UNIVERSITY OF MANITOBA

STRUCTURAL STUDIES ON *RHODOSPIRILLUM RUBRUM* AND *RHODOPSEUDOMONAS PALUSTRIS* CELL-FREE PROTEIN SYNTHESIZING SYSTEMS

by

SUDARSHAN DEVARASHETTY

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for the
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ABSTRACT

Several protein-containing fractions, including the total, the PS-60, the S-100 and the ribosome fractions, have been prepared from both phototrophically and heterotrophically grown Rs. rubrum and Rp. palustris cells. Analyses by isoelectric focusing polyacrylamide gel electrophoresis of the first three fractions as well as the ^{35}S-labeled Rs. rubrum S-100 fractions have revealed that there are no significant differences between the phototrophic and the heterotrophic samples, however, the latter may contain a higher content of acidic proteins.

In addition to the isoelectric focusing PAGE system, the ribosomal proteins have also been analyzed by the SDS and the two-dimensional PAGE systems in which similar number of protein bands (about 18 in SDS gels) or protein spots (about 32 in two-dimensional gels) has been observed for both phototrophic and heterotrophic Rs. rubrum or Rp. palustris samples. The intensity of several acidic protein bands is, however, much higher in the heterotrophic ribosome samples than in the phototrophic ribosome samples, as demonstrated in the isoelectric focusing gels. The possible association of the higher quantity of acidic proteins in the heterotrophic ribosomes and the generally higher protein-synthesizing activity of the heterotrophic bacterial ribosomes will be discussed.

To my brother-in-law, Hanmiah

my sister, Bharathi

and

my fiancee, Sitara

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{260}	absorbance at 260 nm
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetraacetic acid
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethyl sulfonylfluoride
pI	isoelectric point
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
S	Svedburg's unit
TCA	trichloroacetic acid
Tris	tri (hydroxymethyl) aminomethane
TEMED	N, N, N', N' - tetramethylene diamine

H I S T O R I C A L

AND

I N T R O D U C T I O N

The phototrophic bacteria are represented by a large number of species of different morphology, pigmentation and physiological-biochemical properties. These bacteria are encountered in almost every body of water as well as in the soil, but they are most abundant in stagnant bodies of water where organic material is undergoing decay and hydrogen sulfide is present.

The terminology and classification of phototrophic bacteria have changed considerably in recent years. Prior to 1974, the phototrophic bacteria were placed in the sub-order Rhodobacterineae of the order Pseudomonadales, with three families, i.e., Thiorhodaceae (the purple sulfur bacteria), Athiorhodaceae (the purple non-sulfur bacteria), and Chlorobacteriaceae (the green bacteria). However, according to the more recent classification system, as described in the eighth edition of Bergey's manual of Determinative Bacteriology (Pfenning and Trüper 1974), all phototrophic bacteria with the exception of Cyano-bacteria have been placed in the order Rhodospirillales which is described into two suborders, the Rhodospirillineae and the Chlorobiineae. These two suborders can be easily distinguished on the basis of the physical location of their photosynthetic pigments. In the Rhodospirillineae, the

pigments are always located in the intracytoplasmic membrane system which is continuous with the cytoplasmic membrane, whereas members of Chlorobiineae have their pigments located in non-unit-membrane bound, cigar-shaped organelles, known as "chlorobium vesicles", that underlie the cytoplasmic membrane.

Based on the ability to use elemental sulfur as an electron donor, the suborder Rhodospirillineae is further divided into two families, the Rhodospirillaceae, formerly Athiorhodaceae, and the Chromatiaceae, formerly Thiorhodaceae. As suggested by their old but more descriptive names members of the former and the latter families are, respectively, unable and able to use elemental sulfur. The second suborder Chlorobiineae also consists of two families, the Chlorobiaceae, formerly Chlorobacteriaceae and the recently discovered Chloroflexaceae (Trüper 1974). The species of Chloroflexaceae are differentiated from those of the Chlorobiaceae by their filamentous, flexible appearance and by their gliding motility on solid surfaces.

The metabolic activity exhibited by the Rhodospirillaceae members differs from the rest of the phototrophic bacteria in their preference for photoorganotrophic growth (Pfenning and Trüper 1971). Most strains in this family require one or more vitamins as growth factors and are unable to grow with sulfide as the sole photosynthetic electron donor. Furthermore, none of them can utilize elemental sulfur as an electron donor. Their internal

photosynthetic membrane system is continuous with the cytoplasmic membrane and is vesicular, lamellar or tubular in appearance. Their storage materials include polysaccharides, poly-β-hydroxy butyrates and polyphosphates.

Certain phototrophic bacteria of the family Rhodospirillaceae afford an unusual opportunity for the study of the molecular events involved in the control mechanism of gene expression, because they are able to grow both phototrophically and heterotrophically, and during such physiological conversions different proteins are produced. According to the current models, protein synthesis in bacteria is believed to be regulated primarily at the level of transcription. The properties of the messenger RNA species in facultative phototrophs, mainly Rs. rubrum and Rp. sphaeroides, have therefore been investigated. Using DNA-RNA hybridization competition techniques which detect differences in RNA base sequences, Yamashita and Kamen (1968), and later Chow (1976a) have demonstrated that there is little qualitative difference between the RNA species of the phototrophically and the heterotrophically grown Rs. rubrum. In another report, Yamashita and Kamen (1969) have shown that after the transfer from heterotrophic to phototrophic growth conditions, light exerted a stimulatory effect on uracil incorporation in Rs. rubrum cultures and from the pulse and chase results, they have also concluded that this light stimulating effect is associated with the synthesis of chromatophores and bacteriochlorophylls. Based on these

findings, the above investigators have suggested that the regulation of protein synthesis in Rs. rubrum is probably at the translational rather than at the more widely accepted transcriptional level. Similar proposals have been made for another facultative phototroph, Rp. sphaeroides, by Witkin and Gibson (1972).

The concept of translational control has also been proposed for many other groups of microorganisms, including E. coli (Shine and Dalgarno, 1974), Staphylococcus aureus (Martin and Landolo, 1975) and Bacillus subtilis (Legault-Demare and Chambliss 1975, Guha and Szulmajster, 1977). The most interesting findings are, perhaps, in the differentiating, spore-forming Bacillus species. From several antibiotic-resistant mutants of B. subtilis 168, Graham and Bott (1975) have isolated a sub-class of mutants that is conditionally asporogenous. Mutants of this sub-class are resistant to erythromycin, kanamycin, spectinomycin and streptomycin during vegetative growth but become sensitive to these antibiotics after initiation of sporulation. Their results have demonstrated that this antibiotic sensitivity is due to alterations in the structure of the 30 S and the 50 S ribosomal subunits in sporulating cells. This conclusion has also been supported by the results of many other investigators (Smith et al., 1969; Kimura et al., 1972; Hanson and Cocoran, 1969; Tanaka et al., 1973), especially by Fortnagel's group (1973 and 1975) who have demonstrated differences in electrophoretic mobilities

between vegetative and sporulating B. subtilis ribosomal proteins. Another interesting finding has recently been reported by Tipper et al (1977). Using erythromycin resistant and temperature sensitive B. subtilis mutants, they have shown that these cells are not able to sporulate at elevated temperatures, and, at the same time, their 50 S ribosomal protein L17 is either missing or shows an altered electrophoretic mobility. Furthermore, the ribosomes of these mutants bind to erythromycin at a rate much lower (less than 1%) than those of the wild-type cells. Ribosomes of the Spo^+ revertants have, however, regained the original L17 protein and the erythromycin-binding rate. From these results, they have concluded that the ribosomal protein L17 has a special function in sporulation.

On the other hand, Guha et al (1975) have obtained some different results when analyzing the ribosomal proteins of vegetative and sporulating B. subtilis 168 M cells by two-dimensional acrylamide gel electrophoresis techniques. They have identified 22 protein species in the 30 S subunits and 28 protein species in the 50 S subunits in both the vegetative and the sporulating cells and these proteins are all identical in their electrophoretic mobility. The discrepancy between these sets of results is, at present, difficult to explain, however according to Guha et al (1975), it may result from the protease activity in the sporulating cells and the different techniques employed in preparing the ribosomes.

As mentioned earlier, translational control has also been proposed for Rs. rubrum (Yamashita and Kamen, 1968 and 1969, Chow, 1976a) and Rp. sphaeroides (Witkin and Gibson, 1972). Thus far most of the studies on the phototrophic bacterial ribosomes have been concentrated on the determination of their sedimentation constants. According to Taylor and Stork (1964), the ribosomes of Rs. rubrum, Rp. palustris, Rp. sphaeroides and Rp. gelatinosa have a sedimentation constant of 66 S, and the subunits of Rp. palustris (Bhatnagar and Stachow, 1972) and Rp. sphaeroides (Friedman et al, 1966) ribosomes are of 29 S and 46 S types. Preliminary analyses of the purified phototrophic Rp. palustris 29 S and 46 S ribosomal subunit proteins by urea-polyacrylamide gel electrophoresis have revealed 23 protein bands for the former and 28 protein bands for the latter (Bhatnagar and Stachow, 1972). Examination of the Rp. palustris ribosomes by sucrose gradient sedimentation techniques (Mansour and Stachow, 1975) has shown that structural changes in ribosomal subunits occur after shifting the cells from heterotrophic growth conditions to phototrophic growth conditions. From these results, Mansour and Stachow (1975) have concluded that Rp. palustris cells contain two distinct populations of ribosomes, one for the phototrophic cells and the other for the heterotrophic cells. Detailed comparison between these two ribosomal populations and their proteins has, however, not been made.

In this study, we have analyzed the ribosomal proteins of both phototrophic and heterotrophic Rs. rubrum and Rp. palustris cells by several different electrophoresis systems. Similar studies on the bacterial S-100 and PS-60 fractions have also been carried out in attempt to achieve a better understanding of the protein synthesizing system and the possible translational control mechanisms in these cells.

MATERIALS AND METHODS

Organisms

The organisms used throughout this study were as follows:

1. Rhodospirillum rubrum strain VF, a varient strain isolated in this laboratory.
2. Rhodopseudomonas palustris ATCC 17002, obtained from Dr. S. Kaplan of the University of Illinois.
3. Escherichia coli B, a stock culture from the Department of Microbiology, University of Manitoba.

Growth Media

a) The growth medium for Rs. rubrum was of the following composition (Chow 1976a):

l - Glutamic acid	4.0 g
dl - Malic acid	3.5 g
Sodium citrate	0.8 g
$MgSO_4.7H_2O$	0.2 g
KH_2PO_4	0.12 g
K_2HPO_4	0.18 g
$CaCl_2$	33.0 mg
Biotin	5.0 µg
Yeast extract	2.0 g

Distilled, deionized water up to 1000.0 ml

The pH was adjusted to 6.8 with a saturated solution of sodium hydroxide. This medium will hereafter be referred to as the A1 medium in this thesis.

b) The medium used to grow Rp. palustris was a modified, semisynthetic medium of Lascelles (1956) with the following composition:

l - Glutamic acid	1.47 g
dl - Malic acid	2.70 g
KH_2PO_4	0.50 g
K_2HPO_4	0.50 g
$(NH_4)_2HPO_4$	0.80 g
$MgSO_4.7H_20$	0.20 g
$CaCl_2.2H_20$	53.00 mg
$MnSO_4.7H_20$	0.85 mg
$FeSO_4.7H_20$	2.78 mg
Na_3 Nitriloacetic acid	1.00 mg
Thiamine HCl	1.00 mg
Nicotinic acid	1.00 mg
Biotin	10.00 µg
Yeast extract	2.00 g

Distilled, deionized water added up to 1000.0 ml

The pH was adjusted to 6.8 with a saturated solution of sodium hydroxide. This medium will hereafter be referred to as the B2 medium in this thesis.

c) Escherichia coli was grown in the casamino acids (0.2%) - minimal salts - glucose medium of Kurland (1966), containing:

KH_2PO_4	3.0 g
$Na_2HPO_4.H_20$	6.8 g
NH_4Cl	2.0 g
Casamino acids	2.0 g
$MgCl_2.6H_20$	0.2 g
Glucose	5.0 g

Distilled, deionized water up to 1000.0 ml

The pH was adjusted to 7.4 with a saturated solution of sodium hydroxide.

Solid agar media were prepared by adding 15 g to Bacto Agar in every liter of the above liquid media.

Buffers

1. Standard Buffer (Modelell, 1971): This buffer was used for preparations of various fractions of the in vitro protein-synthesis system and had the following composition:

10 mM	Tris-HCl pH 7.8
60 mM	NH_4Cl_2
10 mM	Mg-Acetate
6 mM	2-Mercaptoethanol

2. KCl-PMSF Standard Buffer (Legualt-Demare, L., and G.H. Chambliss, 1974; Chow, 1976b): This buffer was used for washing Rs. rubrum and Rp. palustris cells. It contained 3.54 mM of PMSF and 1.0 M KCl in the standard buffer.

A number of other buffers have also been used in this study, mainly for gel electrophoretic analyses, and will be described in later sections.

Growth Conditions

Both Rs. rubrum and Rp. palustris were routinely subcultured as stab cultures every seven days in the phototrophic mode of growth (Chow, 1976a), because there was a less chance of contamination when the cultures were maintained under semi-anaerobic conditions. The stab cultures were incubated in five inch screw capped tubes in a Conviron Model E7 incubator which was equipped with both incandescent and fluorescent lamps. Growth was allowed to proceed at 28°C for two to three days after which time the tubes were stored in a refrigerator at 4°C.

To grow these organisms phototrophically in large quantities, the following procedure was used: a needleful of bacteria was inoculated into a five inch screw capped tube filled with the liquid medium up to the neck; the tube was incubated in the light chamber at 28°C until the cells had reached mid-logarithmic phase of growth which was determined by measuring optical density (about 200 Klett units). The mid-logarithmic cultures were then transferred as an inoculum (7% v/v) into one-liter Roux bottles filled with about 700 ml of medium. The Roux bottle cultures were similarly incubated and at

mid-logarithmic phase they were transferred into 10 or 15 liter glass carboys. The cultures in the carboys were again incubated under the phototrophic condition until mid-logarithmic phase of growth.

For heterotrophic growth, the initial inoculating cultures were prepared by the same way as described above, i.e., growing in five inch screw capped tubes under phototrophic condition. However, these tube-grown cultures were transferred into one-liter flasks containing 200 ml of medium instead of into Roux bottles. These flasks were wrapped with aluminum foil to prevent exposure to light and were shaken on a shaker in a dark 28°C incubation room until the culture had adapted to heterotrophic growth. When the cultures had reached mid-logarithmic phase of growth (about 130 Klett units), they were used to inoculate 10 or 15 liter glass carboys as a 3% (v/v) inoculum. Similarly, these carboys were wrapped with aluminum foil and incubated with vigorous aeration in a dark 28°C incubation room until mid-logarithmic phase.

Escherichia coli cultures were grown under the standard conditions and were harvested when they had reached an optical density about 200 Klett units.

Harvesting Cells

All cultures were harvested in mid-logarithmic phase of growth. The cells were collected by centrifugation

in a Sharples steam-driven centrifuge at 40 lbs/in.^{2} and at a flow rate of 200 ml/min. The packed cells were first washed in the standard buffer by centrifugation at 12,000 x g for 10 minutes. Thus prepared bacterial pellets were weighed and stored at -76^{o}C. The average yields (wet weight) of various bacterial cultures were:

Phototrophic	Rs. rubrum	1.0	g/l
Heterotrophic	Rs. rubrum	1.2	g/l
Phototrophic	Rp. palustris	1.3	g/l
Heterotrophic	Rp. palustris	1.5	g/l
	E. coli	2.5	g/l

Preparation of S-30, PS-60, S-100 and ribosome fractions

All the following extraction and purification steps were carried out at 4^{o}C. Frozen cell pellets were thawed slowly and ground with 2X (w/w) of alumina until the mixture was homogeneous. The pastes were extracted with 2 volumes of the standard buffer. The alumina and cell debris were then sedimented twice by centrifugation at 27,000 x g for 20 minutes. Thus obtained supernatant fluids were the S-30 fractions.

The PS-60 and S-100 fractions were prepared by the procedure of Chow (1976 c) with some minor modification. The PS-60 pellets were obtained by centrifuging the S-30 fraction at 60,000 x g/50 minutes and the pellets were re-

suspended in small amounts of the standard buffer. Subsequently the supernatants were further centrifuged at 105,000 x g/2 hours and the top two-thirds of the supernatants were carefully aspirated, without disturbing the bottom layers of the fluids, and these samples were the S-100 fractions. All S-30, PS-60 and S-100 samples were stored in small aliquots at -76°C.

Ribosomes were purified from the S-30 extracts by the ammonium sulfate precipitation method of Kurland (1966) with the omission of the puromycin step. The final ribosomal pellets were resuspended in the standard buffer at a concentration of 300 A_{260} units per milliliter, divided into small aliquots, and stored at -76°C.

Extraction of Ribosomal Proteins

Ribosomal proteins were extracted by the acetic acid method of Hardy et al (1969). To one volume of ribosome samples, one-tenth volume of 1.0 M $MgCl_2$ and two volumes of glacial acetic acid were added in rapid succession with constant stirring. After stirring for 45 minutes in an ice bath, the mixture was centrifuged at 20,000 x g for 10 minutes. The RNA pellet thus obtained was washed with 67% acetic acid and recentrifuged at 20,000 x g for 10 minutes. The two supernatants which contained most of the ribosomal proteins were pooled together into which ammonium sulphate was added to a final concentration of 42% (w/v). The

sample was stirred at 4°C for 10 minutes and centrifuged at 25,000 x g for 10 minutes. The ribosomal protein pellet was resuspended in a very small amount of the standard buffer and after extensive dialysis against the standard buffer the sample was stored at -20°C.

Isoelectric Focusing-Polyacrylamide Gel Electrophoresis (PAGE) System of O'Farrell (1975) For Total (T), PS-60 and S-100

In the isoelectric focusing PAGE system two buffers were used for preparation of samples:

a) Sonication Buffer

10 mM Tris-HCl pH 7.4

5 mM $MgCl_2$

50 µg/ml Pancreatic RNase

b) Lysis Buffer

9.5 M Urea

2% (v/v) Ampholines (a mixture of 1.6% pH range 5 to 7 Ampholine and 0.4% pH range 3.5 to 10 Ampholine)

5% (v/v) 2-Mercaptoethanol

Preparation of Samples

One gram (wet weight) of bacterial cell pellet was mixed with 3 ml of the Sonication buffer and the mixture was sonicated in an Insonator, Model 1000 at a power setting of 2.5 for 6 x 30 seconds followed by a further

sonication step of 60 seconds. To the sonicated sample, pancreatic DNase was added to a final concentration of 50 µg/ml. This sample contained all of the bacterial proteins and was therefore called the total (T) fraction. A portion of the T-fraction was used to prepare the PS-60 and the S-100 fractions by the previously described high speed centrifugation technique. For preparation of isoelectric focusing samples, 500 mg of urea and 0.5 ml of the Lysis buffer were added to 0.5 ml of the total, the PS-60 or the S-100 fractions. The samples were either analyzed immediately or stored at -20°C.

Isoelectric Focusing Polyacrylamide Gels

Isoelectric focusing polyacrylamide gels with the following composition were prepared:

Urea	9 M
Acrylamide (w/v)	3.78%
Bis-acrylamide (w/v)	0.22%
Triton N-101 (v/v)	2.0%
Ampholine pH 5-7	1.6%
Ampholine pH 3.5-10	0.4%

The final gel solution contained a total of 4% acrylamide and was polymerized by adding 3.3 µl of a freshly prepared 10% ammonium persulfate solution and 3 µl of TEMED into every 10 ml of the gel solution. The gel tubes were prepared by adding 3.3 ml of the gel mixture into an acid washed glass

tube (130 x 6 mm, inner diameter). To flatten the gel surface, a small amount of 8 M urea solution was overlayed on top of the gel mixture and polymerization was allowed to proceed at room temperature for 1-2 hours. After polymerization, the overlaying urea solution was replaced by 20 µl of the Lysis buffer and the gels were let to stand for another period of 1-2 hours.

The polymerized gels were then electrophoresed without samples at 200 volts/15 minutes, 300 volts/30 minutes and 400 volts/30 minutes (the prerun step) in a Buchler Electrophoresis Apparatus with the anode chamber filled with 10 mM phosphoric acid and the cathode chamber filled with extensively degassed 20 mM NaOH. After the prerun, the gels were then loaded with protein samples, on top of which 10 µl of sample overlay solution (9 M urea, 1% Ampholines) was overlayed. The anode and the cathode chambers were again filled with fresh 10 mM phosphoric acid and 20 mM NaOH solutions, respectively, and electrophoresis was carried out at a constant voltage of 400 volts for 20 hours.

On the completion of isoelectric focusing, the gels were rimmed out, soaked in 10% trichloroacetic acid for 10 minutes and stained in a solution containing 0.2% coomassie Brilliant Blue, 25% ethanol and 10% acetic acid for 30 minutes. The stained gels were then destained and stored in 10% acetic acid solution.