BIOSYNTHESIS AND REGULATION OF POLY-3-HYDROXYBUTYRATE IN *VIBRIO HARVEYI*

by

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ABSTRACT

Lipid-like granules in the luminescent bacterium, *Vibrio harveyi,* grown in complex medium were detected by electron microscopy. On staining with malachite green, sponge-like granules were detected, implicating the existence of poly-3-hydroxybutyrate (PHB). After NaOCl treatment, a homopolymer of 3-hydroxybutyrate was identified by a series of analyses, including Fourier transform infrared, nuclear magnetic resonance and ultraviolet spectrophotometry. A copolymer with 3-hydroxyvalerate was not present according to gas chromatography results. Accumulation of PHB occurred in the late stages of cell growth and was repressed by the presence of glucose. The level of PHB increased by three-fold when the nitrogen content in the medium was decreased by 50%. Syntheses of PHB in the autoinducer-deficient mutants of *V. harveyi* was very low (< 2% of wild type cells). The production of PHB can be stimulated by addition of autoinducer (ß-hydroxybutyryl homoserine lactone) for the luminescence system, supporting the hypothesis that the autoinducer is involved in regulation of pathways other than light-emission. Moreover, it was demonstrated that the autoinducer regulates the accumulation of PHB in *V. harveyi* by working in conjuction with a *lux* regulatory protein, LuxR.

RESUME

Des granules lipidiques dans la bactérie luminescente, *Vibrio harveyi*, cultivée en milieu complex, ont été détectées par la microscopie électronique. Colorées au vert de Malachite, ces granules se présentent sous forme spongieuse, ce qui est caractéristique des granules de poly(3-hydroxybutyrate) (PHB). Après l'extraction avec le NaOCl, l'homopolymère de 3-hydroxybutyrate a été identifié par une série d'analyses, incluant la spectroscopie infrarouge à transforme de Fourier, la résonance magnétique nucléaire, la chromatographie en phase gazeuse et la spectroscopie ultraviolette. Le copolymère avec le 3-hydroxyvalérate n'était pas présent selon les résultats de la chromatographie en phase gazeuse. L'accumulation de PHB a eu lieu aux dernières étapes de la croissance cellulaire, et pouvait être réprimée par la présence du glucose. Le taux de PHB a augmenté trois fois quand le teneur en azote dans le milieu a été diminué de 50 %. Une souche mutante de *Vibrio harveyi*, dans laquelle la production de l'autoinducteur lux est absente, possède un niveau de PHB très bas (< 2% de la souche normale). Toutefois, la synthèse de PHB dans la souche mutante peut être stimulée par l'addition de l'autoinducteur (β-hydroxybutyryl homosérine lactone) dans le milieu de culture, ce qui soutient l'hypothèse que l'autoinducteur est impliqué

dans la régulation d'une voie métabolique autre que la luminescence. De plus, il a été démontré que l'autoinducteur et une protéine régulatrice lux, le LuxR, fontionnent ensemble pour régulariser l'accumulation de PHB dans le *Vibrio harveyi.*

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FORWARD

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The candidate has the option, subject to approval of their department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

I Poly-3-hydroxybutyrate is produced by *V. harveyi*, which was previously described as a PHB negative stain. The production of PHB is cell density dependent. The maximum accumulation is obtained when cell growth has entered its stationary stage.

II The production of PHB in *V. harveyi* is under glucose repression, which can be reversed by adding cAMP to the culture medium. In contrast, the accumulation of PHB is stimulated when cells are grown under nutrient limitation condition.

III Both autoinducer and *lux*R of the luminescent regulatory system are involved in regulation of biosynthesis of PHB in *V. harveyi*. Much higher levels of PHB were obtained when mutant cells transconjugated with *lux*R were grown in medium containing exogenous autoinducer, compared to cells missing one of the regulatory factors, autoinducer or *lux*R.

LIST OF PUBLICATIONS

Sun, W., Cao, J.-G., Teng, K. and Meighen E. A. "Biosynthesis of Poly-3-hydroxybutyrate in the Luminescent Bacterium, *Vibrio harveyi* and Regulation by the lux autoinducer, *N*-(3-hydroxybutyroyl)homoserine lactone". (1994). *J. Biol. Chem.* **269**, 20785-20790.

Sun, W., Teng, K. and Meighen E. A. "Detection of Poly-3-hydroxybutyrate granules by electron microscopy in *Vibrio harveyi* stained with malachite green" Submitted to *Can. J. Microbial.*

Sun, W., J-G. Cao and E. Meighen, "Occurrence of Polyhydroxybutyrate in *Vibrio harveyi"* Submitted to *Proceedings of the VIIIth International Symposium On Bioluminescence and Chemiluminescence*. John Wiley & Sons, New York.

Cao, J.-G., Lee, C. Y., **Sun, W.,** O'Kane, D., Swartzman, E. and Meighen, E. A. Metabolic Pathways Coregulated with Luminescence in Marine Bacteria.

Submitted to *Proceedings of the VIIIth International Symposium On Bioluminescence and Chemiluminescence*. John Wiley & Sons, New York.

Swartzman, E., Miyamoto, C., Cao, J.-G., **Sun, W.** and Meighen, E. A. (1993) *In* "Proceedings of the VIIth International Symposium on Bioluminescence and Chemiluminescence" (Szalay, A., Kricka, L. and Stanley, P. eds.), pp. 168-172. John Wiley & Sons, New York.

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LIST OF ABBREVIATIONS

AI	autoinducer
Am^r	ampicillin resistant
cAMP	adenosine 3',5'-cyclic monophosphate
CoA	coenzyme A
CRP	catabolic repressor protein
dry wt	dry weight
FMN	flavin mononucleotide (oxidized form)
$FMNH_2$	flavin mononucleotide (reduced form)
FTIR	Fourier transform infrared
GC	gas chromatography
Km^r	kanamycin resistant
NADP	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	nuclear magnetic resonance
PHA	polyhydroxyalkanoate
PHB	poly-3-hydroxybutyrate
P(HB-co-HV)	poly-(3-hydroxybutyric-co-3-hydroxyvaleric) acid

Sm^r	streptomycin resistant
TCA cycle	tricarboxylic acid cycle
TMS	tetramethylsilane
UV	ultraviolet

CHAPTER I INTRODUCTION

POLY-3-HYDROXYBUTYRATE

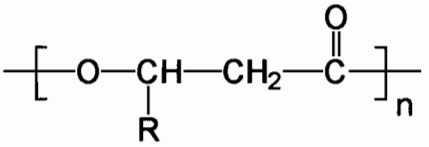
1.1 Introduction

Poly-3-hydroxybutyrate (PHB) was first discovered in bacteria by Lemoigne (1925). It is accumulated as intracellular granules by a wide variety of Gram-positive and Gram-negative bacteria and cyanobacteria. Traditionally, granules in bacteria, which could be stained with Sudan Black, were suggested to contain PHB (Williamson and Wilkinson, 1958; Merrick and Doudoroff, 1961). Staining with malachite green generates characteristic sponge-like PHB granules surrounded by a single electron dense membrane which can be readily detected by electron microscopy (Kushnaryov et al., 1980). Experiments on *Bacillus megaterium* have shown that PHB was synthesized in response to an imbalance in growth caused by nutrient limitation (Macrae and Wilkinson, 1958). As the PHB was used by the bacteria during sporulation, it was suggested that the physiological role of PHB in *Bacillus* species was to serve as a carbon and energy reserve (Splepecky and Law, 1961; Kominek and Halvorson, 1965).

The molecular weight of PHB extracted from bacteria varies from about 50,000 to over 100,000 depending on the source, growth conditions and method of extraction (Lundgren et al, 1965). The polymer is insoluble in most solvents

(e.g. water, acetone and ethanol), however it can be dissolved in hot chloroform. The PHB molecules from *Rhizobium* were found to be in compact left-handed helixes with a two-fold screw axis and a fibre repeat of 0.596 nm (Okamura and Marchessault, 1967; Cornibert and Marchessault, 1972).

Currently, PHB is known to be one of the general class of compounds, referred to as polyhydroxyalkanoates (PHA) and possessing the general formula:



where R = CH_3 in PHB. The other two principal polyesters have R = CH_3CH_2 (polyhydroxyvalerate, PHV) and R = $CH_3(CH_2)_4$ (polyhydroxyoctanoate, PHO).

The presence of long side chain polymers in bacteria was first recognized by Wallen and Rohwedder (1974). They noted that in sewage sludge, the polymers contained 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as major constituents with C_6 and possibly C_7 3-hydroxyacids as minor components. It is now known that many bacteria can produce polymers which contain hydroxyacyl monomer units other than 3-hydroxybutyrate. The production and composition of those polymers are determined by the type of bacteria as well as by the carbon sources and their relative concentrations in the

culture medium during polymer accumulation.

The major applications of PHB are based on its properties of thermoplasticity and biodegradability. From the standpoint of environmental protection, studies of PHB should therefore be of great importance. The production of PHB on a large scale to meet the volume markets is under investigation (Byrom, 1987; Hrabak, 1992). Another important property is that PHB is biocompatible, a property which attracts the interests of the pharmaceutical industry (Korsatko et al., 1983; 1984). Since the PHA heteropolymers (copolymers) possess properties so diverse from those of PHB, they may even be more attractive for commercial exploitation (Holmes, 1988).

1.2 Metabolism of poly-3-hydroxybutyrate

1.2.1 Enzymes involved in poly-3-hydroxybutyrate metabolism

There are three major enzymes involved in PHB synthesis; 3-ketothiolase (acetyl-CoA acetyltransferase; EC 2.3.1.9), acetoacetyl-CoA reductase (hydroxybutyrate-CoA dehydrogenase; EC 1.1.1.36) and poly-3-hydroxybutyrate synthetase, which sequentially catalyze the reactions from acetyl-CoA to PHB.

$CH_3CO\text{-}SCoA$ ---> $CH_3COCH_2CO\text{-}SCoA$ ---> $CH_3CH(OH)CH_2CO\text{-}SCoA$ ---> PHB

In *Alcaligenes eutrophus*, synthesis of PHB is controlled by the 3-ketothiolase. Coenzyme A (CoA) functions as the key effector metabolite, inhibiting the enzyme activity in the condensation direction (Oeding and Schlegel, 1973). In *Zoogloea ramigera*, the production of PHB is also limited by the acetoacetyl-CoA carbon-carbon bond-forming step (Masamune et al., 1989).

The second enzyme involved in PHB biosynthesis is acetoacetyl-CoA reductase, which is a NADPH reductase and produces short chain D-(-)-3-hydroxyacyl-CoAs. For biosynthesis of various PHAs, different pathways are involved to convert the intermediates from the central metabolic pathways into hydroxyacyl-CoA (HA-CoA) thioesters, the substrates of the PHA synthetases.

Unlike the thiolase and the reductase, the synthetase is most likely associated with the PHB granules rather than in the cytoplasm (Griebel et al. 1968; Merrick and Doudoroff, 1961; Ritchie and Dawes, 1969). In *Z. ramigera* as well as in *A. eutrophus*, two forms of PHB synthetase activity were observed depending on the growth conditions, the granule-bound form and the soluble form. The increase of granule bound synthetase activity was observed when PHB accumulation and granule formation were stimulated (Fukui et al., 1976; Tomita et al., 1983).

Two types of depolymerases have been detected in *R. rubrum* and *B.*

megaterium during the intracellular degradation of PHB. Although both activities are associated with the PHB granules, native granules from *R. rubrum* as well as *Azotobacter beijerinckii* are self hydrolysing (Merrick et al., 1962), whereas those from *B. megaterium* are quite stable (Merrick and Doudoroff, 1964). The depolymerase, either along with an activator protein (in *R. rubrum*, Merrick and Yu, 1966) or forming a multienzyme complex (in *B. megaterium*, Griebel et al. 1968), catalyses the hydrolysis of PHB to D-(-)-3HB, which can then be oxidized to acetoacetate by a NAD-specific dehydrogenase and converted to acetoacetyl-CoA via the action of an acetoacetate:succinate CoA transferase. The resulting acetoacetyl-CoA may then serve as a substrate in other metabolic pathways (see review by Steinbüchel, 1992).

1.2.2 Cloning and expression of genes involved in poly-3-hydroxybutyrate biosynthesis

The structural genes for 3-ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHB synthetase from *Z. ramigera* (Peoples et al., 1987; Ploux et al., 1988) and *A. eutrophus* (Slater et al., 1988; Schubert et al., 1988) have been cloned and expressed in *Escherichia coli*. The recombinant strain of *E. coli* was capable of accumulating a substantial amount of PHB as intracellular granules. It

has been shown by sequence analysis that the 3-ketothiolase and the NADPH-linked acetoactyl-CoA reductase genes are clustered and in the same operon for both bacteria. In addition, Peoples and Sinskey (1989) have demonstrated that the PHB synthetase gene of *A. eutrophus* is also in the same operon. Recent studies show that the PHB synthetases are not restricted to the use of 3-hydroxybutyryl-CoA as substrates but can also utilize other hydroxyacyl-CoAs. It has been suggested that these enzymes should be generally referred to as PHA synthetases. So far, at least ten PHA synthetase genes from eight different bacteria have been cloned and sequenced (Steinbüchel, 1992).

1.2.3 Metabolism of poly-3-hydroxybutyrate in bacteria

The most detailed studies of the regulation of PHB synthesis have been conducted with *A. beijerinckii* grown on glucose as the carbon source. Senior et al. (1972) found that much larger amounts of PHB were accumulated under conditions of oxygen limitation than under nitrogen limitation. It is believed that the ratio of NADH/NAD determines the fate of acetyl-CoA which may be oxidized via the tricarboxylic acid (TCA) cycle or serve as a substrate for PHB synthesis. When the bacteria were grown under conditions of oxygen limitation, the NADH/NAD ratio increased, resulting in the citrate synthetase and isocitrate

dehydrogenase in the TCA cycle being inhibited by NADH. In addition, high concentrations of NADH also inhibit the NAD-specific dehydrogenase involved in PHB degradation. As a consequence, acetyl-CoA no longer entered the TCA cycle and instead was converted to acetoacetyl-CoA by 3-ketothiolase, the first enzyme of the PHB biosynthetic pathway resulting in the accumulation of PHB. In contrast, when the bacteria were grown under carbon limitation rather than oxygen limitation, e.g. at the very late stationary stage of growth, the concentration of free CoA increased and inhibited the condensation activity of the 3-keto-thiolase. Therefore, the accumulation of PHB was reduced.

In summary, PHB is such a versatile and important product synthesized by bacteria that it has attracted great interest. For some bacteria, it has been used as a taxonomic character for classification. It was noted that PHB had not been previously detected in *Vibrio harveyi* (Baumann and Baumann, 1981), the bioluminescent strain which was investigated in this study.

BACTERIAL BIOLUMINESCENCE

1.3 Introduction

Bioluminescent bacteria are one of several groups of luminous organisms (Hastings and Morin, 1991). Light-emitting organisms in the marine environment were first documented by Anaximenes around 500 BC (Harvey, 1957). Systematic studies on bioluminescence were started over 300 years ago by Robert Boyle (1668), who reported the requirement of air for light emission by rotten wood and dead fish, presumably due to the presence of luminous bacteria or fungi. Since then, many advances have been made in understanding the mechanism and regulation of bacterial bioluminescence.

Currently, light-emitting bacteria are classified in four genera [*Vibrio*, *Photobacterium*, *Shewanella* (*Alteromonas*) and *Xenorhabdus*] (Meighen and Dunlap, 1993). Most luminous bacteria are distributed in marine habitats, although there are species living in fresh-water (*Vibrio cholerae*, Hada et al, 1985) and terrestrial (*Xenorhabdus luminescens*, Thomas and Poinar, 1979) environments. They can occur as free-living forms, saprophytes, commensal symbionts, parasites of animals and specific light-organ symbionts (Hastings and Nealson, 1981; Hastings et al, 1987; Dunlap and Greenberg, 1991).

It is suggested that the bioluminescence system may function as an alternative pathway for electron flow under conditions of low oxygen tension when the cytochrome oxidase mediated electron transport pathway is inhibited (Ulitzur et al., 1981; Hastings, 1983; Grogan, 1984). In the case of symbiotic association, light emitted by the luminescent bacteria could be used by the host, which supplies the luminescent bacteria with nutrients and protected environments, to attract prey, confuse predators and for interspecies communication (Morin, et al.,1975). However the reason for the persistence of the luminescence system in free-living bacteria is still obscure.

1.4 Bioluminescence reaction

The bioluminescence reaction is catalyzed by bacterial luciferase, which uses molecular oxygen to oxidize a long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$) resulting in the emission of a blue-green light at 490 nm.

$$FMNH_2 + O_2 + R\text{-}CO\text{-}H \longrightarrow FMN + R\text{-}COOH + H_2O + light$$

In the reaction, the bacterial luciferase binds the reduced flavin mononucleotide and then reacts with molecular oxygen to form an enzyme-bound 4a-hydroperoxyflavin (Hastings et al., 1973) which in turn reacts with aldehyde

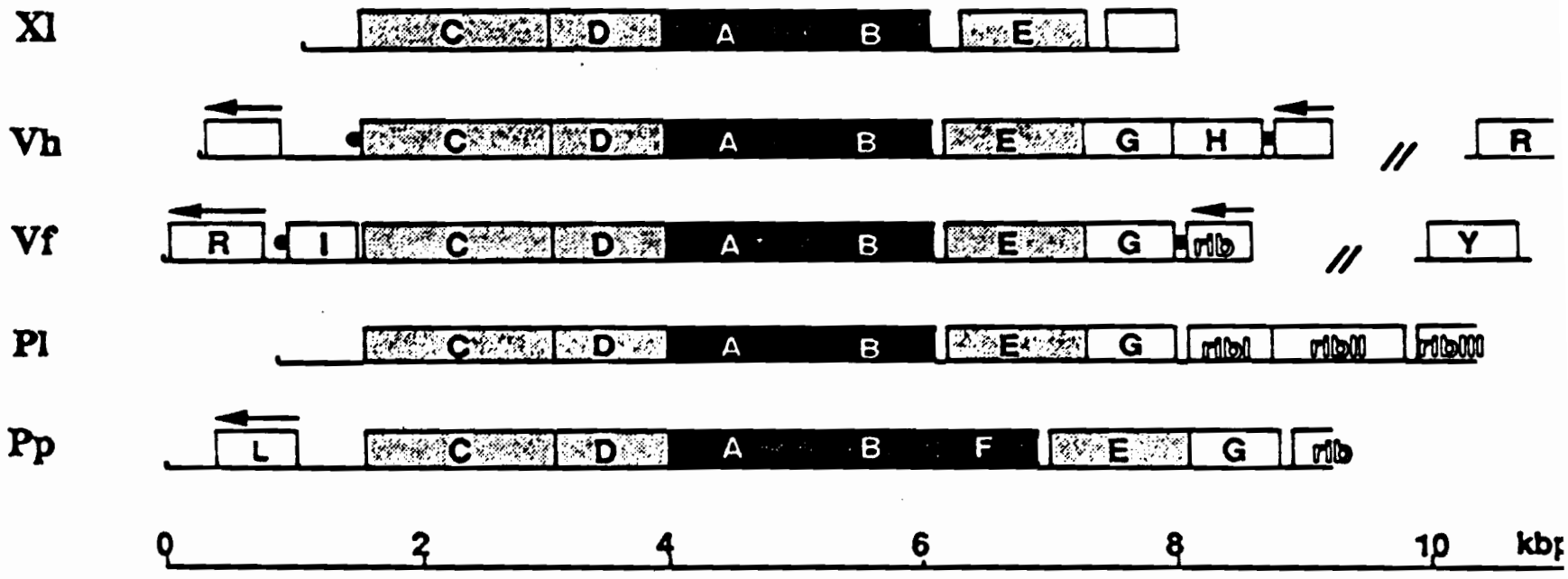
to generate the excited state (Eberhard and Hastings, 1972; Baldwin and Ziegler, 1991). The flavin specificity of the luciferase is very high. Alterations in the reduced flavin ring or removal of the phosphate decreases the activity significantly (Chen and Baldwin, 1984; Macheroux et al., 1987). Long-chain aliphatic aldehydes containing seven or more carbon atoms are required for a high level of light (Hastings et al., 1963; Hastings and Nealson, 1977). Maximum light intensity occurs with tetradecanal, which is believed to be the natural substrate (Meighen et al., 1982).

1.5 Enzymes and corresponding genes involved in the bacterial luminescence system

All bacterial luciferases are 80 kDa heterodimers containing two subunits, a (41 kDa) and ß (37 kDa), coded by the *lux*A and *lux*B genes, respectively. Although the ß subunit appears to have some effect on interaction with the flavin (Meighen and Bartlett, 1980), the primary kinetic properties, including light emission, aldehyde specificity and turnover rate are associated with the a subunit (Meighen et al 1971; Cline and Hastings, 1972). It is believed that the two genes arose by gene duplication as the two polypeptides share approximately 30% identity in amino acid sequence.

Conversion of fatty acid into the long chain aldehyde used in the luminescent reaction is catalyzed by a *lux*-specific multienzyme fatty acid reductase complex. This complex contains three different protein components; the ATP-dependent synthetase subunit (s), the NADPH-dependent acylprotein and acyl-CoA reductase subunit (r), and the acyltransferase subunit (t), coded by the *lux*E, *lux*C and *lux*D genes, respectively. The three polypeptides, r, s, t, with molecular masses of 54, 42, and 33 kDa, respectively, interact to form a large molecular aggregate of approximately 500 kDa with the central core being composed of four reductase subunits (Wall and Meighen, 1986). Each reductase subunit interacts with a synthetase subunit, giving a complex with the structure of $r_4s_4t_{2-4}$.

The order of the genes, [*lux*CDAB(F)E], coding for the luciferase (*lux*AB) and the fatty acid reductase (*lux*CDE) complex is the same in all the species analyzed (Scheme 1), including *V. fischeri* (Vf), *V. harveyi* (Vh), *P. phosphoreum* (Pp), *P. leiognathi* (Pl), and *X. luminescens* (Xl). The gene, *lux*F, located between *lux*B and *lux*E in some *Photobacterium* species, is apparently not necessary for luminescence and also probably arose by gene duplication as its protein product has almost 40% sequence identity with the carboxyl half of the LuxB subunit (Soly et al, 1988).



Sheme 1 **Organization of bacterial *lux* operon**

As shown in Scheme 1, in marine luminescent bacteria, a gene designated as *lux*G was found downstream of the *lux*CDAB(F)E genes (Swartzman et al., 1990). The amino acid sequence of the LuxG protein has been shown to be related to those of a flavin reductase and other enzymes involved in electron transport possibly implicating the function of the LuxG protein in producing $FMNH_2$ for the luminescent reaction (Andrews et al., 1992).

Immediately downstream of *lux*G, genes involved in riboflavin synthesis have been found. In *V. harveyi*, a gene designated as *lux*H is present, whose protein product has 60% sequence identity with an *E. coli* protein catalyzing the synthesis of a riboflavin precursor, 3,4-dihydroxy-2-butanone 4-phosphate (Richter et al., 1992). In *P. leiognathi* and *P. phosphoreum*, a cluster of *rib* genes are closely linked to *lux*G. These genes are denoted as *rib*I, *rib*II and *rib*III, for which the protein products catalyze the conversion of 6,7-dimethyl-8-ribityllumazine (lumazine) to riboflavin and the biosynthesis of 3,4-dihydroxy-2-butanone 4-phosphate and lumazine, respectively (Lee and Meighen, 1992).

Two other genes, *lux*L in *P. phosphoreum* and *lux*Y in *V. fischeri*, code for proteins which affect the wavelength of the emitted light (Lee et al., 1990; Eckstein et al., 1990). Both proteins have sequence similarity to the enzyme catalyzing the conversion of lumazine to riboflavin (Prasher et al., 1990; Baldwin

et al., 1990).

In *V. fischeri*, genes involved in regulation of the luminescence system, namely *lux*I and *lux*R have been detected upstream of the *lux*C gene. It is believed that, the *lux*I gene product is responsible for biosynthesis of the autoinducer (Eberhard et al., 1991), a small molecule which accumulates in the cell culture during growth, whereas the *lux*R gene product is a transcriptional activator, which has C-terminal sequence similarity with the *E. coli* protein, UvrC-28 (Henikoff et al., 1990). The latter protein was thought to belong to a response regulatory superfamily (Deretic et al., 1989; Stock et al., 1989). In *V. harveyi*, a regulatory gene, also designated as *lux*R, was found in another part of the genome (Martin et al., 1989; Showalter et al., 1990). Although the LuxR protein in *V. harveyi* has a similar function to that proposed for *V. fischeri* LuxR, no amino sequence similarity was identified between the two proteins.

1.6 Regulation of bacterial bioluminescence

Both cellular and environmental factors control luminescence in bacteria. The expression of the luminescence system has been found to be cell density-dependent and requires intracellular cAMP. Other factors (e.g. iron, osmolarity and oxygen) also affect light emission in bacteria, although the mechanism is not

yet understood. So far, most studies on regulation of luminescence have been concentrated on *V. fischeri* and *V. harveyi*.

1.6.1 Autoinduction

Expression of the luminescence system in many bacteria is controlled by a cell density-dependent induction, called autoinduction (Eberhard,1972; Nealson,1977; Rosson and Nealson, 1981). At early stages of cell growth, luciferase is expressed constitutively. Luminescence in the cell cultures is very low and actually decreases with growth due to a lack of substrates for the luminescent reaction. During cell growth, a small molecule, referred to as the autoinducer, is accumulated in the cell culture and induction of luminescence occurs when the concentration of the autoinducer reaches a threshold level. About 10 to 100-fold higher levels of luciferase and a 1,000 to 10,000-fold increase in luminescence over pre-induction levels can be obtained in the induced cell cultures (Coffey, 1967; Kempner and Hanson, 1968; Eberhard, 1972). Later work showed that the autoinducers for *V. fischeri* and *V. harveyi* were *N*-(3-oxohexanoyl)homoserine lactone and *N*-(ß-hydroxybutyryl)homoserine lactone, respectively (Eberhard et al., 1981; Cao and Meighen, 1989).

In *V. fischeri*, a model for regulation of luminescence has been proposed

(Engebrecht et al., 1983, Engebrecht and Silverman, 1984; Dunlap and Greenberg, 1991), in which the autoinducer binds to the LuxR protein forming a complex that subsequently interacts with the *lux* promotor and activates the transcription of the downstream genes including *lux*I (*lux*ICDABE*G*). The positive-feedback circuit for synthesis of the autoinducer results in an exponential increase in luciferase and luminescence.

In *V. harveyi*, *lux*I and *lux*R genes similar to those in *V. fischeri* have not been detected (Miyamoto et al., 1988). The expression of the *lux* structural genes from *V. harveyi* can be stimulated by *lux*R, the regulatory gene which is required for the high expression of luminescence in *V. harveyi* and *E. coli* (Martin et al, 1989; Showalter et al, 1990). Recently, genes involved in the reception (*lux*N) and synthesis (luxLM) of the autoinducer for the luminescence system in *V. harveyi* have been discovered (Bassler et al., 1993) as well as a negative regulator of the luminescence sytem, LuxO (Basasler et al., 1994). The specific mechanism remains to be elucidated.

1.6.2 Metabolite Repression

Luminescence in the two *Vibrio* species can be repressed by glucose. In *V. harveyi*, permanent glucose repression is found in batch cultures and is reversed

by the addition of cAMP (Nealson et al., 1972). In contrast, in *V. fischeri*, glucose repression is temporary and eliminated by prior growth of the cells in the presence of glucose (Ruby and Nealson, 1976). Permanent glucose repression can be obtained and reversed by cAMP when *V. fischeri* cells are grown in a phosphate-limited chemostat culture. Experiments showed that the cAMP stimulation of luminescence in *V. fischeri* is mediated by activation of transcription of the *lux*R promotor (Dunlap and Greenberg, 1985, 1988; Dunlap and Ray,1989). Along with the autoinducer, the LuxR protein then induces expression of the lux operon. In *V. fischeri*, a consensus CRP-binding site is present in the regulatory region of the *lux*R gene.

1.6.3 Other regulatory factors

The presence of exogenous iron has been found to repress light-emission in *V. fischeri* (Makemson and Hastings,1982). Although the mechanism of this repression is unknown, the induction of the luminescence system at low concentrations of iron may be important in the symbioses of luminescent bacteria in fish and squid for inducing high levels of luminescence, in which iron-limitation functions as one of the environmental signals (Haywood and Nealson, 1985a, Hastings et al., 1987).

Other environmental factors, such as oxygen and osmolarity, can influence luminescence differentially depending on the species of luminous bacteria (Meighen and Dunlap, 1993). More studies are required to reveal the nature of these effects.

1.7 The potential role of autoinducers as general signal transductants

The possibility that the lux autoinducers in *V. fischeri* and *V. harveyi* are involved in controlling metabolic pathways other than the luminescence system was noted more than a decade ago. Early work shows that the autoinducer for *V. harveyi* is also produced by other bacteria, including non-luminous species (Greenberg et al., 1979). Although the functions of the autoinducer in these species are not known, they are clearly not related to bioluminescence. Recently, the *V. fischeri lux* autoinducer, *N*-(3-oxohexanoyl)homoserine lactone, was found to control both the biosynthesis of the antibiotic, carbapenem (Bainton et al., 1992; Stewart and Williams, 1992) and virulence in the plant pathogen, *Erwinia carotovora* (Pirhonen et al., 1993; Jones et al., 1993). In addition, a gene, *exp*I, whose product has a similar function to the LuxI protein and is related in sequence, was found in *E. carotovora* (Pihonen et al., 1993), implicating a common mechanism for modulation of gene expression in these bacteria. It is also suggested that the autoinducer may play a role in communication between *V.*

fischeri and the sepiolid squid *Euprymna scolopes* during the establishment of the mutualistic symbiotic relationship (Ruby and McFall-Ngai, 1992).

As the structures of the autoinducers from *V. harveyi* as well as *V. fischeri* are analogous to that of A-factor (2*S*-isocapryloyl-3*S*-hydroxymethyl-γ-butyrolactone), which mediates the autoinduction of sporulation, streptomycin biosynthesis and streptomycin resistance in *Streptomycin griseus* (Horinouchi and Beppu, 1990; 1992), it is possible that the autoinducer of *V. harveyi* also controls more than the luminescence system. Recently, the conjugation factor of *Agrobacterium tumefaciens*, which behaves as a secondary messenger by transmitting the environmental information to the *tra* genes, has been identified as *N*-(3-oxo-octanoyl)homoserine lactone, a compound with a close relationship in structure to the autoinducers of the two *Vibrio* species (Zhang et al, 1993). It thus seems that the *lux* autoinducers may belong to a general family of signal transductants with *N*-acyl-homoserine lactone as a conserved moiety in which the length and the nature of the lipophilic acyl chain determines the biological function to be regulated.

1.8 Goal and strategies of the present research

During the investigation of potential morphological changes in *V. harveyi*

on development of luminescence, lipid-like granules were detected in cells by electron microscopy, implicating the presence of PHB. This was surprising because previous work has shown that PHB was not produced in this strain (Baumann and Baumann, 1983). The discovery was of particular interest as the autoinducer, *N*-(3-hydroxybutyryl)homoserine lactone, contains a 3-hydroxybutyric acid moiety. Studies were therefore carried out in the present research to investigate the synthesis and regulation of PHB in *V. harveyi*.

On staining with malachite green, characteristic sponge-like granules similar to the PHB granules found in *Aquaspirillum serpens* (Kushnaryov et al., 1980) were observed by electron microscopy. Further identification of PHB in *V. harveyi* was conducted by a series of analyses including FTIR, NMR and GC, showing that the PHB homopolymer is present. Metabolism of PHB in *V. harveyi* was followed by measurement of the level of PHB at different stages of cell growth. The amount of PHB was determined by UV spectroscopy. The effects of environmental (glucose or nutrition) and other regulatory factors (autoinducer and/or the LuxR protein) on the *in vivo* synthesis of PHB were also investigated.

Details of the work are presented in Chapters 2 and 3.

CHAPTER II MATERIALS AND METHODS

2.1 Materials

Sodium cacodylate, glutaraldehyde and Spurr's epoxy resin for electron microscopy sample preparation were purchased from Polysciences Inc. Malachite green, poly-3-hydroxybutyrate (PHB), D-(-)-3-hydroxybutyric acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, were from Sigma Chemical Company. α-Amino-γ-butyrolactone hydrobromide and poly-(3-hydroxybutyric-co-3-hydroxyvaleric) acid (P(HB-co-HV)) were from Aldrich Chemical Co. Antibiotics, ampicillin, kanamycin and streptomycin were obtained from BDH chemical Inc. Tryptone, yeast extract and agar were from Difco Laboritories. Other chemicals were from J. T. Baker Inc.

The luminescent bacteria used in this study were *Vibrio harveyi*, strain B392 (Reichelt and Baumann, 1973), which was used in most of the experiments, strain BB7, two luminescence regulatory mutants of strain B392, D1 and D34 (Cao et al., 1989) and one mutant of strain BB7, MR1101, which has a Tn5 transposon insertion in the *lux*R gene (Martin et al., 1989). All of them are ampicillin-resistant. The *E. coli* strain MM294 (Hfr, ATCC 33625) and the plasmids pRK2013 (ATCC 37159) were obtained from the American Type Culture Collection. The kanamycin resistant plasmid pRK2013 carries the RK2 transfer system (*tra*$^+$) in a ColE1 replicon (Figurshi and Helinski, 1979).

Recombinant plasmid pKT231 containing the *lux*R gene, a luminescent regulatory gene, was kindly provided by Dr. M. Silverman (Showalter et al., 1990).

2.2. Methods

2.2.1 Cell growth and luminescence measurement

Cells grown overnight were inoculated into 250 ml Erlenmeyer flasks containing 50-60 ml of culture medium, at a starting cell density of 0.05 at 660 nm. Bacteria were incubated in a New Brunswick Scientific Rotary Shaker at a rotary rate of 250 rpm. *V. harveyi*, its mutants and the transconjugants of the mutants were grown at 27°C in complex medium (10 g NaCl, 3.7 g Na_2HPO_4, 1.0 g KH_2PO_4, 0.5 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 ml glycerol, 5 g tryptone and 0.5 g yeast extract per liter). To the transconjugants, the appropriate antibiotics, ampicillin 50 μg/ml, kanamycin 25 μg/ml and/or streptomycin 30 μg/ml were added to the culture medium in order to maintain the selective pressure. *E. coli* strains bearing different plasmids were grown at 37°C in Luria-Bertani (LB) medium (10 g NaCl, 10 g tryptone, 5 g yeast extract per liter) with the appropriate antibiotics. Cell growth was monitored by the optical density at 660 nm, and the in vivo luminescence of 1 ml of culture was measured using a

photomultiplier calibrated with the light standard of Hastings and Weber (1963). One light unit = 1 x 10^{10} quanta/sec. An optical density of 1.0 at 660 nm corresponds to approximately 5 x 10^{8} cells/ml culture (Riendeau and Meighen, 1980). Cells were diluted 10-fold for measurements of cell growth and luminescence when its optical densities were higher than 0.4 at 660 nm.

2.2.2. Bacterial sample preparation for electron microscopy

Bacterial cells were harvested at different time of growth, prefixed in situ at room temperature for 30 min by adding an equal volume of 2% glutaraldehyde fixative buffer containing 0.1 M sodium cacodylate (pH 7.2) only or plus 0.2% w/v malachite green. After centrifugation, the pellets were suspended and fixed in 0.1 M fixative buffer again (with or without 0.1% w/v malachite green) for 2 hours at room temperature.

Following fixation, the samples were washed three times with 0.1 M sodium cacodylate buffer (pH 7.2) and then mixed with 1% osmium tetroxide (OsO_4) plus 1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hours at room temperature. Specimens were then washed twice with water, poststained with 2% uranyl acetate for 1 hour, dehydrated in graded concentrations (first in 70% then 90%, 95% to 100% (v/v)) of acetone and

embedded in Spurr's (Spurr, 1969) epoxy resin by incubating at 70°C overnight. Thin sections of 70 nm were cut and stained in Reynold's lead citrate, and photographed with a Phillips 300 electron microscope.

2.2.3. Poly-3-hydroxybutyrate extraction

PHB samples were extracted by the NaOCl method (Splepecky and Law, 1960). Bacteria were harvested by centrifugation, and resuspended in 10 mM Na_2EDTA. The NaOCl solution, containing at least 5% Cl_2, was added to the cell suspension. Bacteria were digested at 37°C for at least 1 hour. PHB samples were collected by centrifugation at 12,000 rpm for 10 min. The pellets were washed by vortexing in sequence with water, acetone and ethanol. The samples were finally dried with a speed-vac centrifuge or air dried before analysis by UV, FTIR and NMR.

2.2.4. Ultraviolet spectrophotometry (UV) analysis

The NaOCl extracts from *V. harveyi* were converted to crotonic acid by treatment with 1 ml of concentrated sulfuric acid at 100°C for 10 min. The solution was then cooled on ice and subsequently diluted with water or concentrated sulfuric acid to give a final concentration of PHB of less than 16

μg/ml. Reference solutions of PHB were treated in the same way. Ultraviolet absorption spectra from 260 nm to 200 nm were scanned on a Beckman DU-7 Spectrophotometer (Beckman Instruments Inc.). A standard curve was obtained by plotting the maximum absorbance as a function of the concentrations of the reference solutions of PHB.

2.2.5. Fourier Transform Infrared (FTIR) Spectroscopy analysis

FTIR spectroscopy was carried out on a Perkin-Elmer 16PC IR spectrophotometer. PHB films were cast on a KBr window and the spectra recorded. As the carbonyl group (C=O) of PHB gives a very strong and narrow absorption band in the infrared region at 1730 cm^{-1}, the spectra of PHB in $CHCl_3$ were also recorded in a KBr cell (optical path = 15 mm) and used as a quantitative measurement. In both cases, commercial PHB was used as the reference.

2.2.6. Nuclear magnetic resonance (NMR) analysis

^1H NMR was performed on a 300 MHz spectrometer (Varian XL-300). The spectrum of NaOCl extract from *V. harveyi* was recorded in $CDCl_3$ at room temperature and compared with that of commercial PHB. The chemical shift of

tetramethylsilane (TMS) was used as an internal reference.

2.2.7. Gas chromatography (GC) analysis

a) Sample preparation

V. harveyi cells (200 ml) were harvested at late logarithmic growth by centrifugation. The pellets were suspended in a mixture of 2 ml acidic methanol (3% H_2SO_4, v/v) and 2 ml of chloroform in a screw capped tube, and heated at 100°C for 3 1/2 hours. After cooling to room temperature, the reaction mixture was mixed with 1 ml of H_2O and shaken vigorously for 10 min. The two phases were separated by centrifugation at 10,000 rpm in a Sorval RC-5B centrifuge for 5 min, during which cell debris gathered at the interphase. The lower organic phase which is supposed to contain the resulting methyl 3-hydroxybutyrate was transferred into a vial and kept at 4°C before analysis. PHB and poly-(3-hydroxybutyric-co-3-hydroxyvaleric) acid were treated in the same way and used as reference solutions.

b) GC analysis

A gas chromatograph (HP 5890 II Hewlett Packard, USA) with a double flame ionization detector system was used for the analysis. A 25 m capillary column (ID = 0.32 mm) coated with cross-linked methyl silicon film of 0.52 mm

in thickness was used. The flow rate of carrier gas was 30 ml/min. The injection port temperature was 180°C and the detector temperature was 200°C. The holding time was 1 min for the initial oven temperature and 5 min for the final temperature. The temperature was raised from 55°C to 150°C at a rate of 10°C/min. One microliter of sample was injected each time.

2.2.8. Autoinducer synthesis and purification

Autoinducer [*N*-(3-hydroxybutyryl)homoserine lactone] of *V. harveyi* was synthesized by following the procedure of Cao and Meighen (1993). (D)-(-)-3-Hydroxybutyric acid (27 mmol) was incubated with equal molar amounts of α-amino-γ-butyrolactone hydrobromide and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide in 50 ml water at 27°C and shaken overnight.

The autoinducer was purified by extracting the reaction mixture four times with 100 ml of chloroform. After evaporation of the organic solvent in a rotary evaporator at 30°C, the residue was redissolved in 15 ml of chloroform. The solution was then concentrated to 3 ml and 1 ml was taken out to load onto a 20 cm x 1 cm silica gel column, which had been previously washed with at least three times the column volume of chloroform. Autoinducer was eluted in a chloroform-methanol mixture (98:2 v/v) at a flow rate of 1 ml/min. Forty

37°C overnight.

In order to screen for transcojugated D1 or D34 cells, the mated cells were spread on LB agar, and grown for 24 hours at 27°C with ampicillin 50 μg/ml, kanamycin 30 μg/ml and streptomycin 25 μg/ml in the plate medium to ensure that the resulting colonies were *V. harveyi* mutants carrying the recombined *lux*R gene in the pKT231 plasmid (*V. harveyi*, amp^r; pKT, sm^r, km^r; pRK2013, km^r).

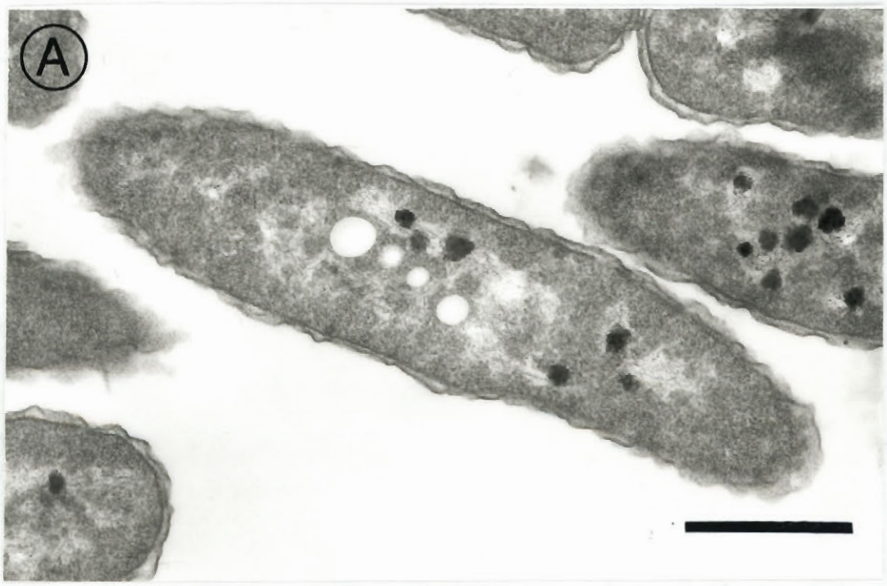
CHAPTER III RESULTS

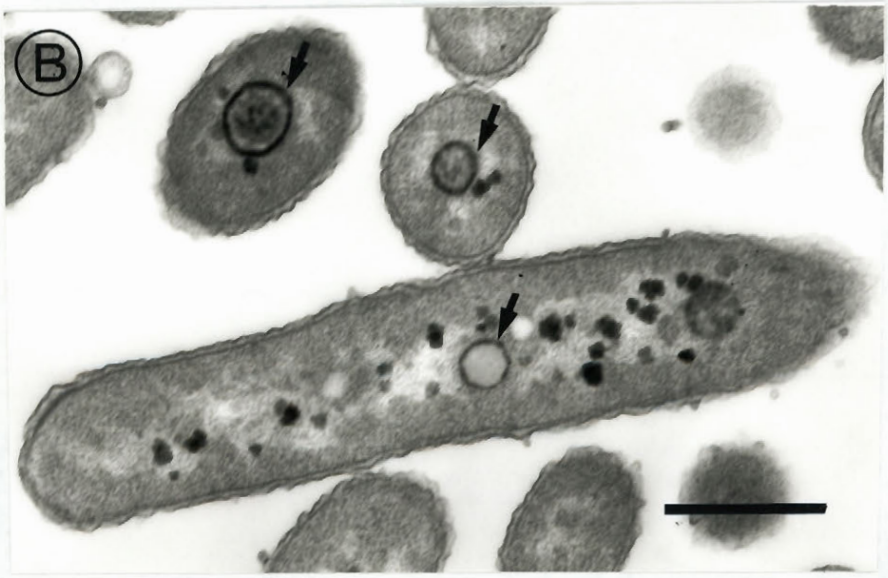
3.1 Visualization of poly-3-hydroxybutyrate granules in *V. harveyi* cells by electron microscopy

During the analyses by electron microscopy of possible ultrastructure changes in *V. harveyi* on induction of the *lux* system, lipid-like granules were detected in cells at high cell densities (Fig. 1A). On staining with malachite green, sponge-like granules with a defined membrane can be detected (Fig. 1B), implicating the occurrence of PHB. These results were surprising as earlier work had indicated that *V. harveyi* did not produce PHB (Baumann and Baumann, 1981).

Fig. 1 shows the electron micrographs of *V. harveyi* cells prepared by two different methods. One is the standard glutaraldehyde fixation procedure (Fig. 1A); the other procedure involved using malachite green and glutaraldehyde as the fixative (Fig. 1B). As shown in Fig. 1A, lipid-like granules (the electron transparent inclusions) of different size can be detected in cells fixed by glutaraldehyde. The variation of the size of those granules is presumably due to the position of the section, with bigger ones corresponding to the granules cut closer to their centre. Alternatively, it is possible that there may be more than one kind of granule or inclusions in cells. Upon adding malachite green to the fixative buffer, granules with variant electron density showing fine granular structures

Fig. 1 Visualization of poly-3-hydroxybutyrate granules in *V. harveyi* by electron microscopy *V. harveyi* cells were collected at OD_{660} = 3.1, and thin sections prepared from cells fixed with (A) glutaraldehyde only or (B) glutaraldehyde combined with malachite green as described in Materials and Methods. Bar = 0.5 μm. Sponge-like granules are indicated by the arrows.


A

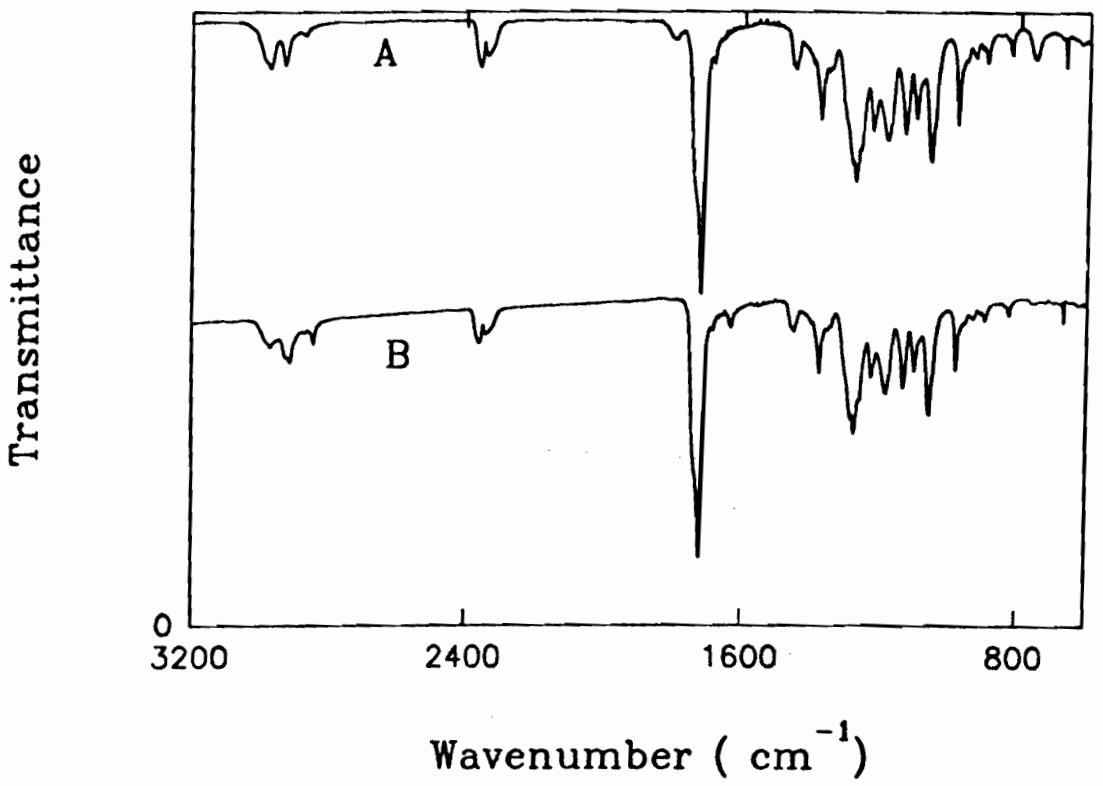

B

surrounded by defined membranes are observed (Fig. 1B). These sponge-like granules are very similar to PHB granules found in *Aquaspirillum serpens* (Kushnaryov et al., 1980). The differences in the appearance of the sponge-like granules could be caused by the variation in section position (Boatman, 1964). The results implicate that PHB was produced in *V. harveyi*. Therefore, the cells were analyzed for PHB by a number of procedures.

3.2 Identification of poly-3-hydroxybutyrate

3.2.1 FTIR spectroscopy analysis

Samples from *V. harveyi* cells were prepared by the NaOCl method. The resulting polymers were dissolved in chloroform, from which a film was cast for FTIR analyses. As shown in Fig. 2, the FTIR spectra of the sample from *V. harveyi* and the reference PHB are essentially identical. Both spectra have the strong absorption band at 1730 cm^{-1} which is the result of the stretching vibrational motion of the ester carbonyl. The absorption bands from 2620 to 2830 cm^{-1} are probably caused by the three different types of C-H bonds on the α- and β- and γ- carbons. The absorption bands at about 2400 cm^{-1} are due to CO_2 in the air. In addition, the peaks in the fingerprint region (800-1500 cm^{-1}) are also identical for the two samples, reference PHB and the NaOCl extract of *V.*


Transmittance
A
B
0
3200
2400
1600
800
Wavenumber (cm^{-1})

harveyi, indicating that PHB was produced in *V. harveyi* cells.

As the carbonyl group of PHB gives a strong and narrow absorption band in the infrared region at 1730 cm^{-1} (Fig. 2) without disturbance by the spectra of $CHCl_3$ (data not shown), FTIR spectroscopy can be used for determination of the amount of PHB in a $CHCl_3$ solution. A standard curve is shown in Fig. 3 for the absorption at 1730 cm^{-1} as a function of the concentration of PHB. An extinction coefficient of 29.3 ml $mg^{-1}cm^{-1}$ was obtained from the slope of the line. The concentration of PHB extracted from 50 ml of *V. harveyi* cells (OD_{660} = 4.5), which was dissolved in 3 ml $CHCl_3$, was found to be 0.91 mg/ml. The total amount of PHB is 2.7 mg. This corresponds to a level of approximately 0.054 mg of PHB per ml of *V. harveyi* cell culture. Further analysis of the *V. harveyi* sample for PHB was conducted by NMR spectroscopy.

3.2.2 NMR spectroscopy analysis

Proton nuclear magnetic resonance of the *V. harveyi* sample was performed on a VARIAN XL-300 spectrometer. Tetramethylsilane (TMS) was used as the standard to designate a chemical shift of 0 ppm. The spectrum of the NaOCl extract which was dissolved in $CDCl_3$, shown in Fig. 4, is identical to that of the PHB standard (not shown). The peaks at 2.50 ppm, 5.35 ppm and 1.25

Fig. 3 Standard curve for determination of poly-3-hydroxybutyrate by FTIR The absorption at 1730 cm^{-1} was measured and plotted as a function of PHB concentration. The optical path is 0.015 cm. The extinction coefficient is 29.3 ml mg^{-1} cm^{-1}.

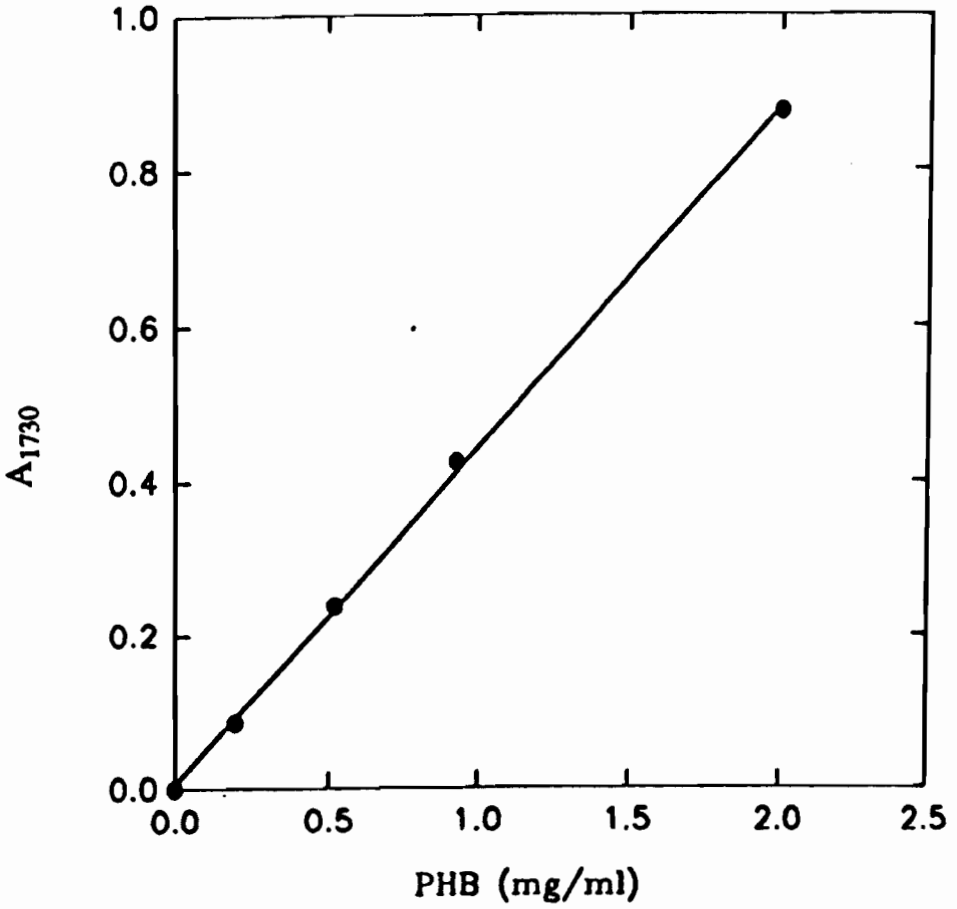
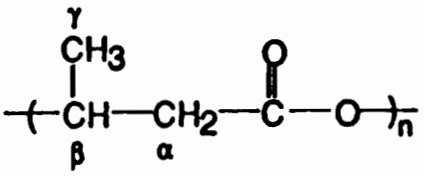
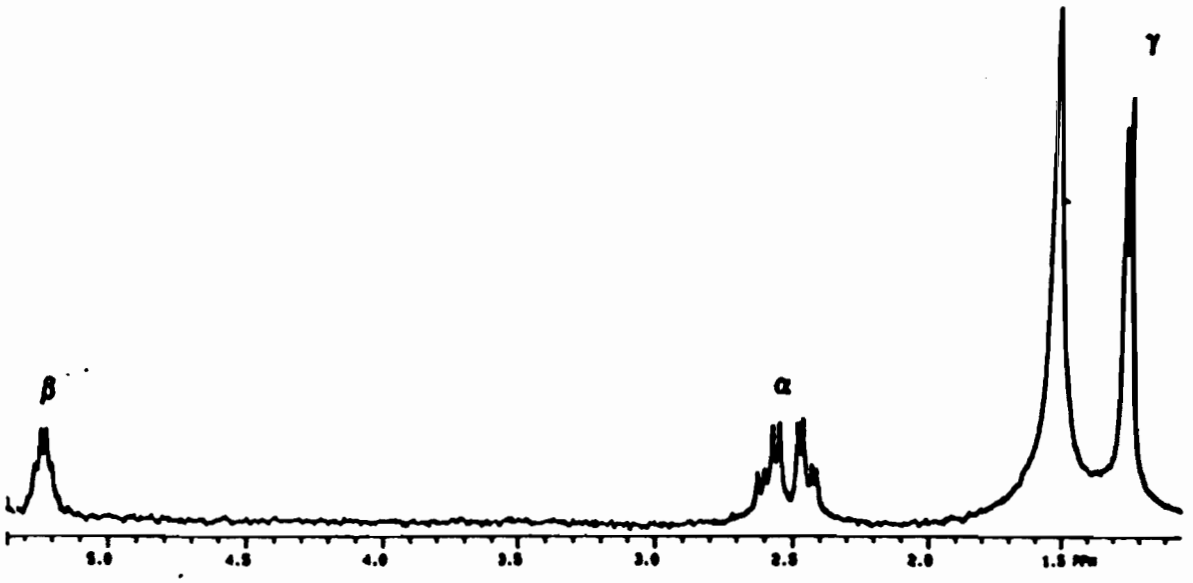

1.0
0.8
0.6
0.4
0.2
0.0
A_{1730}
0.0
0.5
1.0
1.5
2.0
2.5
PHB (mg/ml)

Fig. 4 ^{1}**H NMR spectrum for the NaOCl extract from *V. harveyi* cells**

Fig 4.





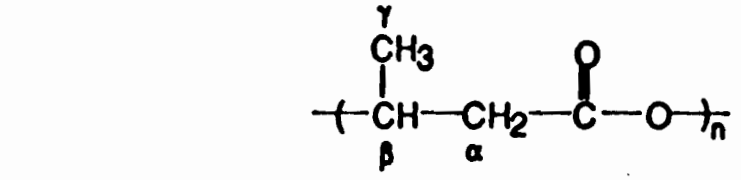
ppm are attributed to the protons on the α-, β-, and γ- carbons, respectively, with the split of the peaks being diagnostic of the spin-spin coupling between the adjacent protons. The peak at 1.5 ppm is due to water trapped in the solvent. The Cosy plot in Fig. 5 clearly shows the spin-spin coupling of the adjacent protons in the PHB molecule. The result confirmed the fact that PHB was present in *V. harveyi*.

3.2.3 GC analysis

Although the NMR and FTIR analyses provide strong proof that PHB was present in the extracts from *V. harveyi* cells, further analysis was carried out by gas chromatography (GC) to determine if any 3-hydroxyvalerate was also present. In general, analysis by gas chromatography is a more sensitive technique for determination of low levels of materials.

V. harveyi cells (OD_{660} = 4.5), the reference PHB and the copolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-co-HV)], were treated with acidic methanol to generate the corresponding methylated esters which were then analyzed by gas chromatography. As shown in Fig. 6, all three reaction mixtures gave the same peak with a retention time of 3.4 min corresponding to methyl 3-hydroxybutyrate. For P(HB-co-HV), a second peak with a retention

Fig. 5 Cosy plot for the NaOCl extract from *V. harveyi* cells


γ
CH3
O
CH
CH2
C
O
n
β
α

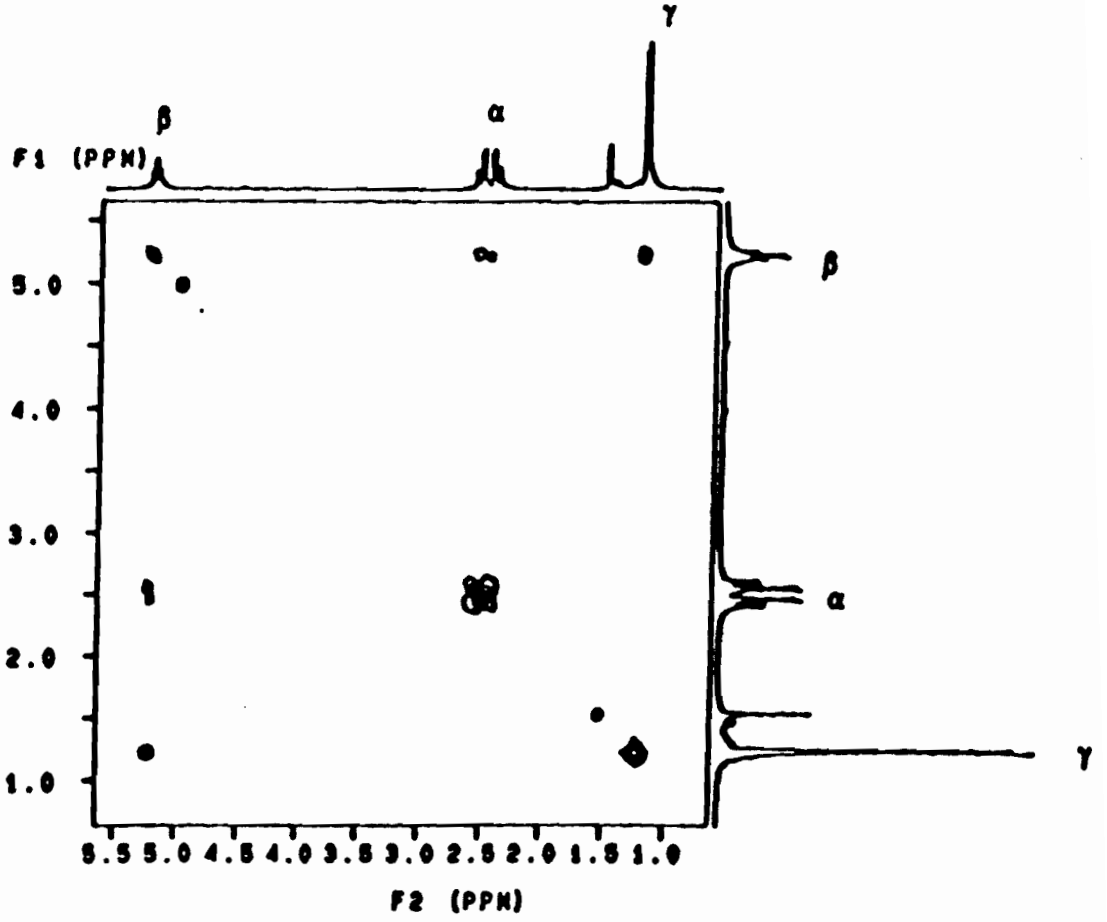
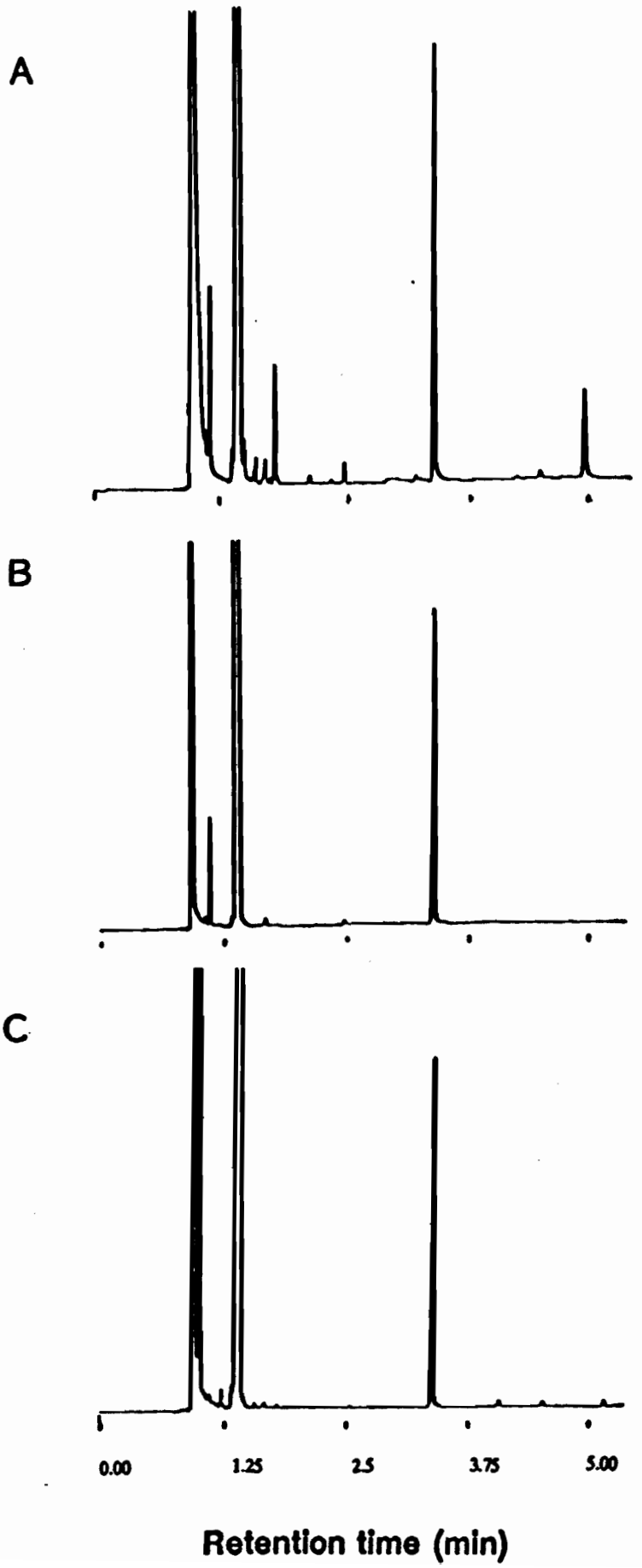

γ
β
α
F1 (PPM)
5.0
4.0
3.0
2.0
1.0
β
α
γ
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
F2 (PPM)

Fig. 6 GC analysis of the production of poly-3-hydroxybutyrate in *V. harveyi* cells P(HB-co-HV) (A), PHB (B) and *V. harveyi* cells (OD_{660} = 4.5) (C) were treated with acidic methanol and the chloroform soluble components were separated by gas chromatography as described in Materials and Methods. Peaks with retention times of 0.8 to 1.5 min correspond to the solvent, while methyl 3-hydroxybutyrate and methyl 3-hydroxyvalerate elute with retention times of 3.4 and 4.9 min, respectively.


A
B
C
0.00
1.25
2.5
3.75
5.00
Retention time (min)

time of 4.9 min was also observed, which can be attributed to methyl 3-hydroxyvalerate. This peak was not detectable in the chromatogram for the extract from *V. harveyi* showing that only the PHB homopolymer was produced by the cells and that the 3-hydroxyvalerate unit was not present.

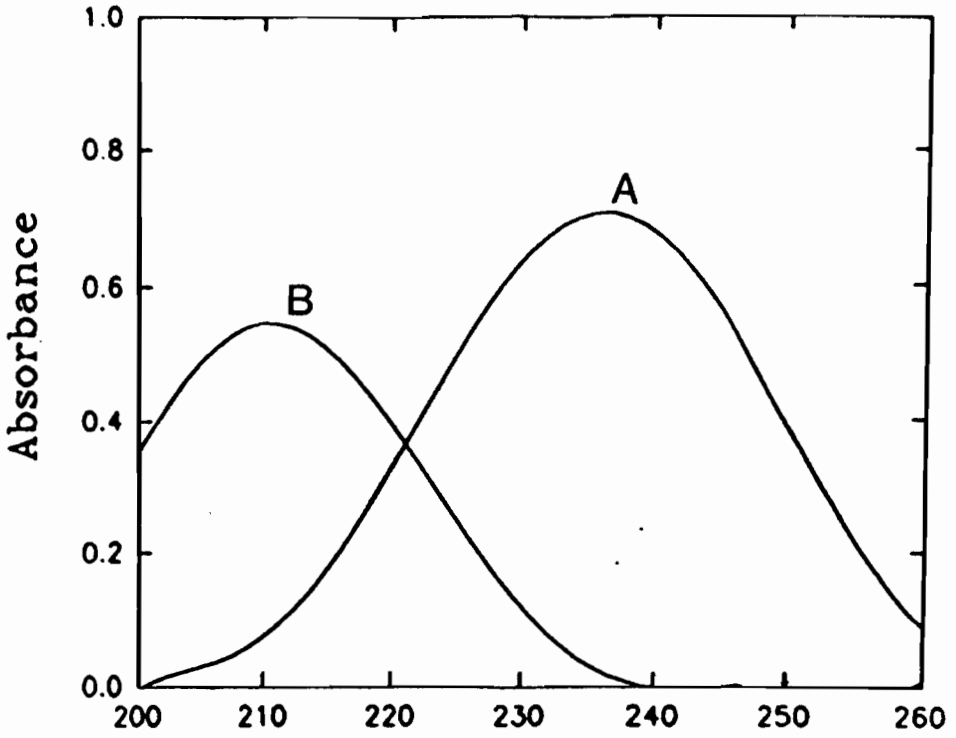
3.3 UV spectrophotometric analysis of poly-3-hydroxybutyrate levels

PHB is converted to crotonic acid on heating in concentrated sulfuric acid. The ultraviolet absorption of the a-ß unsaturated C=C double bond of crotonic acid undergoes a strong bathochromic shift with a maximum absorption at 235 nm if concentrated sulfuric acid is used as the solvent (Splepecky and Law, 1960). Using 10% H_2SO_4 instead of concentrated H_2SO_4, the maximum absorption for crotonic acid shifts to 210 nm (Fig. 7).

Conversion of PHB to crotonic acid and measurement of the maximum absorption at 235 nm in concentrated H_2SO_4 or 210 nm in 10% H_2SO_4 provide an excellent method for the determination of the amount of PHB. A series of PHB samples (from 0 - 160 µg/ml) was treated with concentrated sulfuric acid and the spectra of the samples were recorded after dilution in water or in concentrated H_2SO_4. A linear relationship with an excellent correlation coefficient was obtained between the absorbance at 235 (in concentrated H_2SO_4)

Fig. 7 UV spectra of poly-3-hydroxybutyrate converted to crotonic acid

PHB (20 μl) from a 2 mg/ml stock solution in $CHCl_3$ was dried and converted to crotonic acid by incubation in 1 ml concentrated sulfuric acid at 100°C for 10 min. After cooling on ice, the digested PHB was diluted 10-fold with either concentrated H_2SO_4 or H_2O. Spectra of samples in (A) concentrated H_2SO_4 and (B) 10% (v/v) H_2SO_4 are shown.


Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
200
210
220
230
240
250
260
A
B

or 210 nm (in 10% H_2SO_4) and the amount of PHB over the range indicated (Fig. 8). Although the extinction coefficient decreased from 0.175 ml $\mu g^{-1}cm^{-1}$ at 235 nm in concentrated H_2SO_4 to 0.143 ml $\mu g^{-1}cm^{-1}$ at 210 nm in 10% H_2SO_4, in most cases, when the final concentration of PHB was high enough (> 2 μg/ml) the spectra were recorded in 10% H_2SO_4 as the lower viscosity of these solutions made analyses more convenient.

Comparison of the extinction coefficient and the path length for determination of PHB by ultraviolet spectroscopy (0.175 or 0.143 ml $\mu g^{-1}cm^{-1}$, 1 cm) to those for FTIR determination (29.3 ml $mg^{-1}cm^{-1}$, 0.015 cm), clearly illustrates that UV analysis is much more (about 400 times) sensitive for the determination of PHB. Only 5 ml out of a 3 ml concentrated PHB extract from 50 ml of *V. harveyi* cells which was used for FTIR analysis was required for quantification by the UV method. The absorbance of 0.88 at 235 nm, corresponding to 0.90 mg/ml of PHB in the 3 ml sample, is in excellent agreement with the value of 0.93 mg/ml of PHB obtained from FTIR analysis.

A very good linear correlation was observed between the amount of PHB extracted and the concentration of PHB in the cells (Fig. 9). These results show that PHB can be extracted from the cells and quantified with the same efficiency over a wide range of concentrations. Therefore extraction with NaOCl and

Fig. 8 Dependence of the UV absorption of poly-3-hydroxybutyrate converted to crotonic acid on the amount of PHB Different amounts (0, 20, 40, 60, 80,100, 120, 140 and 160 μg) of PHB were treated as described in Fig. 7. Samples were diluted 1:10 into (A) concentrated H_2SO_4 or (B) H_2O. Absorptions were recorded at the peak of the spectrum, 235 nm for (A) and 210 nm for (B), and plotted versus the amount of PHB. The extinction coefficients are 0.175 in (A) and 0.143 ml μg^{-1} cm^{-1} in (B).

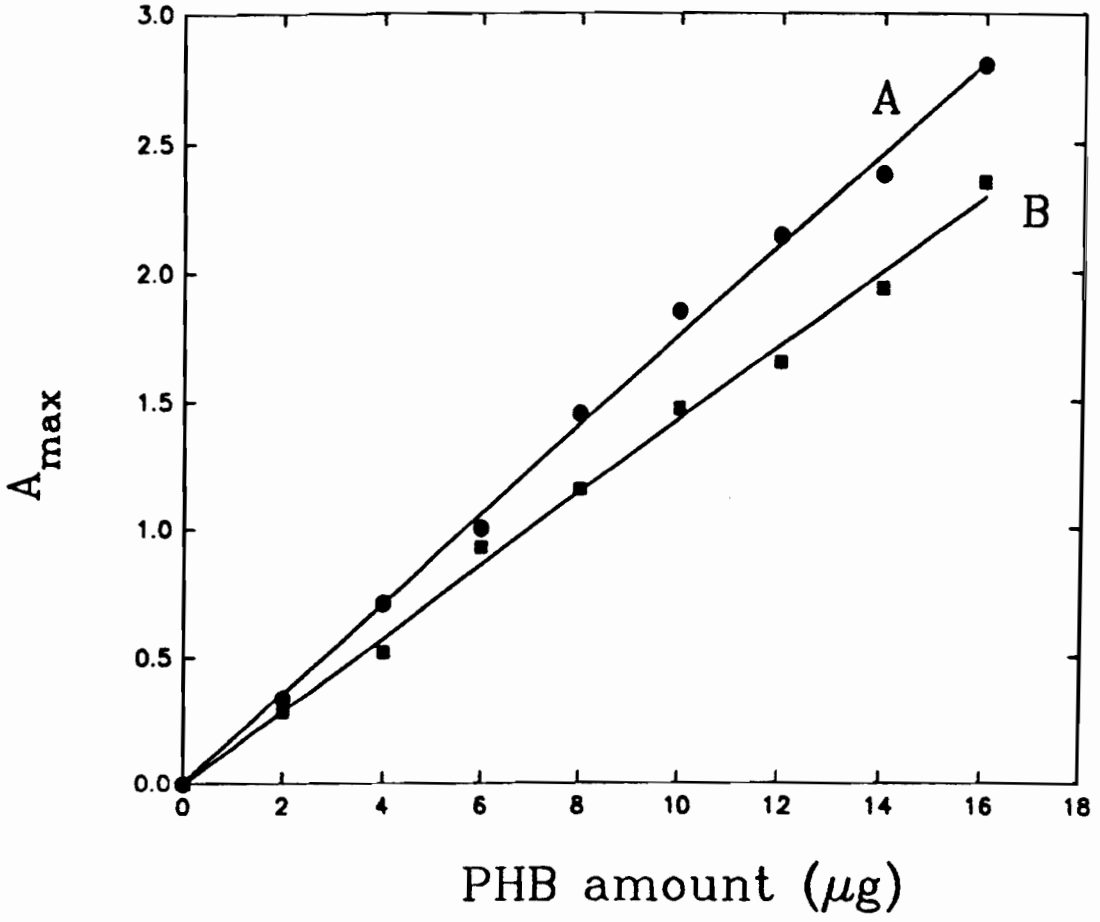
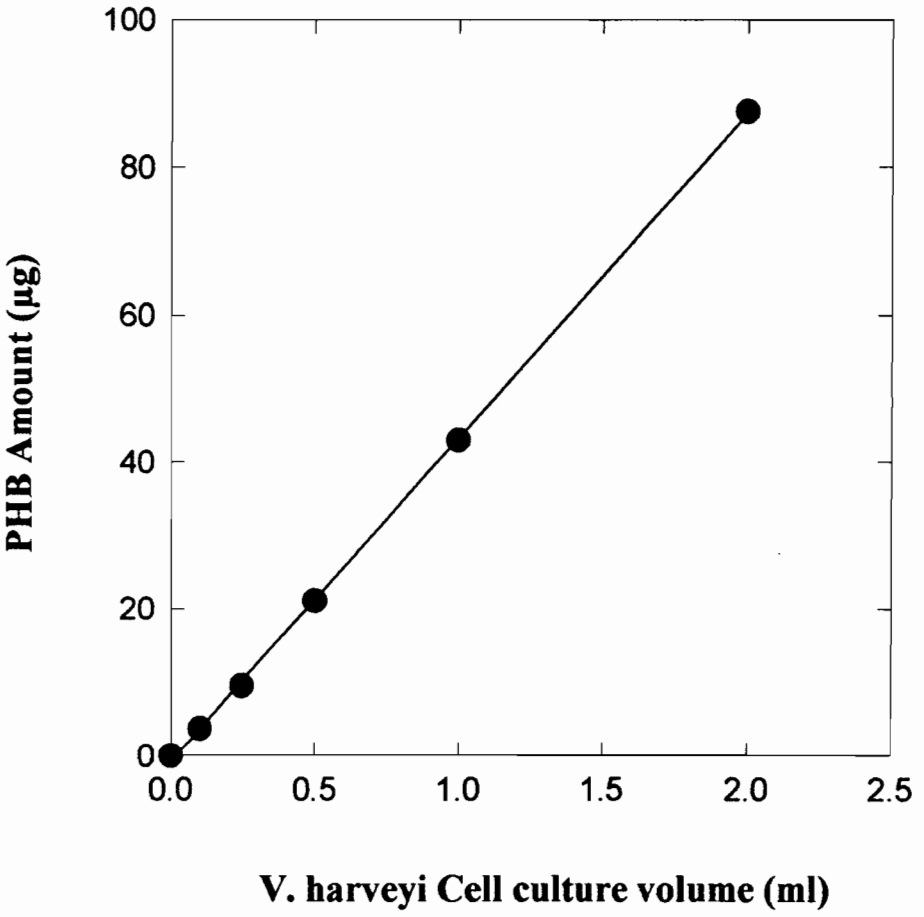

3.0
2.5
2.0
1.5
1.0
0.5
0.0
A_{max}
A
B
0
2
4
6
8
10
12
14
16
18
PHB amount (μg)

Fig. 9 Relationship between the concentration of poly-3-hydroxybutyrate in cell cultures and the amount of PHB measured by UV spectroscopy

Cultures of *V. harveyi* wild type cells and a mutant cell line, D1, which does not accumulate PHB (see Fig. 13) were grown overnight in complex medium to an optical density of ~ 5 at 660 nm. Different volumes of cell cultures of *V. harveyi*, 0, 0.1, 0.25, 0.5, 1.0 and 2.0 ml, were then mixed with 2.0, 1.9, 1.75, 1.5, 1.0 and 0 ml of D1 mutant cells, respectively, and harvested by centrifugation. PHB from these samples was prepared by the NaOCl method and quantitated by UV spectrophotometric analyses. A straight line with a correlation coefficient of 0.999 was obtained for the analysis of the relationship between the amount of PHB and the volume of wild type *V. harveyi* cells analyzed.


100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
PHB Amount (µg)
V. harveyi Cell culture volume (ml)

analysis by UV spectroscopy were chosen as the procedures for determination of the amount of PHB in *V. harveyi* cells throughout this study.

3.4 Dependence of luminescence and poly-3-hydroxybutyrate levels on growth of *V. harveyi*

The growth of *V. harveyi* cells was followed by measurement of the accumulation of PHB and the level of luminescence at different stages of cell growth. A fixed amount of cells (OD_{660} x volume = 10) were analyzed each time for the amount of PHB. Table 1 shows that there is not any significant difference in the amount of material collected based on dry weight at different stages of cell growth. The weights of the samples were between 4.5 and 5.5 mg, which gave an average of 5.1 mg per 10 OD units (OD_{660} x volume) of cells with a standard deviation of 0.4 mg. As a result, the levels of PHB can be readily presented as milligrams of PHB per gram of dry cells.

The dependence of luminescence, PHB levels and cell density on the time of growth of *V. harveyi* cells is shown in Fig. 10. Luminescence was induced after 2 hours of cell growth (OD_{660} = 0.3) and reached a maximum of about 1 x 10^6 LU/g cell dry weight at OD_{660} around 2.5. In contrast, synthesis of PHB appeared to start at a later time, after 3 - 4 hours of cell growth (OD_{660} = 1.5). A

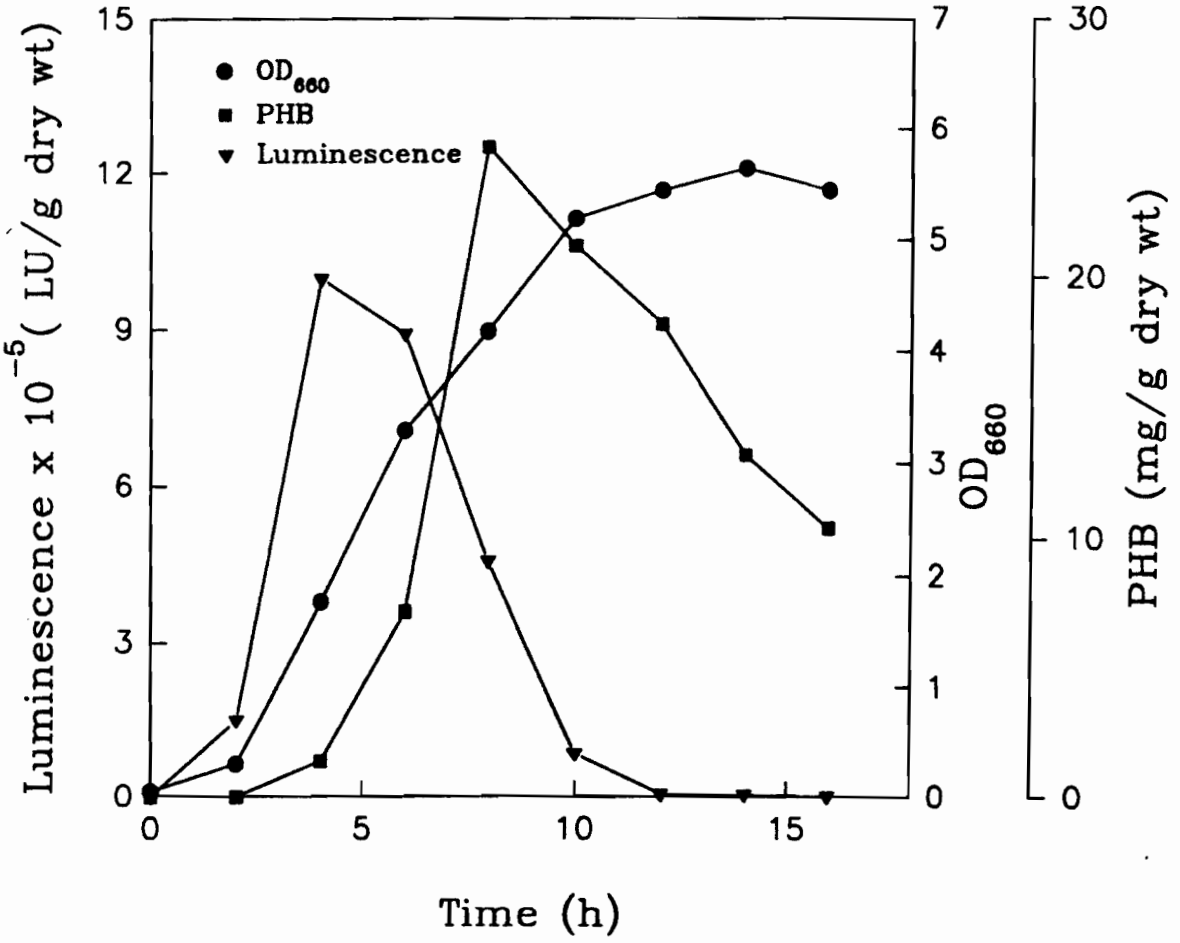
Table 1 Relationship between optical density (660 nm), culture volume and dry weight of *V.harveyi* cells at different stages of growth[a]

Time (h)	OD_{660}	Cell Volume (ml)	Dry weight (mg)[b]
4	1.7	5.6	4.5
6	3.3	3.0	5.4
8	4.2	2.4	5.5
10	5.2	1.9	4.5
12	5.4	1.8	5.1
14	5.6	1.8	5.5
16	5.4	1.8	5.3

a. The same amount of *V.harveyi* cells (OD_{660} x Vol (ml) = 10) were collected at different times of cell growth and dried at 120°C until the weight was constant.

b. Dry weight presented is an average of 4 samples measured in duplicate.

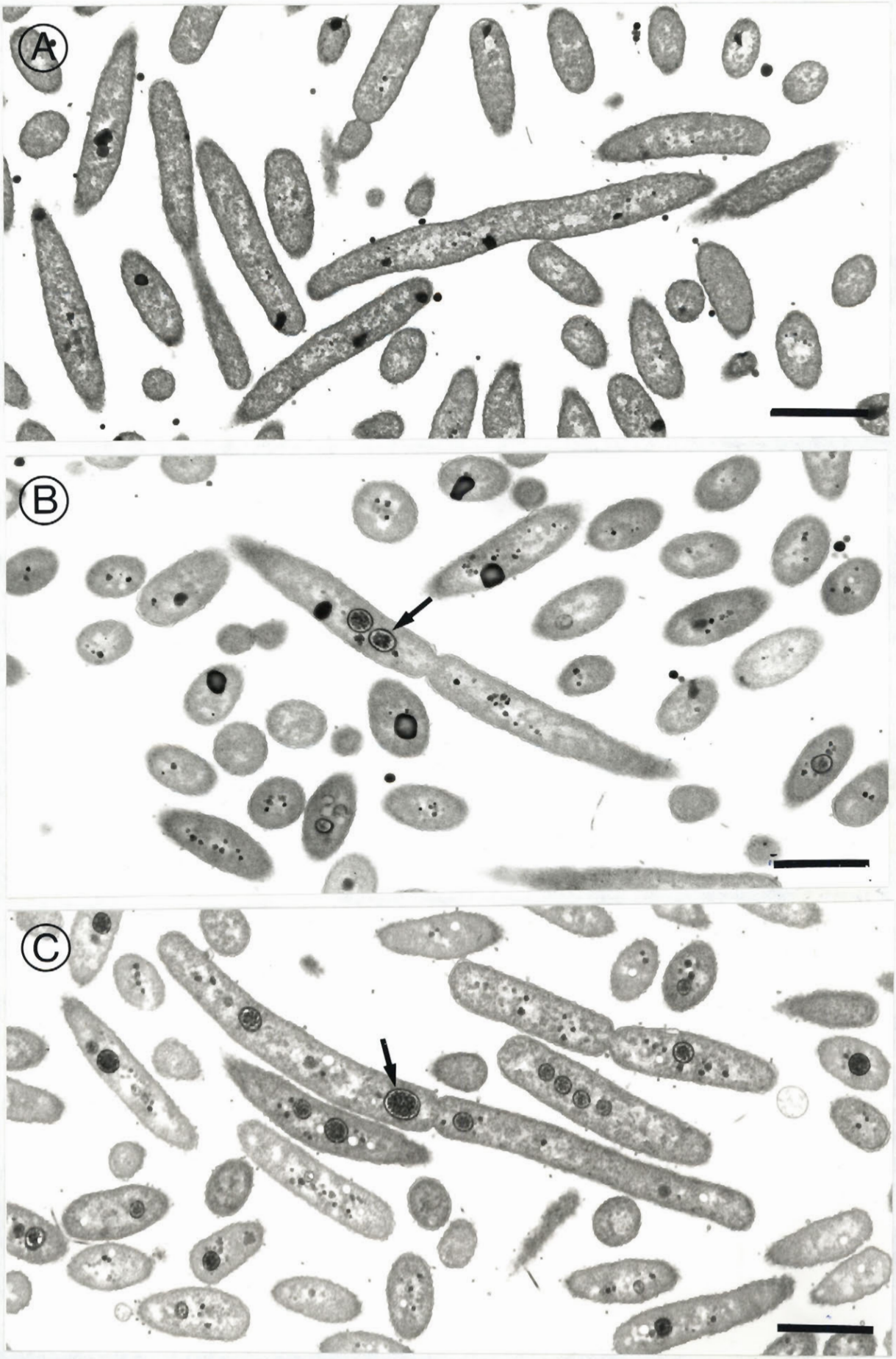
Fig. 10 Dependence of luminescence and poly-3-hydroxybutyrate levels on growth of *V. harveyi* An overnight growth of *V. harveyi* cells, was inoculated into 50 ml of complex medium in a 250 Erlenmeyer flask, with an initial optical density of 0.05. The cell cultures were then incubated at 27°C with shaking at 250 rpm. Samples were taken at different time and the cell density, luminescence and PHB levels measured. Identical amounts (OD_{660} x vol (ml) = 10) of cells were used each time for analysis of PHB.


Luminescence x 10^{-5}(LU/g dry wt)
OD_{660}
PHB (mg/g dry wt)
Time (h)
OD_{660}
PHB
Luminescence
0
3
6
9
12
15
0
1
2
3
4
5
6
7
0
10
20
30
0
5
10
15

maximum accumulation of 26 mg of PHB /g of cells (dry weight) was obtained at an OD_{660} of 4.2, after 9 hours of inoculation, when cells had entered the stationary phase of growth. The level of 26 mg (OD_{660} = 4.2) of PHB per gram of dry cells by UV spectroscopy analysis corresponds well with the previous analyses (total 2.7 mg of PHB in 50 ml of cells, OD_{660} = 4.5, 107 mg of dry weight), in which 25.2 mg of PHB per gram of dry cells could be obtained by FTIR analysis. The amount of PHB in cells decreases at later time, which is possibly due to the depletion of carbon sources in the culture medium.

As the granules detected in the *V. harveyi* cells (Fig. 1) appeared to be due to PHB, it would be expected that the number of sponge-like granules in the cells should increase as the level of PHB rises with cell growth. As shown by the electron micrographs in Fig. 11, the number of sponge-like granules observed on staining with malachite green increased significantly when the optical density of the cells increased from 1.5 to 4.5 at 660 nm. Few sponge-like granules can be observed at a low cell density (OD_{660} = 1.5, Fig. 11A), while a significant increase of the number of sponge-like granules was obtained at higher cell density (OD_{660} = 3.1, Fig. 11B) and especially at OD_{660} = 4.2 (Fig. 11C).

Fig. 11 Increase of the number of sponge-like granules during cell growth as shown by electron microscopy *V. harveyi* cells incubated in complex medium were harvested at OD_{660} of (A) 1.5, (B) 3.1 and (C) 4.2 respectively. Cell samples were fixed for electron microscopy with glutaraldehyde and malachite green. Bar = 1 μm. Sponge-like granules are indicated by the arrows.


A
B
C

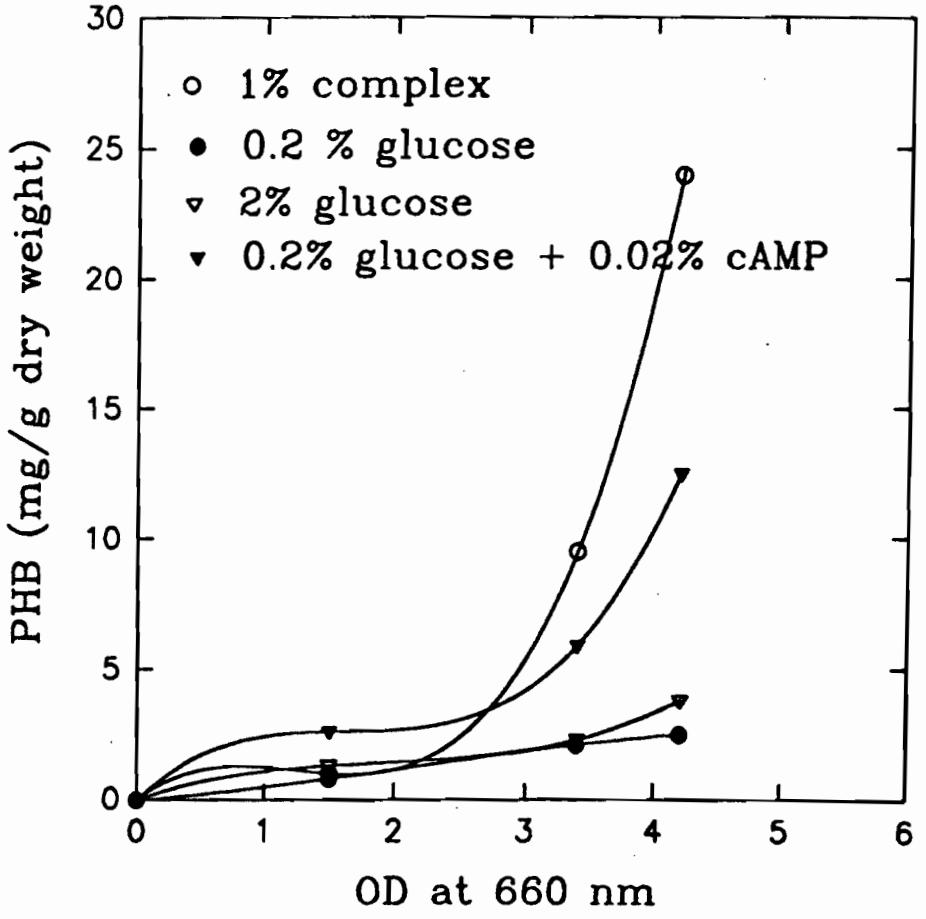
3.5 Glucose repression of poly-3-hydroxybutyrate synthesis in *V. harvey*

So far, the most detailed studies on the regulation of PHB biosynthesis have been conducted with *A. beijerinckii* using glucose as the carbon source. Under nitrogen-limiting conditions, the amount of PHB in the cells could reach up to 70% of the dry weight (Stockdale et al., 1968). Therefore, the amount of PHB in *V. harveyi* cells grown on glucose was examined. Surprisingly, a decrease in the levels of PHB was found when *V. harveyi* cells were grown in medium containing 0.2% or 2% (w/v) glucose (Fig. 12). The repression of PHB production by glucose (0.2% (w/v)) can be reversed by adding 0.02% (w/v) cAMP into the medium. The results that glucose reduces rather than stimulates the production of PHB indicate that in *V. harveyi*, PHB synthesis is under catabolite repression as is the luminescence system (Nealson et al., 1972). It was therefore of interest to determine if any other regulatory factors controlling luminescence could also control PHB synthesis.

3.6 Autoinducer stimulation of poly-3-hydroxybutyrate accumulation in *V. harveyi* autoinducer deficient mutants

Two autoinducer deficient mutants of *V. harveyi* were analyzed for PHB levels and the effect of adding the *lux* autoinducer, *N*-(3-hydroxybutyryl)

Fig. 12 Glucose repression of poly-3-hydroxybutyrate production by *V. harveyi* *V. harveyi* cells were grown in complex media containing glycerol (0.2%), or glucose (0.2% or 2%), or 0.2% glucose plus 0.02% cAMP, and the amount of PHB measured at different stages of cell growth


30
25
20
15
10
5
0
0
1
2
3
4
5
6
PHB (mg/g dry weight)
OD at 660 nm
1% complex
0.2 % glucose
2% glucose
0.2% glucose + 0.02% cAMP

homoserine lactone, which is required for high levels of light emission. Both mutants emit a low level of light and exogenous addition of autoinducer greatly stimulates light emission (Cao et al., 1989; Cao and Meighen, 1993). One mutant, D34, produces about 15% of the autoinducer synthesized by wild type *V. harveyi* cells (~4 μg/ml culture). Addition of relatively high concentrations of exogenous autoinducer to D34 cells can stimulate luminescence over 10^5-fold with the final light intensity reaching close to that of wild type cells. For the second mutant, D1, autoinducer could not be detected (<0.02 μg/ml). Levels as low as 1 μg/ml of exogenous autoinducer could stimulate luminescence of D1 cells to intensities comparable to that of wild type cells.

As shown in Fig. 13, D34 and D1 cells had very low levels of PHB at all stages of growth. Maximum accumulation of PHB was only 0.3 mg/g cells (dry weight) in D34 cells and PHB could not even be detected in D1 cells. In contrast, a level of over 26 mg PHB/g cells (dry weight) could be reached in wild type cells (Fig. 10).

Synthesis of PHB can be greatly stimulated by addition of exogenous autoinducer to the cell cultures of the mutants. Fig. 14 shows the dependence of PHB accumulation of D34 and D1 cells on autoinducer concentration. In D34 a cells, PHB levels can be increased from 0.3 to 7.3 mg/g dry cell weight on

Fig. 13 Poly-3-hydroxybutyrate production in autoinducer deficient mutants of *V. harveyi* D1 and D34 cells were grown to different stages and the amount of PHB analyzed as described for the wild type cells (see Fig. 10). As both mutant cells grow at the same rate, only one set of optical densities at 660 nm is given for the two strains.

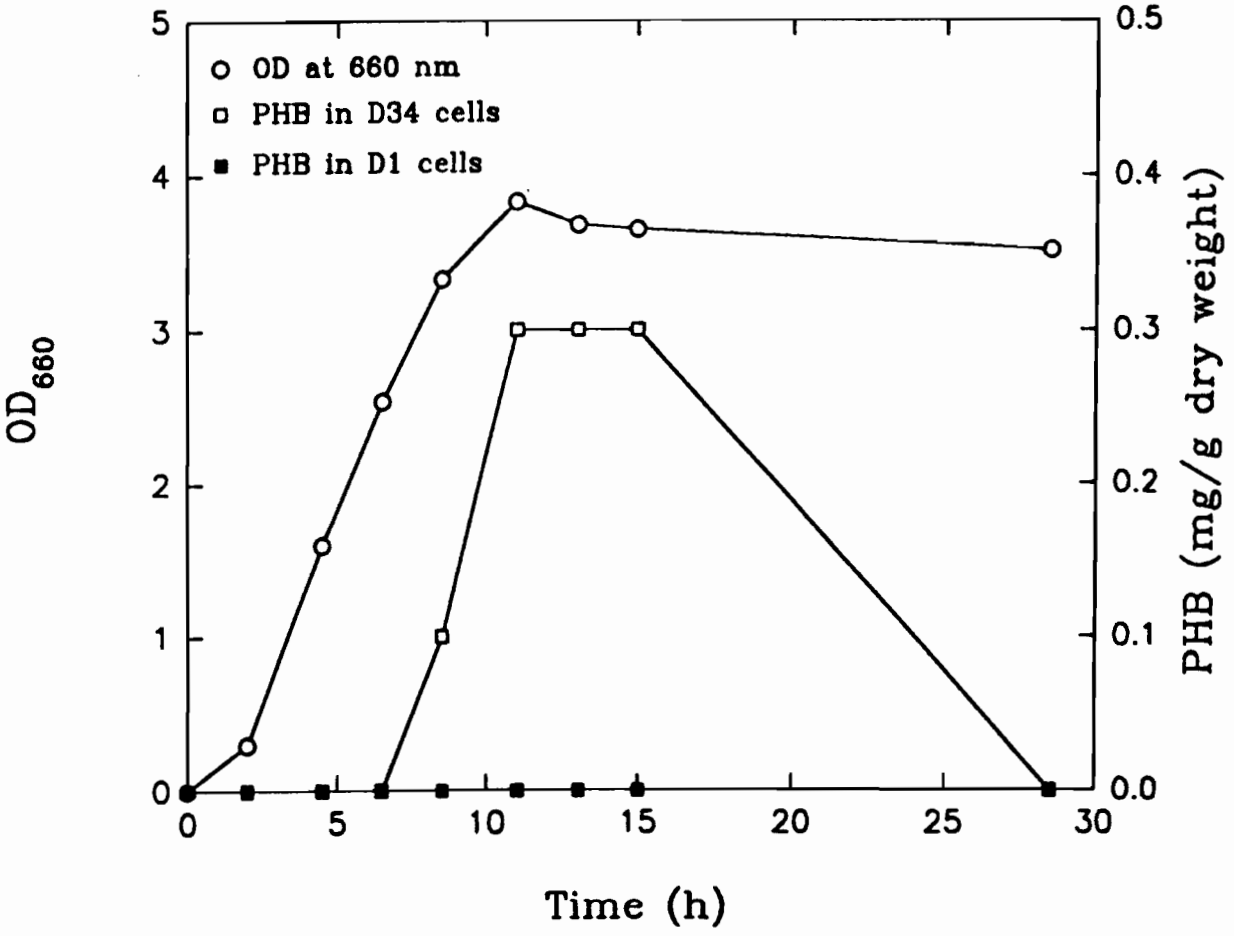
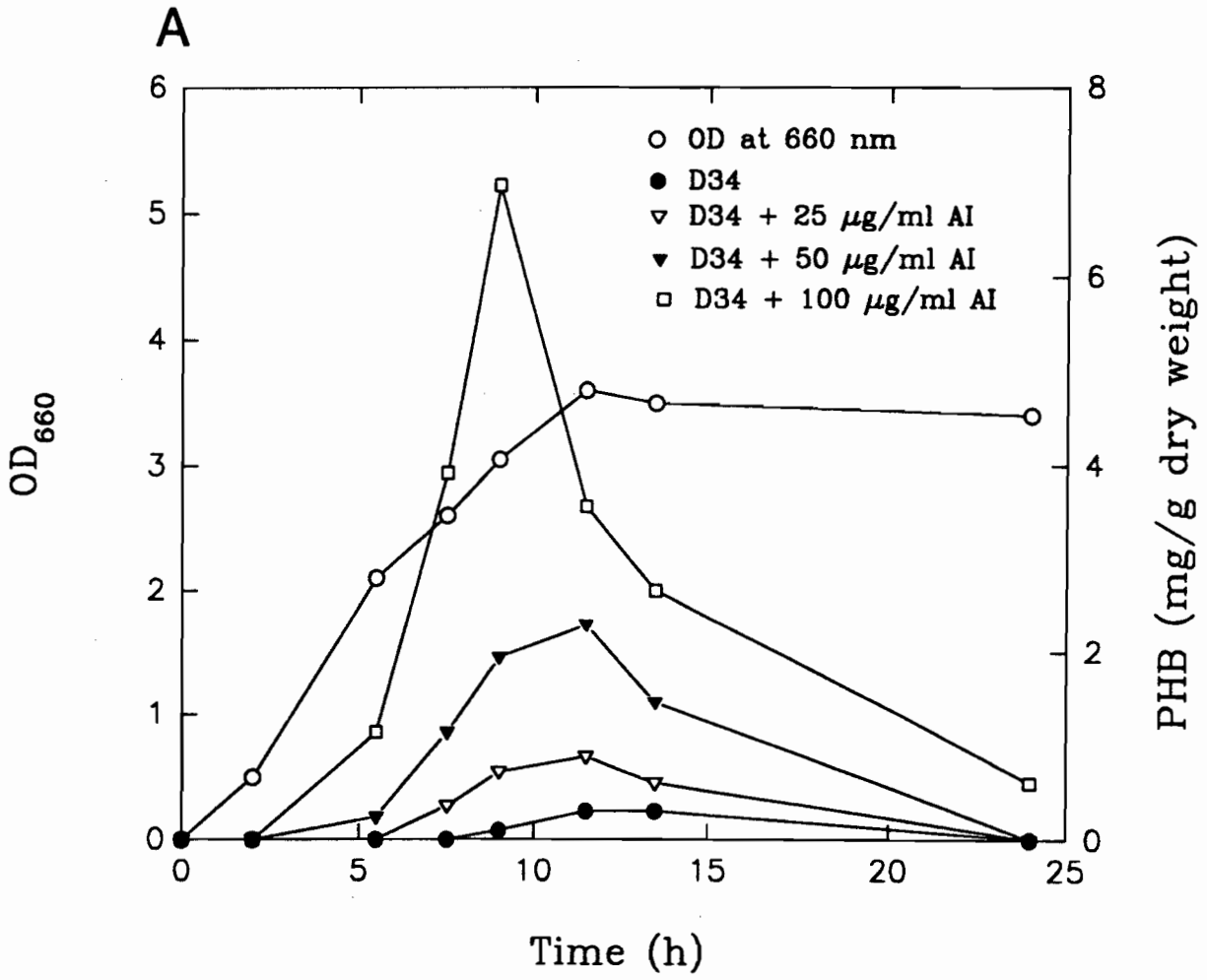
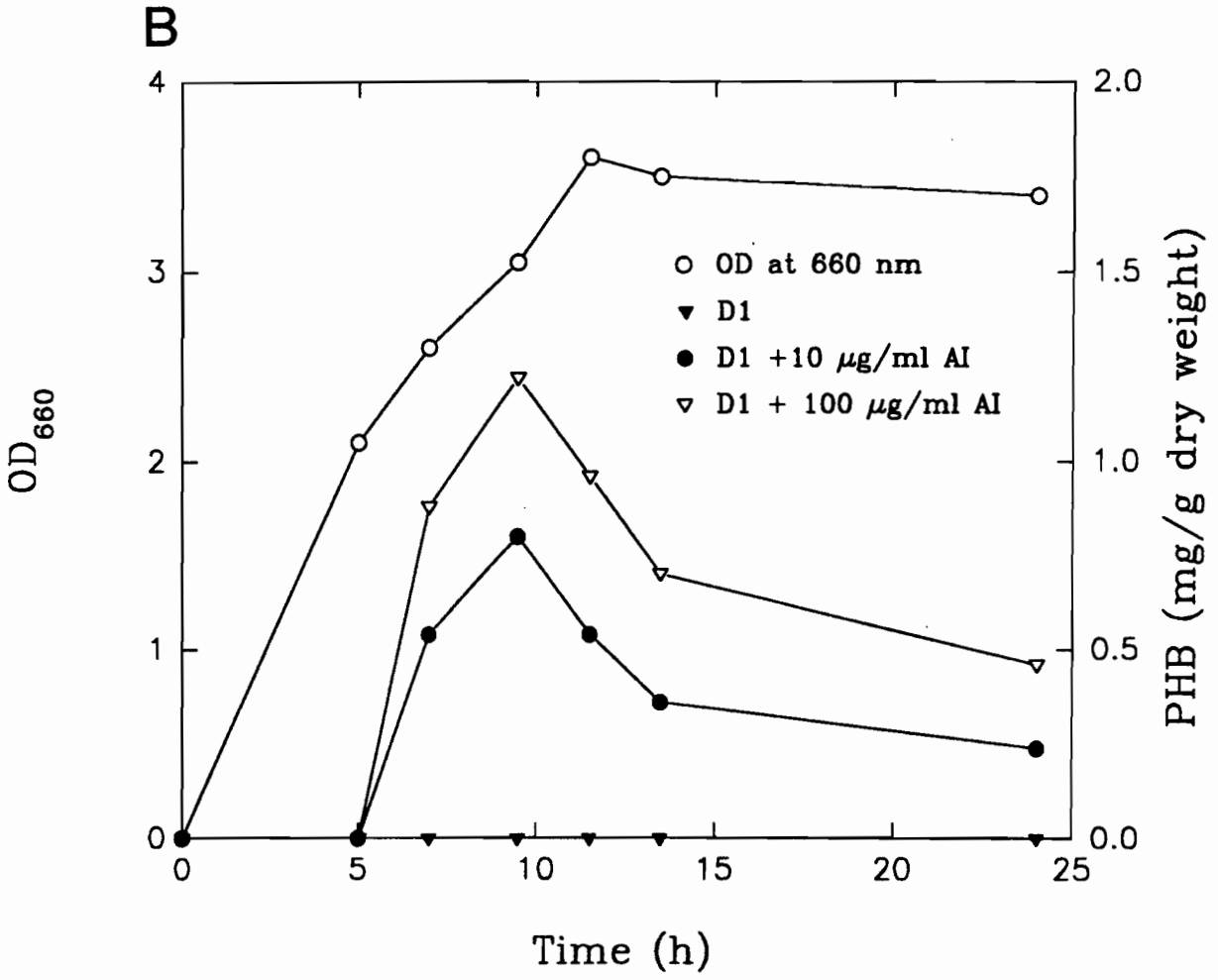

OD at 660 nm
PHB in D34 cells
PHB in D1 cells
OD660
PHB (mg/g dry weight)
Time (h)
0
1
2
3
4
5
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
25
30

Fig. 14 Dependence of poly-3-hydroxybutyrate accumulation in D34 and D1 cells on autoinducer concentration Growth conditions for the mutants are described in Fig. 10. Different amounts of autoinducer (AI) were added to the culture media. The levels of PHB and optical density were monitored as a function of time for D34 cells (A) and D1 cells (B).


A
OD at 660 nm
D34
D34 + 25 μg/ml AI
D34 + 50 μg/ml AI
D34 + 100 μg/ml AI
OD660
PHB (mg/g dry weight)
Time (h)
0
1
2
3
4
5
6
0
2
4
6
8
0
5
10
15
20
25


B
4
3
2
1
0
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
OD660
PHB (mg/g dry weight)
Time (h)
OD at 660 nm
D1
D1 +10 μg/ml AI
D1 + 100 μg/ml AI

addition of 100 μg/ml autoinducer to the cell culture (Fig. 14A). In D1 cells, PHB levels can only be increased to 1.2 mg/g when same amount (100 μg/ml) of autoinducer was added into the cell culture. Although the level of PHB produced by the D1 cells is lower than the D34 cells, those D1 cells were much more sensitive to lower concentrations of the autoinducer. As shown in Fig. 14B, about 0.7 mg PHB/g cells was obtained when 10 μg/ml of autoinducer was added to the D1 cells compared to 25 μg/ml required for the D34 cells to reach the same level of PHB. The difference in sensitivity of these two mutants in response to autoinducer, which is probably due to the different nature of the mutant, has also been observed with regard to stimulation of luminescence.

Corresponding to the increase of the amount of PHB on addition of autoinducer to the culture media of the mutant D34 and D1 cells, an increase of the number of sponge-like granules can be detected by electron microscopy (Fig. 15). Few granules can be detected in the D1 and D34 cells, in agreement with their low levels of PHB.

3.7 Stimulation of poly-3-hydroxybutyrate biosynthesis by *lux*R

Since the *lux* autoinducer can stimulate the production of PHB as well as light emission, It was possible that other regulator(s) of the luminescence system

Fig. 15 Electron micrographs showing increase of the number of sponge-like granules in D1 and D34 cells on addition of exogenous autoinducer

Cells were grown in complex medium as described in Fig. 10. Samples were harvested when the accumulation of PHB was close to its maximum level and prepared for electron microscopy as described in Fig. 11.

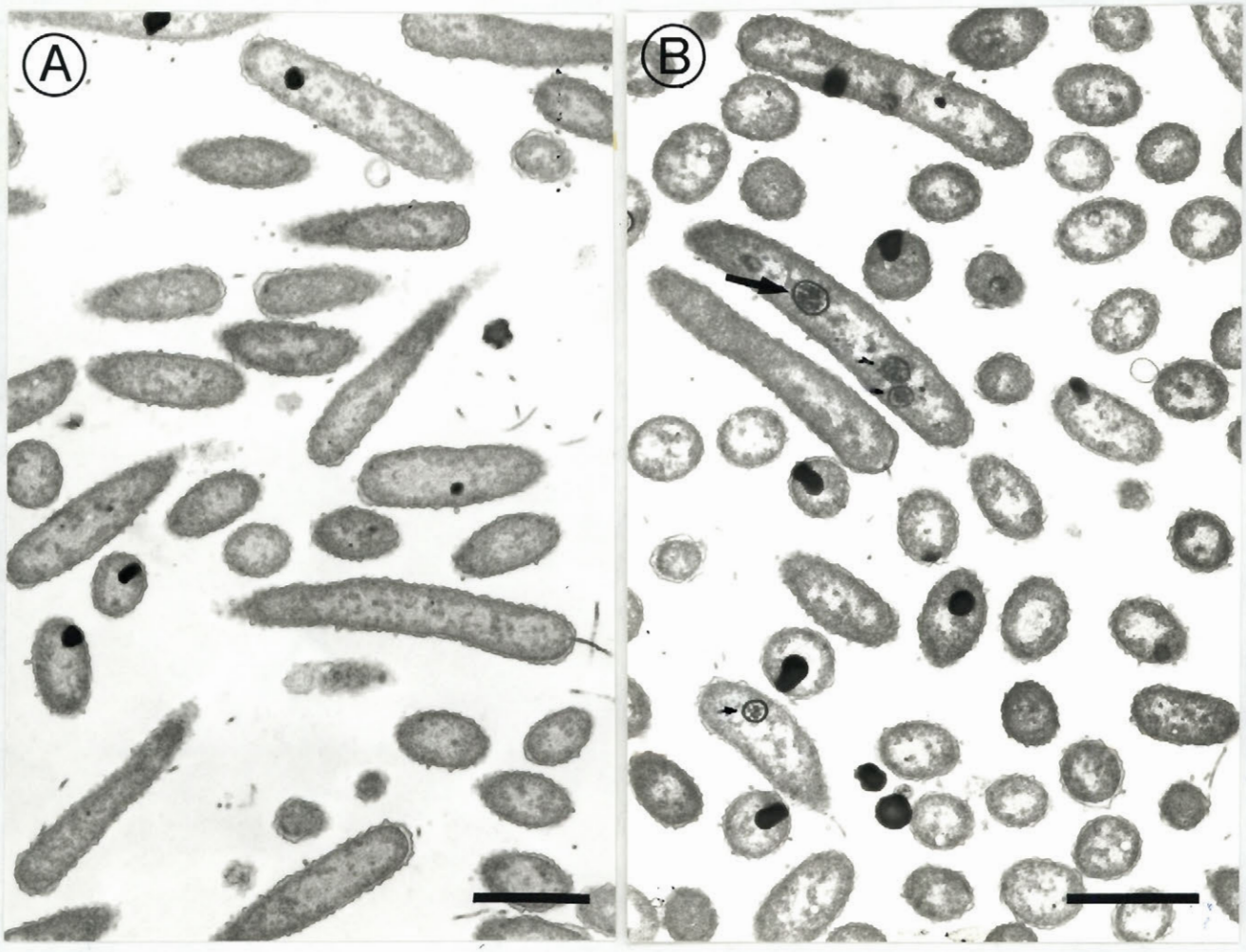
(A) D34 cells (OD_{660} = 3.0)

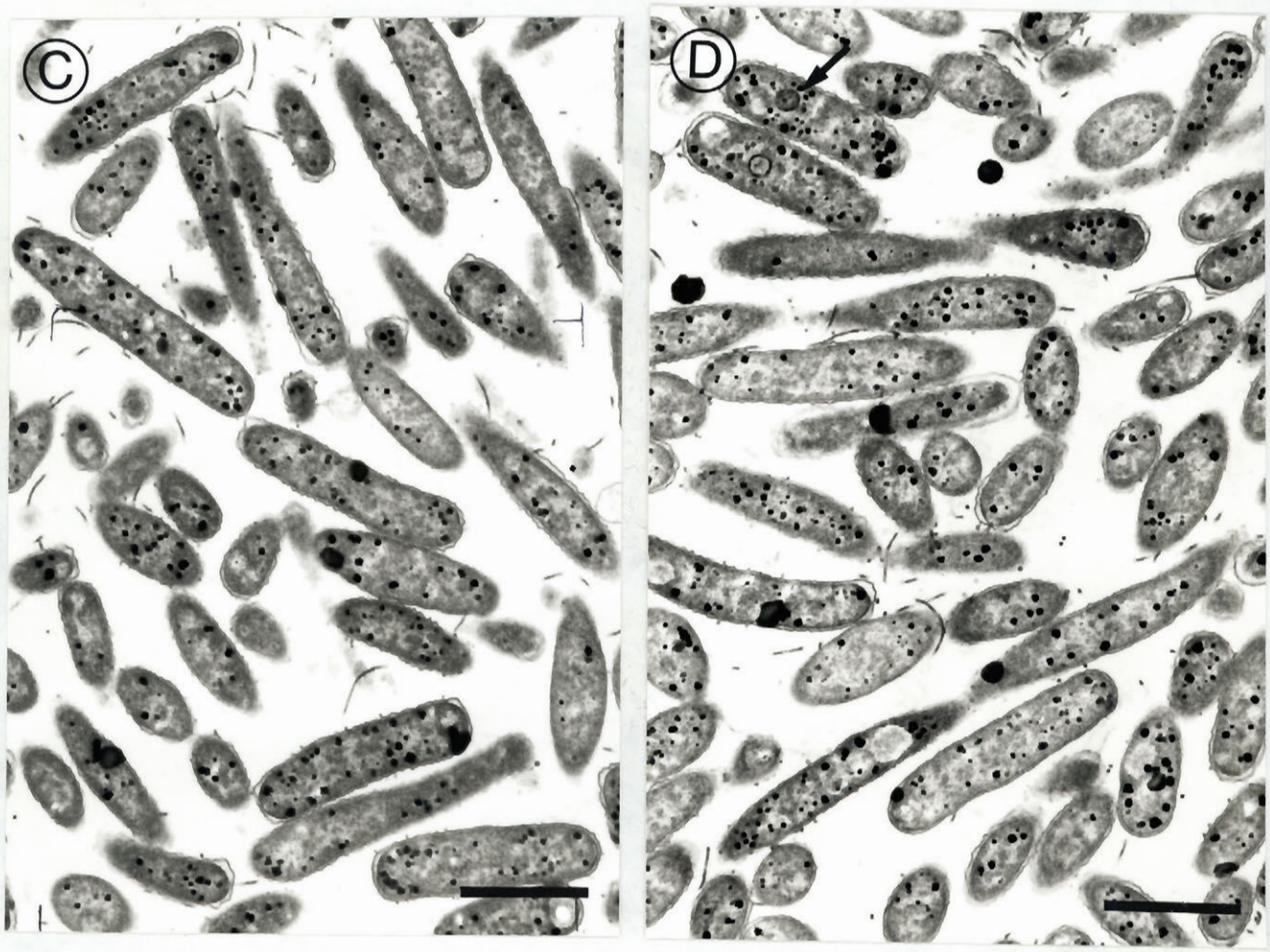
(B) D34 cells with 100 μg/ml of autoinducer in the medium (OD_{660} = 3.3)

(C) D1 cells (OD_{660} = 3.3)

(D) D1 cells with 10 μg/ml of autoinducer in the medium (OD_{660} = 3.5)

Bar = 1 μm. Sponge-like granules were indicated by the arrows.


A
B


C
D

of *V. harveyi* also controls PHB synthesis. A regulatory gene of the luminescence system, designated *lux*R, was found recently, which is required for high expression of the *lux* genes in *V. harveyi* and *E. coli* (Showalter et al., 1990). To study the effect of LuxR on PHB accumulation, the gene was mated into D34 and D1 cells by transconjugation. As shown in Fig. 16, the PHB level in the D34 transconjugant cells containing *lux*R was much higher, 12.4 mg/g cell dry weight, compared to that of the D34 cells. An even higher level of PHB, 61.6 mg/g dry cell, which is more than twice the amount found in the wild type cells, was obtained when autoinducer was also added to these cells. In the D1 cells transconjugated with *lux*R, a maximum amount of only 0.8 mg PHB/g of dry cells was produced. With autoinducer presented in the transconjugated cell culture, 6 mg PHB/g of dry cells could be detected. The electron micrographs in Fig. 17 show the increase of the sponge-like PHB granules in the mutant cells transconjugated with *lux*R on addition of autoinducer.

Direct evidence for regulation of PHB synthesis by the LuxR protein in *V. harveyi* was obtained by examination of the level of PHB in *V. harveyi* strain BB7 and its mutant MR1101 which has a Tn5 transposon insertion in the *lux*R gene. PHB was not detected in mutant MR1101 cells during the cell growth, whereas a maximum of 18 mg PHB/g cells (dry weight) was accumulated in wild

Fig. 16 Poly-3-hydroxybutyrate accumulation in D1 and D34 cells transconjugated with the *lux*R gene Cells were grown in complex medium. Appropriate antibiotics were added to the media to maintain selection pressure on the transconjugated plasmids. Maximum levels of PHB are given for

(1) *V. harveyi* cells

(2) D34 cells

(3) D34 cells transconjugated with *lux*R

(4) D34 cells transconjugated with the *lux*R gene plus 5 μg/ml of autoinducer

(5) D1 cells

(6) D1 cells transconjugated with *lux*R

(7) D1 cells transconjugated with the *lux*R gene plus 5 μg/ml of autoinducer

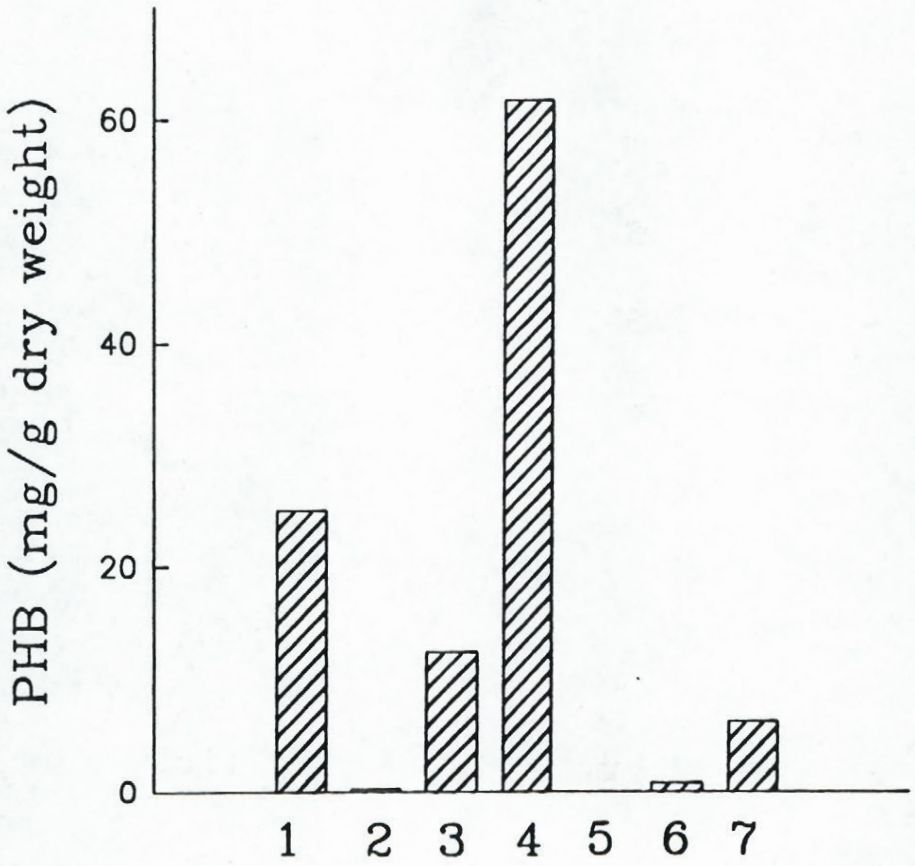

PHB (mg/g dry weight)
60
40
20
0
1
2
3
4
5
6
7

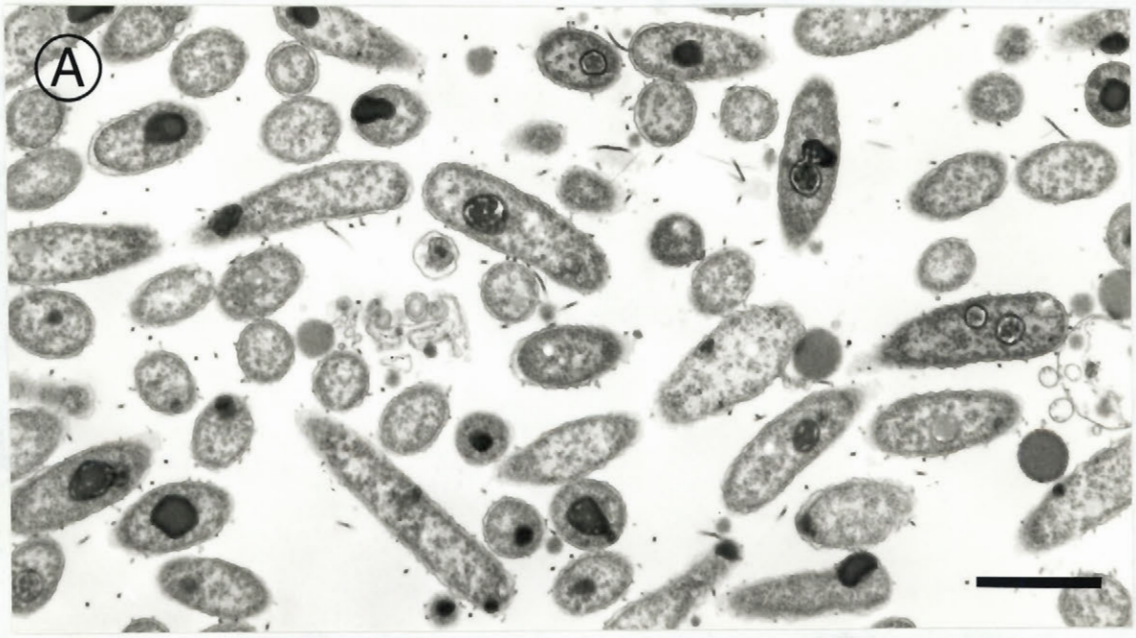
Fig. 17 Increase of the number of sponge-like granules in D34 and D1 cells transconjugated with the *lux*R gene on addition of autoinducer Cell samples were grown and prepared as described as Fig. 16. Bar = 1 μm.

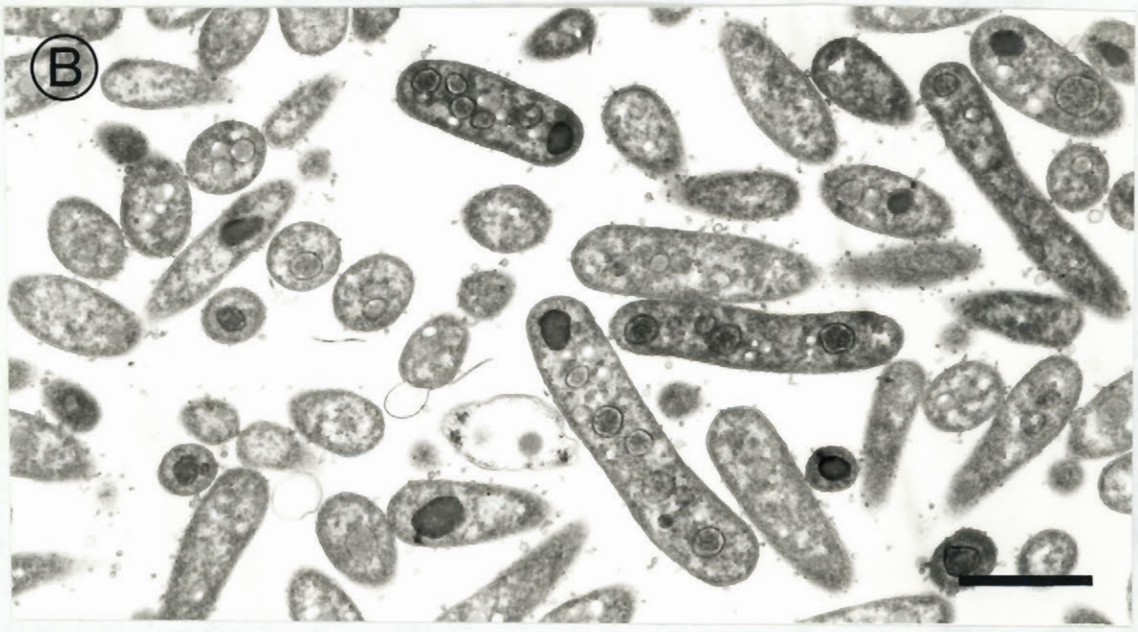
(A) D34 cells transconjugated with *lux*R (OD_{660} = 3.4)

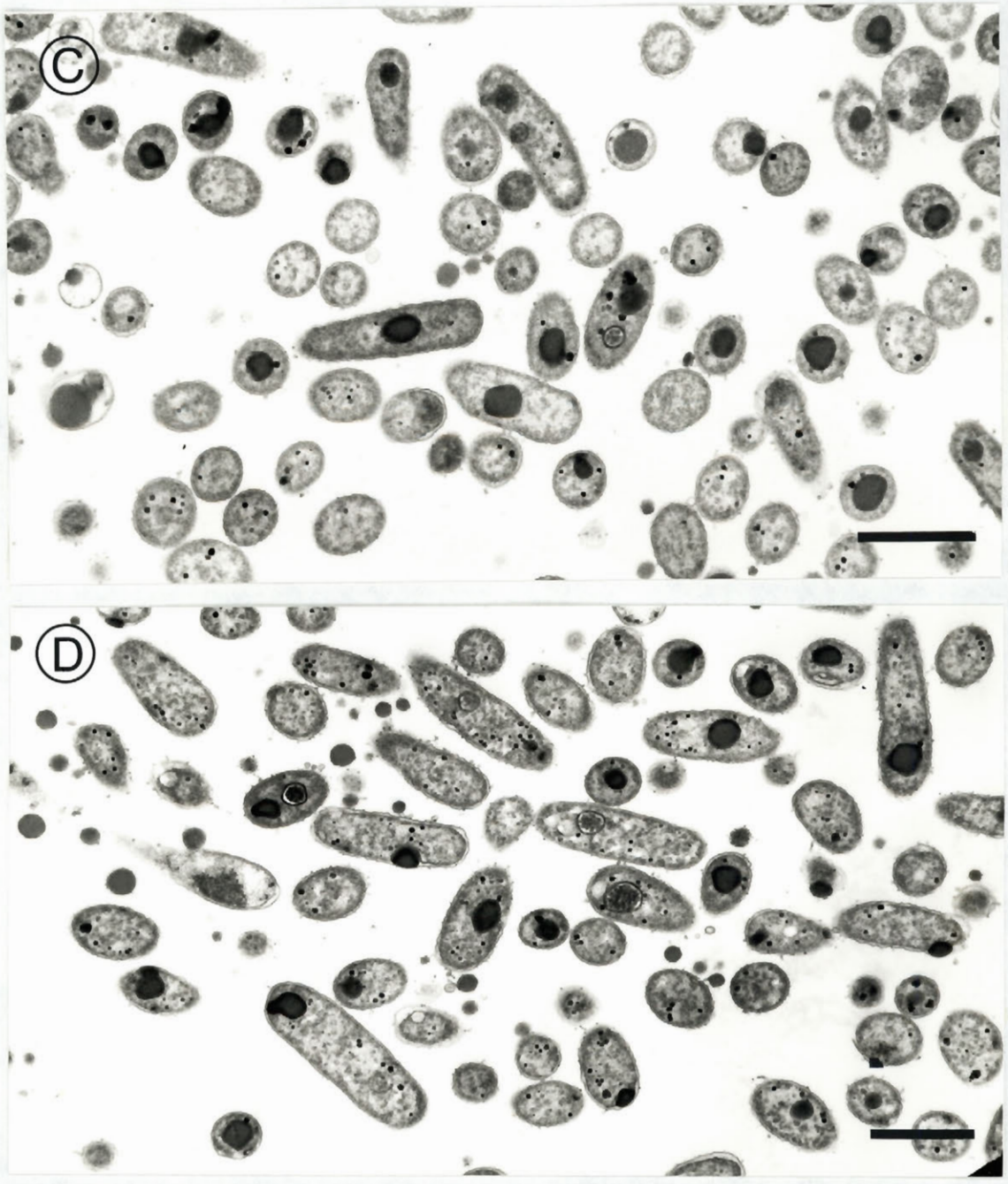
(B) D34 cells transconjugated with *lux*R plus 5 μg/ml autoinducer (OD_{660} = 4.5)

(C) D1 cells transconjugated with *lux*R (OD_{660} = 3.9)

(D) D1 cells transconjugated with *lux*R plus 5 μg/ml autoinducer


A


B


C
D

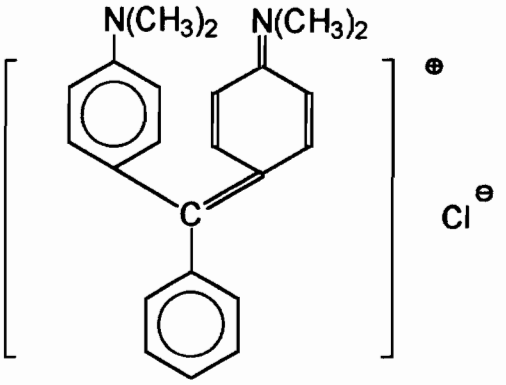
type cells (Fig. 18) indicating that the LuxR protein is essential for PHB synthesis in *V. harveyi*.

CHAPTER IV DISCUSSION

4.1 Occurrence of poly-3-hydroxybutyrate in *V. harveyi*

The present work has shown that PHB was synthesized in the luminescent bacterium, *V. harveyi*, a species previously described as PHB negative (Baumann and Baumann, 1981). The presence of PHB was observed by electron microscopy, in which characteristic sponge-like granules were detected on staining with malachite green, as well as by a series of analyses including GC, FTIR, NMR and UV spectroscopy. These latter experiments not only confirmed the presence of PHB in *V. harveyi* but also eliminated the possibility that the polymer contained units other than 3-hydroxybutyric acid.

It is known that malachite green is a weakly basic diaminotriphenylmethane dye,



which has classically been used by the optical microscopist for detection of fungi and bacterial spores (Lillie, 1977). It can also be added to the standard

glutaraldehyde fixative for detection by electron microscopy of a large group of lipid and lipid-related compounds including fatty acids, fatty aldehydes, phospholipids, glycolipids and cholesterol (Pourcho et al., 1978). Because of the differential affinity of the dye, diverse electron densities are observed for different cell structures thus allowing them to be distinguished. In this study, PHB containing granules, which are opaque in appearance in a standard glutaraldehyde fixation procedure (Fig. 1A), could more readily be identified after staining with malachite green (Fig. 1B) due to their characteristic sponge-like appearance. A granular structure for PHB may arise if PHB is not tightly packed in the granule and interspersed with nonstaining material, e. g. water (Lauzier, et al., 1992). During sample preparation for electron microscopy, cells were dehydrated by acetone, which causes the loss of water from the granules as well as increases the crystallinity of PHB. This process could result in a sponge-like granule with a relatively low electron dense thin halo surrounded by a single membrane reflecting variations in the crystallinity of PHB in the granules after sample preparation.

As shown in Figs. 10 and 11, the number of sponge-like granules as well as the amount of PHB determined by UV spectroscopy increased with cell growth. In order to obtain a direct correlation between the two analyses,

duplicate samples were collected from the same cell cultures and analyzed for PHB by UV spectroscopy and electron microscopy. For comparison, the levels of PHB as well as the number of sponge-like granules are normalized separately to 100% in two sets of experiments (*V. harveyi* at OD_{660} = 4.2 in experiment 1 and D34 + *luxR* + AI in experiment 2). Table 2 shows that the relative amount of PHB and the relative number of granules are in very good agreement. The results indicate that the sponge-like granules are indeed PHB granules and support the use of the malachite green-glutaraldehyde fixation procedure for identification of PHB. It is also noticed that the maximum accumulation of PHB in *V. harveyi* is very low, only 2.6% of cell dry weight in wild type cells and 5.1% in D34 cells transconjugated with *lux*R grown in medium containing 5 μg/ml autoinducer.

By stereological analysis of the electron micrographs of the sponge-like granules, the area density of the granules could be determined. A straight line with a correlation coefficient of 0.993 was obtained when the amount of PHB detected by UV spectroscopy was plotted as a function of the area density of the granules (Fig. 19). As the area density of the granules in cells on the thin sections are equal to their volume density(Underwood, 1967), which in turn is directly proportional to mass, the linear relationship in Fig. 19 clearly establishes the direct link between the sponge-like granules and the amount of PHB.

Table 2 Relationship between the level of poly-3-hydroxybutyrate and the number of sponge-like granules

Samples	N_{cell} [a]	$N_{granule}$ [b]	$N_{granule} / N_{cell}$ x 100 (%)[c]	PHB content, mg/g cell dry weight, (%)[c]
Experiment 1[d]				
$WT_{1.5}$	4252	14	0.3 (1.0)	0.0 (0.0)
$WT_{3.1}$	2170	276	12.7 (39.0)	8.8 (34.0)
$WT_{4.2}$	3436	1136	33.1 (100)	26.0 (100)
Experiment 2[e]				
D1	2140	0	0	0
D1 + AI	2602	31	1.2 (2.2)	1.0 (2.0)
D1 + R	1956	8	0.4 (0.7)	1.0 (1.9)
D1 + R + AI	1890	103	5.5 (10.1)	6.6 (12.8)
D34	3651	7	0.2 (0.4)	0.3 (0.6)
D34 + AI	3530	237	6.7 (12.3)	4.4 (8.5)
D34 + R	1349	90	6.7 (12.3)	7.9 (15.3)
D34 + R + AI	2567	1393	54.3 (100)	51.6 (100)

a. Number of cells counted.

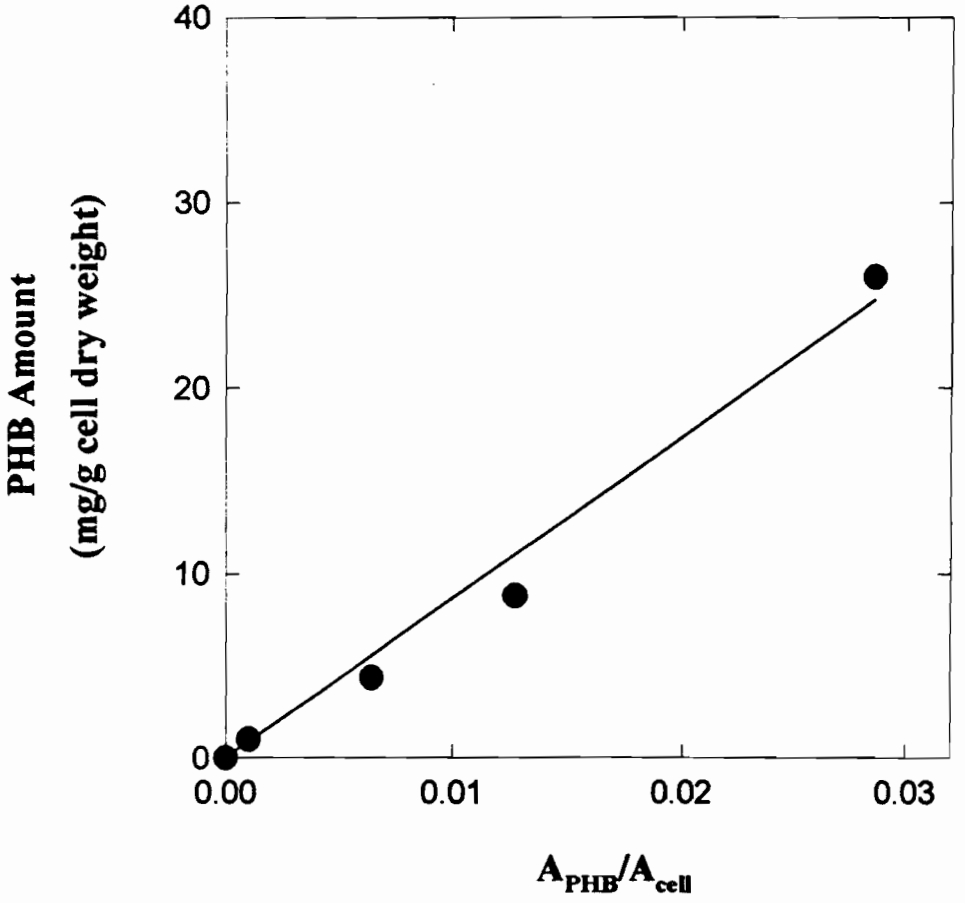
b. Number of sponge-like granules

c. Percentage relative to maximum amount observed in each experiment

d. Wild type *V. harveyi* cells at different stages of cell growth (OD_{660} =1.5, 3.1 and 4.2).

e. Accumulation of PHB in D1 and D34 cells at late logarithm growth, with or without autoinducer (AI) and/or *luxR* (R).

Fig. 19 Relationship between the amounts of poly-3-hydroxybutyrate and area densities of the sponge-like granules The electron micrographs in Figs. 11 and 15 were analyzed for the area of the granules and the cells by a modular system for quantitative digital analysis (MOP-3, Carl Zeiss, Inc.). A total of 1000 to 1500 cells were analyzed for each point on the plot. The area density of the sponge-like granules is represented as A_{PHB}/A_{Cell}, where A_{PHB} and A_{Cell} are the respective sums of the areas of the sponge-like granules and the areas of the cells in a given set of sections. The area densities (A_{PHB}/A_{Cell}) of the sponge-like granules were then plotted as a function of the amount of PHB measured by UV spectrometry using the same set of cells.


PHB Amount
(mg/g cell dry weight)
40
30
20
10
0
0.00
0.01
0.02
0.03
A_{PHB}/A_{cell}

4.2 Regulation of biosynthesis of poly-3-hydroxybutyrate in *V. harveyi*

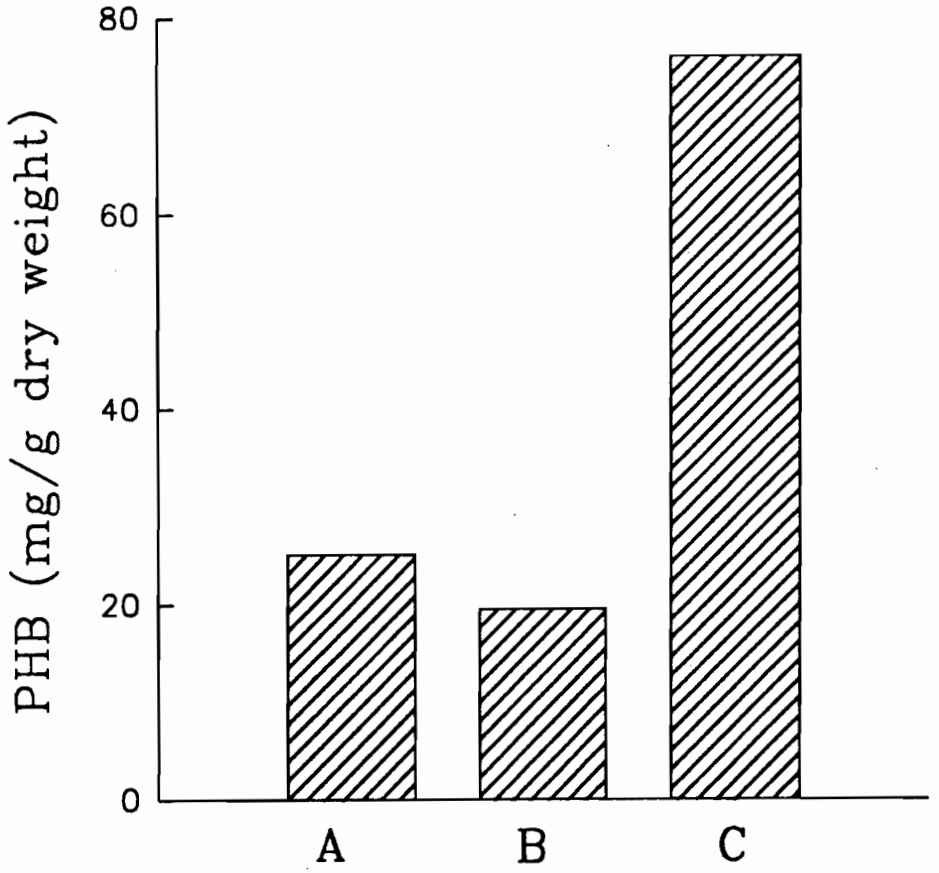
The physiological role of PHB in *Bacillus* species was first reported by Tinelli (1955a; 1955b), who demonstrated the involvement of PHB in the sporulation process. Later work by Splepecky and Law (1961) showed that although the accumulation of PHB is not a prerequisite for spore formation, the polymer could serve as a source of carbon and energy to drive the sporulation process. In *V. harveyi*, as light emission also counts for a large expenditure of energy, the possible relationship between bioluminescence and the synthesis of PHB was considered. It seems unlikely that *V. harveyi* cells use the accumulated PHB as the carbon or energy source for luminescence. As shown in Fig. 10, the production of PHB in *V. harveyi* is initiated at a higher cell turbidity (OD_{660} = 1.5) than the induction of the luminescence system (OD_{660} = 0.3) and reaches its maximum accumulation at a later stage (OD_{660} = 4.2) than the point (OD_{660} = 3.1) at which the highest light emission is obtained. The levels of PHB then decreased. It is possible that the PHB in *V. harveyi* serves as an energy source for maintaining cell viability in the stationary stage of growth.

It is found that both environmental and intracellular factors, including the carbon/nitrogen ratio, oxygen, glucose, autoinducer and LuxR protein are involved in regulation of the synthesis of PHB in *V. harveyi*. The dependence of

the production of PHB on the carbon /nitrogen ratio of the medium for *V. harveyi* cells can be observed in Fig. 20, in which the amount of PHB increased about three times when cells were grown in medium containing only 50% the content of tryptone and yeast extract compared to that of cells grown in normal complex medium. The results indicate that the *in vivo* biosynthesis of PHB in *V. harveyi* is stimulated by growth conditions where nutrients are limited.

A 20% decrease in the level of PHB was obtained when cells were grown in a large volume where the entrance of oxygen by diffusion was more limited (Fig. 20). This result is quite different from that observed in *A. beijerinckii* (Jackson and Dawes, 1976), in which the accumulation of PHB was greatly stimulated under conditions of oxygen limitation. It is known that in *Azotobacter* species, under very high aeration conditions, competition for NADH between PHB formation and the respiration protection of the nitrogen fixation system decreases PHB formation (Page and Knosp, 1989). Therefore oxygen limitation is essential for PHB accumulation in *Azotobacter* cells. In the case of *V. harveyi*, low oxygen tension has only small effects on luminescence or growth rate. It may implicate that the concentration of oxygen is not a critical factor for some physiological activities including PHB production in *V. harveyi* cells. Further experiment should be carried out with an adequate apparatus (e.g. as described

Fig. 20 The accumulation of poly-3-hydroxybutyrate under oxygen and nutrient limiting conditions *V. harveyi* cells were grown in (A) 50 ml of complex medium, (B) 100 ml of complex medium and (C) 50 ml of medium containing only 50% the content of tryptone and yeast extract of the complex medium. The amounts of PHB were measured at different times of growth and the maximum levels were compared.


PHB (mg/g dry weight)
80
60
40
20
0
A
B
C

by Lloyd et al., 1985), from which the oxygen concentration can be precisely controlled.

As shown in Fig. 12, the production of PHB was reduced in cells grown in complex medium containing 0.2% (w/v) or 2% glucose instead of 0.2% (v/v) glycerol. PHB synthesis can be restored by adding cAMP into the glucose medium, indicating that PHB synthesis in *V. harveyi* is under the catabolic repression. The mechanism awaits to be elucidated.

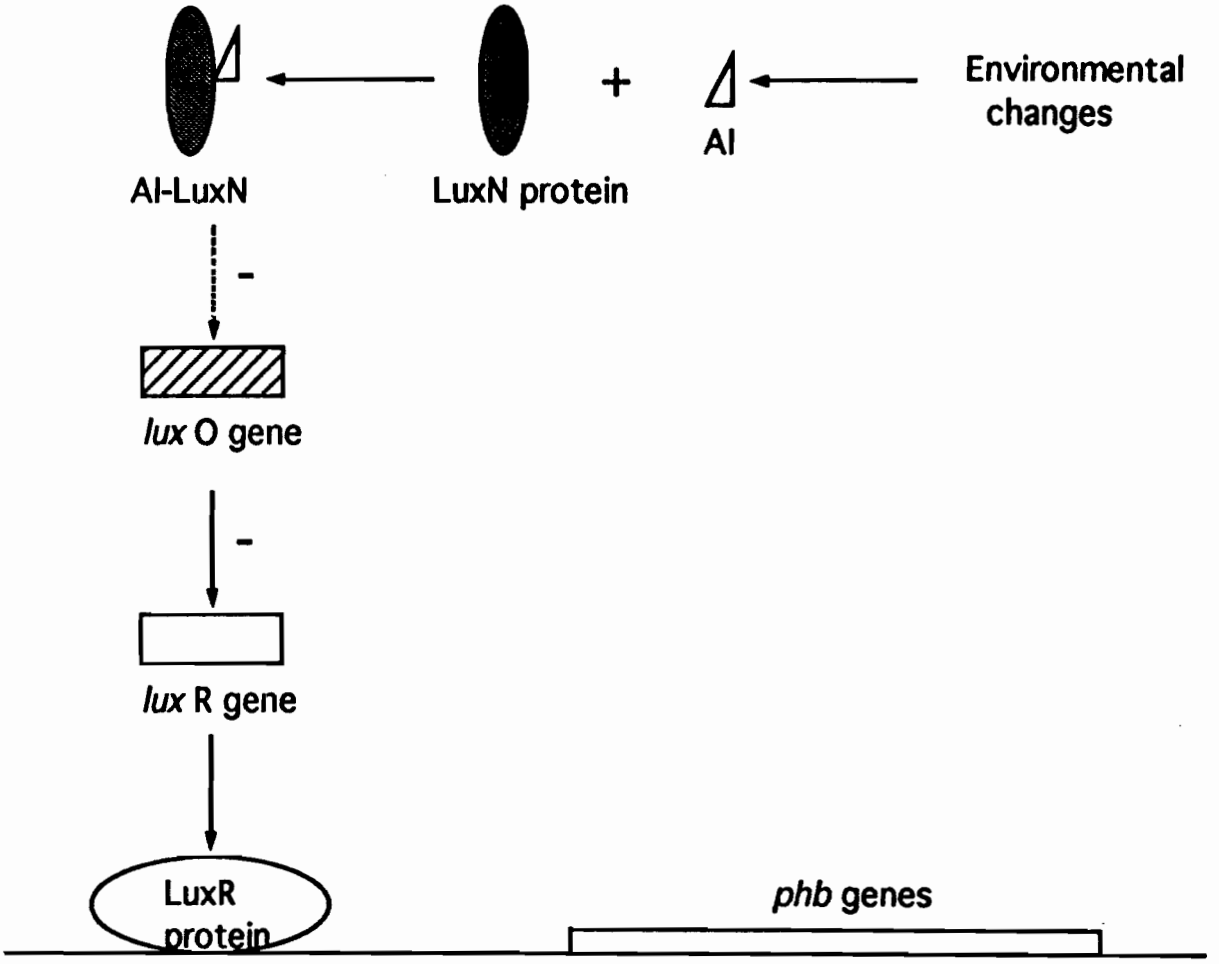
The discovery that the biosynthesis of PHB, as well as the luminescence system, is under the control of the *lux* autoinducer provides strong evidence to support the proposal that the autoinducer for *V. harveyi* is involved in regulation of other metabolic pathways. PHB was not labelled when autoinducer (100 μg/ml) with tritium in the 3-hydroxybutyrate moiety (0.14 mCi/mg) was added to the culture medium. This result demonstrates that the autoinducer functions as a regulator instead of as a substrate in the production of PHB in *V. harveyi*.

It was found that the production of PHB is also stimulated by the LuxR protein (Figs. 16 and 17), which may prove to be the key for understanding the regulation of synthesis of PHB. Addition of autoinducer to the two mutants, D1 and D34, transconjugated with the *lux*R gene, resulted in a large stimulation of the accumulation of PHB compared to cells with *lux*R gene only (Fig. 16),

indicating that both the LuxR protein and the autoinducer are required for biosynthesis of PHB in *V. harveyi*. The regulation by autoinducer likely occurs indirectly via other regulatory proteins (Bassler etal, 1994). In the *lux* system, LuxN has been proposed to interact with the autoinducer repressing LuxO which in turn acts as a negative regulator of the luminescence system (Bassler et al., 1993, 1994). Recently, it has been suggested that LuxO may function by repressing the expression of luxR (Meighen, 1994). A similar mechanism for regulation of PHB is proposed in Fig. 21, with the autoinducer being produced in response to environmental changes (depletion of nutrients, etc.) and binding to the LuxN protein which results in a negative regulation of the *lux*O gene. The LuxR protein then binds to the promoter of the *phb* genes to stimulate the transcription.

In summary, the present study has shown the occurrence of PHB in *V. harveyi* by a series of analyses. The production of PHB is subjected to metabolic repression. The stimulation of the production of PHB by *lux* autoinducer and the LuxR protein provides not only a connection between luminescence and PHB formation in *V. harveyi* but also direct proof that the autoinducer and the LuxR protein are involved in controlling multiple metabolic pathways. The results also provide the first evidence for the regulation of PHB biosynthesis in bacteria. It

Fig. 21 **Potential roles of autoinducer and LuxR in regulation of the *phb* genes in *V. harveyi***


Environmental changes
AI
AI-LuxN
LuxN protein
-
lux O gene
-
lux R gene
LuxR protein
phb genes

will be of great interest in future work to clone and sequence the *phb* operon and its promoter/operator region from *V. harveyi* to

1. find a CRP (catabolic repressor/activator protein) binding site providing direct proof for catabolic repression of PHB synthesis.

2. determine whether the LuxR protein and other regulatory proteins bind to the operator region of the *phb* genes.

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