T4 DNA kinase. Two hundred pmol of purified oligonucleotide was diluted to either 20 µL or 40 µL with ddH_2O containing 1 x polynucleotide kinase buffer, 10 mM ATP, and 10 units of enzyme. The reaction was incubated at 37°C for 1 hr, and then heated at 68°C for 10 min to inactivate the kinase.

2.6.5 Oligonucleotide-directed mutagenesis

Site-directed mutagensis was carried out using double primers consisting of a mutagenic oligonucleotide and the M13 forward sequencing primer. The mutagenesis reaction was accomplished in two steps: 1) annealing of the primers and 2) *in vitro* extension and ligation.

The annealing reaction contained 0.5 pmol U-DNA, 10 pmol phosphorylated mutagenic oligonucleotide, 10 pmol phosphorylated forward sequencing primer, and 2 µL of 10 x PE 1 buffer diluted to a final volume of 20 µL with ddH_2O. The primers were annealed for 10 min at 80°C and then allowed to slowly cool to room temperature.

The extension and ligation reaction was performed by addition of the following to the above annealing mixture: 1.5 µL of PE 2, 1.0 mM dNTPs, 1.0 mM ATP, 10 units

of T4 DNA ligase, 3.0 units of T4 DNA polymerase, and 3.5 μL of ddH_2O. The reaction was incubated sequentially under the following conditions: 5 min on ice, 5 min at room temperature, and 2 hr at 37°C. The entire extension/ligation mixture was then transformed in DH5α and plated with TG2 cells as described in section 2.3.5. The resulting plaques were screened for site specific mutations by dideoxy sequencing (section 2.4.5).

2.7 Northern blot analysis of *pufQ*-specific transcripts

2.7.1 RNA preparation

RNA was isolated from 10 mL cultures of the following strains: TG2(pSF1), QY13(pSF2), and TB1(pSF3) and control strains TG2(pKK223-3), QY13(pLcII-FX), and TB1(pMal-c). Cultures of these strains were prepared that were both induced and un-induced for gene expression. Cells were pelleted by centrifugation at 12,000 x *g* for 10 min at 4°C and resuspended in 10 mL of protoplasting buffer containing 80 μL of a 50 mg mL^{-1} stock solution of lysozyme. The cells were incubated on ice for 15 min and re-centrifuged at 5,900 x *g* for 5 min at 4°C. The sedimented protoplasts were then resuspended in 0.5 mL of gram-negative lysing buffer containing 15 μL of diethylpyrocarbonate (DEPC), a potent RNase inhibitor (89). The solution was incubated for 5 min at 37°C, and the SDS, protein, and DNA were then coprecipitated on ice for 10 min by the addition of 250 μL saturated NaCl. Following centrifugation in a microfuge at 12,000 rpm for 10 min, the RNA from the supernatant was precipitated with 1 mL of 100 % ethanol. The mixture was incubated for 30 min on dry ice and re-centrifuged. The pelleted RNA was washed with 70 % ethanol, resuspended in 100 μL of DEPC-treated ddH_2O and stored at -70°C.

2.7.2 Separation of RNA by electrophoresis

RNA samples mixed with RNA Electrophoresis Sample Buffer were resolved on 1.5 % (w/v) agarose gels containing 0.66 M formaldehyde and 0.5 mg mL^{-1} EtBr. The buffer used for preparing the agarose gels and electrophoresis was 1 x MOPS/EDTA buffer. Electrophoresis was performed in a horizontal gel apparatus at 75 volts for 3 hr. An RNA ladder of 0.16 to 1.77 Kb lengths (BRL) was used to estimate band size.

2.7.3 Transfer of mRNA to Zeta-probe membranes

Prior to transfer of RNA to Zeta-probe blotting membrane, the gel was soaked in ddH_2O for 10 min and then in 10 x SSC for 20 min at room temperature with gentle shaking. Transfer of RNA was achieved either by capillary transfer or by spin blotting (89) for 45 min at 1250 rpm in a HN-S rotor. Following transfer, the membrane was soaked briefly in 20 x SSC and the RNA fixed to the membrane by exposing the RNA to ultraviolet light at 254 nm for 1 min in a Stratagene UV Stratalinker 2400.

2.7.4 Preparation of radiolabeled DNA probe

The DNA used to generate a hybridization probe was obtained from a 2.3 Kb *Xba*I -*Hind*III restriction fragment excised from the plasmid pUC-19 (*250-Xba*I) and purified by electro-elution from an agarose gel. The concentration of purified template DNA was approximated by agarose gel electrophoresis. Radiolabeled probe was prepared by random hexa-primer extension as follows: Twenty five ng of DNA was diluted to 34 µL with ddH_2O and denatured by heating to 98°C for 5 min. The sample was then cooled on ice and the following added: 5 µL of 10 X Labelling Buffer, 5 µL dNTPs, 5 µL α-^{32}P dATP and 1 µL (5 units) of the Klenow Fragment of DNA

polymerase I. The reaction mixture was incubated at 37°C for 1 hr and the reaction terminated with the addition of 5 µL of 0.2 M EDTA. The radiolabeled probe was then denatured by heating to 95°C for 5 min and cooled on ice for an additional 5 min prior to hybridization.

2.7.5 Hybridization of DNA probe to RNA-bound membranes

Membranes were placed in a 50 mL Falcon tube containing 40 mL of Hybridization Buffer and prepared for hybridization by incubation in a Robbins Scientific model 310 hybridization oven at 55°C for 1 hr with gentle rotation. An aliquot of radiolabeled probe corresponding to approximately 10^7 cpm per 100 cm^2 of membrane was then added and the membranes re-incubated for an additional 16 hr. The membranes were subsequently washed as follows to remove any excess and non-specifically bound probe: 1) 2 x SSC containing 0.3 % SDS for 20 min twice at room temperature, 2) 1 x SSC containing 0.5 % SDS for 20 min at 65°C, and 3) 0.3 x SSC containing 1.0 % SDS twice at 65°C.

For autoradiography, the blots were sealed in a bag and placed in a film cassettte containing an intensifying screen and Kodak XAR film. The film was developed using either developing tanks or an automated developer usually after overnight exposure of the blot at -70°C.

2.8 Protein analysis and separation

2.8.1 Protein determination

2.8.1.1 Bradford micro-assay

Apolipoprotein A-I and immunoglobulin G concentrations were determined using the Bradford assay (16). Samples were diluted to 0.8 mL with ddH_20 and mixed with 0.2 mL Bio-Rad dye reagant. The mixture was then incubated at 37°C for 15 min, after which the A_{595} was measured. Bovine serum albumin in the range of 1-15 μg was used for generating a standard curve.

2.8.1.2 Lowry assay

A slight modification of the Lowry method was used for protein determination of sucrose gradient fractions. The alkali reagant was supplemented with 1.0 % (w/v) SDS so that samples could be assayed directly without prior solubilization or delipidation. The assay procedure was performed as described by Lowry et al. (68) on 1.0 mL samples previously diluted with ddH_20 to a concentration of sucrose not exceeding 0.20 M. Bovine serum albumin was used to generate a standard curve in the range of 10-100 μg (diluted from a 1.28 mg mL^{-1} stock solution).

2.8.2 Separation of proteins by gel electrophoresis

Mini-PROTEAN II (Bio-Rad) dual slab gels (7 (l) x 8 (w) cm gel size) were prepared with either 12 % (w/v) or 15 % (w/v) polyacrylamide and were electrophoresed using the SDS-buffer system of Laemmli (64). Protein samples were

denatured and solubilized prior to electrophoresis by heating at 75°C for 5 min in SDS sample buffer followed by centrifugation at 12,000 rpm for 5 min. Electrophoresis was performed at 200 volts in SDS Electrophoresis Buffer until the bromophenol blue marker dye had reached the bottom of the gel (approximately 45 min). Gels were stained in Coomassie blue R-250 in 40 % methanol/10 % acetic acid for 20 min, destained in 'fast' destain (40 % methanol/10 % acetic acid) for 10 min, and further destained in 'slow' destain (10 % acetic acid) until the background was removed. Destained gels were stored by first immersing them in SDS electrophoresis buffer containing 50 % (v/v) glycerol for 1-2 min, and then placing them between two pieces of 'gel wrap'. The gels were then dried using a Bio-Rad gel dryer for 1 hr at 60°C.

2.9 Protein expression from *pufQ*-containing vectors

2.9.1 Test expression of pKK223-3 derived *pufQ* constructs

Small scale test expressions were performed on the *pufQ* containing constructs pKK223-3(*Sma*I), pKK223-3(*Hind*III), and pSF1. Two mL cultures of TG2 cells harboring each of these constructs were grown to an OD_{600} of 0.6, following which they were induced with 1.0 mM IPTG. Five hundred µL aliquots were withdrawn from the culture at 1, 2, and 3 hr intervals post induction and analysed for PufQ protein expression as follows: The cells were harvested at 12,000 rpm for 5 min in a microfuge, resuspended in 50 µL of TE8, and lysed using a combination of repeated freeze-thawing (three times), followed by sonication for 10 min in a bath sonicator. The cells were re-centrifuged and both the cellular debris (pellet) and crude cell lysate (supernatant) were analysed by SDS-PAGE.

2.9.2 Test expression of pSF2

Two mL cultures of *E. coli* strain QY13 transformed with plasmids pLcII-FX and pSF2 were grown at 30°C until an A_{600} of 0.6 was reached, at which point they were transferred to a water bath at 42°C and agitated for 15 min. Cultures were then incubated at 37°C for an additional 2 hr following which the cells were pelleted by centrifugation at 12,000 rpm for 5 min. The cells were then resuspended in 50 µL of TE8 and lysed as described above in section 2.9.1. The lysed cells were re-centrifuged and the crude cell-free extract in the supernatant was analysed by SDS-PAGE.

2.9.3 Test expression of pSF3

Small scale (2 mL) test expression of *E. coli* strain TB1 transformed with vector pSF3 was performed identically to that described in section 2.9.1 for vector pSF1 except that the concentration of IPTG used for induction was 0.3 mM.

2.9.4 Large scale expression of pSF3

Fifteen L of 2 x TY media containing 100 µg mL^{-1} ampicillin was inoculated with a 200 mL culture of pSF3-transformed *E.coli* strain TB1, and the culture was incubated at 37°C in a Chemap fermentor at 535 rpm while bubbling air at a rate of 10 L min^{-1} until a cell density at A_{600} of 0.6 was reached. Gene expression was then induced by the addition of IPTG, and the cells were grown an additional 2 hr. After incubation, the culture was cooled to 18°C and the cells concentrated to a final volume of 2 L using a Pellicon tangential flow filtration unit (Millipore Corp., Bedford, MA) equipped with a 0.45 mm filter cassette. The concentrated cells were then harvested by centrifugation at 4000 x *g* for 20 min in a Sorvall GS3 centrifugation rotor at 4°C.

Cell pellets were resuspended in a total volume of 500 mL of Buffer A. Cell suspensions were incubated with lysozyme (1 mg mL^{-1}) for 30 min at 4°C, followed by sonication with three 20-s bursts at maximum power from a probe sonicator (FisherSonic-Dismembrator Model 300) fitted with a medium-sized probe. Finally, NaCl was added to lysates to a final concentration of 0.5 M and the lysed cells were centrifuged at 9000 x *g* for 30 min in a Sorval SS34 rotor. The supernatant was diluted 5-fold with Buffer B and the MBP-FX-PufQ fusion protein purified (section 2.10).

2.10 Purification of PufQ protein

2.10.1 Affinity column chromatography

The diluted cell-free lysate was loaded onto a 50 mL amylose column (New England BioLabs) which had been previously washed with 50 mL of Buffer B. The column was eluted at a flow rate of 1 mL min^{-1} with 150 mL of Buffer B followed by 250 mL of Buffer B minus Tween 20. Fusion protein was recovered in a single fraction by elution with 10 mM maltose. The fusion protein (in 30 mL) was then dialyzed against 4 L of Buffer C, using dialysis membranes having a molecular weight cutoff of 12,000-14,000. Identical methods were used to express and purify the gene product of the unaltered pMal-c vector (*malE-FX-lacZα* gene fusion), the MBP-FX-β-galactosidase' fusion protein.

2.10.2 Solubilization of PufQ protein

A variety of detergents were used to attempt to solubilize the PufQ protein following digestion of the MBP-FX-pufQ fusion protein with Factor X_a. The detergents tested were either the non-ionic detergents n-octylglucoside and Triton X-100, or the zwitterionic detergents sodium cholate, sodium deoxycholate, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Small scale solubility tests of each of these detergents were done by adding incremental amounts of detergent to 100 µL of the digest and centrifuging at 12,000 rpm for 30 min. The criterion for successful solubilization was the absence of precipitated PufQ protein. In the case of sodium cholate, solubilization was more rigorously tested by centrifugation at 100,000 x *g* for 1hr.

2.10.3 Site-specific proteolysis of the MBP-FX-PufQ fusion protein

Factor X_a purified from bovine serum was obtained from Dr. T.J.Borgford (SFU). Conditions for the digestion of each preparation of the MBP-FX-PufQ fusion by Factor X_a were determined empirically. Two sets of buffers were used to prepare PufQ protein for further analysis. Digests were performed either in Buffer C with an 800:1 (w/w) ratio of fusion protein to Factor X_a, or in Buffer C containing 20 mM sodium cholate with an 80:1 (w/w) ratio of fusion protein to Factor X_a. The sodium cholate was necessary in order to maintain solubility of the PufQ protein. Typically, complete digestion was achieved in 12 hr at 23°C in both buffers. The digests were terminated by the addition of PMSF (from a 100 mM stock solution in 2-propanol) to a final concentration of 1 mM. Following digestion in Buffer C, the unconjugated MBP was purified by affinity chromatography on an amylose column as described in section 2.10.1 for the MBP-FX-PufQ fusion protein.

2.10.4 Purification of PufQ protein by HPLC

Fusion protein digested in Buffer C with Factor X_a (as described in section 2.10.3 in the absence of sodium cholate) was solubilized in 60 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid. The sample was then applied to a 10 mm x 25 cm Vydac C4 reverse phase high performance liquid chromatography (HPLC) column previously equilibrated in 40 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid. The HPLC system was a Spectra-Physics model 8810 equipped with an Applied Biosystems model 757 absorbance detector set at 280 nm. The column was eluted for 60 min at room temperature with a gradient of 40-60 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid at a flow rate of 1.0 mL min^{-1}, followed by an additional 20 min with isocratic 60 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid. For analysis of the peak fractions by SDS-PAGE, samples were concentrated under vacuum, washed with 0.5 mL water to remove any traces of trifluoroacetic acid, and concentrated again under vacuum.

2.10.5 N-terminal sequence analysis

For amino-terminal sequence analysis, two HPLC fractions having retention times of 68 min and 73 min, and having similar M_r-values to the PufQ protein, were concentrated under vacuum to a final volume of 20 µl and sequenced in the Microsequencing Center at the University of Victoria, Victoria, B.C., using an Applied Biosystems model 473 protein sequencer.

2.11 Fractionation of *R. capsulatus*

2.11.1 Preparation of crude chromatophores

R. capsulatus cells from 1 L of culture medium were harvested at the end of the exponential growth phase by centrifugation in a Sorvall GS3 rotor at 13,500 x *g* for 20 min at 4°C, and washed twice with Buffer E. All of the following steps were performed at 4°C. The washed cells were resuspended in Buffer G and sonicated 3-4 times at maximum power for 30 s (with 1 min intervals between sonications) with a FisherSonic-Dismembrator model 300 sonicator fitted with a medium-size probe. The crude homogenate was centrifuged at 17,500 x *g* for 20 min to remove the unbroken cells and cell debris. The resulting supernatant was centrifuged in a Beckman Ti75 rotor at 110,000 x *g* for 90 min. After ultracentrifugation, the soluble proteins in the supernatant was stored at -20°C, and the pellet, which contained chromatophores and other components of the cell envelope, was washed in Buffer E using a hand-held glass homogenizer. These two fractions were used in the initial identification of PufQ protein by Western blot anlaysis.

2.11.2 Dissociation studies of chromatophores

Purified chromatophores from wild type *R. capsulatus* were prepared and extracted with the assistance of D. Huang from 1 L of fully photosynthetic cells as follows: Cells were harvested and lysed as described in section 2.11.1, and the cell lysate applied on a continuous gradient of sucrose composed of 10-50 % (w/w) sucrose in Buffer G. Following centrifugation in a Beckman SW40 rotor at 30,000 x *g* for 90 min at 4°C, a visible band in the approximately middle of the gradient was collected that corresponded to the chromatophore fraction. This fraction was then treated with

3M sodium bromide and incubated for 30 min on ice. The sample was then diluted with an equal volume of 50 mM glycylglycine (pH 7.8) and ultracentrifuged at 300,000 x *g* for 60 min to dissociate any peripheral proteins associated with the chromatophores. The proteins remaining in the chromatophores were then solubilized by treating the pellet with 1 % (w/v) octylglucoside and incubating the extract for 20 min on ice. The sample was again ultracentrifuged and the resulting pellet re-extracted with 3 % (w/v) octylglucoside.

All of the soluble and insoluble fractions were subsequently analysed for the presence of PufQ protein as described in section 2.12.4. Aliquots of each of the detergent-solubilized chromatophore proteins were further purified by applying the samples on linear gradients of 10-50 % (w/w) sucrose and centrifuging in a Beckman SW40 rotor at 30,000 x *g* for 19 hr at 4°C. In both samples, upper and lower bands were visible and analysed by visible spectroscopy (section 2.16.2) and Western blotting (section 2.12.4).

2.11.3 Preparation of membranes from mutants of *R. capsulatus*

Membrane fractions of the *R. capsulatus* mutant strains ΔRC6, ΔRC6(pΔ4), and CB1200 were isolated as follows: One L of fully grown anaerobic cells were harvested by centrifugation in a Sorvall GS3 rotor at 13,500 x *g* for 20 min at 4°C. All of the following steps were performed at 4°C. The cell pellet was washed twice with Buffer E, resuspended in 10 mL of Buffer G, and lysed as described in section 2.11.1. The resulting crude cell lysate was layered on top of six 11 mL tubes containing a discontinuous gradient of sucrose composed of 10 % , 20 %, and 35 % (w/w) sucrose in Buffer G and centrifuged in a Beckman SW40 rotor at 30,000 x *g* for 90 min. Fractions located at the interface between the 20 and 35 % sucrose zones were

collected and pooled. One hundred μL aliquots of these samples were assayed for MT activity (section 2.15), while the remaining samples were dialysed extensively against 8 L of buffer E (with 3 changes of the same volume of buffer), followed by additional dialysis against 8 L of ddH_2O. The dialysate was then freeze-dried and the resulting pellet resuspended in 1.5 x SDS sample buffer. The concentrated protein samples were then used in subsequent PufQ protein localization studies (section 2.12.4).

2.11.4 Adaption of *R.capsulatus* from aerobic to phototrophic growth conditions

A 200 mL mid-log culture of wild type *R. capsulatus* grown under photosynthetic conditions was used to inoculate 15 L of RCV in a Chemap fermentor. This culture was initially grown under aerobic conditions for 48 h using a stir rate of 300 rpm and bubbling air at a rate of 6-8 L min^{-1} until the cells reached an A_{680} of 0.64. The cells were then induced to grow photosynthetically by lowering both the stirring and air flow rates to 200 rpm and 1-2 L min^{-1} respectively. One L aliquots of cells at time intervals of 0h, 1h, 3h, 4h, and 34h following induction were harvested and processed as described in section 2.11.3.

2.11.5 Fractionation of adapted *R.capsulatus* cells for PufQ protein localization studies and MT assays

Following induction (2.11.4), 1 L of *R. capsulatus* cells were harvested by centrifugation in a Sorvall GS3 rotor at 13,500 x *g* for 20 min at 4°C, and washed twice with Buffer E. The cells were then lysed and fractionated on a gradient of discontinuous sucrose as described in section 2.11.3. Fractions corresponding to the

very top layer of the gradient (termed soluble fraction), at the interface between the 10 and 20 % sucrose zones (termed upper pigmented band or chromatophore precursor fraction), at the interface between the 20 and 35 % sucrose zones (termed lower pigmented band or chromatophore fraction), and the pellet (termed cell envelope fraction) were collected. One hundred μL aliquots of these samples were assayed for MT activity (section 2.15), while the remaining samples were concentrated as described in section 2.11.3 in preparation for PufQ protein localization studies (section 2.12.4).

2.12 Preparation of antibodies and immunoblotting of PufQ protein

2.12.1 Immunization of rabbit

Antibodies raised against the MBP-FX-PufQ fusion protein were elicited in a rabbit by subcutaneous injections of a thoroughly mixed solution consisting of 100 μg purified fusion protein combined with either Freund's complete (used for the initial injection) or incomplete adjuvant (used for all subsequent injections). Injections were performed over an approximate 10-week period. Typically, about 25 mL of blood was collected.

2.12.2 Isolation of rabbit anti-MBP-FX-pufQ immunoglobulin G by FPLC

Antiserum was prepared for purification of immunoglobulin G (IgG) by fast protein liquid chromatography (FPLC) as follows: antiserum proteins were precipitated overnight at 4°C with an equal volume of saturated ammonium sulfate. The precipitate was recovered by centrifugation at 3000 x *g* for 30 min, resuspended in 50 mL of Buffer D, and dialysed against 4 L of Buffer D (with 3 changes of buffer)

using dialysis membranes having a molecular weight cutoff in the range of 12,000-14,000. The dialysate was then applied to a 2.6 x 31 cm anion-exchange column packed with Mono Q which had been previously washed with 20 mL of Buffer D containing 1 M NaCl. IgG was eluted in a 500 mL linear gradient from 10 mM NaCl to 1 M NaCl at a flow rate of 2 mL min^{-1}. Fractions were screened for relative activity against both MBP-FX-PufQ fusion protein and MBP by performing enzyme-linked immunosorbant assays (ELISA) as described in section 2.12.3. Based on the ELISA results, a major component of the antiserum, which eluted at a salt concentration of 55 mM NaCl, was identified as IgG. It demonstrated a 4 to 7-fold increase in activity against the fusion protein when compared to the unconjugated MBP.

2.12.3 Enzyme linked immunosorbent assay of FPLC fractions

Purified MBP and MBP-FX-PufQ fusion protein were used to coat ELISA plates. One hundred µL of 10 µg mL^{-1} stock protein solutions diluted in PBS/Tween Buffer were added to each of the wells and the plates incubated overnight at 4°C. Excess protein was then removed by washing the plates with ddH_2O, and the uncoated regions of the plate were blocked by the addition of 200 µL of PBS/Tween Buffer containing 1 % (w/v) ovalbumin. The plates were incubated at 37°C for 1 hr and washed again with ddH_2O. One hundred µL of FPLC fractions containing IgGs (diluted by factors of 5 x 10^{-3}, 10^{-4}, and 2 x 10^{-4} in PBS/Tween Buffer containing 0.5 % ovalbumin) were then added in duplicate to each of the wells. Following re-incubation for an additional 2 hr, the plates were washed and 100 µL of goat anti-rabbit serum conjugated to horseradish peroxidase (diluted by a factor of 4 x 10^{-3} in PBS/Tween Buffer containing 0.5 % ovalbumin) was added. The plates were again incubated for 2 hr and washed. The antibodies were detected by colour development at 37°C by the addition of 100 µL of a stock substrate solution consisting of 10 mM 1,2-diaminobenzene in 0.1 M sodium

acetate, pH 6.0, and containing 0.030 % (v/v) hydrogen peroxide. When the colour was adequately intense, 20 µL of 2.5 M sulfuric acid was added to terminate the reaction. The reactivity was quantified by measuring the A_{490} on a Bio-Tek Instruments model Ceres 900 HDi ELISA plate reader.

2.12.4 Immunoblotting of proteins

Two sets of Western immunoblot assay conditions were used for blotting and detecting either PufQ protein or MBP and its derivatives. Samples were electrophoresed on SDS denaturing gels (section 2.8.2) composed of either 12 % polyacrylamide for the latter or 15 % polyacrylamide for the former. Proteins were blotted onto Immobilon-p filters composed of polyvinylidene diflouride (PVDF) previously equilibrated with Buffer F. Electroelution was performed using a LKB multipore transfer apparatus. The transfer conditions for MBP and PufQ protein were 1.2 mA cm^{-2} for 2 hr and 2.5 mA cm^{-2} for 6 hr, respectively. It was found that the PufQ protein successfully transferred to the membranes when the direction of migration was towards the cathode. The filters were blocked in 3 % (w/v) fetal calf serum (FCS) in PBS Buffer, shaking either for 1 hr at room temperture or overnight at 4°C. The filters were then washed for 30 min in PBS Buffer containing 0.1 % (w/v) FCS, and probed for MBP by incubation with a 10^5-fold dilution of rabbit anti-MBP serum in PBS Buffer containing 1.0 % (w/v) FCS and 0.05 % Tween-20. Alternatively, blots were probed for PufQ protein using either a 2 x 10^3-fold dilution (for chromatophore homogenates) or a 5 x 10^4-fold dilution (for sucrose gradient fractions) of rabbit anti-MBP-FX-PufQ IgG (prepared as described in section 2.12.2) in the same buffer. The wash with PBS Buffer containing 0.1 % FCS was repeated and the filters were then incubated at room temperature with a 4 x 10^3-fold dilution of goat anti-rabbit serum conjugated to horseradish peroxidase in PBS Buffer containing 1.0 % (w/v) FCS and

0.05 % Tween-20. After a final wash in PBS Buffer containing 0.1 % FCS, the filters were developed with 1.0 mM 3,3'-diaminobenzidine in 50 mM Tris-Cl, pH 7.6, containing 0.01 % (v/v) hydrogen peroxide.

2.13 Reconstitution of PufQ protein into liposomes in the presence and absence of Pchlide

2.13.1 Preparation of liposomes

Liposomes were prepared by sonicating 175 mg soybean phospholipids in 2.5 mL Buffer C at 80% power output with a Branson sonicator fitted with a medium-size probe until the turbid solution was clear. Sonication was performed at 4^{o}C under a stream of nitrogen gas. The liposomes were then centrifuged at 12,000 rpm for 5 min to remove any pelleted titanium and residual multilamellar vesicles. For preparing radiolabeled liposomes, a 1.5 µL aliquot of 0.15 µCi of dipalmitoylphosphatidyl [*methyl*-^{3}H] choline from a toluene:methanol (1:1) stock solution was dried under nitrogen and resuspended in a 1.5 mL suspension of unlabeled Asolectin prior to sonication. Soybean Asolectin contains a mixture of 39% phosphatidylcholine, 23% phosphatidylethanolamine, 20% phosphatidylinositol, 5% phosphatidic acid, and 13% unidentified phospholipids.

2.13.2 Reconstitution of PufQ protein into liposomes

Aliquots containing 0.54 mL of the labeled liposomes (38 mg phospholipids) were added to 1.4 mg of the MBP-FX-PufQ fusion protein digested in buffer C (as described in section 2.10.3) in the presence of 10 mM sodium cholate. The digested mixture was then incubated for 15 min at 37^{o}C, and the cholate was removed by extensive dialysis

against 4 L of buffer C (with 3 changes of the same volume of buffer). Dialysed samples (1.2 mL) were then layered on top of centrifuge tubes containing discontinuous sucrose gradients formed by successive layers (bottom to top) of 3.0 mL of 50%, 3.0 mL of 35%, and 4.5 mL of 15% (w/w) sucrose, and centrifuged in a Beckman L8-80 ultracentrifuge using a SW40Ti rotor for 20 h at 30,000 x *g* and 13°C. For comparison, a control sample containing radiolabeled liposomes, formed as described above, was added to solutions of 10 mM sodium cholate containing 1.4 mg of MBP, and centrifuged as above. Following centrifugation, the gradients were divided into equal 1 mL fractions and 50 µL aliquots of each were analyzed in a Beckman LS6000 Series liquid scintillation counter for the presence of ^{3}H-labeled phospholipids. Protein determination on each of the fractions was performed using a modified Lowry assay (section 2.8.1.2).

2.13.3 Preparation of Pchlide from etiolated wheat seedlings

^{14}C-labeled Pchlide was isolated from etiolated wheat seedlings by Dr. S.B. Hinchigeri using the procedure of Griffiths (42) with slight modifications. The concentration of Pchlide was calculated by measuring its absorbance in methanol, using an extinction coefficient at 434 nm of 289.5 mM^{-1} cm^{-1}. It was found to have a specific activity of 348 mCi mol^{-1} (compared to a value of 383 mCi mol^{-1} expected from the specific activity of ^{14}C-ALA added).

2.13.4 Solubilization of Pchlide with sodium cholate

Solubilization of Pchlide was performed as described by Griffiths (42) with slight modifications. For use in reconstitution experiments, Pchlide samples were initially solubilized in 8 mL of peroxide-free ether. An equal volume of petroleum ether (bp 30-

60°C) was then added, followed by 4 mL 90% aqueous methanol containing 1.2 mM sodium cholate. The mixture was shaken vigorously, followed by an immediate addition of petroleum ether to facilitate the transfer of the sodium cholate-pigment solution into the methanolic layer. The Pchlide extraction was repeated twice, and the pooled methanolic extracts were finally dried with a stream of nitrogen. The sodium cholate solubilized Pchlide residue was used immediately in all of the subsequent reconstitution experiments.

2.13.5 Binding of Pchlide to liposomes containing the PufQ protein.

Aliquots containing 0.54 mL of either the labeled or unlabeled liposomes (38 mg phospholipids) were added to 1.4 mg of the MBP-FX-PufQ fusion protein digested in buffer C (as described in section 2.10.3) in the presence of 10 mM sodium cholate. Subsequently, 44 nmol of ^{14}C labelled Pchlide (348 mCi mol^{-1}) was added to unlabeled liposomes, while 44 nmol of unlabeled Pchlide was added to ^{3}H-labeled liposomes. The mixtures were then incubated for 15 min at 37°C, and the cholate was removed by extensive dialysis against 4 L of buffer C (with 3 changes of the same volume of buffer). Dialysed samples (1.2 mL) were then layered on top of 11 mL of continuous linear gradients formed between of 10 % and 35% (w/w) sucrose, and centrifuged in a Beckman L8-80 ultracentrifuge using a SW40Ti rotor for 20 h at 30,000 x *g* and 13°C. For a comparison control, unlabeled liposomes containing ^{14}C-labeled Pchlide and 1.4 mg of Apolipoprotein A-I in place of the digested fusion protein were prepared and centrifuged identically. Following centrifugation, the gradients were divided into equal 1 mL fractions and 500 µL aliquots of each were analyzed in a Beckman LS6000 Series liquid scintillation counter for the presence of ^{3}H-labeled phospholipids or ^{14}C-labeled Pchlide. Protein determination on each of the fractions was performed using a modified Lowry assay (section 2.8.1.2). The sucrose

concentration of fractions was determined by a Milton Roy model Abbe-3L refractometer.

2.14 Analysis of *R. capsulatus* strains ΔRC6 and ΔRC(pΔ4)

The mutant *R.capsulatus* strains ΔRC6 and ΔRC(pΔ4) were used to measure the effect of *pufQ* on the levels of biosynthetic intermediates synthesised. Both mutants were grown anaerobically in 50 mL of RCV$^+$ media using a 0.5 mL innoculum. The media was supplemented with either of the following: N-methyl protoporphyrin (NMP), ALA, PBG, or a combination of nicotinamide and either ALA or NMP. The concentrations of NMP, ALA, PBG, and nicotinamide were 0.070 mM, 1.0 mM, 0.25 mM and 12 mM respectively. A control set of cultures was also grown in RCV$^+$ media without any additions.

Following growth for 36 hrs, the cell density of the cultures and any porphyrins excreted into the medium were analysed by visible spectroscopy (section 2.16.2). The relative amounts of Bchl and Pchlide were determined by measuring the absorption at 860 and 631 nm respectively. For determining the Coprogen levels, the cells were harvested in a Sorval SS34 rotor at 12,000 rpm for 20 min at 4^{o}C, and the Coprogen in the supernatant chemically oxidized to coproporphyrin (copro) by treatment with benzoquinone (53) as follows: 0.2 mL of the supernatant was mixed with 50 μL of 5 M HCl and 100 μL of benzoquinone (1.0 mg mL^{-1} in methanol). The samples were then incubated at 23^{o}C in the dark for 30 min., after which time 100 μL of saturated sodium bisulfite was added to decolourise the remaining benzoquinone. After dilution to 1.0 mL with 1 M HCl, the spectra was measured and the A_{405} recorded.

The effect of exogenous PBG on porphyrin synthesis was also tested using levulinic acid. Twenty-five mL fully grown cultures of both strains were harvested and the cell pellets resuspended in the same volume of 50 mM Tris, pH 8.0 buffer

containing either 1.0 mM ALA or a combination of 0.25 mM PBG and 10 μM levulinic acid. The cells were then incubated for 14 hrs at 30°C with gentle shaking (100 rpm) and spectrophotometrically analysed for porphyrin synthesis.

2.15 Analysis of MT activity

The specific activity of MT in various subcellular fractions of *R. capsulatus* mutant and wild type strains was measured by Dr. S.B. Hinchigeri using the method of Gibson et al. (37).

2.16 Spectroscopic methods

2.16.1 Fluorescence spectroscopy

The fluorescence emission spectra of samples were recorded in 1.0 cm^2 quartz cuvettes at an excitation wavelength of 454 nm, using a Photon Technology Model LS-100 fluorometer equipped with a cell assembly thermostated at 25 ± 1°C, and interfaced with a NEC Information Systems model 286 Powermate microcomputer.

2.16.2 Visible spectroscopy

Absorption spectra were recorded in a Beckman DU 640 spectrophotometer. For detecting Bchl in whole cells, the absorption due to light scattering at wavelengths between 650 and 900 nm was estimated using the equation below and subtracted from the sample spectrum.

$$A_S = a \lambda^{b}$$

where A_S is the absorption due to scatter at a particular wavelength λ, and a and b are constants.

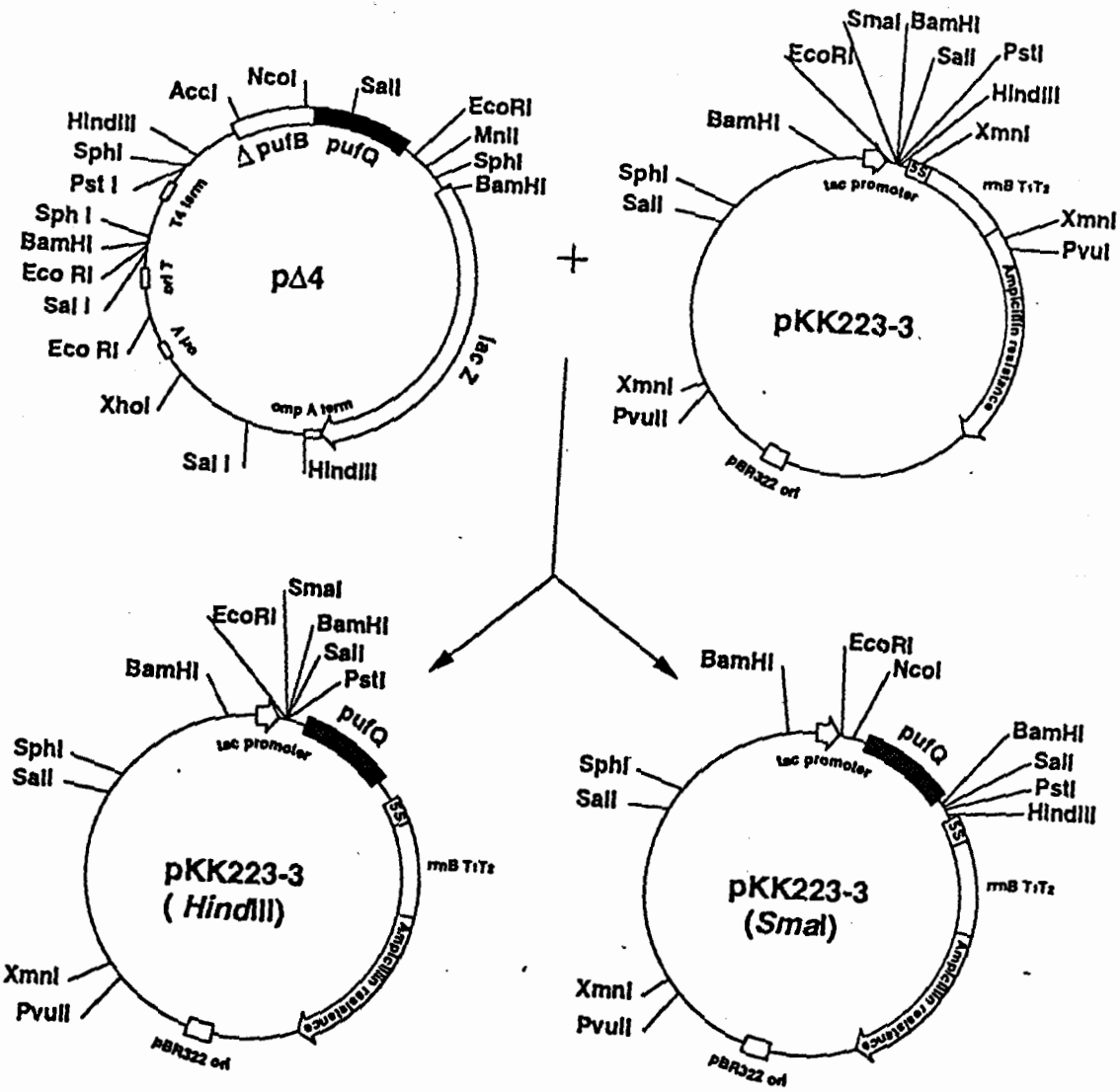
Treatment with the above light scatter correction function produced a Bchl absorption spectrum in the absence of light-scattering due to cell turbidity.

3. RESULTS

3.1 Cloning summary

Various *E.coli* expression vectors have been used for cloning the *pufQ* gene. A summary of the expression vectors assembled and the cloning scheme used is illustrated in fig.13. During the course of the cloning steps, site-directed mutagenesis was used to introduce a novel restriction site (*Xba*I), improve the native ribosome binding site for better recognition in *E.coli* (105), and to correct spontaneous mutations. All of the final expression constructs were confirmed by dideoxy sequencing.

fig.13 Summary of cloning scheme. A diagram outlining the scheme used for sub-cloning the *pufQ* gene into various plasmids is shown. See text (section 2.5.4) for a more complete description of individual steps.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XhoI
Sal I
HindIII
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ori T
ori V
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+
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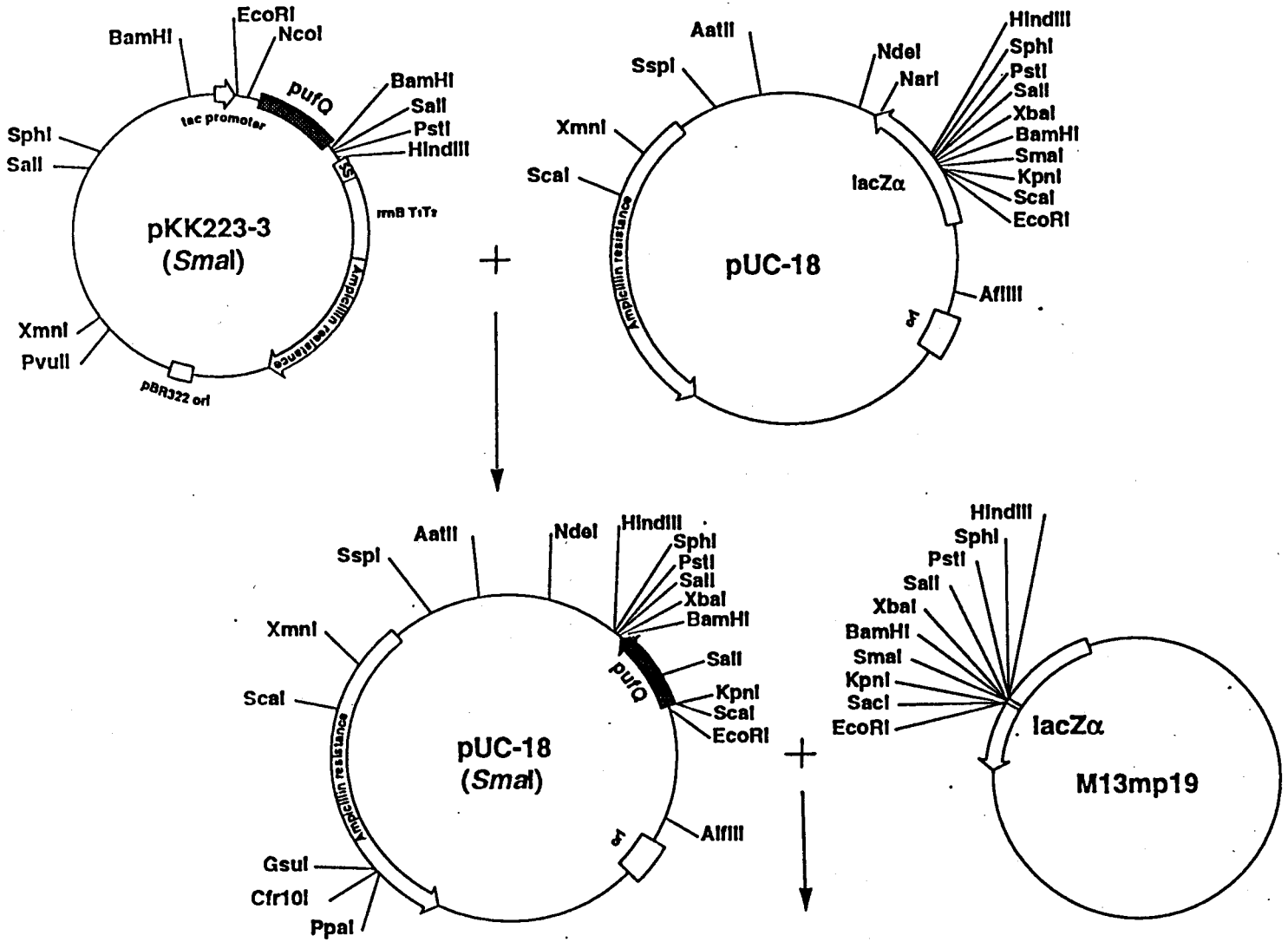
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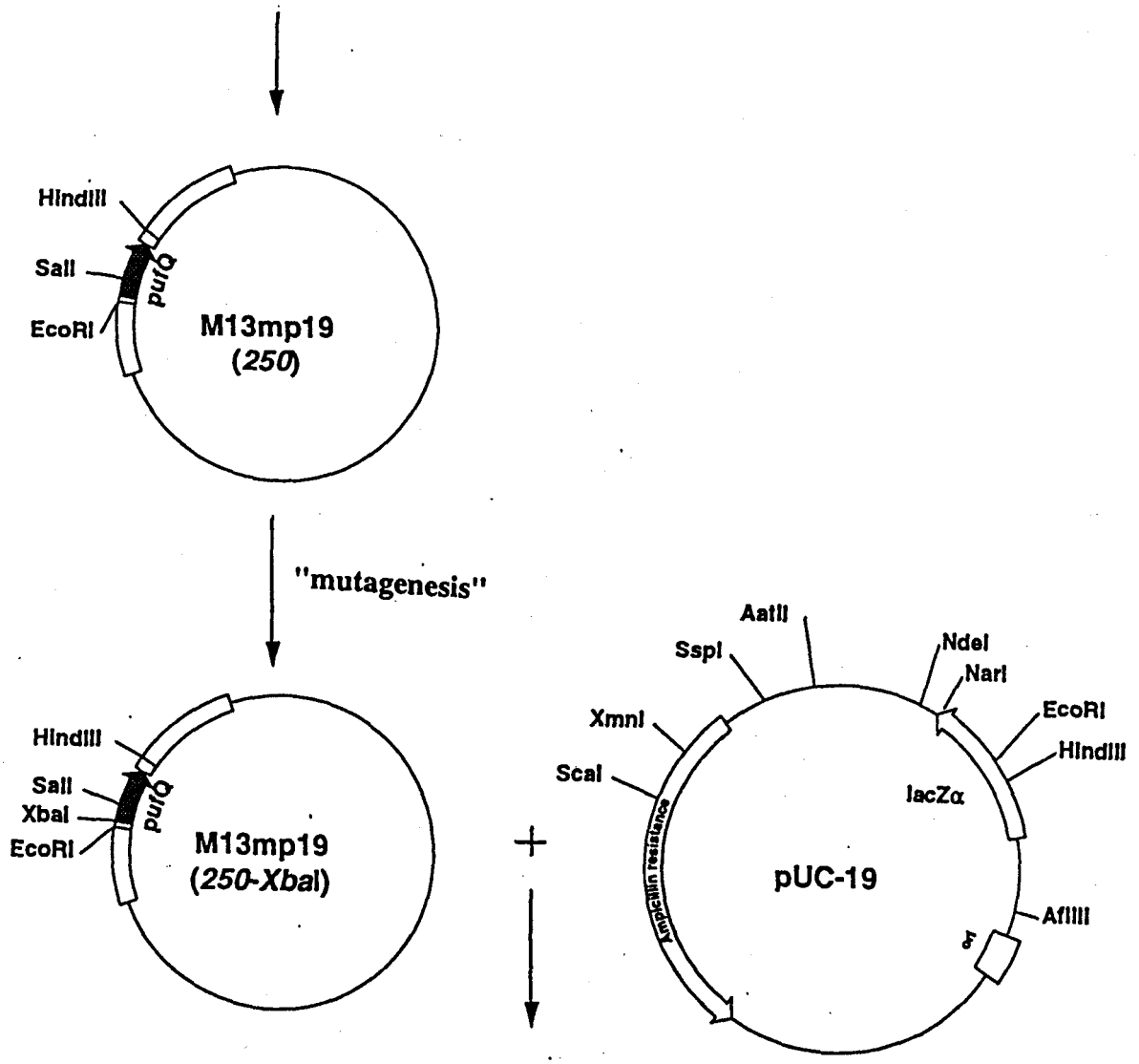
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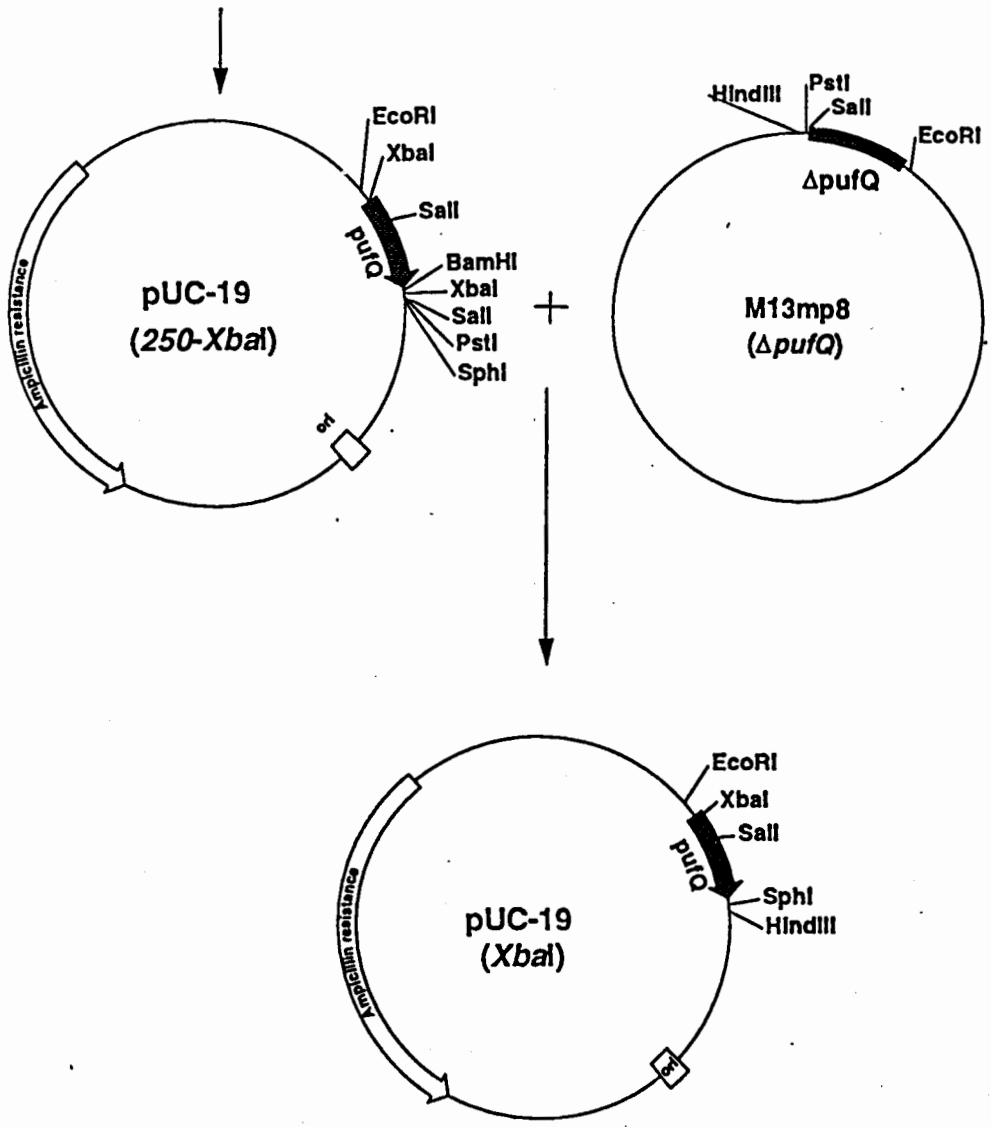
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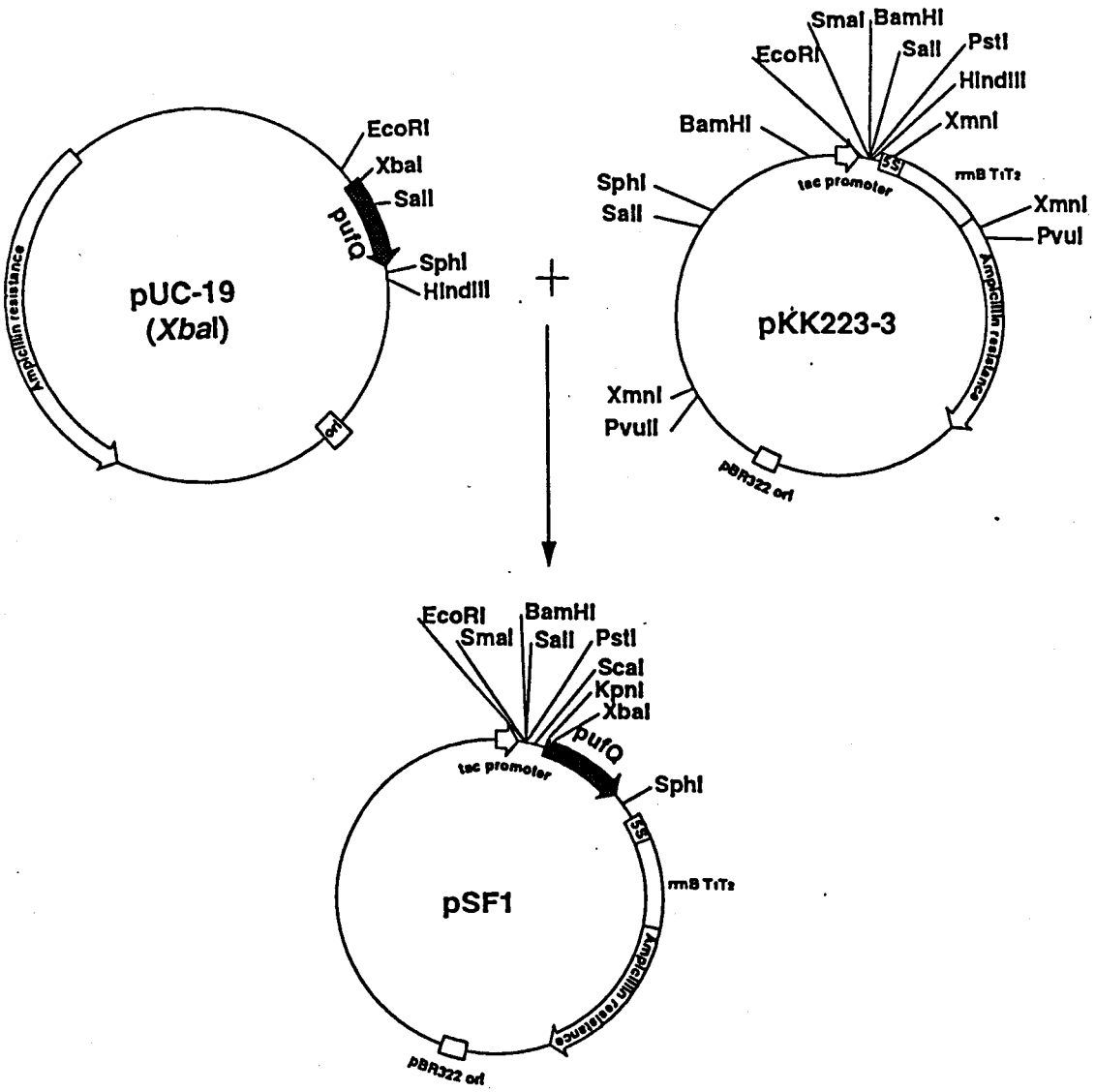
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Sphl
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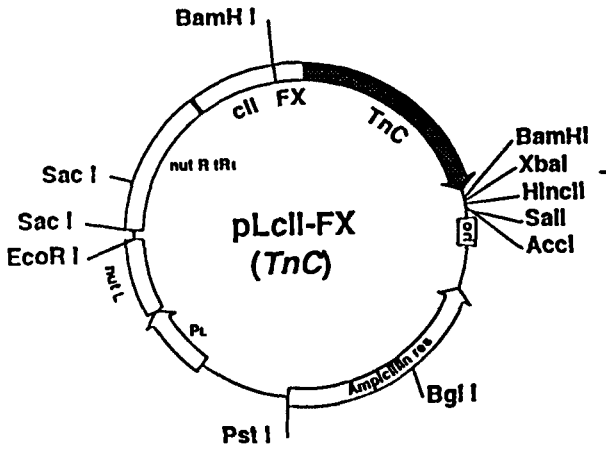

*Bam*HI Ser Ile Glu Gly Arg

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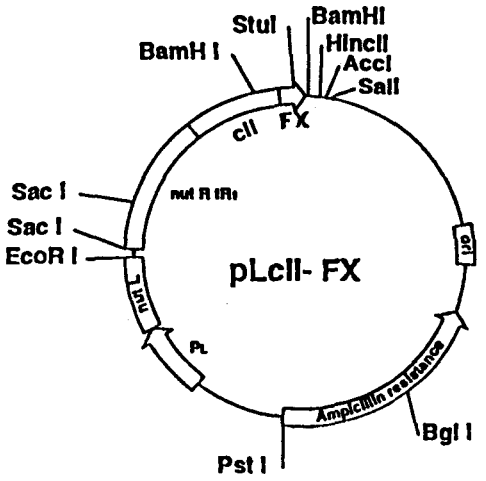
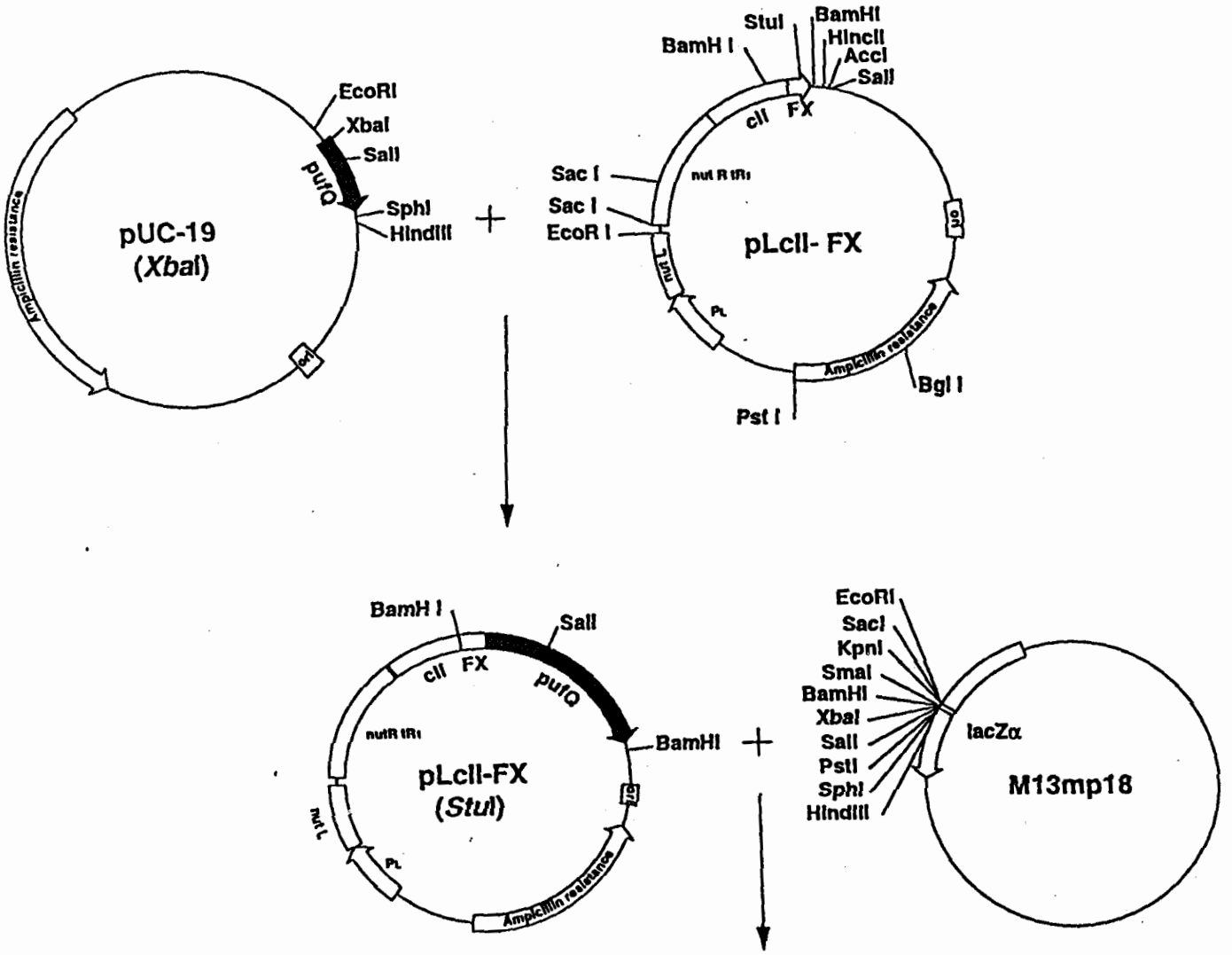
3'-G TAG CTC CCA TCC GGA TGG GAG CTA CCTAG-5'

Arg Gly Glu Ile Ser *Bam*HI

30 mer oligonucleotide

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SalI
cII
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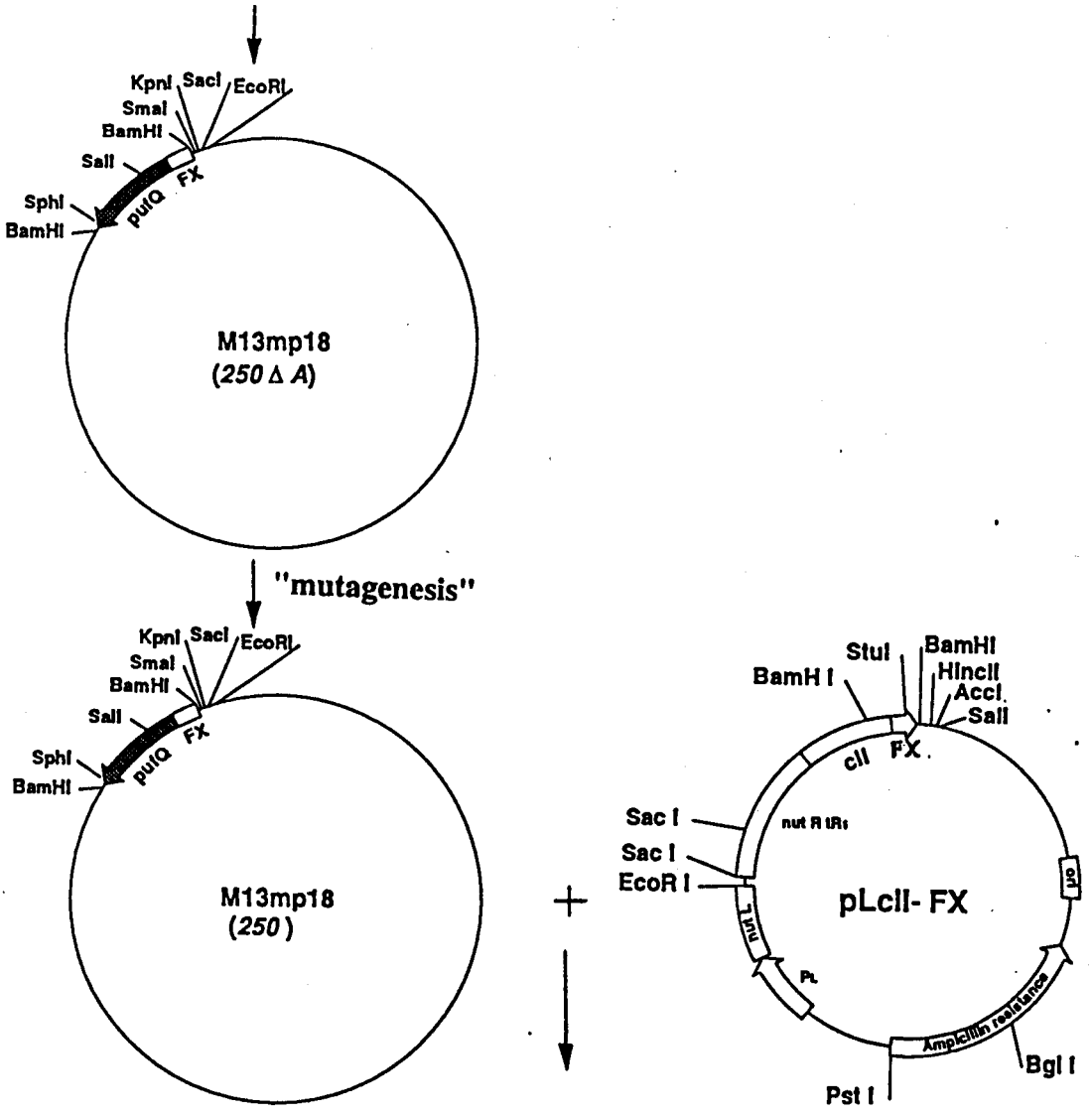
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BamHI
BamH I
HincII
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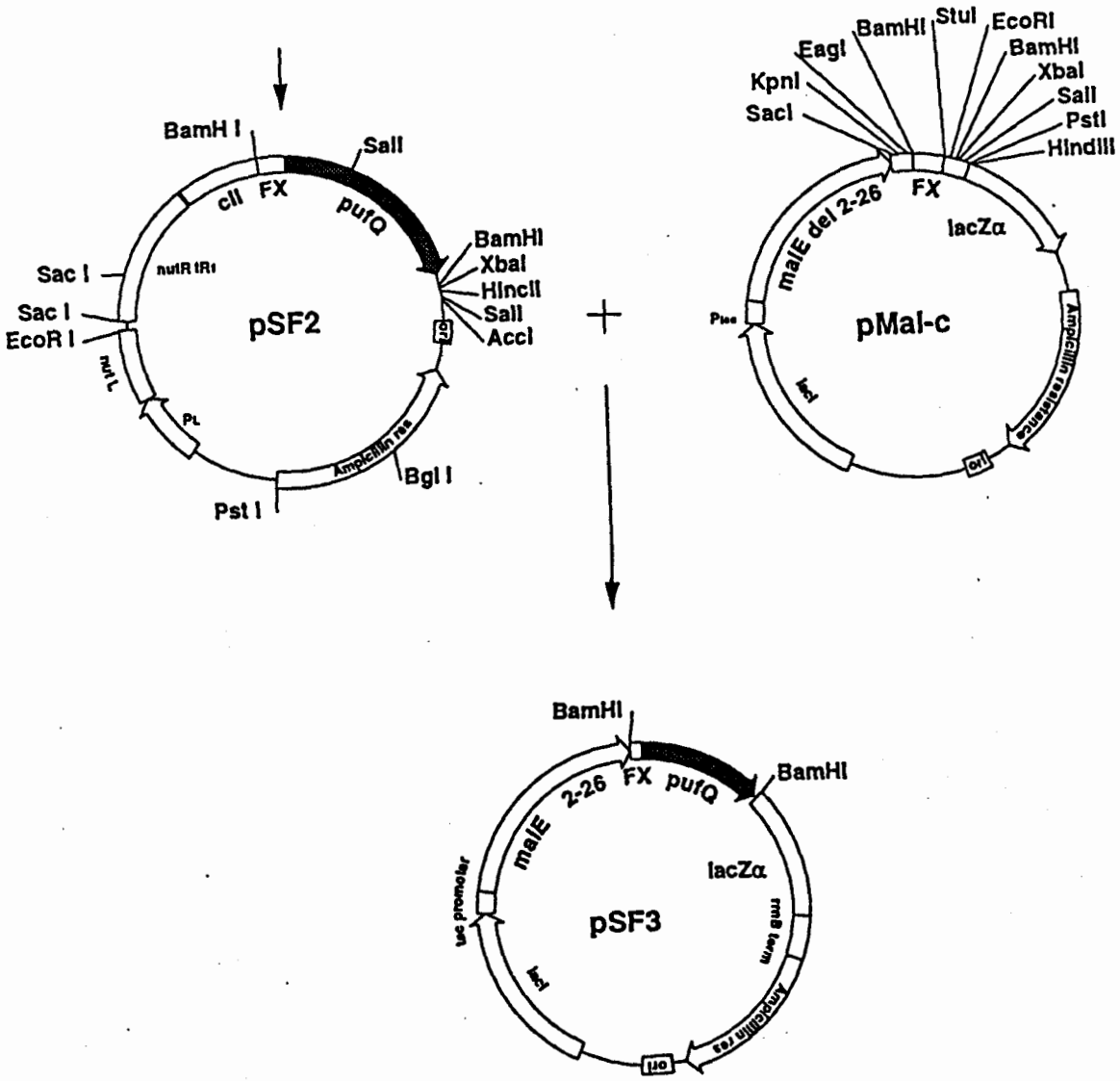
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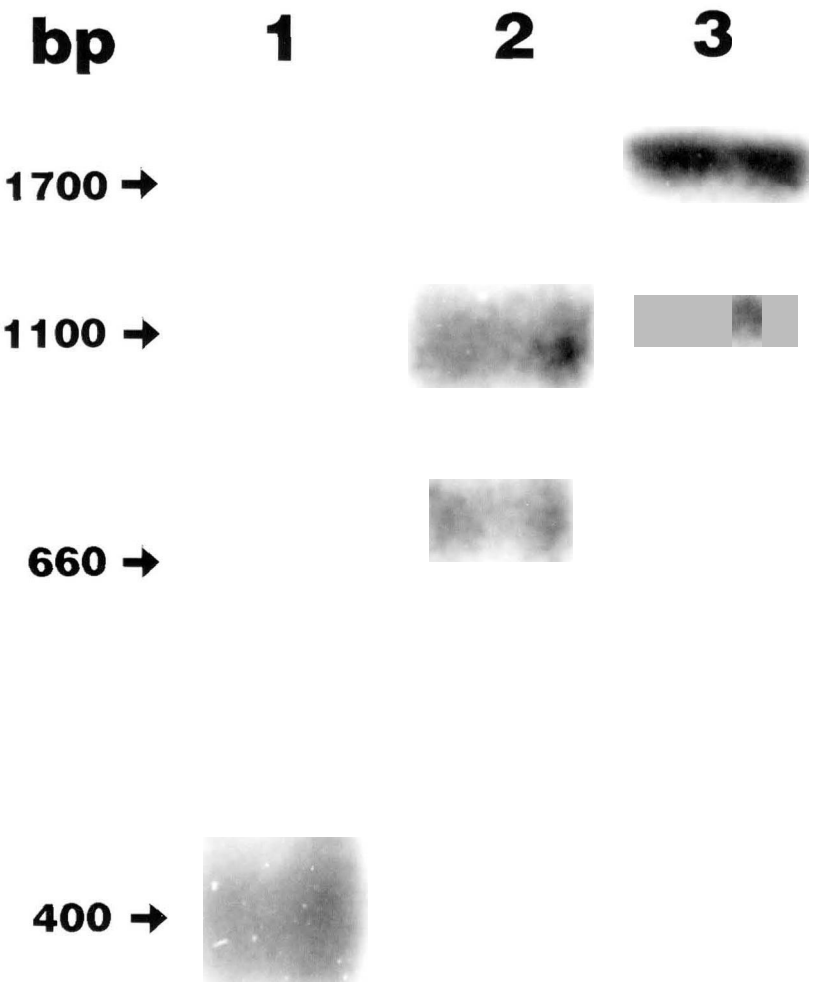
pSF2
nutL
PL
Amp ellin ras
Bgl I
Pst I
EagI
BamHI
StuI
EcoRI
KpnI
BamHI
SacI
XbaI
SalI
PstI
HindIII
malE del 2-26
FX
lacZα
pMal-c
Ptac
lacI
Ampicillin resistance
ori
BamHI
malE
2-26
FX
pufQ
BamHI
lacZα
pSF3
lacI
Ampicillin res
ori

3.2 Northern blot analysis

Transcription of *pufQ* genes in the recombinant constructs pSF1, pSF2, and pSF3 was evaluated by Northern blot analysis. In each of the three constructs, *pufQ*-specific transcripts were undetectable prior to induction, but were detected following induction (fig.20). The probe specificity was determined by performing Northern blots on each of the expression vectors lacking the *pufQ* gene. In these control vectors, no *pufQ*-specific RNA species were detected either before or after induction.

In vector pSF1, transcripts of approximately 400 and 660 bases were detected (fig.20, lane1). Transcripts of these lengths are consistent with the anticipated 410 and 580 bases lengths which correspond to termination of transcription at either the *rrnB* T_1 and *rrnB* T_2 sites on the vector pKK223-3. In studies of vector pSF2, three distinct *pufQ*-specific mRNA species were detected having lengths of approximately 740, 1100, and 1500 bases (fig.20, lane2). Since the transcription termination sites of the vector pLcII-FX are not known (75), distances from the end of the *pufQ* gene to transcription termination sites on the vector cannot be calculated. However, the minimum *λcII-FX-pufQ* mRNA length of 720 bases for vector pSF2 is consistent with the observed transcript length of 740 bases (fig.20, lane 2), suggesting that the 1100 and 1500 base mRNA species also observed (Fig.20, lane 2) are poorly terminated "read-through" transcripts. Vector pSF3 produced two *pufQ*-specific transcripts of 1260 and 1700 bases (fig.20, lane 3), the latter of which corresponded to the anticipated transcript length for the *MBP-FX-pufQ* mRNA.

fig.14 Northern hybridization analysis of *E.coli* strains harboring expression vectors containing the *pufQ* gene. Total RNA (100 μg) from the following induced strains of *E.coli* was electrophoresed and blotted using a *pufQ*-specific probe. Lane 1, TG2(pSF1); lane 2, QY13(pSF2); lane 3, TB1(pSF3). Uninduced strains expressed negligible amounts of transcript, while no transcripts were detected in expression vectors lacking inserts containing the *pufQ* gene (data not shown).


bp
1
2
3
1700 →
1100 →
660 →
400 →

3.3 Protein expression

3.3.1 pSF1

SDS-PAGE analysis of lysates of *E.coli* TG2 containing constructs pKK223-3(*Sma*I), pKK223-3(*Hind*III) (data not shown), and pSF1 (fig.14) indicated no PufQ protein overexpression either before or after IPTG induction. Expression was not evident when either the *R. capsulatus* ribosome binding site in construct pKK223-3(*Hind*III) or the endogenous pKK223-3 ribosome binding site in construct pKK223-3(*Sma*I) was utilized.

3.3.2 pSF2

Protein expression in cell lysates of *E.coli* QY13 harboring vector pSF2 was determined by SDS-PAGE. The results indicated that a protein with an M_r-value of approximately 15 kDa was present in the crude cell-free extract of induced (fig.15, lane 2), but not uninduced cultures of strain QY13 (pSF2). A polypeptide of approximately 12 kDa corresponding to the cII-FX-PufQ fusion protein is expected from a fusion of the gene sequences indicated in fig.9. Although expression was observed, the levels of expression were insufficient for attempts at subsequent protein purification.

3.3.3 pSF3

The fusion expression vector pMal-c used for construct pSF3 maintained compatible cloning sites with vector pSF2. Expression of the *pufQ* gene fusion in vector pSF3 was expected to yield a MBP-FX-PufQ fusion protein with a molecular

weight of 50.5 KDa. Analysis of *E. coli* TB1(pSF3) lysates by SDS-PAGE following IPTG induction indicated the presence of a protein with an M_r-value of 50 kDa in the crude cell-free extract (fig.16, lane 1).

fig.15 SDS-PAGE of crude cell-free extracts of *E. coli* strain TG2 transformed with plasmids pKK223-3 and pSF1. Crude cell-free lysates of TG2 (pKK223-3) before (lane 2) and after (lane 1) IPTG induction, and of TG2(pSF1) before (lanes 3, 5, and 7) and after (lanes 4, 6, and 8) IPTG induction are shown. Protein standards and their M_r-values are indicated.

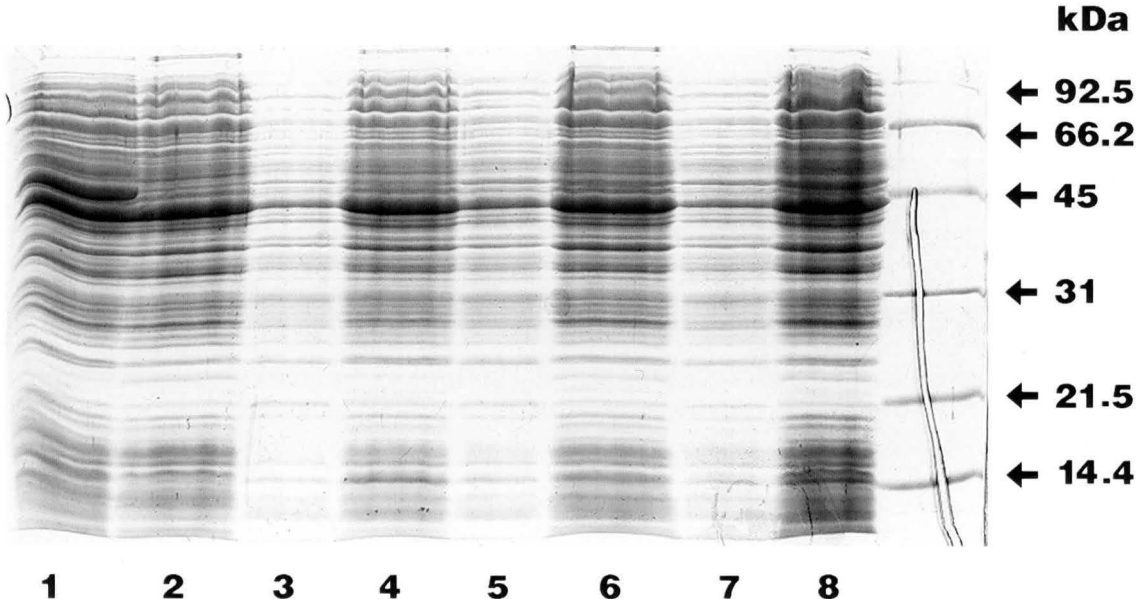

kDa
92.5
66.2
45
31
21.5
14.4
1
2
3
4
5
6
7
8

fig.16 SDS-PAGE of crude cell-free extracts of *E. coli* strain QY13 transformed with plasmids pLcII-FX and pSF2. Crude cell-free lysates from QY13(pLcII-FX) (lane 1) and QY13(pSF2) (lane 2) were analysed following heat induction. A band with a M_r of 15 kDa (corresponding to the cII-FX-PufQ fusion protein) is marked in lane 2.

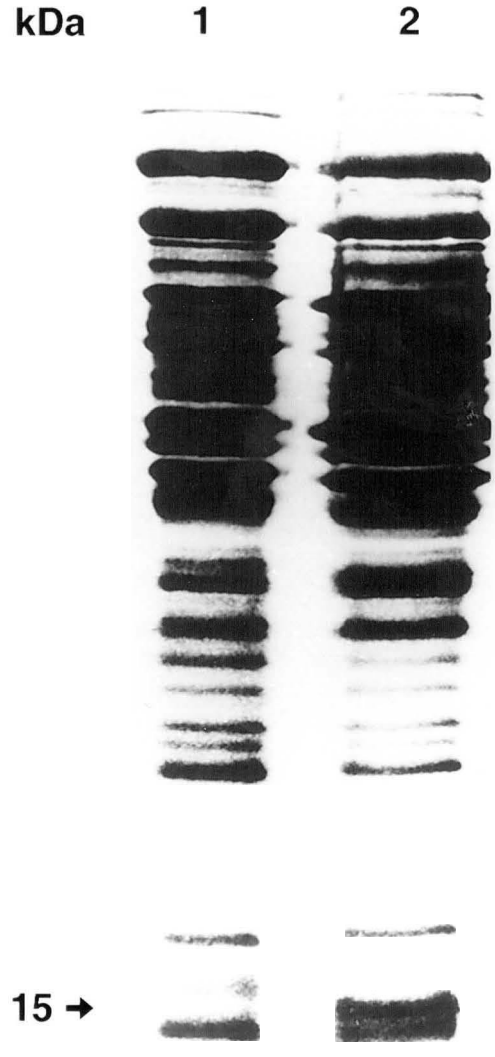
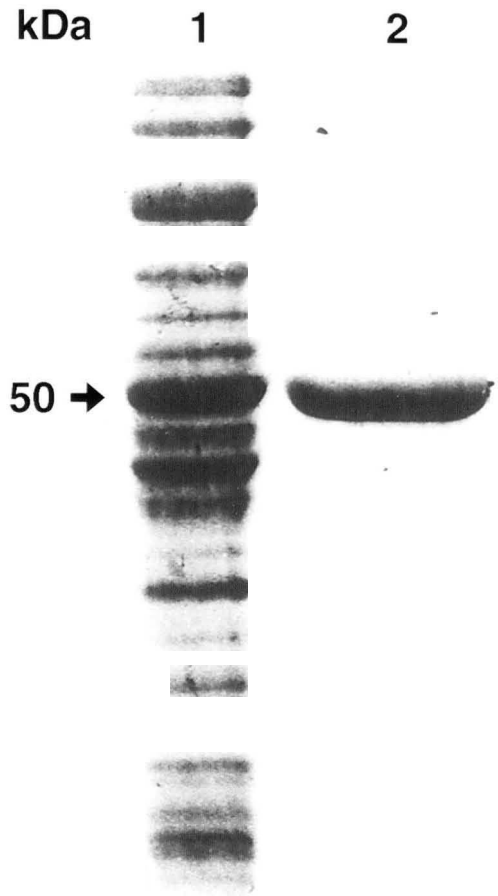

kDa
1
2
15 →

fig 17 SDS-PAGE of crude cell extracts of *E. coli* TB1 transformed with plasmid pSF3 before and after affinity chromatography. Following induction with IPTG, crude cell lysates were analysed before (lane 1) and after (lane 2) purification on an amylose affinity column (section 2.10.1). A band with a M_r of 50 kDa (corresponding to the MBP-FX-PufQ fusion protein) is marked for both lanes.


kDa
1
2
50

3.4 Isolation and purification of the PufQ protein

A large scale (15 L) growth of *E.coli* TB1(pSF3) was carried out (section 2.9.4), and the overexpressed fusion protein purified by affinity chromatography on an amylose column (section 2.10.1). Elution of proteins from the column was monitored by measuring the A_{280} and is shown in fig.17. A single peak was detected in the void volume following the addition of 10 mM maltose. SDS-PAGE analysis of this peak revealed a single band with a M_r-value of 50 kDa (fig.16, lane 2). Western blot analysis using rabbit anti-MBP serum demonstrated that the overexpressed fusion protein contained the maltose-binding domain (fig.18).

The MBP-FX-PufQ fusion protein was hydrolyzed by the sequence-specific Factor X_a protease, which was expected to cleave exactly at the N-terminal methionine residue of the PufQ protein. SDS-PAGE analysis of the hydrolysate indicated that the hydrolysis had gone essentially to completion, since very little unhydrolyzed fusion protein was visible with the Coomassie blue stain (fig.24, lane C).

The digested fusion protein was purified by HPLC on a C4 reversed phase column as described in section 2.10.4. The MBP eluted from the HPLC column in the 40-60 % (v/v) acetonitrile gradient at 14.3 min. Peak fractions collected at 68 and 73 min during elution with isocratic 60 % (v/v) acetonitrile contained small polypeptides of similar, although not identical, M_r-values of approximately 7600, 8700 Da respectively, as determined by SDS-PAGE (fig.19, inset). Five cycles of N-terminal sequence analysis were performed with each of the polypeptides. The sequence of the 68 min peak fraction was identical to the first 5 amino acids fo the PufQ protein, while the 73 min peak fraction contained a polypeptide derived from the PufQ protein by the deletion of the first 7 amino acids (referred to as Δ_{1-7}PufQ).

fig.18 Affinity chromatography of MBP-FX-PufQ fusion protein. Following induction, the crude cell-free lysate from TB1(pSF3) was applied on an amylose column and the elution of proteins monitored by measuring the A_{280}. The MBP-FX-PufQ fusion protein eluted in the void volume after addition of maltose (indicated by the arrow) to the column buffer and was collected in the fraction indicated by the shaded peak.

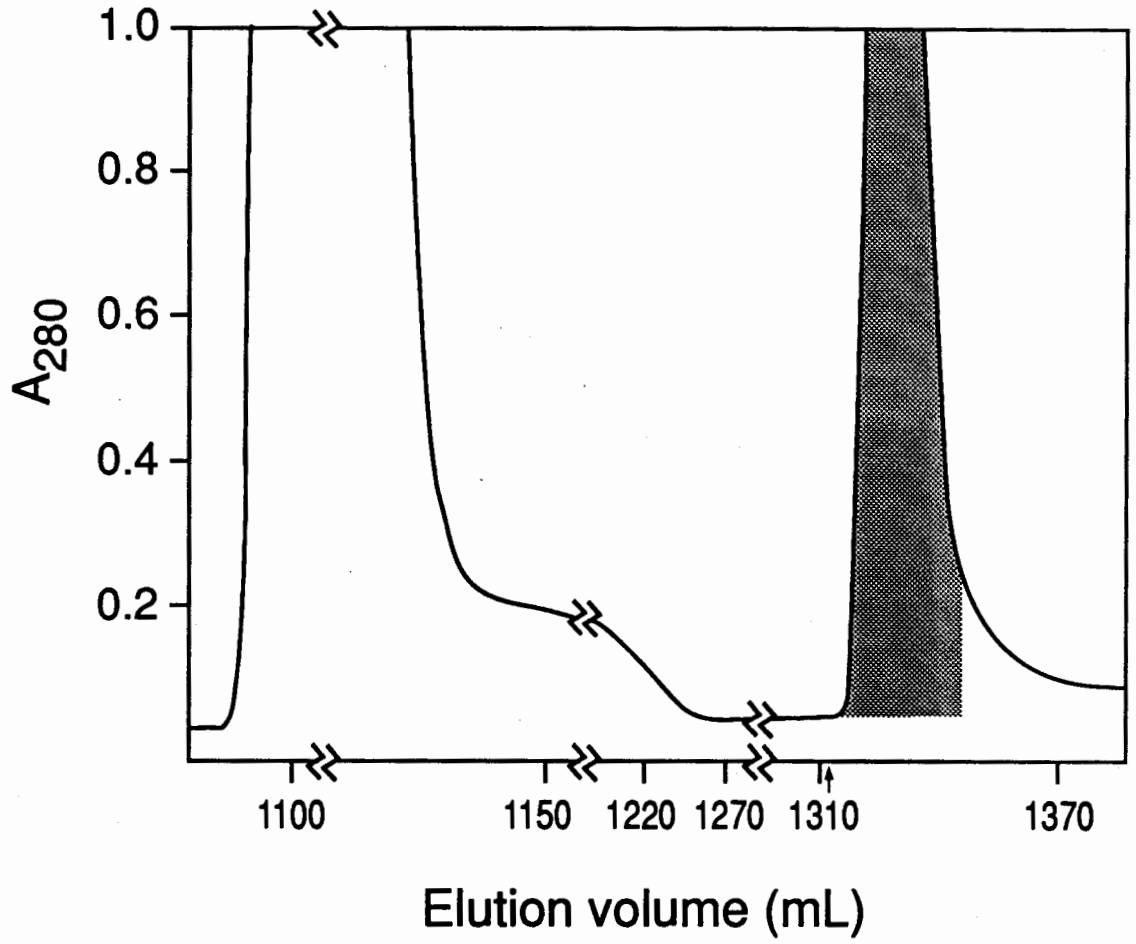

1.0
0.8
0.6
0.4
0.2
A_{280}
1100
1150
1220
1270
1310
1370
Elution volume (mL)

fig.19 Western immunoblot analysis of the maltose-binding domain of MBP and MBP fusion proteins. The following proteins were analysed with rabbit anti-MBP serum. Lane 1, MBP-FX-PufQ fusion protein; lane 2, MBP-FX-β-galactosidase fusion protein; lane 3, MBP. Unconjugated MBP was prepared by digestion of MBP-FX-PufQ fusion protein with Factor X_a protease, and it and the two fusion proteins were purified by affinity chromatography as described in section 2.10.1.

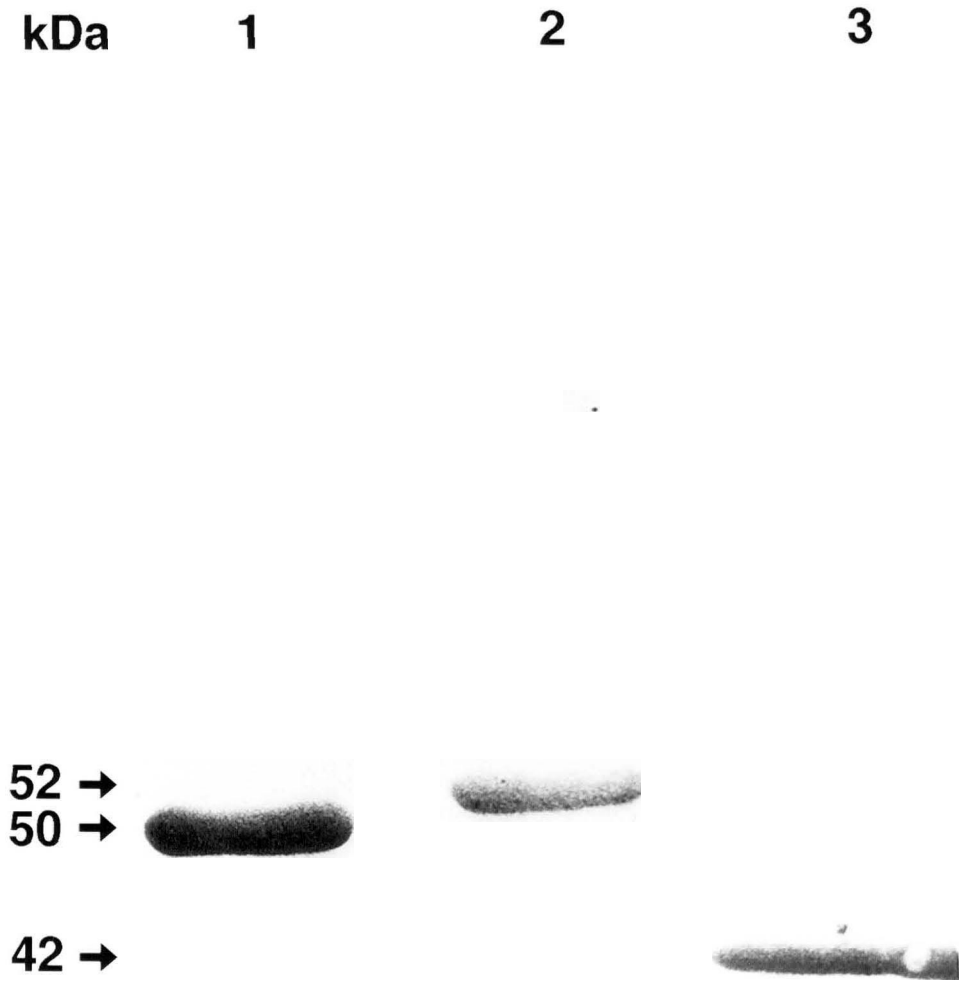
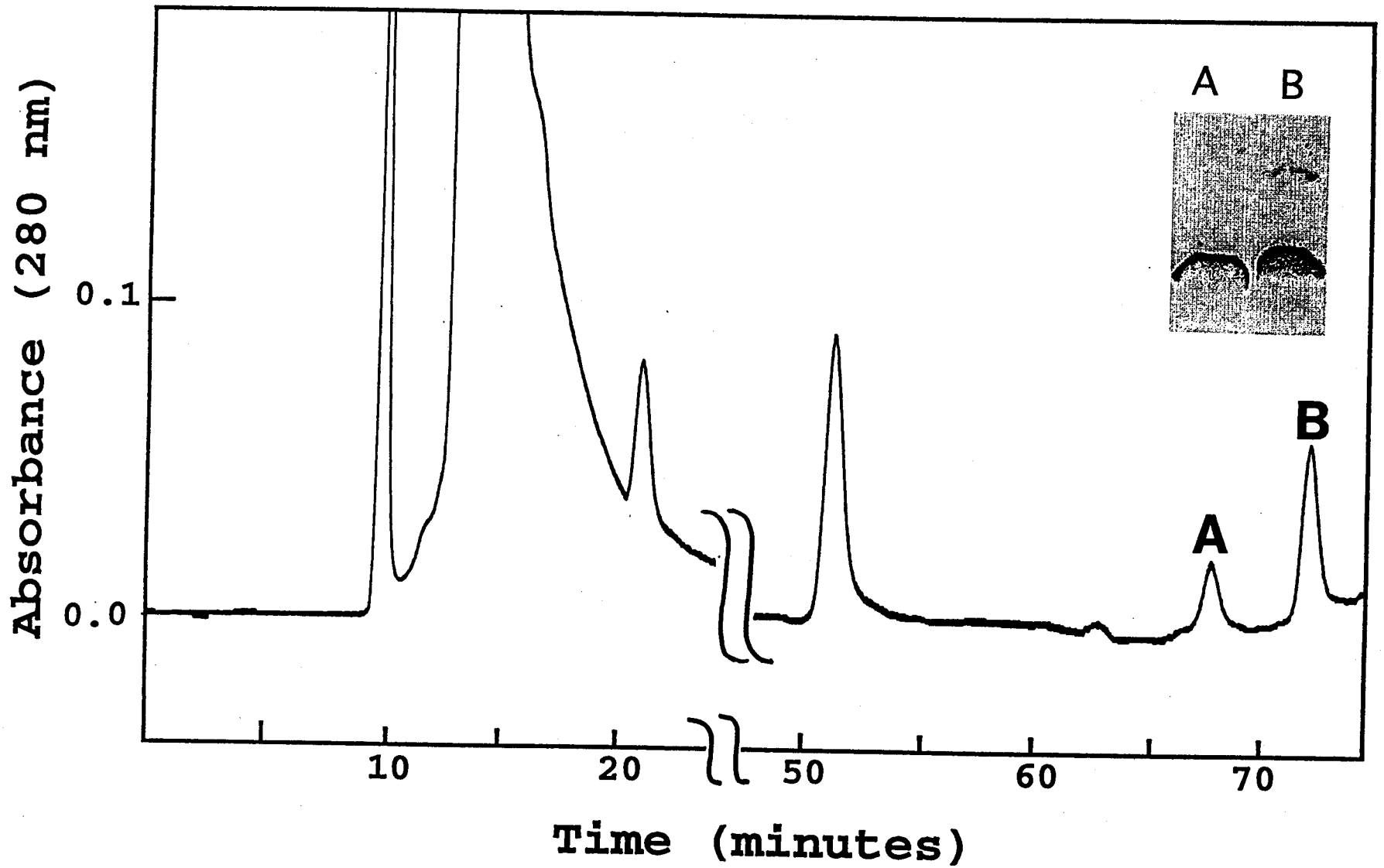

kDa
1
2
3
52
50
42

fig.20 Purification of MBP, PufQ protein, and Δ_{1-7}PufQ by HPLC. Samples of MBP-FX-PufQ fusion protein digested with Factor X_a protease were solubilized and run on a Vydac C4 reversed-phase HPLC column (section 2.10.4). Peaks which were identified contained MBP (14.3 min) and either PufQ protein (68 min) (B) or Δ_{1-7}PufQ (A). The inset shows the analysis of the two latter fractions by SDS-PAGE.


Absorbance (280 nm)
0.1
0.0
10
20
50
60
70
Time (minutes)
A
B
A
B

3.5 Purification of rabbit anti-MBP-FX-PufQ immunoglobulin G

Antiserum proteins were precipitated by ammonium sulfate and the anti-MBP-FX-PufQ IgG purified by FPLC on a Mono Q anion-exchange column. Eluted proteins were monitored by measuring the absorbance at 280 nm. The FPLC chromatogram is shown in fig.21. A major peak was detected at a concentration of 55 mM NaCl. SDS-PAGE analysis of this peak revealed the presence of two bands having M_r-values of approximately 55 kDa and 25 kDa (fig.22), corresponding to the H and the L antibody chain fragments due to the reduction of disulfide bonds. A Bradford micro-assay (16) was performed on the peak fraction and the antibody concentration was determined to be 0.176 mg mL^{-1}. The activity of the purified IgG against PufQ protein was determined by ELISA analyses. ELISA plates containing equal amounts (1.0 μg) of either MBP-FX-PufQ fusion protein or MBP were compared for their activity against the purified IgG (see table below). An approximate 4 to 7-fold increase in activity towards the fusion protein over MBP was found, which was presumed to be due to antibody recognition of the PufQ protein. As seen from the table, the absorbance ratios of MBP to MBP-FX-PufQ fusion protein are approximately constant for antibody dilutions of $2x10^{-4}$ and $1x10^{-4}$. The ratio however deviates significantly when the antibody is further diluted to $5x10^{-5}$, probably as a result of non-linear absorbance values at the low antibody concentration.

fig.21 Purification of anti-MBP-FX-PufQ IgG by FPLC. Anti-serum proteins were separated by FPLC on a Mono Q (sepharose) column. ELISA analysis of peak fractions indicated that anti-MBP-FX-PufQ IgG eluted at a salt concentration of 55 mM NaCl. The hatched peak denotes the fraction that was collected.

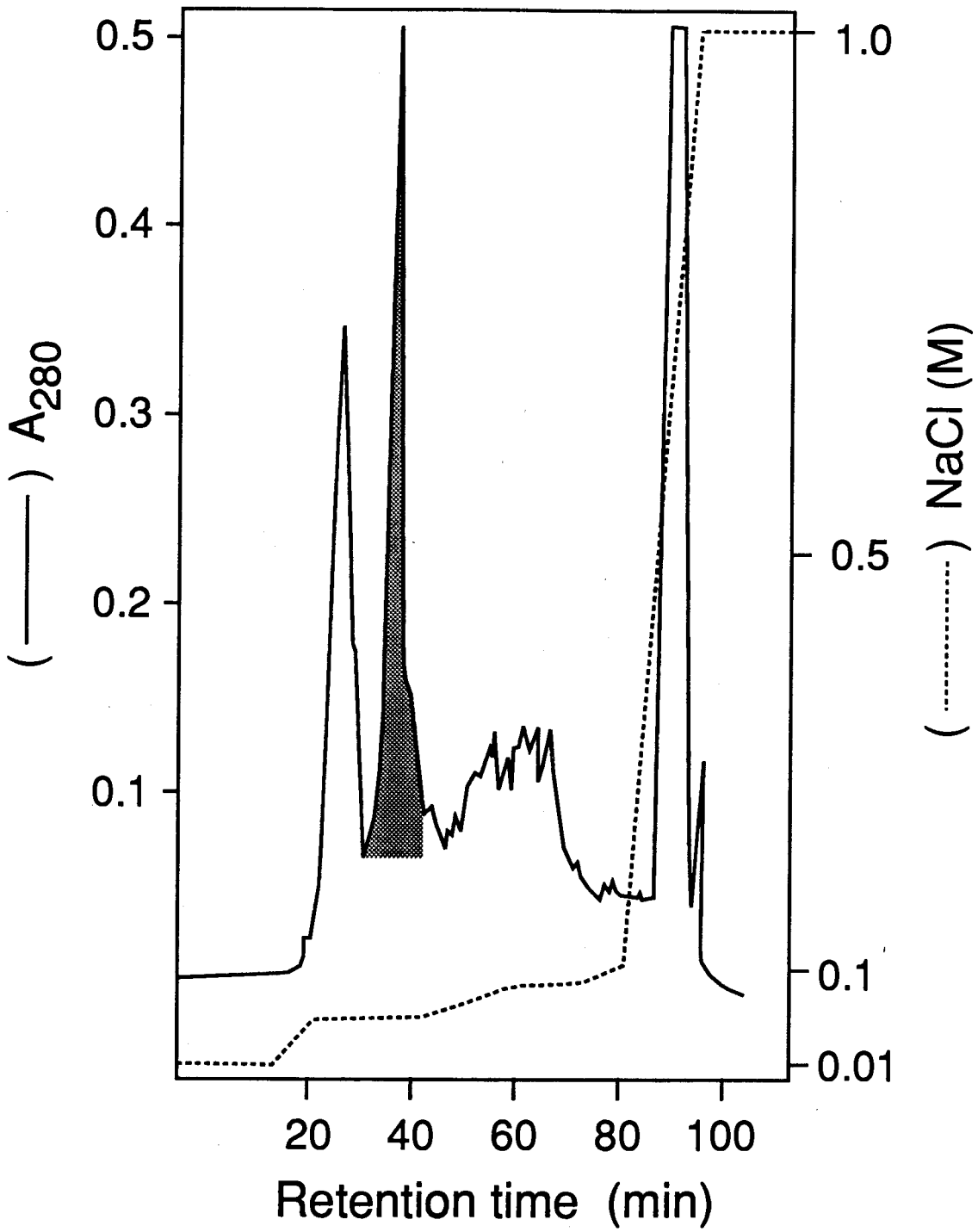

0.5
0.4
0.3
0.2
0.1
(——) A_{280}
1.0
0.5
0.1
0.01
(------) NaCl (M)
20
40
60
80
100
Retention time (min)

fig.22 SDS-PAGE of the peak fraction collected by FPLC. Aliquots of 10 μL (lane1) and 30 μL (lanes 2,3) of the anti-MBP-FX-PufQ IgG fraction collected in fig.21 were analysed by SDS-PAGE. The upper and lower bands have relative M_r-values of 55 and 25 kDa respectively. Protein standards with the indicated M_r-values are shown.

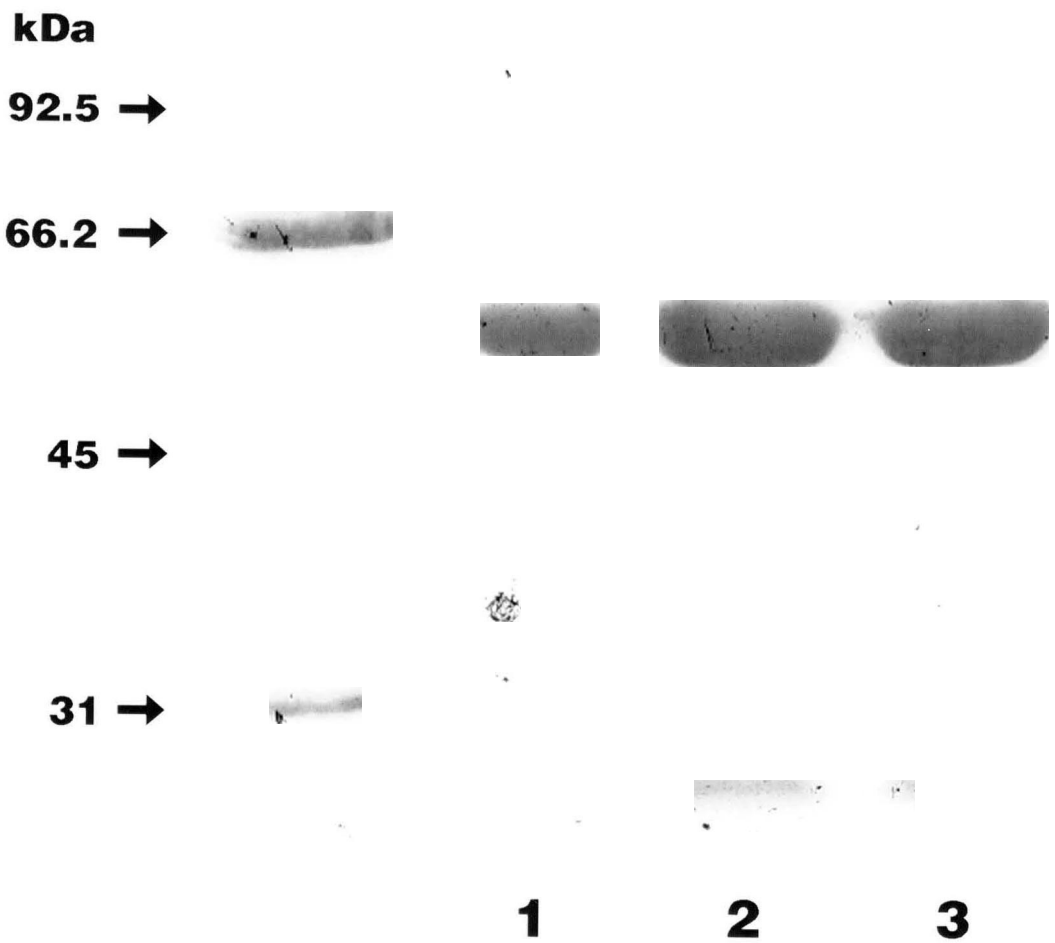

kDa
92.5
66.2
45
31
1
2
3

Table of ELISA plate absorbance values

Antibody dilution	Absorbance			Ratio of MBP-PufQ/MBP absorbances
	MBP	MBP-PufQ fusion protein	none	
2×10^{-4}	0.172	0.628	0.028	4.17
1×10^{-4}	0.098	0.376	0.027	4.92
5×10^{-5}	0.056	0.212	0.031	7.24

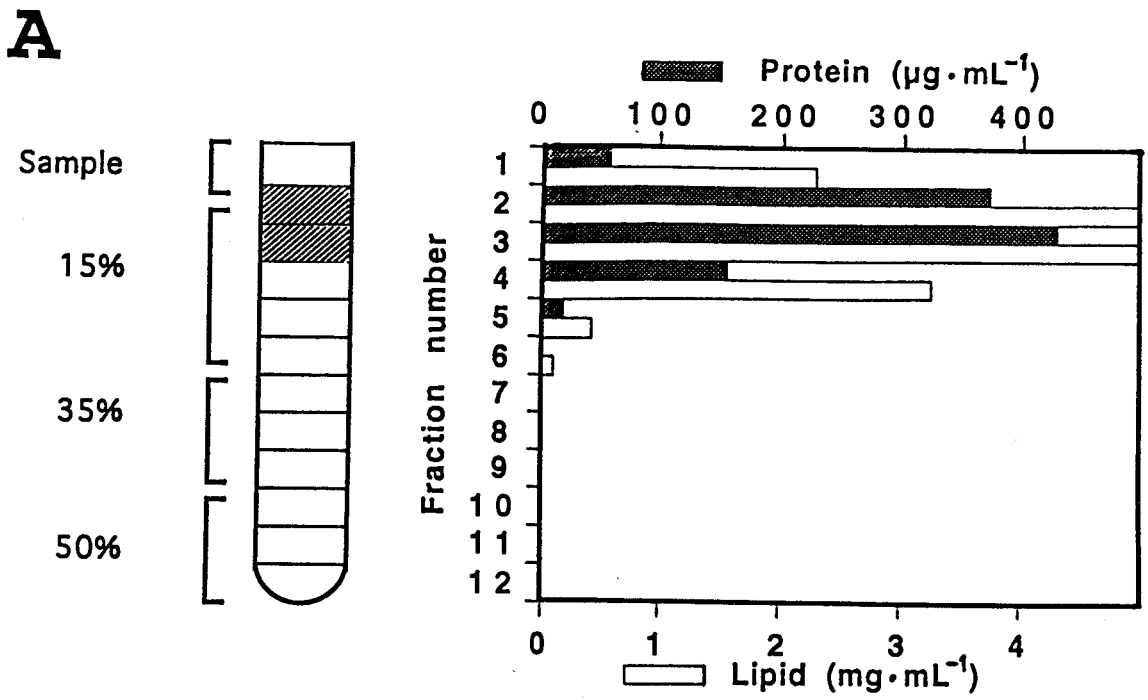
3.6 Binding of the PufQ protein to liposomes

Although the MBP-FX-PufQ fusion protein was soluble in aqueous buffers, the isolated PufQ protein was completely insoluble unless sodium cholate was present. It was also seen to be soluble in phospholipid vesicles after digestion of the fusion protein under conditions whereby the fusion protein and soybean phospholipids were first sonicated together. However, complete digestion of the fusion protein could not be achieved under these conditions, presumably because some of the protease hydrolysis sites were on the inside of the vesicles. If the fusion protein was added to pre-formed liposomes prior to digestion with factor X_a protease, the PufQ protein aggregated during digestion due to poor incorporation into liposomes.

In order to maintain the solubility of PufQ protein while ensuring complete proteolytic digestion, preformed ^{3}H-labeled liposomes were added to cholate micelles of the digested fusion protein. Following dialysis of the cholate, the liposomes and

protein were centrifuged on step gradients of sucrose as described in section 2.13.2. The protein and phospholipid profiles of fractions from this sucrose gradient (fig.23) were compared to a control gradient containing radiolabeled liposomes and unconjugated MBP only. When gradient fractions were compared, only the gradient containing hydrolyzed MBP-FX-PufQ fusion protein contained a band at the 35% sucrose interface (fig.23B, fraction 7). This band was found to contain a significant amount of both phospholipid and protein. SDS-PAGE analysis of this fraction indicated the presence of only PufQ protein and Δ_{1-7}PufQ (fig.24, lane7).

fig.23 Sucrose density gradient centrifugation of phospholipid vesicles reconstituted with either unconjugated MBP (A) or hydrolyzed MBP-FX-PufQ fusion protein (B). Sucrose gradient fractions were analysed for protein (shaded bars) and phospholipid (open bars) content. The protein content of protein-containing fractions of the tube containing the hydrolyzed fusion protein was analysed by SDS-PAGE (see fig.24).


A
Sample
15%
35%
50%
Protein (μg·mL⁻¹)
0 100 200 300 400
Fraction number
1 2 3 4 5 6 7 8 9 10 11 12
0 1 2 3 4
Lipid (mg·mL⁻¹)

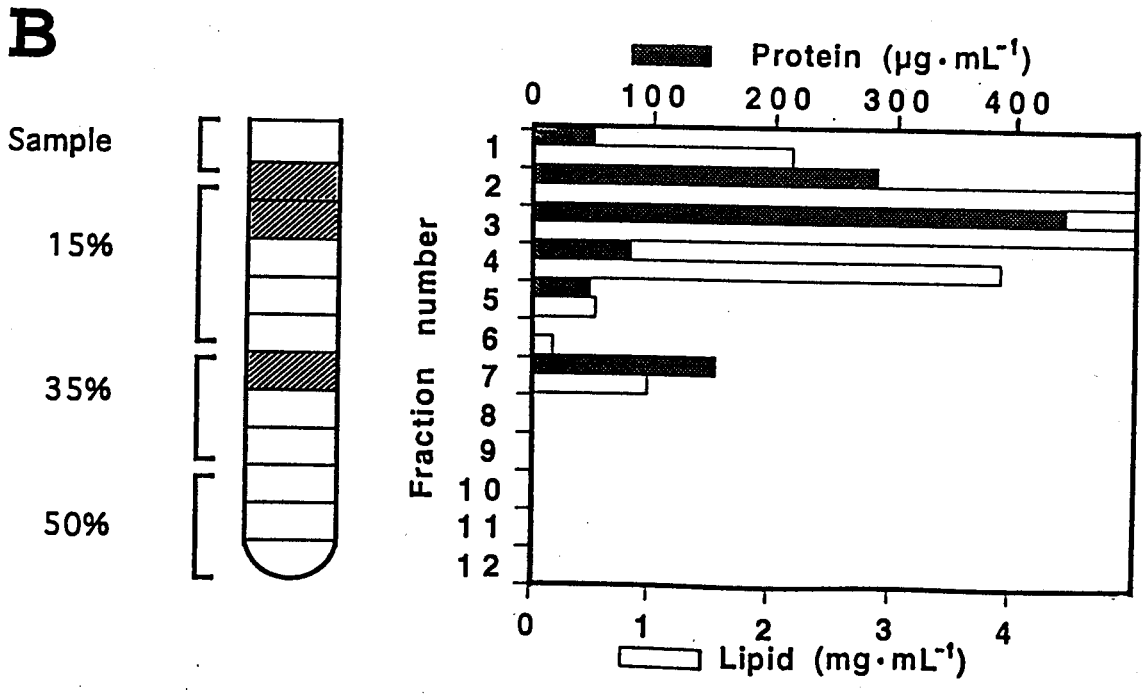
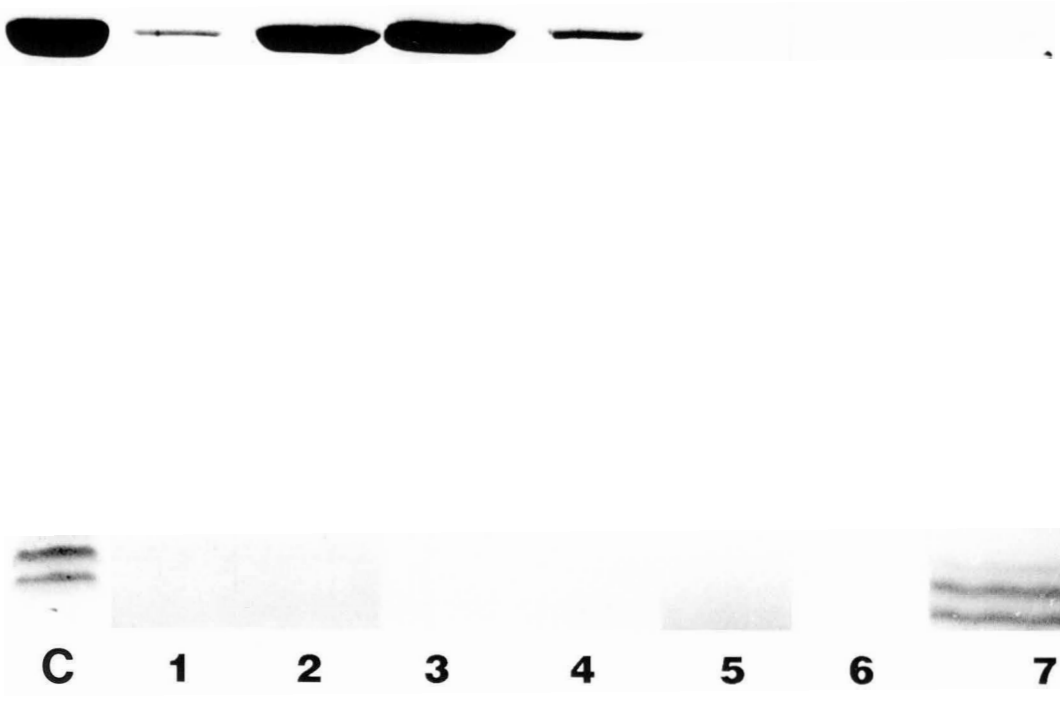

B
Sample
15%
35%
50%
Protein (μg·mL⁻¹)
0 100 200 300 400
Fraction number
1 2 3 4 5 6 7 8 9 10 11 12
0 1 2 3 4
Lipid (mg·mL⁻¹)

fig.24 SDS-PAGE of selected sucrose density gradient fractions. Protein-containing fractions from a sucrose gradient tube containing hydrolyzed MBP-FX-PufQ fusion protein and phospholipid vesicles (fig.23B) were analysed by SDS-PAGE. Lanes 1 to 7 correspond to fractions 1 to 7 in fig. 23B. The top fractions (lanes 2 to 4) located near the 15 % sucrose boundary are seen to contain the MBP only, while the PufQ protein and Δ_{1-7}PufQ appear in the fraction (lane 7) located at the 35 % sucrose boundary. A control sample (lane C) containing the unfractionated hydrolyzed fusion protein is also shown.


C
1
2
3
4
5
6
7

3.7 Binding of Pchlide to liposome-reconstituted PufQ protein

^{14}C-labeled Pchlide was prepared from etiolated wheat as described in section 2.13.3. The absorption maxima at 432 and 630 nm of the Pchlide spectrum in ether was characteristic of Mg-bound Pchlide (fig.25). The Pchlide was made soluble in aqueous solution by the addition of sodium cholate (section 2.13.4). This solution was then added to mixtures of preformed liposomes containing either the digested fusion protein also solubilized in sodium cholate or Apolipoprotein A-I. Following dialysis of the cholate, the samples were centrifuged on continuous gradients of sucrose as described in section 2.13.5. The protein, phospholipid, and Pchlide profiles of each of the fractions from the sucrose gradients were calculated from gradients containing either ^{3}H-labeled liposomes and unlabeled Pchlide, or ^{14}C-labeled Pchlide and unlabeled liposomes. The results indicated a clear increase in the levels of phospholipid and Pchlide at sucrose concentrations of 23-32% (fig.26 C) when compared to the corresponding fractions in the control gradient (fig.26 A). Subsequent Western immunoblot analysis of these fractions (data not shown) revealed the presence of predominantly PufQ protein.

fig.25 Absorption spectrum of ^{14}C-labeled Pchlide in ether. Pchlide was labeled using ^{14}C-ALA and isolated as described in section 2.13.3. Two peaks with absorption maxima at 434 and 630 nm are marked. The Pchlide concentration was calculated using an extinction coefficient of 289 $mM^{-1}cm^{-1}$ at 432 nm.

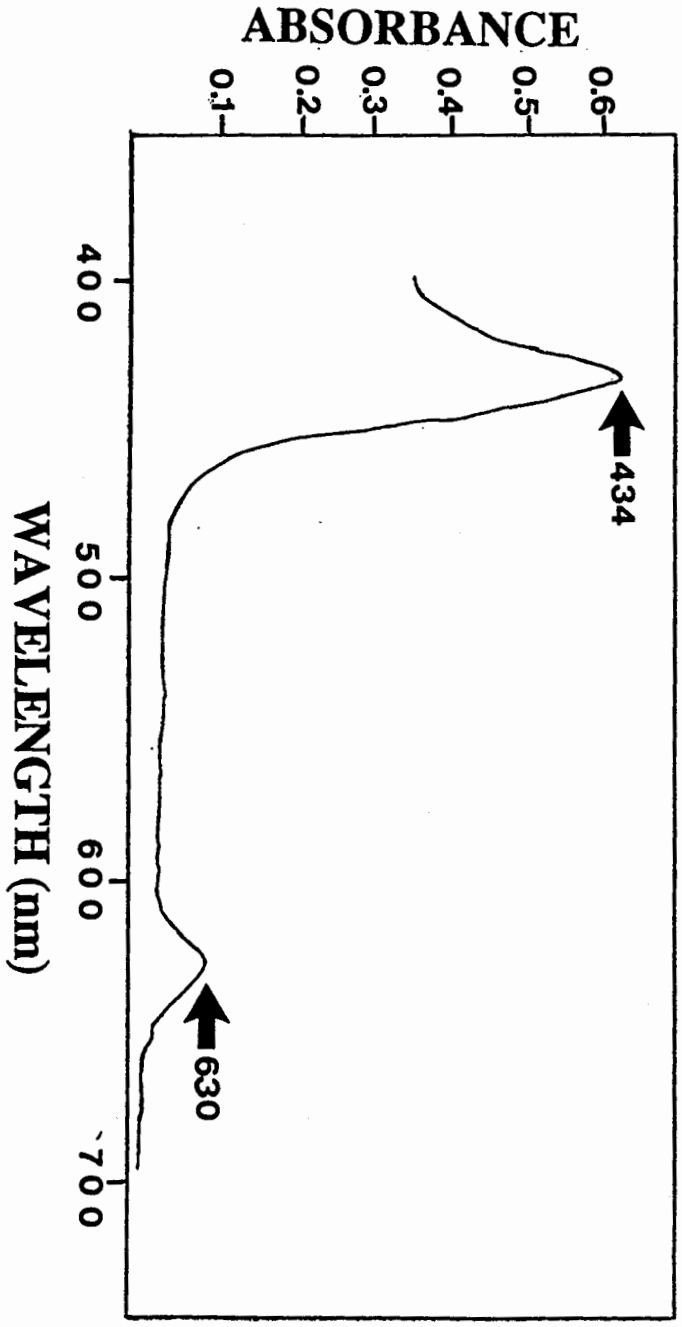
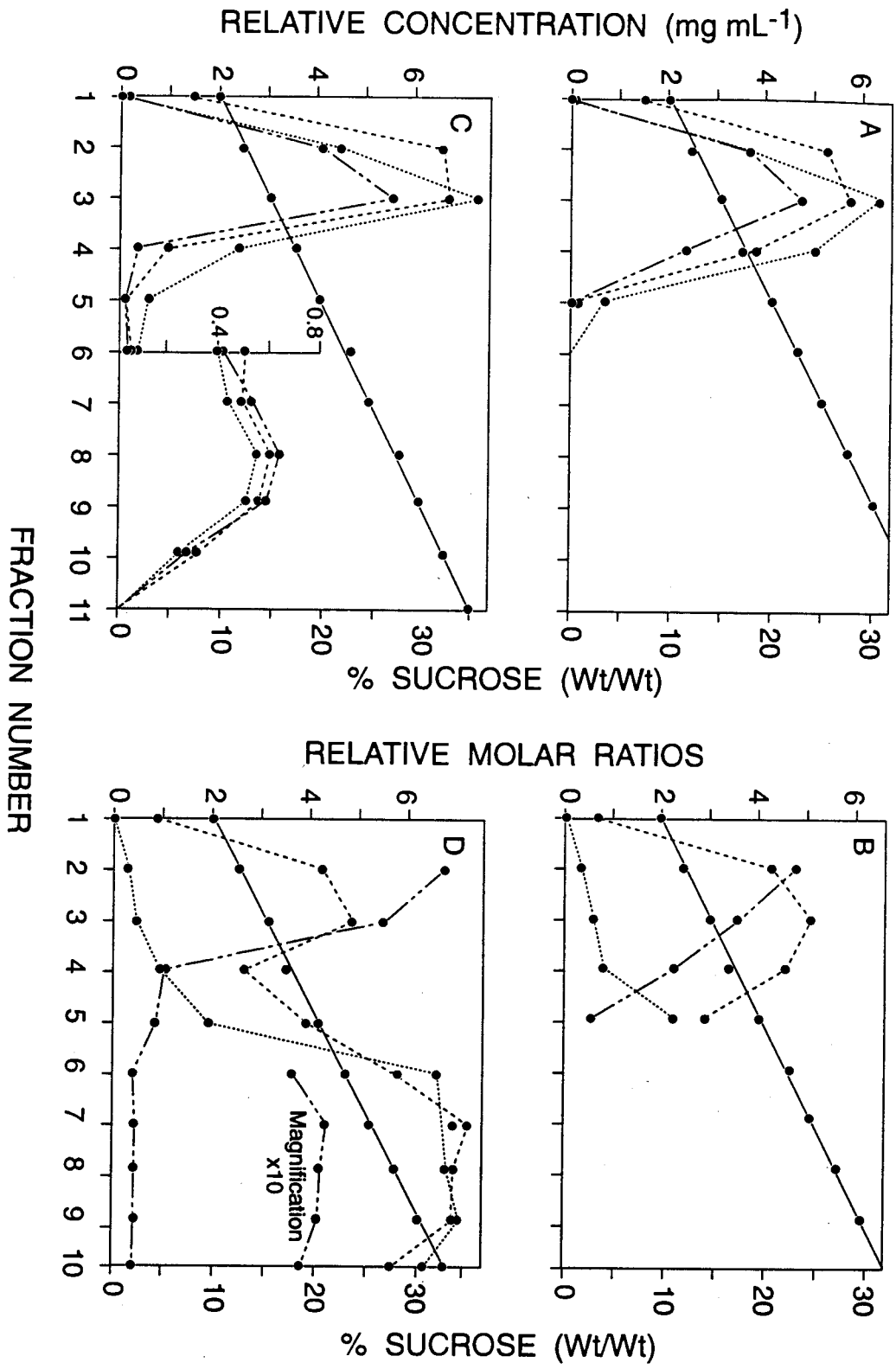

ABSORBANCE
0.1
0.2
0.3
0.4
0.5
0.6
400
500
600
700
WAVELENGTH (nm)
434
630

fig.26 Sucrose density gradient centrifugation of phospholipid vesicles reconstituted with Pchlide and either Apolipoprotein A-I (A and B) or hydrolyzed MBP-FX-PufQ fusion protein (C and D). In figures A and C, fractions from a linear gradient of sucrose (———) were analysed for lipid (x 0.5) (------), protein (x 10) (·····), and Pchlide (x 10^3) (·———·). The relative concentrations (mg mL^{-1}) of each of the components in fractions 1 to 11 are shown. In figures B and D, the relative molar ratios of Pchlide/lipid (x 10^4) (------), protein/lipid (x 500) (·····), and Pchlide/protein (x 10) (·———·) for each of the gradient fractions analysed are shown. In all of the above figures, the data were multiplied (x) by the factors indicated before being plotted on the relative scales.


RELATIVE CONCENTRATION (mg mL-1)
A
B
C
D
0.8
0.4
Magnification x10
% SUCROSE (Wt/Wt)
RELATIVE MOLAR RATIOS
% SUCROSE (Wt/Wt)
FRACTION NUMBER

3.8 Fluorescence analysis of Pchlide-containing gradient fractions

Mixtures of either liposomes, Pchlide, and the hydrolysed MBP-FX-PufQ fusion protein or liposomes, Pchlide, and Apolipoprotein A-I were centrifuged on linear gradients of sucrose and fractionated as described in section 2.13.5. Aliquots of each of the fractions were diluted with Buffer C until their A_{434} was < 0.05 and their fluorescence emission spectra recorded. The diluted fractions exhibited approximately equal fluorescence emission intensities. Representative spectra containing either Apolipoprotein A-I, MBP, or PufQ protein (as determined by either SDS-PAGE or Western blot), and that also contained significant amounts of phospholipid and Pchlide (as determined by either ^{3}H or ^{14}C counts) were overlayed for comparison (fig.27). A 3 nm blue shift in the emission maximum from 642 to 639 nm was detected only in the fractions from the gradient containing PufQ protein. In all of the fractions from control gradients containing Apolipoprotein A-I or MBP, the emission wavelength maximum remained unchanged at 642 nm.

3.9 Immunoblotting of *R.capsulatus* subcellular fractions

3.9.1 Western blot analysis of crude chromatophores from phototrophically grown *R.capsulatus*

FPLC purified rabbit polyclonal antibodies raised against the MBP-FX-PufQ fusion protein were used to detect the presence of the PufQ protein in phototrophically grown *R.capsulatus*. A band corresponding to PufQ protein was detected both in uncentrifuged homogenates and in crude chromatophore preparations (fig.28). In contrast, no PufQ protein was detected in the supernatant following sedimentation of the chromatophore fraction (data not shown).

fig.27 Fluorescence emission spectra of selected sucrose density gradient fractions. Fractions from sucrose gradient tubes containing Pchlide, phospholipid vesicles, and either PufQ protein, MBP, or Apolipoprotein A-I were analysed by fluorescence spectroscopy. Samples were excited at a wavelength of 454 nm and the spectra overlayed for comparison. A 3 nm blue shift of the PufQ protein-containing fraction is shown.

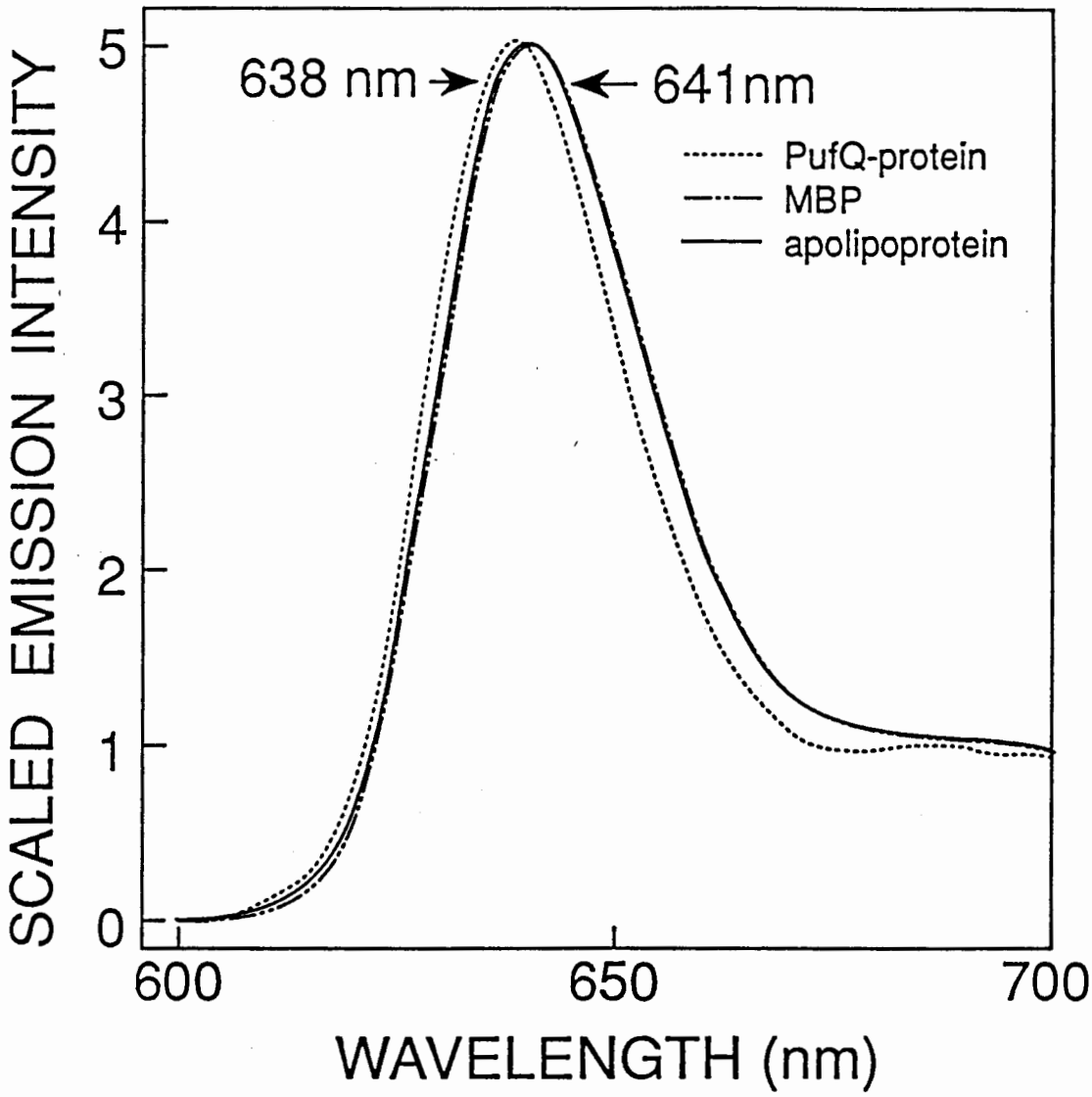
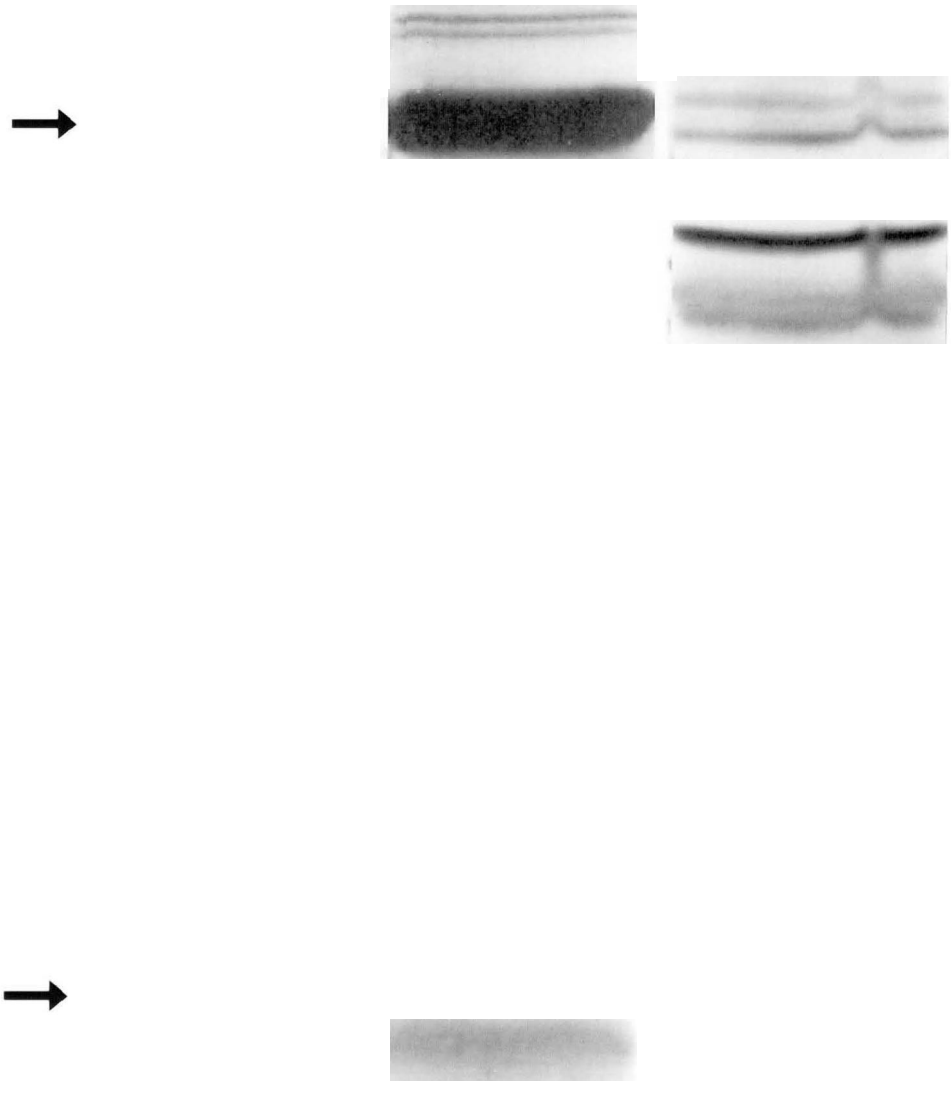

638 nm
641nm
PufQ-protein
MBP
apolipoprotein
SCALED EMISSION INTENSITY
0
1
2
3
4
5
600
650
700
WAVELENGTH (nm)

fig.28 Western immunoblot analysis of *R. capsulatus* cells. Samples of a crude cell-free lysate (lane 3) and a crude chromatophore preparation (lane 1) from phototrophically grown *R. capsulatus* cells were electrophoresed, blotted, and probed using anti-MBP-FX-PufQ IgG as described in section 2.12.4. A control sample containing partially hydrolyzed MBP-FX-PufQ fusion protein (lane 2) was used to identify the PufQ protein (indicated by the lower arrow) and MBP (indicated by the top arrow).


1
2
3

3.9.2 Western blot analysis of subcellular fractions from *R.capsulatus* adapting from aerobic to phototrophic growth conditions

Adaptation experiments were carried out with a mid-log phase culture of *R.capsulatus* cells in which the growth conditions were shifted from semi-aerobic to semi-anaerobic by varying the stir rate and rate of air flow in the culture medium. The growth curve and the levels of Bchl synthesised under these conditions is shown in fig.29. Cells were harvested at various time intervals following induction (0h, 3h, 4h, 34h) and the cell lysate of each fractionated by sucrose density gradient centrifugation (section 2.11.5). Fractions corresponding to the proteins that were soluble, or located either in the chromatophore precursors or in fully developed chromatophores were resolved by SDS-PAGE (fig.30 A and fig.31 A) and probed for PufQ protein expression by Western blot analysis (fig.30 B and fig.31 B). From the results of the Western blot, a band corresponding in molecular weight to the PufQ protein first appears in the UPB (lanes 2, 3, 4 in fig.30 B) and the chromatophore fractions (lanes 3, 4, 5 in fig.31 B) after 3h of induction, and continues to increase over the 34h time interval. No PufQ protein was ever detected in the soluble fractions.

fig.29 Adaptation of *R. capsulatus* from aerobic to phototrophic growth conditions. The growth of cells (—○—) was compared to the levels of Bchl (—□—), and the specific activity of MT in the chromatophore fraction (—●—) after oxygen induction (beginning at time zero). Cell growth and Bchl synthesis were monitored by measuring the A_{680} and A_{860} respectively.

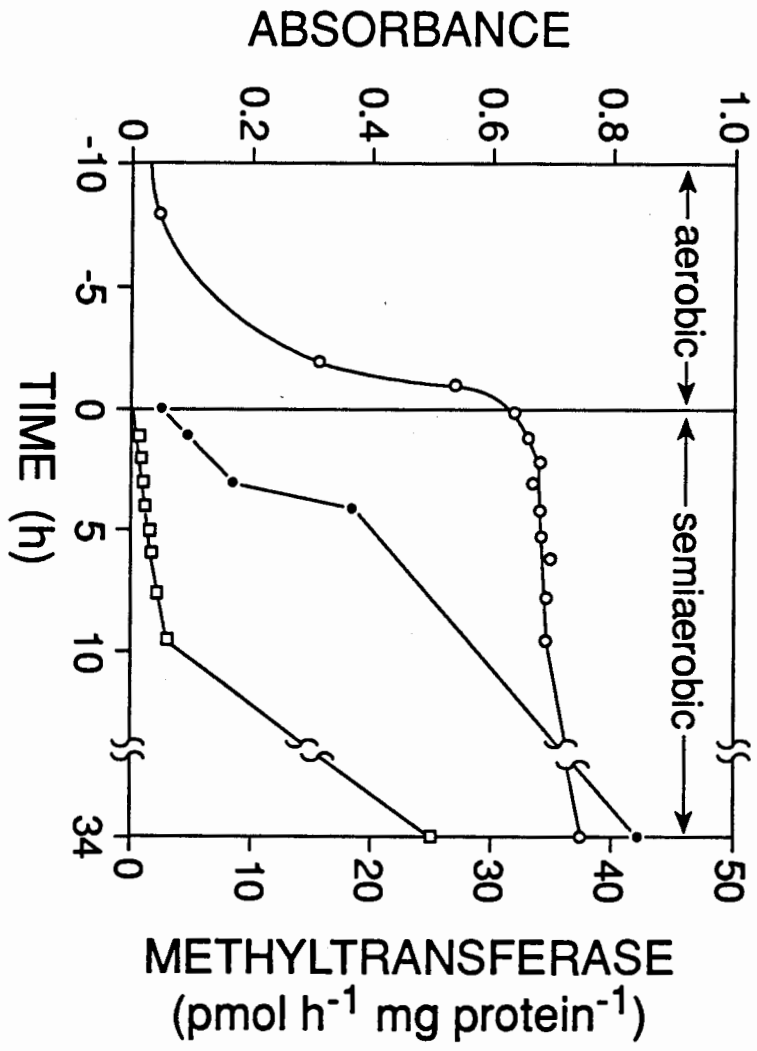
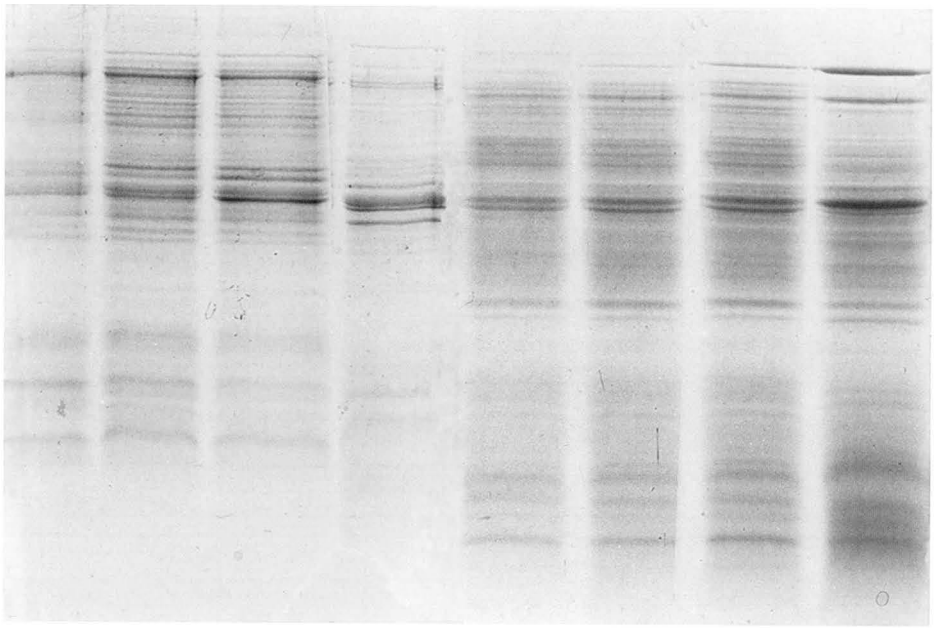

ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
aerobic
semiaerobic
TIME (h)
-10
-5
0
5
10
34
0
10
20
30
40
50
METHYLTRANSFERASE
(pmol h-1 mg protein-1)

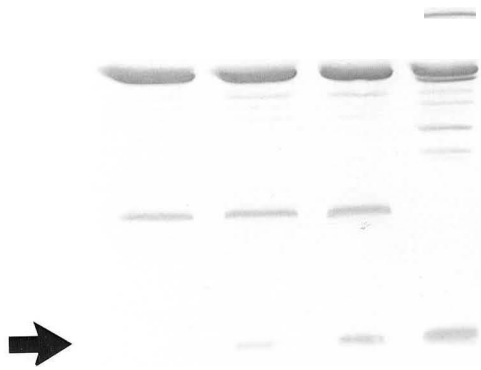
fig.30 SDS-PAGE (A) and Western immunoblot (B) analyses of the UPB and soluble fractions of *R. capsulatus* during adaptive growth. The UPB (lanes 1-4) and soluble (lanes 5-8) fractions were analysed at 0hr (lanes1,5), 3hr (lanes 2,6), 4hr (lanes 3,7), and 34hr (lanes 4,8) following anaerobic induction. The arrow in (B) shows the appearance of a band corresponding in molecular weight to the PufQ protein.

A



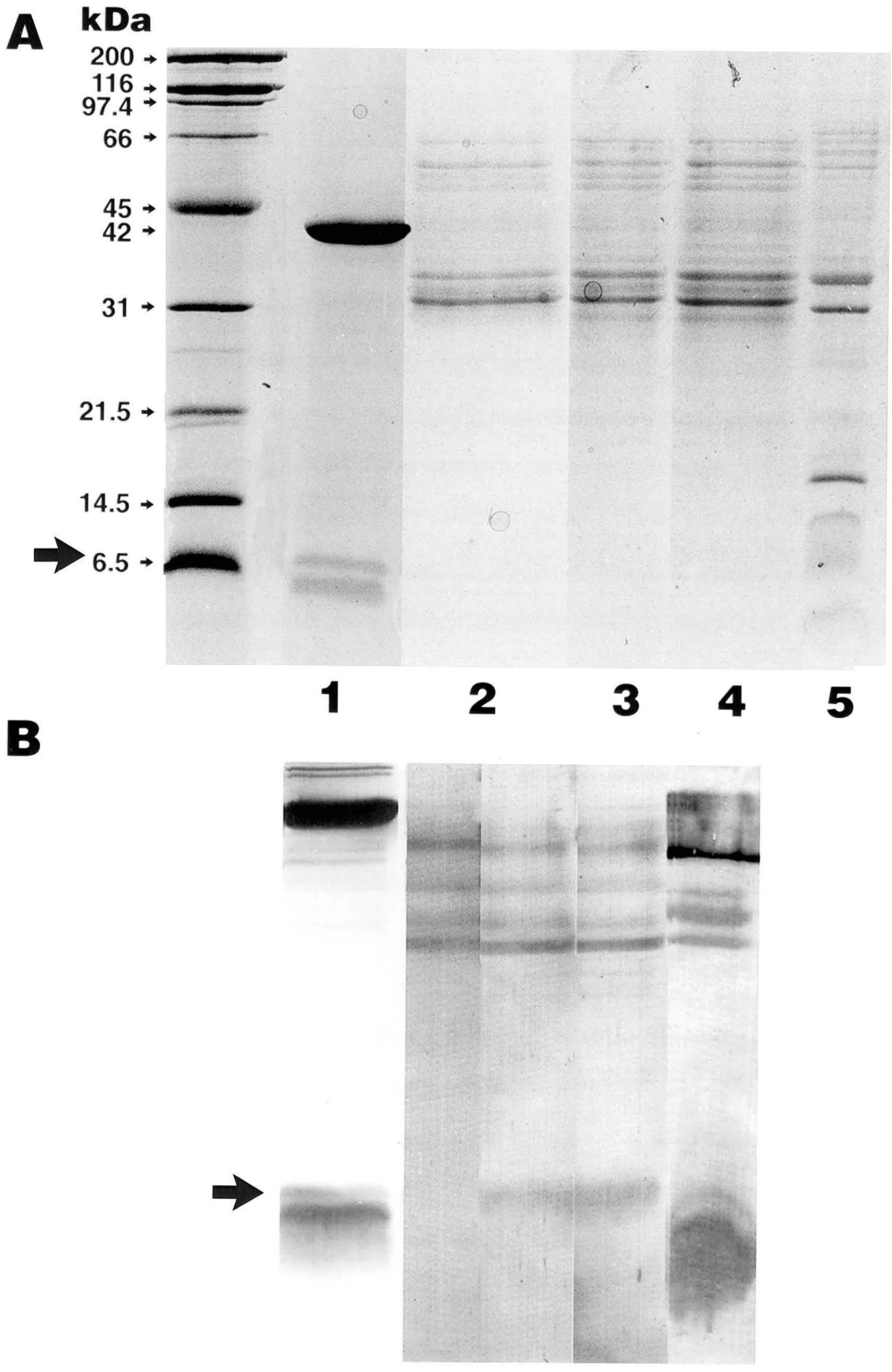
1 2 3 4 5 6 7 8

B



1 2 3 4 5 6 7 8

fig.31 SDS-PAGE (A) and Western immunoblot using anti-MBP-FX-PufQ IgG (B) analyses of the chromatophore fractions of *R. capsulatus* during adaptive growth. The chromatophore (lanes 2-5) fractions were analysed at 0hr (lane 2), 3hr (lane 3), 4hr (lane4), and 34hr (lane 5) following anaerobic induction. A control sample containing hydrolyzed MBP-FX-PufQ fusion protein (lane 1) in figures (A) and (B) was included for identifying PufQ protein (as shown by the arrow). Protein standards and their M_r-values are indicated on the far left lane of figure (A).


A
kDa
200
116
97.4
66
45
42
31
21.5
14.5
6.5
1
2
3
4
5
B
1
2
3
4
5

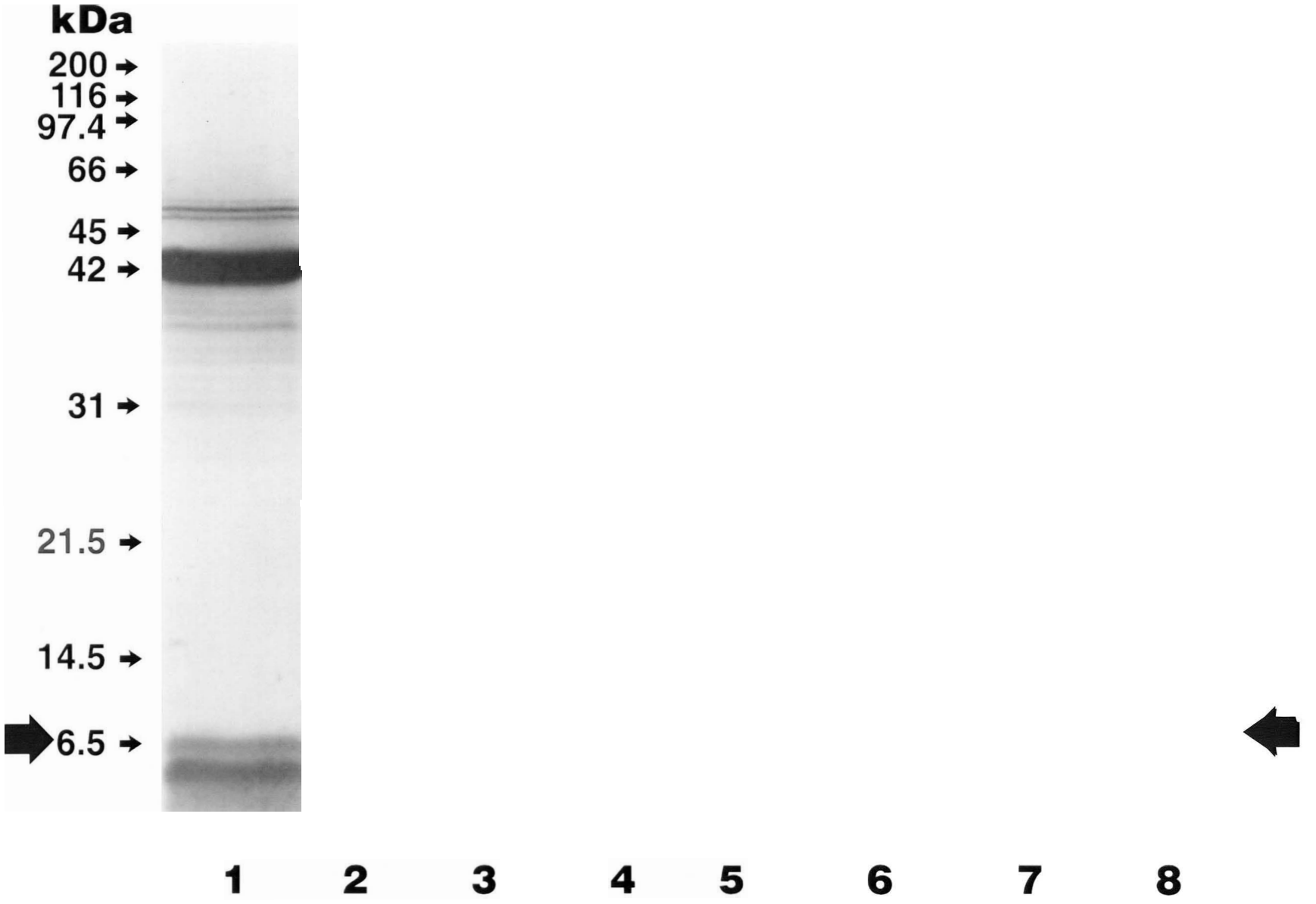
3.9.3 Western blot analysis of subcellular fractions of mutant *R. capsulatus* strains

While the analysis of *R. capsulatus* cellular fractions demonstrated the presence of a protein corresponding in molecular weight to PufQ (section 3.9.2), bands other than that due to PufQ protein were also visible on the Western blots. In order to resolve whether these higher molecular weight bands were specific to PufQ protein (ie- as either PufQ protein aggregates or as a tightly associated PufQ protein-containing complex), the cell envelope and chromatophore fractions of three mutant strains, ΔRC6, ΔRC6(pΔ4), and CB1200 were compared to the UPB of wild type *R. capsulatus* by Western blot analysis (fig.32). Of the mutant strains tested, ΔRC6(pΔ4) and CB1200 both contain the *pufQ* gene, while strain ΔRC6 is deleted for *pufQ*. When fractions from all three strains were analysed, very little differences in the pattern of the higher molecular weight bands were observed, indicating that these bands were non-specific to PufQ protein. Interestingly however, an additional band showing high cross-reactivity and having an approximate M_r-value of 33 kDa was detected in the ICM fraction of strain CB1200 (lane 7) that was not present in any of the fractions from the strains tested. Furthermore, in this same strain, the PufQ protein band was notably more intense.

Although the results obtained from the analysis of strain CB1200 were clearly unexpected, a possibile explanation may be attributed to the nature of the strain itself. In *bch* mutant strains such as CB1200, one way cells are able to tolerate the large amounts of pigment which accumulate is by excreting excess amounts into the medium. The mechanism whereby the cells remove these pigments to the outside of the cell membrane probably involves transport via the outer membrane porin proteins. However, the pigment must first be shuttled to the porin proteins from the cytoplasmic membrane, a process which may require the aid of a membrane-bound protein such as

PufQ. It is conceivable therefore, that the additional band that was detected in the ICM fraction may be due to PufQ protein that is complexed with a pigment.

fig.32 Western immunoblot analyses of mutant and wild type strains of *R. capsulatus*. Fractions corresponding to the chromatophore and cell envelope fractions, respectively, of strains ΔRC6(pΔ4) (lanes 3,4), ΔRC6 (lanes 5,6), and CB1200 (lanes 7,8) were compared to the UPB of wild type *R. capsulatus* (lane 2) and a control sample containing hydrolyzed MBP-FX-PufQ fusion protein (lane 1). The M_r of protein standards are also shown and the band corresponding to the PufQ protein is indicated by the arrow.



114 b

3.10 Solubilization of PufQ protein bound to chromatophores

The extent to which PufQ protein is associated with chromatophores was tested using a chaotropic agent (sodium bromide), and a non-ionic detergent (octylglucoside). Chromatophores were first treated with 3M sodium bromide, followed by successive additions of 1% and 3% octylglucoside to the insoluble chromatophore pellet. All of the insoluble and soluble extracts were analysed by Western blot for the presence of PufQ protein. In every case, PufQ protein was present only in the insoluble samples (fig.33), indicating neither sodium bromide nor octylglucoside were able to dissociate PufQ protein from the chromatophores. The octylglucoside-soluble samples were further purified by an additional sucrose density gradient centrifugation, and two of the resulting pigmented fractions were shown, by absorption spectroscopy (fig.34), to contain either the LH-I or LH-II pigment-protein complexes. As expected, no PufQ protein was detected by Western blot analysis in these fractions (data not shown).

fig.33 Western immunoblots of chromatophore extracts. Chromatophores were treated with successive additions of NaBr and octylglucoside as described in section 2.11.2 and analysed by Western blots. The insoluble extract following treatment with 3 % (w/v) octylglucoside (lane 2) was compared to the hydrolyzed MBP-FX-PufQ fusion protein (lane 1) for identifying PufQ protein.

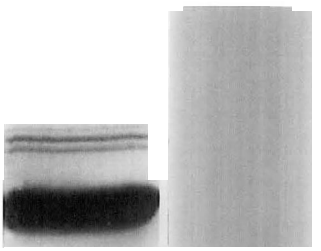
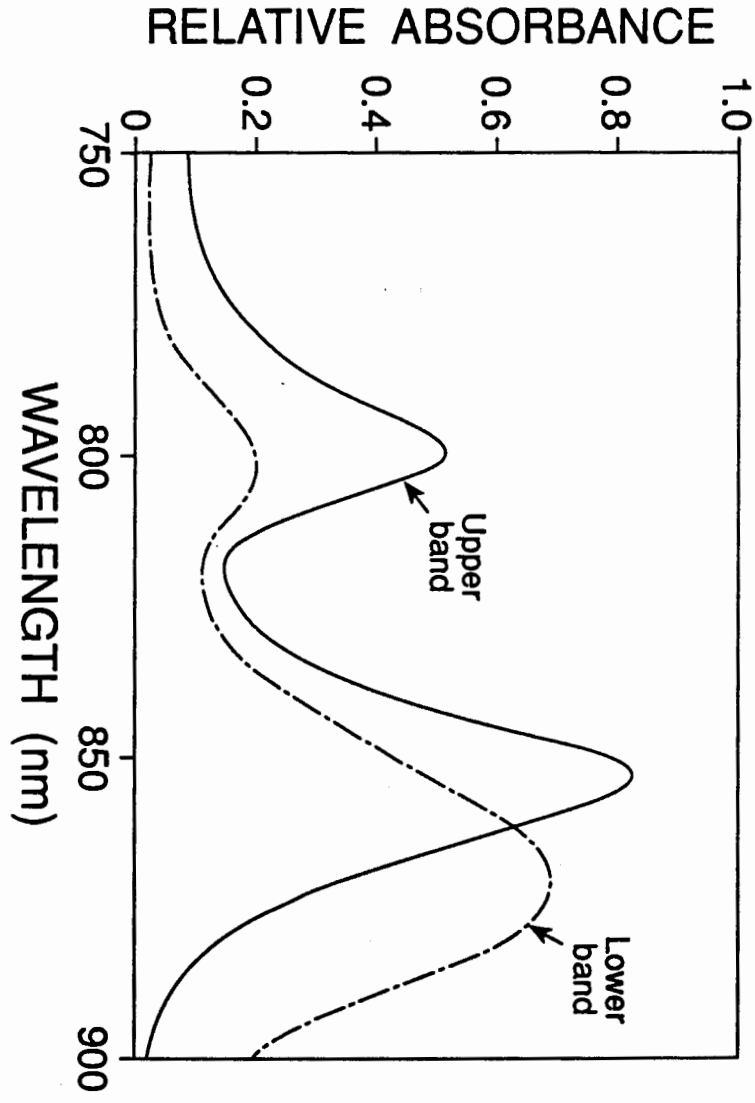


fig.34 Absorption spectra of the B800-850 LH-II and B870 LH-I complexes. The soluble extract of purified chromatophores was treated with 3 % octylglucoside and centrifuged on a sucrose density gradient. Fractions corresponding to the upper and lower bands were identified as the B800-850 LH-II and B870 LH-I complexes respectively by absorption spectroscopy. The band corresponding to PufQ protein is indicated by the arrow.


RELATIVE ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
750
800
850
900
WAVELENGTH (nm)
Upper band
Lower band

3.11 Porphyrin accumulation in *R. capsulatus* strains ΔRC6 and ΔRC6(pΔ4)

The effect of *pufQ* on the levels of Bchl and its tetrapyrrole intermediates was studied using both a *pufQ*$^-$ mutant strain (ΔRC6) of *R. capsulatus* and the same strain containing an extra-chromosomal copy of the *pufQ* gene (ΔRC6(pΔ4)). Growth incubations were performed using a variety of precursors and inhibitors of tetrapyrrole synthesis in an attempt to narrow down the point at which PufQ protein was exerting its effect on the flow of tetrapyrroles.

The inhibitors added to the growth medium included nicotinamide, NMP, and levulinic acid. Nicotinamide has been shown in *R. sphaeroides* to cause the accumulation of divinyl Pchlide, and it was proposed that this occurred through inhibition of the enzyme (4-vinylreductase) responsible for the reduction of the 4-vinyl group of divinyl Pchlide to the 4-ethyl group of monovinyl Pchlide (94), although it more likely is due to inhibition of the reduction of either divinyl or monovinyl Pchlide to the corresponding chlorophyllide (Dr. W.R. Richards, personal communication). NMP blocks heme synthesis by inhibiting the enzyme (ferrochelatase) responsible for the insertion of Fe into protoporphyrin (46). Since heme is a feedback inhibitor of ALA synthase, the addition of NMP has an overall effect of enhancing the levels of porphyrin intermediates. The inhibitory effect of levulinic acid on PBG synthesis has been well documented (57). An additional consequence of levulinic acid however, is to inhibit cell growth, probably as a result of inhibiting heme synthesis. To overcome this toxic effect of levulinic acid, cell cultures were initially grown in its absence and subsequently incubated in a buffer containing levulinic acid.

An alternative method to enhance porphyrin synthesis was the inclusion in the growth medium of either ALA or PBG, which are common early precursors of tetrapyrroles. These precursors have the added benefit of circumventing the feedback

inhibition of heme on ALA synthase, resulting in a greater overall yield of porphyrins, including Bchl.

In cultures containing no additions, there was an average 7-fold increase in Bchl in the strain containing *pufQ* (fig.35 A and fig.36 B). The Bchl levels in both strains increased in the presence of either ALA (fig.35 A and fig.36 A) or NMP (fig.35 A). The greatest enhancement in Bchl synthesis was observed when cultures were grown in the presence of PBG (fig.36 A). While Bchl in both strains increased, the difference in relative levels of Bchl between both strains was more pronounced in the presence of PBG compared to any other additions.

A dramatic effect on the relative levels of Pchlide synthesis between both strains was seen when nicotinimide was added in combination with either ALA or NMP (fig.35 B). In the case of ALA and nicotinimide co-incubation, there was a 8-fold increase in Pchlide due to the presence of *pufQ*, while a 28-fold increase in Pchlide was observed in the *pufQ*-containing strain when the cultures were co-incubated with NMP and nicotinimide.

In order to compare the total Coprogen synthesised, it was chemically oxidized to copro with benzoquinone (section 2.14). The results indicated that the relative levels of Coprogen were 3-fold higher (fig.36 B) under both sets of incubations in the *pufQ*-containing strain.

To demonstrate that the increase in porphyrin synthesis was due to the conversion of exogenous PBG and not due to a secondary effect caused by PBG, fully grown cells were incubated in a buffer containing both PBG and levulinic acid. Since the levulinic acid inhibits PBG synthesis, any *de novo* Coprogen synthesis can therefore be attributed to the added PBG. Under these conditions, the levels of Coprogen were 7-fold higher (fig.36 C) in the presence of *pufQ*.

fig.35 Analysis of Bchl and Pchlide synthesis in *R. capsulatus* strains ΔRC6 and ΔRC6(pΔ4). The strains ΔRC6 (−) and ΔRC6(pΔ4) (+) were grown in the presence of the indicated inhibitors and precursors of Bchl synthesis. The relative *in vivo* levels of Bchl (A) and Pchlide (B) were determined by measuring the A_{860} and A_{635} respectively (section 2.16.2), and normalizing the values with respect to a cell density at A_{680} equal to 1.

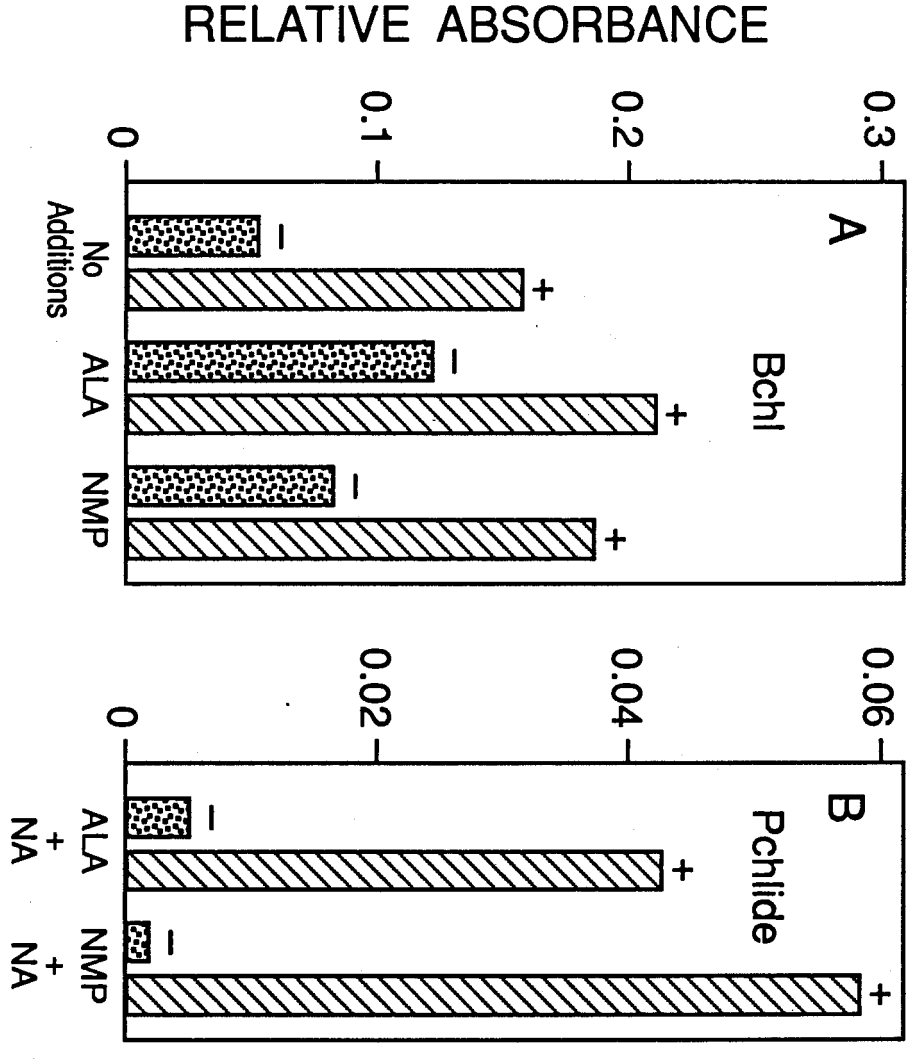
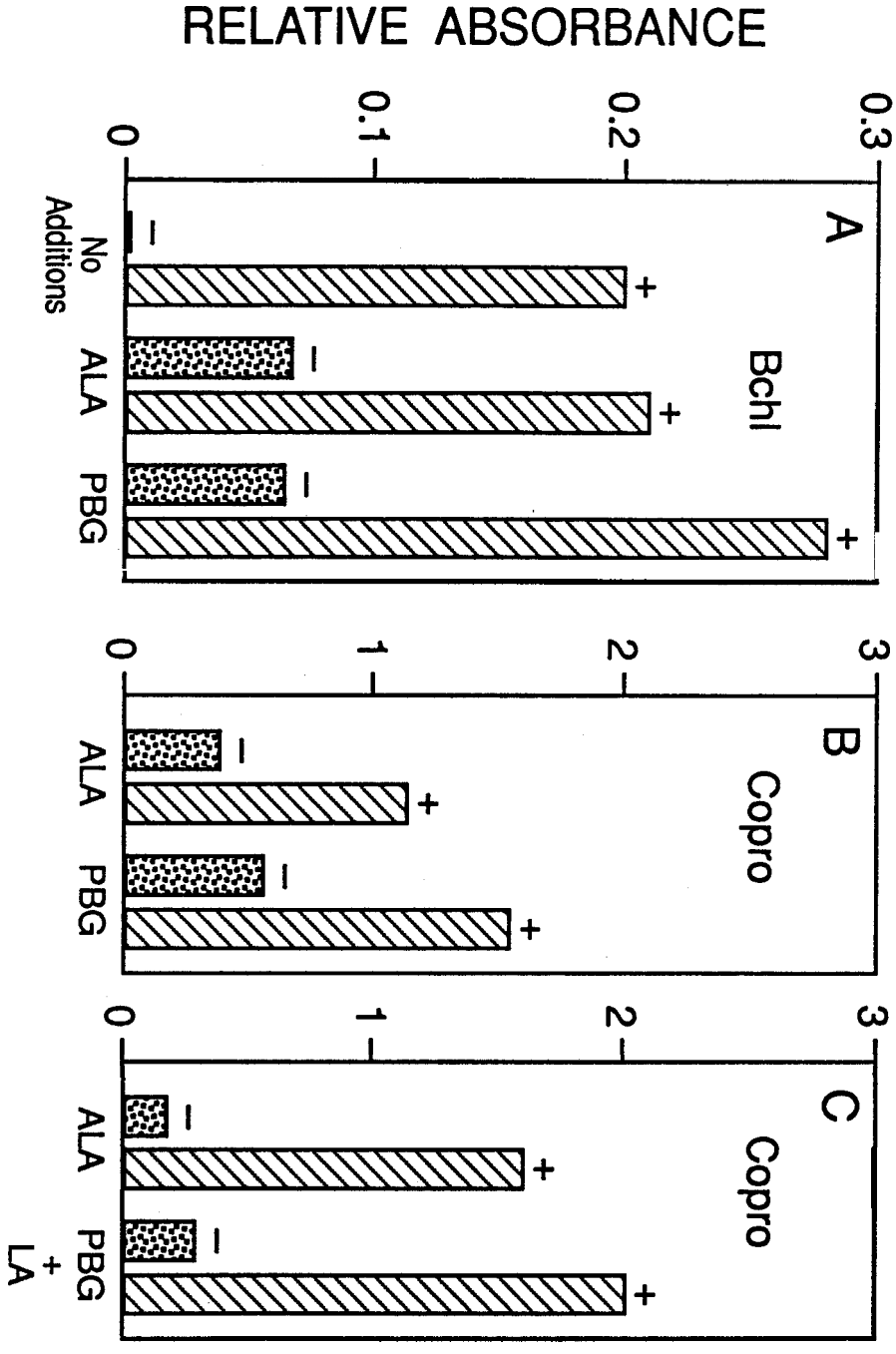

RELATIVE ABSORBANCE
0
0.1
0.2
0.3
A
Bchl
No Additions
ALA
NMP
−
+
0
0.02
0.04
0.06
B
Pchlide
ALA + NA
NMP + NA

fig.36 Analysis of Bchl and Coprogen synthesis in *R. capsulatus* strains ΔRC6 and ΔRC6(pΔ4). The strains ΔRC6 (–) and ΔRC6(pΔ4) (+) were either grown in the presence of ALA or PBG (A and B), or fully grown cells were incubated in the presence of ALA or both PBG and LA (C). For purposes of quantification, Coprogen was chemically oxidized to Copro as described in section 2.14. The relative levels of Bchl and Copro were determined by measuring the A_{860} and A_{405} respectively, and normalizing the values with respect to a cell density at A_{680} equal to 1.


RELATIVE ABSORBANCE
0
0.1
0.2
0.3
A
Bchl
No Additions
ALA
PBG
B
Copro
C
Copro
1
2
3
LA

4. DISCUSSION

4.1 Regulation of Bchl synthesis by the PufQ protein

Bchl in purple non-sulfur bacteria such as *Rhodobacter capsulatus* is an essential component of the light harvesting and reaction centre complexes that convert light energy into useful forms of cellular energy. In addition, it is important in maintaining the stability of light harvesting and reaction centre proteins, as evident from analysis of mutant strains that are unable to synthesize Bchl. In these strains, very little Bchl-binding proteins are ever detected, presumably due to rapid degradation of these proteins when they are not present in a Bchl-bound form. However, despite their interdependance, the Bchl biosynthesis genes and the light harvesting and reaction centre genes are not regulated in the same manner.

The single most important stimuli for cells to undergo phototrophic growth is a drop in the oxygen partial pressure below a threshold level. During these adaptive transitions, the synthesis of Bchl and the light harvesting and reaction centre proteins is coordinated such that none of the components are uncomplexed. Consequently, the genes encoding these polypeptides have been thought to be highly regulated.

There is a large body of evidence for the regulation of transcription of the genes of Bchl biosynthesis and Bchl-binding proteins by oxygen. The latter proteins include the α and β subunits of the B870 LH-I complex and L and M subunits of the reaction centre complex (encoded by genes located on the *puf* operon), as well as the α and β subunits of the B800-850 LH-II complex (encoded by genes located on the *puc* operon). Although insights into the mechanism whereby oxygen is able to regulate promoters of photosynthetic genes have emerged recently (see section 1.4.2), it remains an active area of research. In contrast, the effect of oxygen on the levels of these gene transcripts is well documented. In the presence of high oxygen tensions,

actively growing semi-aerobic cultures undergo a dramatic 10-30 fold reduction in the levels of the *puf* and *puc* transcripts. However, under these same oxygen conditions, the *bch* transcripts are repressed only 2-3 fold. Thus, while oxygen has a direct effect on the transcription of the *puf* and *puc* operons, its repression of transcription of the *bch* genes is much less, a fact that is suggestive of regulation of both sets of genes occurring via different mechanisms.

While it is expected that the reduced levels of *bch* gene transcripts would result in a decrease in the levels of Bchl synthesised, the lack of any detectable Bchl suggests there is a post-transcriptional mode of Bchl regulation. There are several possible mechanisms for this type of regulation including direct regulation by oxygen on one or more of the Bchl biosynthetic enzymes. Alternatively or in addition, it may involve the action of a regulatory protein that modulates activity of these enzymes. The putative regulatory protein may either be directly or indirectly activated by oxygen, or be one that is synthesised in response to oxygen.

Genetic studies first demonstrated a link between Bchl synthesis and the *pufQ* gene product. However, no additional evidence has yet been presented on the exact nature of this relationship. To address questions concerning the function of the *pufQ* gene, two different approaches have been used in this study. In the first case, experiments were done using purified PufQ protein to 1) test its binding to radiolabeled pigment, 2) determine its subcellular location, and 3) investigate its role in the conversion of Bchl intermediates. The second approach was to monitor the effects of the *pufQ* gene on the levels of pigments in the presence of early Bchl precursors and various enzyme inhibitors of heme and Bchl biosynthesis.

4.2 Overexpression of the *pufQ* gene

In order to characterize PufQ protein, it was necessary to generate quantities of the protein from non native sources. A hydropathy plot based on the deduced sequence of the *pufQ* gene (1) revealed a predominantly hydrophobic protein, containing a possible membrane-spanning domain of 25 amino acids located at its center (fig.5). With a molecular weight of 8,556 Da, the protein was considered too large to be produced economically by peptide synthesis but perhaps large enough to be made by recombinant DNA methods. Three different vector systems of *E.coli* were examined for their ability to overexpress *pufQ*, allowing therefore, a direct comparison of each of the systems to be made. The recombinant expression and purification of PufQ protein was made difficult by the lack of a ready assay. Consequently, our only criterion for overexpression of PufQ protein was the appearance of a protein in extracts with the anticipated molecular weight. Amino terminal analysis of isolated peptides was used in the final identification of the products.

Derivatives of the expression vector pKK223-3 were assembled and tested in which the *pufQ* gene was either under the control of its own ribosome binding site or under the control of the vector-borne ribosome binding site. Since neither of the constructs expressed the PufQ protein to the extent that it could be identified by SDS-PAGE, a genetically engineered ribosome binding site was constructed which most closely resembles an ideal *E. coli* ribosome binding site with respect to a consensus sequence and distance from the initiation codon (105). The resulting plasmid, termed pSF1 (fig.8), was used in the subsequent transcription studies (fig.20).

The expression vector pLcII-FX differs in several respects from pKK223-3. It possesses a promoter that is regulated by the temperature sensitive repressor cI857 and provides better repression of uninduced genes. Moreover, the gene products of pLcII-FX-derived clones are, unlike pKK223-3, produced as fusions with a 31 amino

acid portion of the lambda cII protein. A Factor Xa protease recognition sequence is present in the fusion at the junction between the recombinant peptide and the cII peptide. Hence, the fusion protein may be processed with Factor Xa to separate the two peptide components. Expression studies were performed with pSF2 (fig.10) and there was evidence of a low level of cII-FX-PufQ protein fusion in cell extracts (fig.15). However, the levels of expression were too low to facilitate purification and useful quantities of PufQ protein were never obtained from this system.

The pMal-c expression vector system shares features of both the pKK223-3 and pLcII-FX systems. The vector has a strong IPTG-inducible promoter, and recombinant proteins are produced as fusions which can be processed with Factor Xa. An added benefit to the system is that recombinant proteins are fusions with a maltose-binding domain rather than the cII peptide. Therefore, fusion proteins can be purified from cell extracts in one step by chromatography on an amylose column. The expression vector pSF3 (fig.12), derived from pMal-c, produced high levels of a MBP-FX-PufQ fusion protein. Western blot analysis using anti MBP antisera confirmed the presence of a maltose binding domain in the expressed fusion protein. The PufQ protein was then separated from the maltose-binding domain by limited proteolysis with Factor X_a. Peptides produced in the protease digest were resolved by reversed-phase HPLC (fig.19), and the identity of the PufQ protein was verified by amino-terminal analysis.

An unexpected product of the Factor Xa digestion was a truncated PufQ protein (designated Δ_{1-7}PufQ), seven amino acids shorter than the full length PufQ protein. The expected M_r of PufQ and Δ_{1-7}PufQ proteins deduced from the amino acid sequence (8,556 and 7,530 respectively) was consistent with the apparent M_r of both proteins based on their mobilities on SDS-PAGE (8,700 and 7,600 respectively). Though the amino acid sequence in the region of the protein where the unexpected cleavage occurred does not resemble a Factor X_a site, there is in common with a Factor X_a site, an arginine residue which precedes the point of cleavage. The amino acid

sequence of the FX-PufQ junction and the amino terminal residues of the PufQ protein are shown below. The arrows indicate the observed cleavage sites.

----Ile Glu Gly Arg Met Gln Ser Glu Arg Leu Arg Ala----

Factor X_a site ^ PufQ protein ^

Despite the loss of seven amino acids, the truncated protein behaved in a manner similar to the full length protein in terms of its membrane-binding properties. It should also be noted that any possible cleavage by Factor X_a at the extreme carboxy terminal would not be detected based on its electrophoretic mobility.

4.3 Northern blot analysis of expression constructs

It was of some interest to establish reasons for differences in the success of the three expression vector systems. One possible cause for difference was the level of gene transcription. As well, there may have been differences in the stablity and/or turnover of *pufQ* mRNA. Therefore, a direct comparison of transcription by Northern blot analysis (fig.20) was conducted. In every case the *pufQ* messenger RNA was abundant and the anticipated lengths of transcript were found.

The pSF1 system produced *pufQ*-specific transcripts with apparent lengths of 410 and 580 bases. The pSF2 system produced transcripts with the lengths 740, 1100 and 1500 bases and pSF3 gave lengths of 1260 and 1700. The results of this analysis suggested that differences in the levels of *pufQ* expression resulted, not from significant differences associated with transcription, but from differences in the translation and/or stability of the protein.

The fusion expression systems pSF2 and pSF3 were successful in producing quantities of PufQ protein whereas the pKK223-3-derived vector pSF1 was not.

Though no obvious explanations for the differences in protein expression were found, several possibilities presented themselves. A small protein such as PufQ protein may be more susceptible to degradation than larger fusion proteins. Another explanation is also possible due to the highly hydrophobic nature of the PufQ protein (see below). As a result, it is likely to aggregate and associate in the membranes when overexpressed in *E. coli*. This may have the effect of lysis of the cell at an early stage of expression. Of the two fusion systems, pSF3 was clearly the superior. The success of the pSF3 vector in producing PufQ protein may be accounted for by the sheer size of the maltose-binding portion of the fusion protein, such that it is simply large enough to compensate for the PufQ protein's non-aqueous solubility.

4.4 Reconstitution of the PufQ protein in phospholipid vesicles

The isolated PufQ protein showed a marked tendancy to aggregate in the absence of the detergent sodium cholate. This observation was consistent with predictions that the PufQ protein is an intrinsic membrane protein. The likelihood that the native PufQ protein is membrane-bound was examined by more direct methods as follows: The fusion protein was digested with Factor X_a in the presence of sodium cholate and the digest was combined with preformed membrane vesicles. The detergent was then removed by dialysis and the components of the mixture were fractionationed on a discontinuous sucrose density gradient (fig.24). In mixtures containing the PufQ protein, there was a visible banding of lipid and protein at the interface between the 15% and 35% sucrose. The protein component of this interface fraction was comprised of almost exclusively PufQ protein (together with the truncated Δ_{1-7}PufQ) as determined by SDS-PAGE. Similar banding behaviour was not evident in control mixtures contained MBP and lipid.

4.5 Binding of Pchlide to reconstituted PufQ protein

In order to test the ability of PufQ protein to bind Bchl intermediates, it was necessary to have PufQ protein in a form which would yield the greatest binding activity. Based on the model of PufQ protein acting as a membrane-bound carrier or regulatory protein, a reconstituted form of PufQ protein in phospholipid vesicles was used for the binding assay. The procedure was similar to that of the reconstitution of PufQ protein described above except for the addition of a cholate mixture containing Pchlide, and the fractionation being performed on a linear sucrose gradient.

Binding of PufQ protein to Pchlide was observed in the higher density liposome fractions between 22 and 32% sucrose (fractions 6, 7, 8, 9, 10 in fig.26 C). In these fractions, PufQ protein, and significant levels of Pchlide and lipid were detected. This was in contrast to control tubes containing Apolipoprotein A-I in place of PufQ protein, in which none of these components were present in the corresponding fractions (fig.26. A). The presence of Pchlide in the PufQ-containing fractions was considered particularly significant since it raised the possibility that its presence was due specifically to the presence of PufQ protein. However, an equally likely possibility was that the Pchlide was simply soluble in the phospholipid vesicles also present in these fractions. An analysis of *specific* binding between PufQ protein and Pchlide was therefore performed in order to differentiate between these two possibilities. This was achieved by calculating the molar ratios of Pchlide/lipid, protein/lipid, and Pchlide/protein and re-plotting these relative ratios (fig.26 B and fig.26 D). Specific binding was based on the following two criteria: a constant ratio of Pchlide/protein and an increase in the Pchlide/lipid ratio with increasing sucrose densities. A constant Pchlide/protein ratio indicates specific binding of PufQ protein, and an increase in the Pchlide/lipid is indicative of an increase in the ability of liposomes to bind Pchlide in the presence of PufQ protein.

The fact that Pchlide was indeed soluble in the phospholipid vesicles was demonstrated by an increase in the ratio of Pchlide/lipid to a maximum value between approximately 1:2000 to 1:2500 in both the control gradient (fractions 2 to 4 in fig.26 B) and in the top fractions of the PufQ-containing gradient (fractions 2 and 3 in fig.26 D). However, the molar ratio of Pchlide/lipid in the presence of PufQ protein increased to 1:1400 (fractions 7 to 9 in fig.26 D). Thus the presence of PufQ protein contributed to an increase in the overall capacity of the liposomes to bind Pchlide. The molar ratio of Pchlide/lipid in these fractions therefore, consists of the Pchlide that is soluble in the phospholipid vesicles, and the Pchlide that is specifically bound to PufQ protein.

Since the Pchlide is soluble in the phospholipid vesicles, it was not possible to obtain a value for the specific interaction of Pchlide with PufQ protein. However, a crude approximation of the Pchlide that is bound to PufQ protein was calculated based on the ratio of Pchlide/protein. This ratio (1:25), while relatively constant for all of the PufQ protein-containing fractions, was indicative of poor binding and found to be in the order of only one molecule of Pchlide for every 25 molecules of the PufQ protein. A possible cause for this low apparent binding affinity may be attributed to a non-active state of PufQ protein during the course of the binding experiment, either due to denaturation or aggregation while in its reconstituted form. An alternate explanation may simply be a lack of specific binding between PufQ protein and Pchlide.

4.6 Fluorescence analysis of Pchlide bound to PufQ protein

Shifts in the fluorescence emission spectra of either proteins or protein-bound probes have proven to be a sensitive method for detecting changes in its environment (19). This technique was used to monitor changes in the environment of Pchlide in the presence of PufQ protein. Fluorescence emission spectra of aliquots of fractions containing PufQ protein and Pchlide from the sucrose gradient described above were

compared to control fractions containing either MBP or Apolipoprotein A-I. When the spectra were overlayed (fig.27), a blue shift of 3 nm was observed only in the presence of PufQ protein. In general, a blue shift (i.e, a shift in the emission spectrum to a shorter wavelength) is indicative of a decrease in the polarity of the environment sensed by the fluorescent molecule. In the case of the Pchlide spectrum containing PufQ protein, this would indicate a change in the orientation of Pchlide from perhaps one in which it was partially exposed to the aqueous environment, to a less polar environment in which the Pchlide is now partially buried in the hydrophobic core of the PufQ protein.

4.7 Subcellular localization of the PufQ protein

Polyclonal antibodies were generated against the MBP-FX-PufQ fusion protein following its expression and purification in *E. coli*. The anti-MBP-FX-PufQ IgG was used as a probe to detect the presence of PufQ protein in various subcellular fractions of *R. capsulatus*. In initial blotting studies, the PufQ protein was detected in the membrane fraction (fig.28), a result that was not unexpected considering its hydrophobic nature (section 4.4). Subsequent studies were performed to test whether the PufQ protein is expressed specifically in the ICM along with other essential protein components of the photosynthetic apparatus. Also of considerable interest was to determine the stage during photosynthetic growth that PufQ protein is expressed. To investigate these possibilities, advantage was taken of the fact that in *R. sphaeroides*, an ICM precursor can be isolated by sucrose density gradient fractionation of cell lysates obtained during adaptation from aerobic to phototrophic growth conditions. This precursor has been shown by Niederman (77) to contain newly-formed, Bchl-containing reaction center and B870 LH-I complexes, and is thought to represent an ICM invagination site on the cell membrane.

Similar adaptation studies were therefore performed on *R. capsulatus*. The cells from a culture induced to grow photosynthetically were harvested at various time intervals and analysed for the presence of PufQ protein (fig.30 and fig.31). PufQ protein was first detected after 3hr of induction in both the chromatophore and precursor fractions. While the levels of PufQ protein remained fairly constant during phototrophic growth in the chromatophore fraction, the PufQ protein levels increased during the same growth period in the precursor fraction. The presence of PufQ protein in the precursor fraction is particularly significant in view of the possibility that this fraction may be a site for Bchl synthesis. Evidence for this is due to the observation that the Bchl intermediate, MgPME, was shown to accumulate in this fraction (79). Although not directly assayed, the enzyme responsible for MgPME synthesis, MT, was assumed to also be present in this fraction.

To investigate the possibility of PufQ protein being involved in enzyme activity, MT assays were performed by Dr. S.B. Hinchigeri on the various fractions analysed by Western blots. The results indicated that although MT appeared in the precursor fraction, its specific activity was approximately the same as in the chromatophore fraction and in the cell envelope fraction (which contained most of the cell membrane) during the early stages of induction. Furthermore, after fully-photosynthetic growth, the specific activity of MT was significantly higher in the chromatophore than in the precursor fractions. Thus, a linear relationship between MT activity and the levels of PufQ protein was not evident.

The association between PufQ protein and the chromatophores was further tested by performing a series of extractions on purified chromatophores. Under conditions where intrinsic proteins such as the LH-I and LH-II polypeptides were extracted, the PufQ protein demonstrated its highly hydrophobic nature by remaining in the particulate fraction (fig.33).

4.8 The role of *pufQ* in the Bchl biosynthetic pathway

The observation by Bauer and Marrs (9) that a *pufQ* mutant of *R. capsulatus* failed to form substantial levels of the Bchl-containing B800-850 LH-II complex demonstrated the importance of the *pufQ* gene and prompted questions regarding its function. The phenotype of a *pufQ*$^-$ strain can be attributed to either a lack of Bchl (encoded by the *bch* genes), a lack of the LH-II complex polypeptides (encoded by the *puc* genes), or a failure to assemble both components.

Whereas *pufQ* was shown not to effect either transcription or translation of the *puc* genes (34), experiments involving *bch* mutants suggested a role of *pufQ* in the Bchl biosynthetic pathway (9). In these studies, it was demonstrated that *pufQ* had an effect on the Mg branch of the pathway as early as the insertion of Mg into protoporphyrin. However, it was not clear whether *pufQ* was exerting its effect at this step or earlier. As an extension of these results, studies are described which suggest that *pufQ* influences the synthesis of intermediates upstream of protoporphyrin.

In mutant strains of *R. capsulatus* where the *pufQ* gene is either deleted (ΔRC6) or is complemented *in trans* on a plasmid (ΔRC6(pΔ4)), the Bchl intermediates and their relative abundance were compared (fig.35 and fig.36). Specific enzymes of the pathway were either stimulated or inhibited in order to define the step in the pathway where *pufQ* is having an effect.

In studies of the early steps in the pathway beginning with the enzymes responsible for PBG synthesis and its subsequent conversion to uroporphyrinogen III, similar effects of *pufQ* on porphyrin biosynthesis were observed. In common was an increase in the levels of Coprogen in the presence of *pufQ* (and to a much lesser extent in the absence of *pufQ*). When combined with the earlier results of *pufQ* being involved at or before the protoporphyrin step, these results suggest that PufQ protein may be activating one of the three enzymes responsible for the conversion of PBG to

Coprogen. It should be noted however, that the identification of Coprogen as the accumulated intermediate remains to be unambiguously resolved.

Based on these preliminary incubation studies, it is not clear exactly how the PufQ protein might enhance enzyme activity. One possibility however, is that the PufQ protein directly interacts with the enzyme by acting as a positive allosteric effector. Alternatively, the PufQ protein may activate a downstream enzyme whose product could subsequently serve as a feedback stimulator of one of the earlier enzymes. Yet another possibility is that PufQ protein may facilitate catalysis by binding to the intermediate such that it is oriented in a manner which is more accessible for the enzyme. A closer kinetic examination of the effect of PufQ protein on each of the earlier enzymes is therefore necessary to address these possibilities.

During the course of this study, experiments were performed to biochemically demonstrate a possible role of PufQ protein in the Mg branch of the Bchl pathway (data not shown). In these studies, PufQ protein was reconstituted in phospholipid vesicles and added to cell-free extracts of *bch* mutants of *R. capsulatus*. Attempts were then made to assay several enzymes of the Mg branch by monitoring the conversion of the accumulated Bchl intermediates to downstream products using fluorescence spectroscopy. While it is possible that the assay conditions were inadequate for reconstituting activity, the addition of PufQ protein did not appear to influence the activity of the Mg branch enzymes tested, a result which is consistent with *pufQ* acting at an earlier step in the pathway.

4.9 Conclusion

To gain a better understanding of its role in Bchl biosynthesis, the *pufQ* gene was successfully expressed in *E. coli* using recombinant methods. Subsequent purification of the PufQ protein allowed the preliminary characterization of its properties and

function. The membrane-binding ability of PufQ protein was demonstrated both by its ability to be reconstituted in phospholipid vesicles and in dissociation studies of chromatophore proteins. The results strongly suggest it is a highly intrinsic protein. To explore its possible function, the binding of PufQ protein and Pchlide was studied. While the binding assay and fluorescence shift data suggest a specific association, tight-binding between PufQ protein and Pchlide was not evident. In a preliminary study, a search for the step in the Bchl pathway that *pufQ* first functions was initiated using mutants of *R. capsulatus* that were either deficient in or which contained the *pufQ* gene. The results suggested that *pufQ* is involved at a step in the pathway before Mg insertion, possibly during the conversion of PBG to Coprogen. Additional studies using PufQ protein-specific polyclonal antibodies localized the PufQ protein in the ICM as well as a fraction of the cell membrane which is a precursor to the ICM.

5.REFERENCES

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