VITREOSCILLA HEMOGLOBIN: GENE STRUCTURE AND REGULATION, FUNCTION, AND APPLICATIONS TO AEROBIC BIOPROCESSES

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Chemical Engineering

California Institute of Technology

Pasadena, California USA

1990

(Submitted December 8, 1989)

To my father,

who inspired me

and taught me that learning can be fun -

Thank you for showing me the way.

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

Robert Frost,

The Road Not Taken

ACKNOWLEDGEMENTS

As I write these lines, the names and faces of several people come to mind, who have contributed intellectually, materially, or emotionally to this work, as well as to my overall growth in the world of science. It is impossible for me to acknowledge each one explicitly; however, it would be unfair to overlook a few of them. Perhaps a chronological approach could be useful in this context.

First, and above all else, I would like to thank my parents and my brother, Raman, for nurturing me to independence, and for their unflagging support and encouragement in all my escapades. It is to their love, which seeks to humble me in my moments of success and to sustain me through my failures, that this work is dedicated.

I have indeed been very fortunate to have come across several outstanding teachers right from my early school years. Under their tutelage I have experienced the incredible excitement of learning new things. Their examples have made me look forward to the opportunity of passing on this feeling in some small measure to future generations.

In particular, I would like to thank my advisor, Jay Bailey. The entire list of things for which thanks are due to him is too long to attempt to summarize. However, two items ought to be mentioned, for without them this thesis would not exist. His tremendous enthusiasm for this research and his incisive comments at various points along this work have made a lasting impression on me.

Equally important, he has provided me with a unique and incredibly stimulating environment in his lab, which has influenced the course of this work.

While on the subject of the Bailey world and its influence on me, I would like to acknowledge some of my colleagues in particular. My classmates, Roger (Howdeedoodie) Hart and Mike ("Baby") Kosinski, have been wonderful friends, collaborators, and brothers-in-arms against the unknown elements of graduate life at Caltech. Among my officemates, Doug (Dude) Axe deserves special mention for his roles as friend, philosopher, critic, SoCal jargon teacher, and smartass, while Pete (unmentionable) Licari needs to be thanked for his valiant attempts to bring excitement in a mellowing lab. The steady-state flow of European postdocs has also contributed to the novelty of the Bailey lab; in particular I'd like to thank Uschi Rinas for introducing me to the art of 21st century gel-electrophoresis. Finally, of course, there is a long list of names associated with pleasant memories: Ken, Nancy, Alex, Karl, Brent, Jackie, Mike P., Todd, Dane, ... While some of them helped me find my bearings on the third floor, others were more successful at doing so outside Caltech.

Thanks are due to George Weinstock, Peter Berget, Russ Maurer, and 16 other moderate maniacs (some not so moderate) for the good times and good learnings at Fort CSH, and to Mel Simon for introducing me to the experience.

I'd also like to thank the crew at Exogene for collaborating with me during the latter part of this work. Joseph, Dallas, John (Durian king), Sharon, Jorge: it was fun working with you all. So long and all the best.

Several people have helped elevate the quality of my life in Pasadena, for which I am grateful: my former housemates and other animals at 1488 Rose

Villa, Bill and Janet Dreyer, Chris and Bev Anderson, the folks and beasts at San Pascual Stables, and above all Rebekah (thank you for everything while it lasted).

Last, but not the least, perhaps it would be appropriate to thank Robert Fulghum for reminding me lucidly (one whole week before submitting my PhD thesis) that All I Really Need to Know I Learned in Kindergarten!

ABSTRACT

Vitreoscilla sp. is a Gram-negative obligately aerobic bacterium, which is capable of synthesizing a soluble, homodimeric hemoglobin-like molecule (VHb) in response to hypoxic environments. Although the mechanism of action of VHb is not understood, it has been hypothesized that the heme-protein enables the bacterium to survive in oxygen-limited environments. The functional role of this bacterial globin has been studied with the aim of exploiting such a naturally evolved strategy to improve oxygen-limited bioprocesses. Furthermore, the regulation of expression of the *VHb* gene in response to changes in environmental conditions has also been investigated. This has provided insights into mechanisms of microaerobic gene regulation, and has enabled the development of oxygen-dependent expression systems for high-level synthesis of recombinant proteins.

The gene encoding the VHb polypeptide was isolated from a *Vitreoscilla* genomic library. The nucleotide sequence of the gene and its flanking regions was determined and analyzed. Synthesis of active VHb was shown to occur in *E. coli* from the natural expression signals of the *VHb* gene.

Studies in fed-batch fermentations demonstrated that under oxygen limitation, the presence of the *VHb* gene on a multicopy plasmid enhanced the growth and respiratory characteristics of a recombinant *E. coli* host as compared to equivalent plasmid-carrying and plasmid-free cells. These results illustrated the

possibility of studying the mechanism of VHb action in *E. coli* as a surrogate host, and were also indicative of the potential applicability of such a genetic strategy in organisms other than *Vitreoscilla*. Furthermore, it was observed that *VHb* expression is under oxygen-dependent control in *E. coli*, suggesting that the mechanism of regulation of the gene in *Vitreoscilla* is also functional in *E. coli*.

Biochemical studies revealed that a considerable fraction, but not all, of the intracellular VHb is localized in the periplasm of *E. coli* and *Vitreoscilla*. The activity of the two fractions was identical, as judged by visible spectroscopy. Genetic evidence for the role of the N-terminal domain of the VHb polypeptide in protein translocation was also obtained; however no cleavage was detected at this end as a result of translocation. Based on available biochemical and biophysical data, it was suggested on theoretical grounds that periplasmic VHb is capable of supporting an additional oxygen flux to the respiratory chain, which may be physiologically significant (the facilitated diffusion hypothesis).

In *E. coli* strains containing *VHb* integrated into the chromosome in single copy, the presence of VHb improved cellular energetics under oxygen-limiting, but not oxygen-excess conditions. Indirect evidence was obtained, suggesting that the net effect of VHb in *E. coli* is to improve the efficiency, rather than the kinetics of oxygen-limited aerobic metabolism. Although the facilitated diffusion hypothesis could not be confirmed or ruled out, an alternative hypothesis (the intracellular redox effector hypothesis) was also proposed. This implies that oxygenated VHb influences the activity of a key redox-sensitive cell function, which is affected under hypoxic conditions.

Genetic studies demonstrated the presence of an oxygen-responsive element (ORE) upstream of the *VHb* gene. Gene expression is maximal under microaerobic conditions, and is also influenced by catabolite repression. Mechanisms responsible for oxygen-dependent regulation act at the level of transcription initiation from two overlapping promoters within the region upstream of the *VHb* gene.

Protocols were developed for the use of ORE-based expression systems for regulated, high-level synthesis of recombinant proteins in high cell density fermentations. A 30-fold modulation of promoter activity could be achieved in these experiments. By simply decreasing the air supply to cells carrying a *VHb-lacZ* fusion, β- galactosidase was expressed to a level of about 10% of total cellular protein in a culture containing 20 g dry cell weight/L.

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CHAPTER 1

INTRODUCTION

1.1 Genetic Manipulation of Cellular Metabolism: A Background

Within less than a decade following the emergence of recombinant DNA technology it became clear that the *in vivo* catalytic activity of a given protein was not necessarily restricted to its natural host organism. For example, mutationally inactivated metabolic functions in *E. coli* could be complemented with corresponding structural genes from diverse organisms including other bacteria (1), yeasts (2,3), other fungi (4) and mammals (5). Inter-species complementation is not limited to procaryotic hosts (6).

These results suggested the possibility of using genetic tools to transfer any metabolic activity from one species to another which lacks its homolog. In recent years a few such attempts have been reported (7-12). For example, the conversion efficiency of ammonia and methanol to single cell protein by the bacterium *Methylophilus methylotropus* was improved by transforming it with the *E. coli* glutamate dehydrogenase gene, which has no counterpart in wild-type *M. methylotropus* (7). More recently, the combination of several enzymes from three distinct bacterial species has led to the development of a bacterial strain capable of degrading a complex mixture of aromatics (12). The eventual goal of most of these examples was to bring together a set of enzymes, each retaining its original substrate specificity, that were capable of providing a 'patchwork' metabolic pathway when put together. However, in at least one case the combination of two sets of genes resulted in the synthesis of novel 'hybrid' compounds, since the enzymes apparently have affinities for each other's reaction intermediates (10). Again, such exercises have also found applications with both procaryotic (7-10, 12) and eucaryotic (11) cells.

The scope of genetic manipulation of metabolism is not necessarily restricted to the introduction of new enzymatic activities. By selectively inactivating, attenuating, or amplifying existing enzymes, or modifying their regulatory mechanisms, one can perturb the kinetics, stoichiometry, product distribution, and even product structures produced by biosynthetic and catabolic pathways (13-15).

1.2 Motivation for this work.

A careful study of the above examples, as well as several others, reveals a correlation between the depth of knowledge about a metabolic function and one's ability to manipulate it genetically. Typically, the minimum prerequisite information is a knowledge of all the individual steps in an existing pathway. Furthermore, if the exercise requires cross-cloning of enzymatic activities, these activities (and their genes) must be identified in a potential donor organism, and their functional role in the recipient organism must be qualitatively predicted *a priori*. The availability of additional information, such as bottlenecks in the pathway of interest and mechanisms of regulation, is also needed to reduce trial-and-error efforts. Interest in the development of new theoretical and experimental tools to obtain such information is illustrative of its importance (16,17).

An alternative approach, based on an evolutionary argument, is suggested here. This approach may be particularly useful in guiding genetic manipulation of imperfectly understood metabolic functions. The motivation for such an approach is based on two tenets. First, there exists enormous genetic diversity in

nature. In other words, the strategies that have evolved in different organisms to cope with diverse environmental niches appears to be almost limitless. Second, in most biocatalytic processes cells are grown and/or utilized in environments that are quite different from their natural habitats. Hence, it could be argued that these cells are not necessarily 'evolutionarily optimized' for the processes in which they are used. In themselves these two points may appear to be somewhat trivial; however, when taken together, they provide a fairly compelling reason to seek and exploit naturally evolved strategies in the hope of ameliorating potential bottlenecks in important, but imperfectly understood metabolic functions.

The specific question addressed here is the adequate supply of oxygen to aerobically growing cells. The problem, which is central to a variety of aerobic bioprocesses, was first highlighted by Louis Pasteur around the middle of the 19th century. Since then, numerous solutions have been proposed, all having a common theme: The aim has been to improve the oxygen *transfer* properties of the *environment* in which the cells are grown. An alternative approach is genetic manipulation to improve the oxygen *utilization* properties of the *cells* themselves. It should be remembered, however, that the mechanisms of aerobic respiration and its regulation are not entirely clear, even in well-studied model systems such as *E. coli*. Such a problem is therefore well suited to exploring the applicability of naturally evolved strategies.

A few years ago a potential genetic solution to this problem suggested itself. The bacterium, *Vitreoscilla* sp., is a Gram-negative obligate aerobe. Species of this genus live in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. The bacterium *Vitreoscilla* sp. synthesizes a soluble

hemoglobin- like molecule (VHb) in response to hypoxic environments (18). Biochemical studies have revealed a striking similarity between VHb and eucaryotic globins. For example, at the level of its primary amino acid sequence, the protein contains conserved residues at almost all positions that are important to the structure of eucaryotic globins (18,19). VHb has been isolated in a homodimeric form, and its CO-binding property shows cooperativity (20). It is stable in an oxygenated (reduced) state (21), and is spectroscopically similar (as judged by visible and IR spectroscopy) to other globins (22,23). Hence, although the mechanism of action of VHb remains unknown, it has been hypothesized that VHb enables the bacterium to survive in oxygen-limited environments (18,19). The principal motivation of this work was to explore the applicability of this solution to the improvement of oxygen-limited growth of other organisms.

1.3 Scope of this work.

Chapter 2 describes the cloning and nucleotide sequencing of the *VHb* gene. When cloned on a multicopy plasmid, the *VHb* gene is expressed from its natural promoter as a major polypeptide in *E. coli*.

Two reasons dictated the selection of *E. coli* as a surrogate host of choice in these, as well as further studies. First, as a necessary prerequisite, it was shown that VHb is expressed in *E. coli* in its active form. Second, studies have shown that the cytochrome composition of *Vitreoscilla* is remarkably similar to that in *E. coli* (24). In Chapter 3, the effect of VHb on oxygen-limited growth of *E. coli* is investigated via fed-batch fermentations.

Chapter 4 describes the results of various biochemical and genetic experiments aimed at determining the sub-cellular location of VHb. Such information is important in understanding the mechanism of action of this heme-protein. Using available biophysical and biochemical data, a quantitative analysis is presented to explore the validity of the facilitated diffusion hypothesis in explaining the *in vivo* physiological role of VHb.

Using *E. coli* strains carrying the *VHb* gene integrated into the chromosome in a single copy, a detailed investigation on the physiological influence of VHb in *E. coli* is presented in Chapter 5. This has led to the proposal and evaluation of two alternative classes of models for the mechanism of VHb action. These results also provide a basis for projecting the applicability of this genetic strategy to other aerobic bioprocesses.

During the course of studies presented in Chapter 3 it was observed that expression of the *VHb* gene from its natural promoter is regulated in *E. coli* in response to oxygen availability. In Chapter 6 a genetic analysis of the oxygen-responsive promoter element (ORE) is presented.

The insights obtained through the results of Chapter 6 provide a basis for the development of protocols for the use of ORE-based expression systems for regulated, high-level synthesis of heterologous proteins in *E. coli*. Efforts in this direction are reported in Chapter 7.

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CHAPTER 2

THE *VITREOSCILLA* HEMOGLOBIN GENE: MOLECULAR CLONING, NUCLEOTIDE SEQUENCE AND GENETIC EXPRESSION IN *ESCHERICHIA COLI*

Source: Khosla, C. and Bailey, J.E. (1988) Mol. Gen. Genet. 214:158-161.

2.1 Summary.

Vitreoscilla hemoglobin is involved in oxygen metabolism of this bacterium, possibly in an unusual role for a microbe. We have isolated the *Vitreoscilla* hemoglobin structural gene from a pUC19 genomic library using mixed oligodeoxy-nucleotide probes based on the reported amino acid sequence of the protein. The gene is expressed in *E. coli* from its natural promoter as a major cellular protein. The nucleotide sequence, which is in complete agreement with the known amino acid sequence of the protein, suggests the existence of promoter and ribosome binding sites with a high degree of homology to consensus *E. coli* upstream sequences. In the case of at least some amino acids, a codon usage bias can be detected which is different from the biased codon usage pattern in *E. coli*. The downstream sequence exhibits homology with the 3' end sequences of several plant leghemoglobin genes. *E. coli* cells expressing the gene contain greater than fivefold more heme than controls.

2.2 Introduction.

The filamentous bacterium, *Vitreoscilla*, a member of the Beggiatoa family, is a strict aerobe that is found in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. Growth of this bacterium under hypoxic conditions results in severalfold induction of the synthesis of a homodimeric soluble heme protein (subunit MW 15,775) (Tyree and Webster 1978, Boerman and Webster 1982) which has a remarkable spectral (Webster and Liu 1974), structural (Wakabayashi et al. 1986), and kinetic (Orii and Webster 1986) homology with eucaryotic hemoglobins. The conservation of most features characteristic

for eucaryotic hemoglobins as well as its possible role in oxygen utilization in *Vitreoscilla* strongly suggest that this protein is indeed a bacterial hemoglobin.

We report here the molecular cloning and nucleotide sequence of the *Vitreoscilla* hemoglobin gene and some of its flanking regions. Interestingly, functional expression of this gene improves the aerobic growth properties of recombinant *E. coli*, as determined by fed-batch fermentations and respirometer measurements on whole cells (Khosla and Bailey 1988).

2.3 Materials and Methods.

Media and growth conditions: E. coli cells were grown at 37°C in L broth (Maniatis et al. 1982). *Vitreoscilla sp.* was grown in a medium containing 1.5% yeast extract, 1.5% peptone, and 0.02% sodium acetate (pH 8.0 with NaOH) at 30°C. Plasmid-containing cells were grown in medium supplemented with 100 mg/l ampicillin. All cells were grown in culture tubes or flasks in a New Brunswick shaker incubator set at 250 rpm.

DNA Manipulations: Vitreoscilla genomic DNA was isolated according to the protocol of Silhavy et al. (1984). λgt10 and pUC19 genomic DNA libraries were made according to standard protocols (Huynh et al. 1985, Maniatis et al. 1982). For all other DNA manipulations, routine methods were used (Maniatis et al. 1982), unless otherwise stated below.

DNA Sequencing: The *Hin*dIII - *Sph*I fragment from plasmid pRED2 (Figure 2), which contains the entire structural gene (see results), was subcloned in plasmid pUC19 in the same orientation as in pRED2. After digesting this new plasmid (pRED4) at the unique *Hin*dIII and *Mlu*I sites, the ends were 5'-labeled

and 3'-labeled. Purified *Hin*dIII - *Mlu*I and *Mlu*I - *Sph*I fragments were then subjected to G-specific (Maxam and Gilbert 1980) and A-specific (Iverson and Dervan 1988) cleavage reactions. Samples were run on 8% and 20% polyacrylamide gels. The nucleotide sequence was read in the directions indicated by dashed arrows in Figure 2.

Heme assay: Heme extracts were prepared and assayed according to the method of Lamba and Webster (1980). In each case 0.1g cells (wet weight basis) were used. The heme concentration was estimated on a Shimadzu UV160 spectrophotometer using the extinction coefficient $E_{556-541} = 20.7\ mM^{-1}cm^{-1}$ for reduced minus oxidized spectra.

2.4 Results and Discussion.

Cloning the Vitreoscilla hemoglobin gene: Three sets of mixed oligonucleotide probes were synthesized which had a predicted homology to one 5' and one 3' domain in the hemoglobin gene (Fig. 1). Southern blot hybridization to *Eco*RI-digested genomic DNA established that the structural gene was present in a single fragment of around 7.24 kb and also enabled the discrimination between probe sets 1 and 2 (Fig. 1). Probe set 1 was identified as the correct set. Further, the minimum selective wash temperatures for probe sets 1 and 3 were also determined to be 50°C and 46°C respectively. We were unable to isolate the gene from a λgt10-*Eco*RI library, probably due to the sharp reduction in packaging efficiency of this vector for inserts bigger than 7.6 kb (Huynh et al. 1985). Hence, Southern blot hybridizations to genomic DNA digested with *Bam*HI, *Hin*dIII and various combinations of these three enzymes

were performed. The smallest gene-containing fragment was a *Hin*dIII fragment that was 2.2 kb. A pUC19-*Hin*dIII library of *Vitreoscilla* DNA was test-plated on rich media containing ampicillin (100 mg/L), X-gal (40 mg/L) and IPTG (1 mM). More than 70% of the colonies were probable recombinants, as estimated by visual inspection. About 10,000 colonies were then screened. Three positive clones were identified and restreaked for single colonies. The plasmids from a small number of clones from each group were examined by *Hin*dIII digestion. One of these, pRED1, carried three *Hin*dIII inserts including one of 2.2 kb. This 2.2 kb fragment was purified and cloned into pUC19 in both orientations (pRED2 and pRED3). Digestion of pRED2 with various enzymes and Southern blot analysis confirmed that the entire hemoglobin structural gene is present on a *Hin*dIII -*Sph*I fragment (approx. 1.1 kb). The restriction map of plasmid pRED2 is shown in Fig. 2.

Interestingly, the *Vitreoscilla* genome had a statistically small number of restriction sites for *Bam*HI. There were only 9 observable distinct bands smaller than 5.5 kb and 4 such bands smaller than 4 kb. A similar observation has also been made in the case of the genome of *Methanococcus voltae* which has an unusually small number of sites for the 4-base pair recognizing enzyme, *Sau*3AI, which is an isoschizomer of *Bam*HI (Jarrell et al. 1987).

Transformation of Vitreoscilla: Attempts to transform *Vitreoscilla* with plasmid pRED2 using both the $CaCl_2$ (Silhavy et al. 1984) and the Hanahan (1983) protocols were unsuccessful. However, neither of the competent cell-making and transformation processes had resulted in a noticeable decrease in the viability of the cells. Since the existence of a natural plasmid in this *Vitreoscilla*

strain has recently been reported (Dikshit and Webster 1987), this difficulty may have resulted from plasmid incompatibility.

Genetic expression of the hemoglobin gene in E. coli: E. coli cells containing plasmids pRED1, pRED2, pRED3 and pUC9 as well as *Vitreoscilla* cells were grown to stationary phase and cell extracts were examined on an SDS-polyacrylamide gel for the existence of the hemoglobin polypeptide. As can be seen in Fig. 3 (lanes 2-5), hemoglobin is expressed as a major cellular protein in all the recombinant cells. Since both plasmids pRED2 and pRED3 express about equal amounts of this polypeptide and since the level of expression from pRED2 is about the same with and without IPTG, the gene is probably expressed from its natural promoter in *E. coli*. This conclusion is reinforced by an examination of the upstream sequences of the gene (see below). Such high level expression of the gene product in *E. coli* contrasts to the low level of expression obtained under better oxygenated growth conditions (Khosla and Bailey 1988) and suggests that the oxygen-dependent regulatory mechanism of this gene is functional in *E. coli*. We are presently studying this regulatory system.

Effect of the cloned gene on heme metabolism: Recombinant cells containing the hemoglobin gene were all observed to be red in color compared to JM101 or JM101/pUC9. Spectrophotometric assays for the heme content of these cells showed elevated heme concentrations in cells containing the *Vitreoscilla* hemoglobin gene. The heme content of JM101/pRED2 was 21 nanomoles/g cells compared to 3.8 nanomoles heme/g cells for JM101/pUC9. Correspondingly, the hemoglobin activity of JM101/pRED2 was fivefold higher than JM101/pUC9 (Khosla and Bailey 1988).

Nucleotide sequence of the structural hemoglobin gene and flanking regions: The complete nucleotide sequence of the structural hemoglobin gene along with its amino acid translation is shown in Figure 1. The deduced amino acid sequence is in perfect agreement with the sequence reported by Wakabayashi et al. (1986). The region downstream of the structural gene has been computer-aligned with the untranslated 3' ends of four leghemoglobin genes which are known to have conserved sequence motifs (Kuhse and Puehler 1987).

Several interesting observations can be made from the nucleotide sequence shown in Figure 1. Firstly, a significant homology can be observed between the 3' end sequence of this gene and other plant globin genes. As shown in the figure, regions 1,2 and 3 represent highly conserved sequence motifs in several plant Lb genes (Kuhse and Puehler 1987) which are also fairly conserved in *Vitreoscilla.* This suggests common evolutionary ancestry between this gene and plant leghemoglobin genes. It should be noted, however, that no functional significance has been assigned to this region in the case of the plant genes (Stougaard et al. 1987). Earlier, Wakabayashi et al (1986) have indicated that amongst several animal and plant globin amino acid sequences, the *Vitreoscilla* hemoglobin shows the maximum sequence homology (24%) with lupin leghemoglobin. Within the 3' region indicated in Figure 1, the *Vitreoscilla* hemoglobin shows the greatest homology (45%) with the soybean leghemoglobin A gene (indicated by dots in Fig.1). Since the corresponding figure between the soybean LbA gene and the kidneybean LbA gene is 52%, this figure is indicative of significant homology. It could be noted that a putative procaryotic terminator, TTTTTA (Glass 1982), exists within region 2 (Fig.1) with a possible stem-loop region,

ACCAtaaggTGGT, preceding it (corresponding to another conserved region in the plant sequences). The *Vitreoscilla* and the soybean LbA sequences share a greater than 60% homology downstream of this putative terminator.

On examining the codon usage pattern within this structural gene, a distinct bias towards the usage of specific codons for some of the more abundant amino acids in the protein can be observed. For example, out of 14 leucine codons, 13 are UUG and one is UUA. Similarly, all phenylalanine codons are UUU and 5 out of 7 proline codons are CCU. In all three cases the preferred triplet for more abundant proteins in *E. coli* is different (Gouy and Gautier 1982).

Expression of the hemoglobin polypeptide as a major cellular protein in *E. coli* from its own promoter suggests a homology between the expression control sequences of *E. coli* and *Vitreoscilla.* In Figure 1, putative Pribnow and -35 boxes as well as ribosome-binding sites have been underlined. These show near-perfect homology to consensus *E. coli* sequences (Glass 1982). Comparison of lanes 2-5 and lane 7 in Fig. 3 indicates that the amount of hemoglobin in *E. coli* is much greater than that in *Vitreoscilla* grown under similar conditions. In our experience, growth of *Vitreoscilla* to early stationary phase in culture tubes placed in an air-circulated shaker incubator is usually not sufficient to induce a detectable reddish tint to the cells; however such a tint is observed in cases where the *Vitreoscilla* cells are grown under more oxygen-limited conditions. Whether this difference in expression in *E. coli* and *Vitreoscilla* is a gene dosage effect or due to a difference in regulation of gene expression in these two strains is as yet unclear. Introducing this gene into *Vitreoscilla* on a plasmid would be useful in this context.

Since the protein is active in *E. coli* and confers growth enhancement to *E. coli* in oxygen-poor environments (Khosla and Bailey 1988), molecular cloning and sequencing of this gene will facilitate a better understanding of its exact *in vivo* metabolic role. Work in this direction is currently in progress in our laboratory.

2.5 Acknowledgements.

This work has been supported by the Energy Conversion and Utilization Technology (ECUT) program of the United States Department of Energy. The authors are grateful to Dr. Webster for providing the *Vitreoscilla sp.* strain.

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2.7 Captions to Figures.

Figure 1: Nucleotide sequence of the Vitreoscilla hemoglobin gene: The nucleotide sequence along with a scheme of the sets of mixed oligonucleotides used to isolate the gene as well as the amino acid translation of the structural gene are shown. Also shown is an alignment of the 3' ends of leghemoglobin (Lb) genes from soybean (LbA, LbC$_3$), broadbean, and kidneybean (LbA). Three sets

of mixed oligonucleotide probes (numbered 1,2, and 3) are indicated by their IUB nomenclature below their respective target sequences. The alignment of the 3' ends of the Lb genes is essentially the same as that of Kuhse and Puehler (1987) with a few changes to maximize base-for-base similarity. The *Vitreoscilla* sequence was computer-aligned to these sequences to maximize similarity using a standard difference-matrix algorithm. Regions of 2 or more adjacent bases which are conserved in at least 4 out of 5 species are boxed. Dots are used to indicate base similarity between the *Vitreoscilla* and soybean LbA sequences (see text). The putative ribosome binding site, Pribnow box and the -35 box have been underlined in the upstream region of the structural gene.

Figure 2: Restriction map of plasmid pRED2. This plasmid is a derivative of pUC19 (28). The position and orientation of the *Vitreoscilla* hemoglobin structural gene is as shown. The symbols Aat, Hin, Mlu, Sal, and Sph indicate approximately the recognition sites for the restriction enzymes *Aat*II, *Hin*dIII, *Mlu*I *Sal*I, and *Sph*I, respectively. The *Hin*dIII insert does not contain any recognition sites for the enzymes *Bam*HI, *Eco*RI, *Hae*II, *Nde*I, *Pst*I, *Pvu*I, *Sma*I, *Sst*I, or *Xba*I. The dashed arrows near the *Hin*dIII and *Mlu*I sites represent the directions of sequencing of labeled fragments (see methods section).

Figure 3: Expression of hemoglobin in E. coli. The total cellular protein content of cells containing various plasmid constructs was resolved on a 12.5% SDS-polyacrylamide gel. Lane 1 shows the molecular weight standards. Lanes 2-4 and 6 represent *E. coli* cells containing plasmids pRED1, pRED2, pRED3 and pUC9 respectively. Lane 5 represents *E. coli* cells containing plasmid pRED2

(which has the hemoglobin gene cloned in the same orientation as the *lac* promoter, see Fig. 3) grown in the presence of 1 mM IPTG. Lane 7 shows the protein content of *Vitreoscilla*. Gels were run according to the standard protocol of Laemmli (1970). Protein in the gel was visualized by the silver staining method of Merril et al. (1983).

Figure 1.

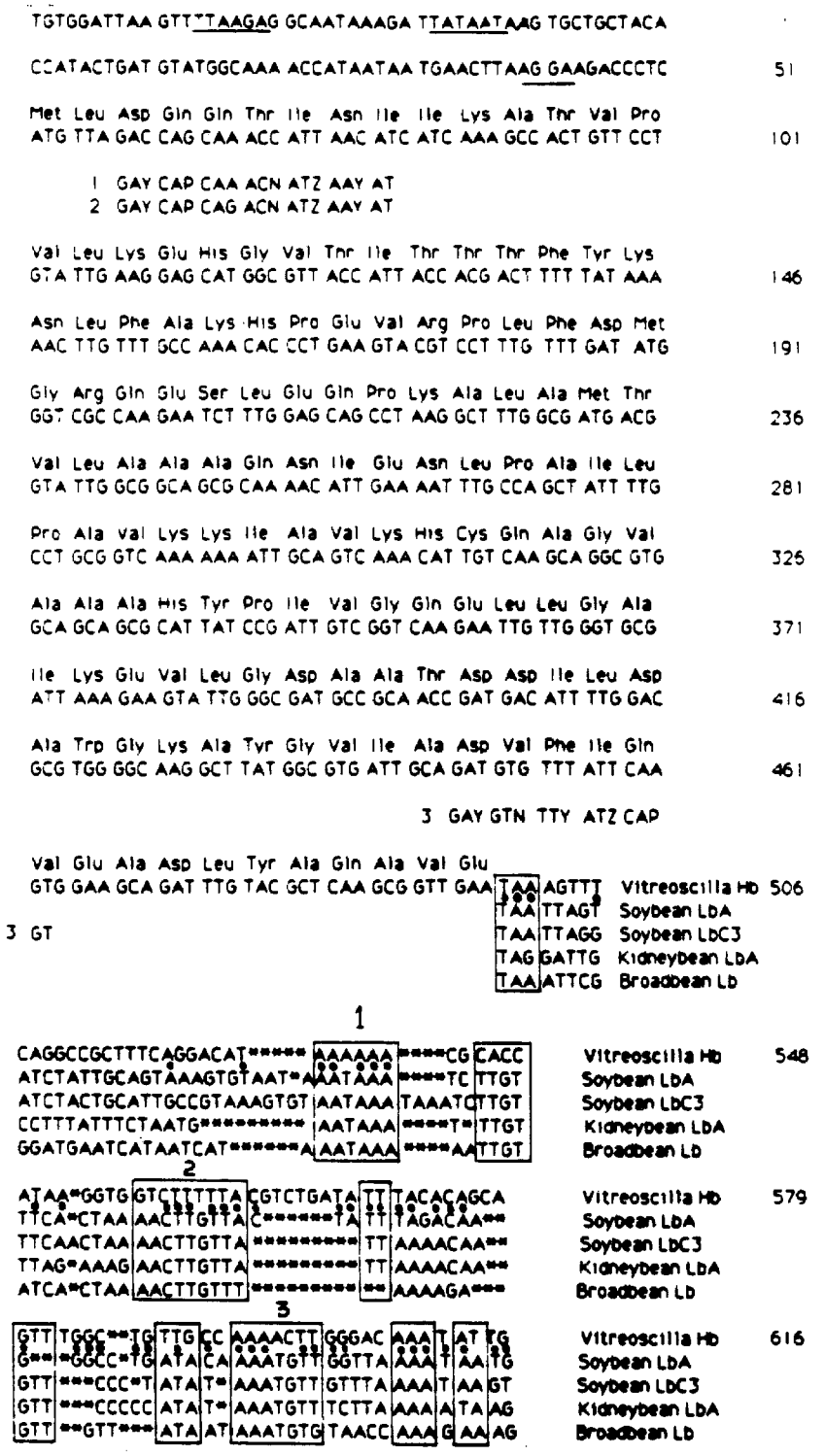
TGTGGATTAA GTTTTAAGAG GCAATAAAGA TTATAATAAG TGCTGCTACA
CCATACTGAT GTATGGCAAA ACCATAATAA TGAACTTAAG GAAGACCCTC 51
Met Leu Asp Gln Gln Thr Ile Asn Ile Ile Lys Ala Thr Val Pro
ATG TTA GAC CAG CAA ACC ATT AAC ATC ATC AAA GCC ACT GTT CCT 101
1 GAY CAP CAA ACN ATZ AAY AT
2 GAY CAP CAG ACN ATZ AAY AT
Val Leu Lys Glu His Gly Val Thr Ile Thr Thr Thr Phe Tyr Lys
GTA TTG AAG GAG CAT GGC GTT ACC ATT ACC ACG ACT TTT TAT AAA 146
Asn Leu Phe Ala Lys His Pro Glu Val Arg Pro Leu Phe Asp Met
AAC TTG TTT GCC AAA CAC CCT GAA GTA CGT CCT TTG TTT GAT ATG 191
Gly Arg Gln Glu Ser Leu Glu Gln Pro Lys Ala Leu Ala Met Thr
GGT CGC CAA GAA TCT TTG GAG CAG CCT AAG GCT TTG GCG ATG ACG 236
Val Leu Ala Ala Ala Gln Asn Ile Glu Asn Leu Pro Ala Ile Leu
GTA TTG GCG GCA GCG CAA AAC ATT GAA AAT TTG CCA GCT ATT TTG 281
Pro Ala Val Lys Lys Ile Ala Val Lys His Cys Gln Ala Gly Val
CCT GCG GTC AAA AAA ATT GCA GTC AAA CAT TGT CAA GCA GGC GTG 326
Ala Ala Ala His Tyr Pro Ile Val Gly Gln Glu Leu Leu Gly Ala
GCA GCA GCG CAT TAT CCG ATT GTC GGT CAA GAA TTG TTG GGT GCG 371
Ile Lys Glu Val Leu Gly Asp Ala Ala Thr Asp Asp Ile Leu Asp
ATT AAA GAA GTA TTG GGC GAT GCC GCA ACC GAT GAC ATT TTG GAC 416
Ala Trp Gly Lys Ala Tyr Gly Val Ile Ala Asp Val Phe Ile Gln
GCG TGG GGC AAG GCT TAT GGC GTG ATT GCA GAT GTG TTT ATT CAA 461
3 GAY GTN TTY ATZ CAP
Val Glu Ala Asp Leu Tyr Ala Gln Ala Val Glu
GTG GAA GCA GAT TTG TAC GCT CAA GCG GTT GAA
3 GT
TAA AGTTT Vitreoscilla Hb 506
TAA TTAGT Soybean LDA
TAA TTAGG Soybean LDC3
TAG GATTG Kidneybean LDA
TAA ATTCG Broadbean Lb
1
CAGGCCGCTTTCAGGACAT***** AAAAAA ****CG CACC Vitreoscilla Hb 548
ATCTATTGCAGTAAAGTGTAAT*A AATAAA ****TC TTGT Soybean LDA
ATCTACTGCATTGCCGTAAAGTGT AATAAA TAAATC TTGT Soybean LDC3
CCTTTATTTCTAATG********* AATAAA ****T* TTGT Kidneybean LDA
GGATGAATCATAATCAT******A AATAAA ****AA TTGT Broadbean Lb
2
ATAA*GGTG GTCTTTTTA CGTCTGATA TT TACACAGCA Vitreoscilla Hb 579
TTCA*CTAA AACTTGTTA C******TA TT TAGACAA** Soybean LDA
TTCAACTAA AACTTGTTA ********* TT AAAACAA** Soybean LDC3
TTAG*AAAG AACTTGTTA ********* TT AAAACAA** Kidneybean LDA
ATCA*CTAA AACTTGTTT ********* ** AAAAGA*** Broadbean Lb
3
GTT TGGC**TG TTG CC AAAACTT GGGAC AAA T AT TG Vitreoscilla Hb 616
G**-*GGCC*TG ATA CA AAATGTT GGTTA AAA T AA TG Soybean LDA
GTT ***CCC*T ATA T* AAATGTT GTTTA AAA T AA GT Soybean LDC3
GTT ***CCCCC ATA T* AAATGTT TCTTA AAA A TA AG Kidneybean LDA
GTT **GTT*** ATA AT AAATGTG TAACC AAA G AA AG Broadbean Lb

Figure 2.

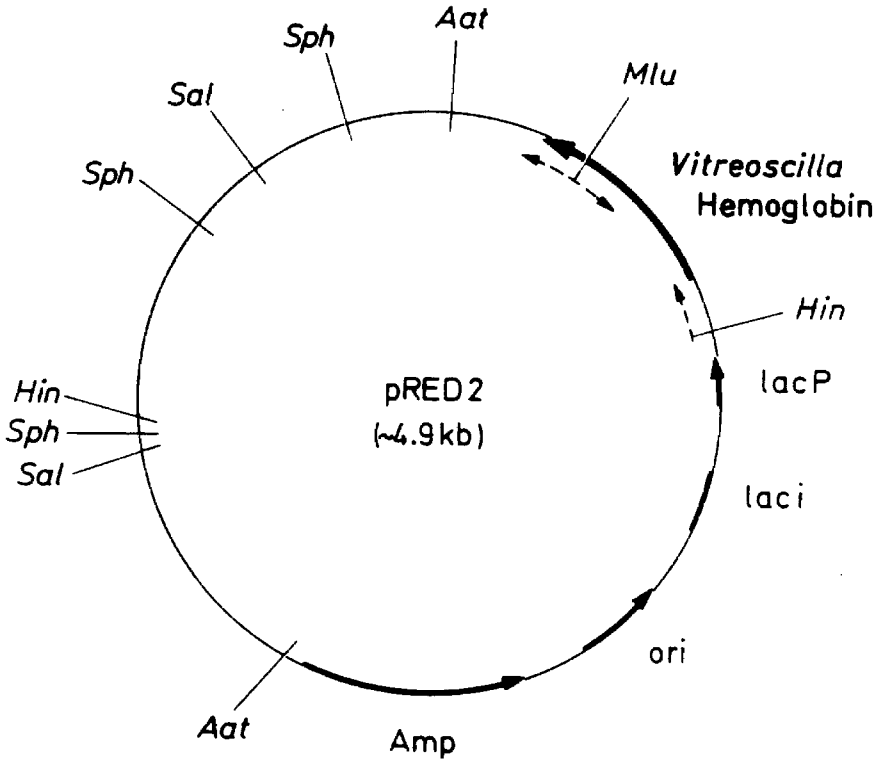
Aat
Sph
Mlu
Sal
Sph
Vitreoscilla
Hemoglobin
Hin
lacP
pRED2
(~4.9kb)
Hin
Sph
Sal
laci
ori
Aat
Amp

Figure 3.

CHAPTER 3

HETEROLOGOUS EXPRESSION OF A BACTERIAL HAEMOGLOBIN IMPROVES THE GROWTH PROPERTIES OF RECOMBINANT *ESCHERICHIA COLI*

Source: Khosla, C. and Bailey, J.E. (1988) Nature 331:633-635.

3.1 Summary.

In poorly oxygenated environments, the aerobic bacterium *Vitreoscilla* synthesizes a haemoglobin-like molecule (1,2). We have isolated the gene encoding this protein and expressed it in *E. coli* (3). Enhanced haem content as well as active haemoglobin can be detected in the recombinant cells. Anticipating the potential significance of haemoglobin under oxygen limitation, which is a central problem in dense cell cultures, we studied the metabolic effects of this protein in fed-batch *E. coli* fermentations. Cells containing this gene grow faster and to considerably greater cell densities than comparable plasmid-containing cells which do not express haemoglobin. Respiratory studies indicate that this haemoglobin increases oxygen utilization rates, especially when dissolved oxygen is less than 5% of air saturation.

3.2 Text.

Rational design of novel as well as improved cellular biocatalysts by genetic manipulation of cellular metabolism has recently attracted considerable interest. A wide range of bacteria have been genetically modified by integrating hitherto non-existent enzymatic functions into their metabolic network, thereby endowing them with new catalytic abilities (4-9) or improving upon existing ones (10). A central problem in the aerobic growth of any cell culture is the maintainence of dissolved oxygen (DO) concentrations above growth-limiting levels. This problem becomes particularly acute in high cell density fermentations which are usually of a fed-batch type. The optimal rate of

nutrient addition (and consequently the productivity) is ultimately limited by the rate at which cells can aerobically catabolize the carbon source without generating growth-inhibitory metabolites such as lactate and acetate (11,12). All approaches thus far have concentrated on improving the oxygen mass transfer rates by manipulating various environmental parameters such as the reactor configuration, inlet oxygen concentrations, agitation rates, sparging rates and fermentor back pressure. We describe here a fundamentally different approach to this problem.

Recently, Wakabayashi, *et al* (1) reported the primary amino acid sequence of a soluble dimeric haemoglobin found in the obligately aerobic bacterium, *Vitreoscilla*, and demonstrated significant homology of this protein with known globin sequences. The synthesis of this protein increases severalfold in response to a drop in DO levels, as evidenced by a rise in intracellular haem content (2). Although the exact molecular mechanism is unclear, several *in vitro* and *in vivo* biochemical studies on this protein suggest that it plays a haemoglobin-like role in the cell and is not a cytochrome as was initially suggested (for review, see (1)).

Protohaem IX, the prosthetic group of all haemoglobins, is synthesized by wild type *E. coli* as a component of its membrane-bound aerobic respiratory chain proteins (13). Mutants which overproduce this pigment without any apparent haem or cytochrome deficiency have been isolated (14), suggesting that protehaem IX biosynthesis

may be under repressive control, and hence amenable to amplification. Thus, bearing in mind the apparent *in vivo* role of the *Vitreoscilla* haemoglobin as well as the importance of *E. coli* hosts for high cell density recombinant fermentations, we attempted functional expression of this gene in *E. coli*. Using mixed oligonucleotide probes, we have isolated and cloned into pUC19 a *Hin*dIII fragment from a *Vitreoscilla* genomic library that codes for the haemoglobin gene (3). The gene expressed a polypeptide of the correct molecular weight as a major cellular protein in *E. coli* when grown under culture tube or shake flask conditions (3). This expression is apparently regulated by endogenous *Vitreoscilla* upstream sequences (3). More significantly, greater than fivefold overproduction of intracellular haem was spectrophotometrically recorded (3). Such elevated haem levels impart a distinct reddish tint to these recombinant cell pellets compared to controls. The haemoglobin activity of these cells was correspondingly fivefold higher than controls as measured by the carbon monoxide difference spectrum method of Webster and Liu (15).

To determine whether the functional expression of this protein had any metabolic effect(s) on the host, we compared the growth properties of this recombinant strain (JM101:pRED2 (3)) to similar plasmid-containing (JM101:pUC9) and plasmid-free (JM101) strains under typical fed-batch fermentation conditions (Figure 1). In each case the fed-batch phase of the experiment was conducted under oxygen-limited conditions (12). The growth parameters correspond-

ing to the data illustrated in Figure 1 are summarized in Table 1. In all cases, the corelation between the optical density and the dry cell weight was linear, as determined by intermittent dry cell weight measurements, thus justifying the relevance of optical density as an acceptable measure of growth. The JM101:pRED2 strain significantly outgrew the control recombinant strain JM101:pUC9 throughout the experiment and also exhibited a higher specific growth rate than the plasmid-free strain JM101 during the fed-batch phase. Figure 3 illustrates important differences between haemoglobin-containing and haemoglobin-free cells with respect to their oxygen utilization kinetics, particularly at low DO concentrations. It should be pointed out that though the measured DO levels in the fed-batch phases are around 5% (Figure 2), spatial microheterogeneity in bioreactors can result in pockets of considerably lower DO levels and poor mixing (16,17); these effects are of greater significance at higher cell densities and in larger reactors.

Haemoglobin activity of JM101:pRED2 samples withdrawn from the fermentor could not be spectrophotometrically detected by the above-mentioned method (15) using whole-cell suspensions; these were considerably more dilute than recommended due to sample size limitations. However, small quantities of the polypeptide were observed relative to controls on a silver-stained SDS polyacrylamide gel. (This analysis required considerable darkening of the gel such that it is unpresentable. We are currently working on the development of a

more sensitive assay for the active protein.) This led us to perform a series of shake-flask experiments to study the oxygen-dependent regulation of this gene (Khosla, C. and Bailey, J.E., *manuscript in preparation*). The following two observations from those experiments are relevant in this context: First, expression of the haemoglobin gene in *E. coli* is under oxygen-dependent regulation in a manner similar to that in *Vitreoscilla*. The major difference seems to be that in extreme oxygen-poor conditions, such as those typically encountered in culture tubes and flasks, the gene in recombinant *E. coli* is overexpressed to form inclusion bodies. This is consistent with a larger median cell size observed for the JM101:pRED2 inoculum (which came from stationary phase of a shake flask preculture) compared to those of growing cells in the fermentor (Figure 2). Second, and perhaps more importantly, presence of the cloned haemoglobin gene has provided growth enhancement in terms of dense culture growth rates as well as final cell densities under all conditions studied so far. These experiments suggest a possible utility for this haemoglobin or similar molecules in any aerobically grown cell-lines in which the haemoglobin can be functionally expressed and appropriately localized.

The exact mechanism underlying these phenomena is presently unknown. The haemoglobin protein likely does not function only as an oxygen storage trap; it is probably involved in enhancing the oxygen uptake rate of the membrane-bound aerobic respiratory apparatus, a

role which the haemoglobin might conceivably play even in low concentrations. Since the regulatory mechanism of this gene is functional in *E. coli*, its genetic dissection should be relatively straightforward.

3.3 Acknowledgements.

This work has been supported by the Energy Conversion and Utilization Technology (ECUT) program of the United States Department of Energy. The authors are grateful to Dr. Webster for providing the *Vitreoscilla sp.* strain.

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TABLE 1: Growth parameters of JM101, JM101pUC9 and JM101:pRED2

	JM101	JM101:pUC9	JM101:pRED2
Batch log-phase growth rate[a] (h^{-1})	0.95	0.73	0.95
Batch stat-phase[b] dry cell mass[c] (g/L)	2.6	1.6	2.6
Fed-batch log-phase growth rate[a] (h^{-1})	0.056	0.033	0.064
Final dry cell mass[c] (g/L)	5.8	2.8	5.9

[a]As calculated from the data presented in Fig. 1.

[b]Corresponding to vertical arrow positions in Fig. 1a

[c]Dry cell mass was determined for 10mL aliquots taken directly from the fermentor. Cells were centrifuged, washed once and resuspended in deionized water, all below 4°C. Samples were dried overnight in a 100°C oven.

3.6 Captions to Figures.

Figure 1: Comparison of growth dynamics of JM101 (—···—···—), JM101:pUC9 (– – – – –) and JM101:pRED2 (————) in fed-batch fermentations. Time courses of cell densities in batch (Fig. 1a, left) and fed-batch (Fig. 1b, right) modes of growth are shown.

Method. Cells were grown in a New Brunswick Microferm fermentor at 37±0.5°C and a pH of 7±0.05 with an initial working volume of 2.5L. A constant air flow rate of 4.5L/min and agitator speed of 300 rpm were maintained throughout each run. Foam control was manually achieved by intermittent addition of silicone AF60 antifoam emulsion as required. The batch medium and feed medium 1 of Table 2 in ref. (12) were used as batch and feed media compositions, respectively. For plasmid-containing cells, 100mg/L ampicillin was added to the batch medium. At t=0, the fermentor was inoculated with a 100mL overnight LB culture grown in a shake flask in each case. Cells were initially grown in batch mode till nutrient was exhausted, as indicated by a sudden increase in DO (Fig. 2). Thereafter, feeding was commenced (as indicated by vertical arrows, Fig. 1a; this is the initial point of each curve in Fig. 1b) at a constant rate of 10mL/h and continued till the O.D. (optical density at 600 nm) reached a constant value.

Figure 2: Dissolved oxygen and median cell size profiles for JM101 (—···—···—), JM101:pUC9 (– – – – –) and JM101:pRED2 (————) in fed-batch fermentations.

Method: Details of the fermentation experiments are described in the caption to Fig. 1. DO was calibrated and recorded with reference to air saturation The median cell size was determined for various samples (as indicated by the discrete points) on a Coulter counter, as described in (18).

Figure 3: Respiratory behaviour of JM101 (—···—···—), JM101:pUC9 (– – – – –) and JM101:pRED2 (————).

Method. 1mL stationary phase cells (from samples indicated by vertical arrows in Fig. 1a) were centrifuged and resuspended in 25μL 100mM phosphate buffer (pH 7). This was then injected into fresh medium prewarmed to 37°C in a Gilson respirometer (approx. 2mL). The above curves represent the time course of DO recordings normalized to a unit OD_{600} basis.

Figure 1.

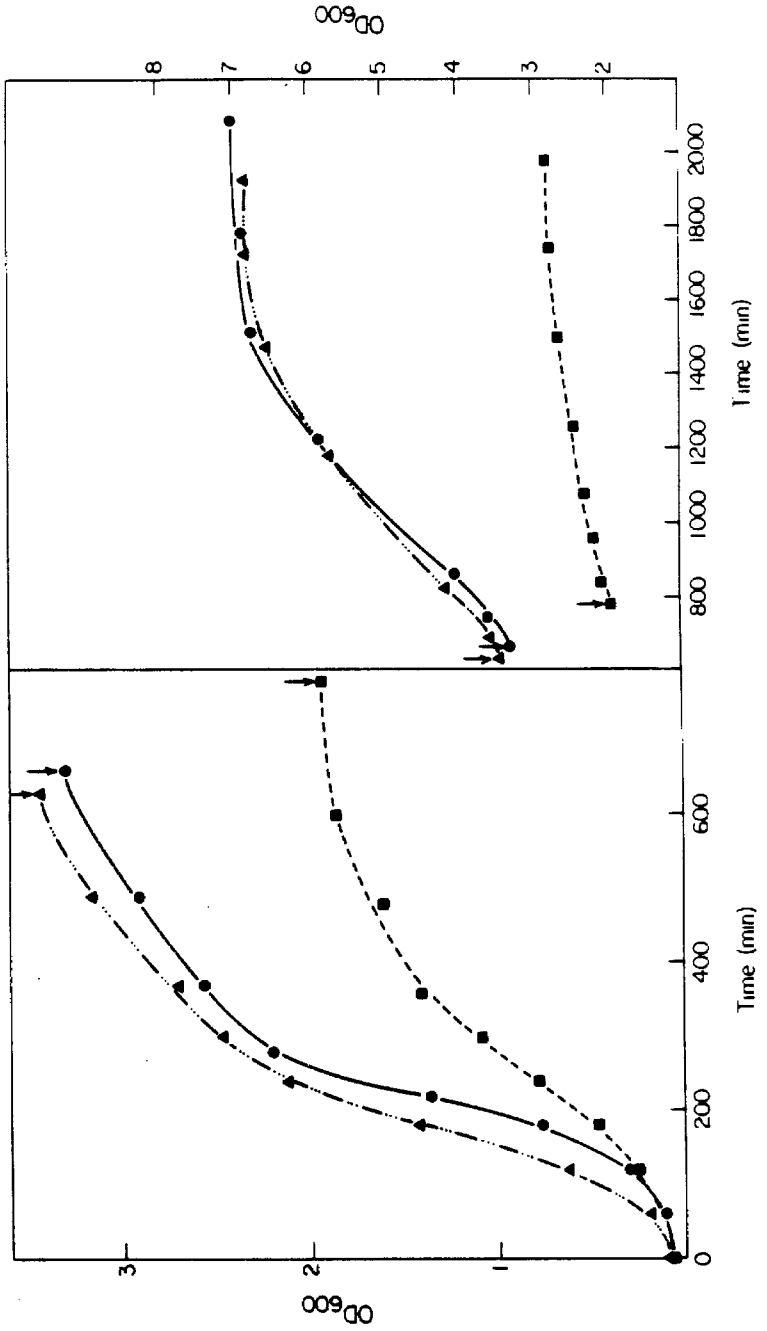
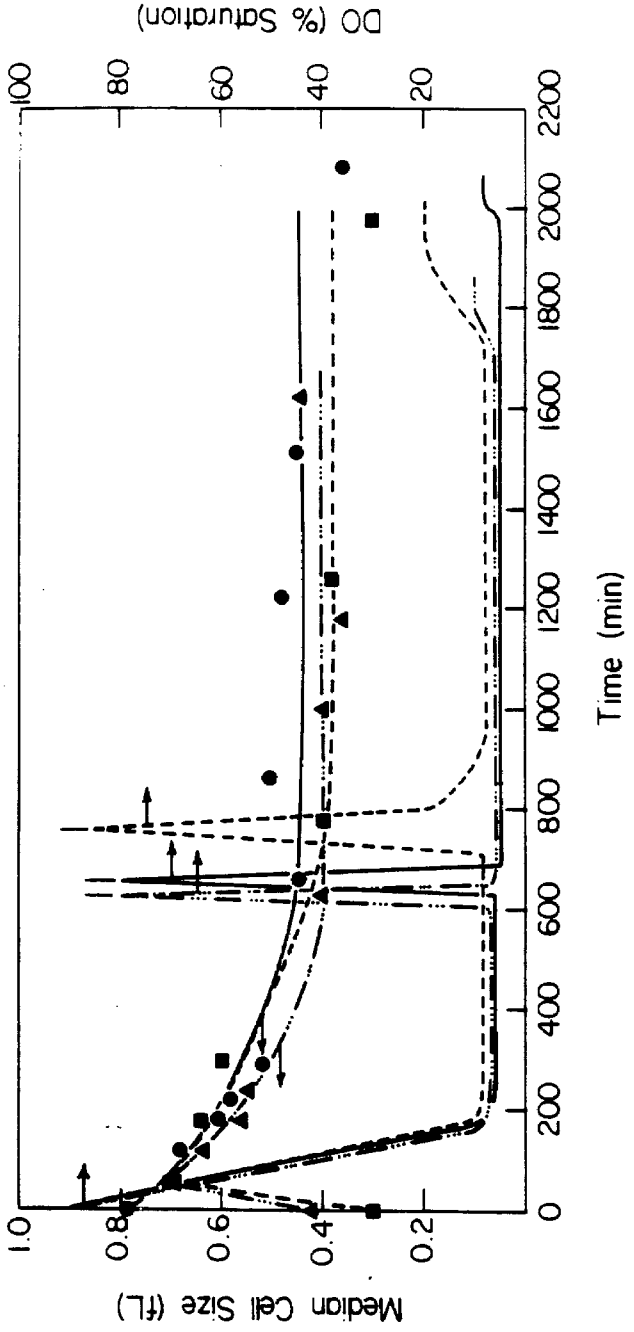

OD600
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
1
2
3
4
5
6
7
8

Figure 2.


DO (% Saturation)
100
80
60
40
20
Median Cell Size (fL)
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0.8
0.6
0.4
0.2
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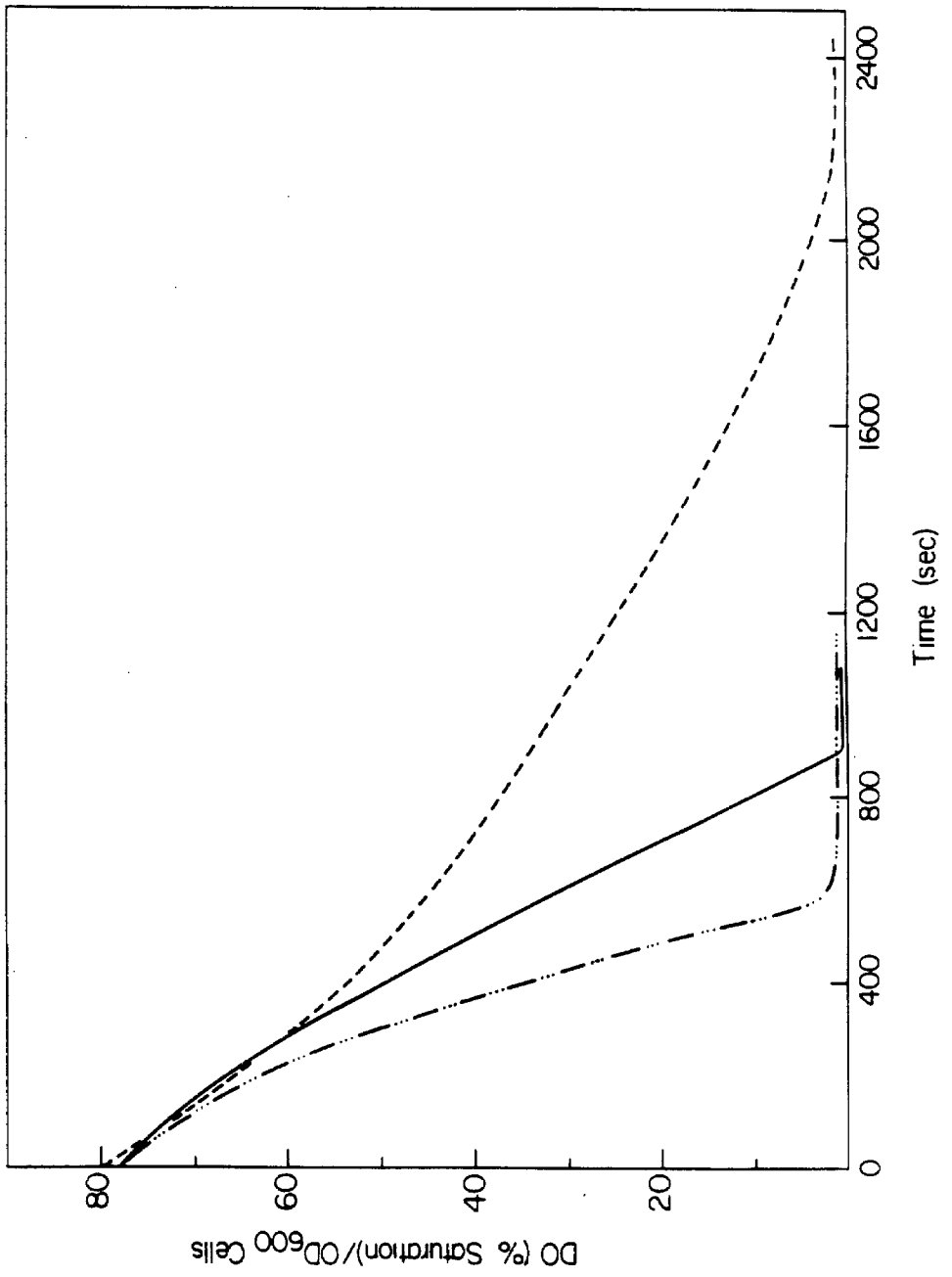
1800
2000
2200
Time (min)

Figure 3.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60
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20
0
400
800
1200
1600
2000
2400
Time (sec)

CHAPTER 4

EVIDENCE FOR PARTIAL EXPORT OF *VITREOSCILLA* HEMOGLOBIN INTO THE PERIPLASMIC SPACE IN *ESCHERICHIA COLI*. IMPLICATIONS FOR PROTEIN FUNCTION

Source: Khosla, C. and Bailey, J.E. (1989) J. Mol. Biol. 210:79-90.

4.1 Summary.

The *Vitreoscilla* hemoglobin protein has been implicated in earlier studies to serve a globin-like function under oxygen-limited growth conditions. Evidence is presented using fractionation as well as proteinase K accessibility techniques to prove that a considerable amount of this protein is localized in the periplasmic space of the cell. Genetic evidence points towards the existence of information within the N-terminal domain of the protein that plays a role in the process of protein export. However, this sequence is not cleaved in the process of translocation. Analysis of the primary structure of this region reveals several unusual features. Instead of positively charged residues at its amino terminus, it has a negative charge. The overall hydrophobicity of the central region of this sequence is significantly lower than in typical leader peptides due to the presence of a charged residue. In keeping with the likelihood that such an export signal may not be very efficient, a substantial fraction of the total cellular hemoglobin can also be detected in the cytoplasm. Heme is incorporated in both cytoplasmic and periplasmic globin as indicated by ability of protein from both fractions to bind carbon monoxide. The secretion of this protein into the periplasm raises questions concerning the physiological significance of its localization. Dimensional analysis of a model based on the facilitated diffusion hypothesis, which was initially proposed to account for the the effects of eucaryotic globins on oxygen transport, suggests that periplasmic globin can support an additional

oxygen flux to the respiratory apparatus that may be physiologically significant.

4.2 Introduction:

The mechanisms involved in translocating proteins into and across membrane barriers have been subjects of intensive investigation. While several differences exist in the processes involved in protein export in different model systems such as bacteria (reviewed in Randall & Hardy, 1984; Duffaud *et al.*, 1985; Ferenci & Silhavy, 1987), mitochondria (reviewed in Schatz & Butow, 1983; Eilers & Schatz, 1988), and the endoplasmic reticulum (reviewed in Wickner & Lodish, 1985), available information suggests common underlying mechanisms (Wickner & Lodish, 1985). In *Escherichia coli* in particular, the development of elegant genetic and biochemical techniques have led to the elucidation of many features of the sequence information required for protein translocation, the cellular factors involved in the pathway(s), as well as the events that occur in the translocation process.

Vitreoscilla is a gram-negative, obligately aerobic bacterium which synthesizes a soluble heme-protein in response to hypoxic conditions (Boerman & Webster, 1982). The amino acid sequence, when properly aligned, shows marked homology to eucaryotic globins (Wakabayashi *et al.*, 1986). It was noted, however, that the first 11 residues of the protein show no homology with the A helix of eucaryotic globins. This helix is unimportant to globin activity (Perutz, 1986).

The gene coding for the VHb protein has been cloned and functionally expressed in *E. coli* (Khosla & Bailey, 1988a; Dikshit & Webster, 1988). Nucleotide sequencing revealed a strong putative promoter upstream of the structural gene (Khosla & Bailey, 1988a), which is responsible for high-level expression of *Vitreoscilla* hemoglobin (Khosla & Bailey, 1988a), as well as other fusion products under oxygen-dependent regulation in *E. coli* (Khosla & Bailey, 1989).

Recently, it has also been shown that heterologous expression of *Vitreoscilla* hemoglobin improves the growth properties of recombinant *E. coli* under low-oxygen conditions (Khosla & Bailey, 1988b). Although *in vivo* respirometric studies suggested that this property may have been due to enhanced rates of oxygen consumption at rate-limiting dissolved oxygen concentrations, the mechanism of such an effect remains unknown. Several plausible hypotheses could be proposed for the role of the protein. For one, the protein could serve a storage function, although the rapid rate of bacterial respiration would exhaust such an oxygen buffer in far less than a single generation time in cells starved for oxygen. Although such a storage function could be useful during temporary oxygen starvation, it cannot explain elevated respiration and specific growth rates over sustained periods of oxygen deprivation as has been observed. A more likely explanation invokes the facilitated diffusion hypothesis, proposed earlier in the context of globin-mediated oxygen transport (for review, see Wittenberg, 1970). Briefly stated, this hypothesis suggests how the

presence of globins can enhance the steady-state rate of oxygen diffusion, even though the diffusivity of the protein is substantially smaller than that of free oxygen. Recent results have also provided evidence for a direct flux of myoglobin-bound oxygen to mitochondria (Wittenberg & Wittenberg, 1987) and suggest the presence of a terminal oxidase that is capable of recognizing and interacting with oxymyoglobin as a terminal electron acceptor in addition to free oxygen. A similar mechanism could be envisaged for the bacterial globin.

The aerobic respiratory apparatus of *E. coli* is localized in the inner (cytoplasmic) membrane of the cell, with the oxygen- binding site oriented towards the cytoplasm (Anraku & Gennis, 1987). Hence, determination of the sub-cellular localization of *Vitreoscilla* hemoglobin is critical to the exploration of the *in vivo* mechanism of its function. In this paper, we present biochemical as well as genetic evidence that a sizeable fraction of the protein is exported into the periplasmic space of *E. coli*. Qualitatively similar results were also obtained by protein localization studies in *Vitreoscilla*. During the course of this study, several interesting features of the translocation mechanism have emerged which are discussed here.

Since secretion of this protein localizes it between the source (the culture medium) and the sink (the terminal cytochromes) for oxygen, it might be intuitively appealing to propose a mechanism for this system that is analogous to its eucaryotic counterparts such as muscle cells or root nodules of leguminous plants. However, the geometric

dimensions as well as the kinetic and thermodynamic properties of these different systems are very different; consequently, an extrapolation of the same model in the absence of detailed measurements need not be correct. In this particular situation, a rudimentary mathematical description that incorporates the essential features of the the hemoglobin function and localization can be useful. Given reasonable estimates of the magnitudes of a few biochemical and biophysical parameters, the mathematical model can be subjected to dimensional analysis in order to assess the feasibility of the above mechanism. For example, Wyman's (1966) theoretical analysis of the facilitated diffusion hypothesis (Wittenberg, 1966) was instrumental in rationalizing its features. Equally important, such an approach enables the identification of potentially useful genetic modifications and biochemical measurements that could test the model and its underlying assumptions.

4.3 Materials and Methods.

Bacterial strains, phages and plasmids: Strains JM101 (*supE, thi, Δ(lac-proAB), [F', traD36, proAB, lacI^{q}ZΔM15]*) (Yanisch-Perron *et al*, 1985) and CC118 (*araD139, Δ(ara, leu)7697, ΔlacX74, phoAΔ20, galE, galK, thi, rpsE, rpoB, argE$_{am}$, recA1*) (Manoil & Beckwith, 1985) were also used. GRO21 and GRO22 are derivatives of *E. coli K12* strain MG1655 (Cold Spring Harbor Laboratory, NY) containing single copies of the *Vitreoscilla* hemoglobin (*VHb*) gene integrated in the *lac* and *xyl* operons,

respectively (Khosla & Bailey, 1989). The construction of λTn*phoA* has been described elsewhere (Manoil & Beckwith, 1985). *Vitreoscilla sp* is the Murray strain No. 389.

Media and Growth Conditions: E. coli cells were grown in 1% bactotryptone, 0.5% yeast extract, 0.5% sodium chloride, 0.3% K_2HPO_4, and 0.1% KH_2PO_4 (pH 7). *Vitreoscilla sp* was grown in 1.5% peptone, 1.5% yeast extract and 0.02% sodium acetate (pH 7.5). Wherever needed, ampicillin was used at a concentration of 50 mg/L and kanamycin at 40 mg/L. Growth of *E. coli* cells in experiments involving induction of the oxygen-regulated *VHb* promoter (discussed above) are described elsewhere (Khosla & Bailey, 1989).

DNA manipulations and sequencing: All *in vitro* DNA manipulations were done according to standard protocols (Maniatis *et al*, 1982). Deletion endpoints and fusion junctions were determined by dideoxy sequencing of the respective plasmids using the Sequenase kit (US Biochemicals) and a modified protocol (Kraft *et al*, 1988). Universal and reverse primers (US Biochemicals) for pUC19-derived plasmids and the *EcoRI* primer for pBR322-derived plasmids (Promega) were used.

Cell fractionation: Quantitative fractionation of cells into periplasmic and cytoplasmic + membrane fractions was done according to the osmotic shock method (Neu & Heppel, 1965). β-lactamase (MW_r 39.6 kd) and isocitrate dehydrogenase ($NADP^+$) (MW_r 46.9 kd) were assayed (described below) as periplasmic and cytoplasmic

control markers, respectively. Alternatively, the release of periplasmic proteins was effected by chloroform treatment of intact cells (Ames *et al*, 1984). SDS-PAGE on protein samples was done by the method of Laemmli (1970).

Proteinase K accessibility experiments: Cultures of GRO22 were grown to late log phase (OD_{600} = 0.65). 5 mL samples were taken at this point with the aim of demonstrating translocation of VHb across the *E. coli* cytoplasmic membrane. The procedure used was identical to that described by Rasmussen *et al* (1984). However, instead of immunoprecipitating the protein prior to analysis, samples were directly solubilized in SDS sample buffer and assayed via Western blotting.

N-terminal sequence analysis: Periplasmic and cytoplasmic fractions of JM101/pINT2 were prepared by the osmotic shock method described above (see Fig. 1). Samples were electrophoresed in a 12.5% SDS polyacrylamide gel and blotted on PVDF membranes (Immobilon P, Millipore) using the protocol referenced below. The PVDF membrane was rinsed in distilled water several times, stained with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 10 min. After a final rinse in distilled water, the membrane was air dried and the VHb bands (periplasmic and cytoplasmic) were cut with a clean scalpel. For each strip the sequence of the first 15 residues of the membrane-bound protein (approx. 300-500 pmol) was determined on an Applied

Biosystems sequenator. Note that this procedure is essentially the same as that originally described by Matsudaira (1987).

Construction of VHb-phoA fusions: The method of constructing phoA fusions on plasmid- encoded genes using λTn*phoA* has been described elsewhere (Manoil & Beckwith, 1985). The plasmid used for this purpose was pOX1, which was constructed by inserting the *HindIII-BamHI* fragment containing the entire hemoglobin gene from pRED4 into the corresponding sites of pBR322 (Khosla & Bailey, 1989). Purity of clones that expressed active phoA (as indicated by blue colonies) was checked by restriction mapping of the resulting plasmid, as well as transforming CC118 to obtain all blue colonies on 5-bromo-4-chloro-3-indolyl phosphate (Sigma) plates.

Assays: Hemoglobin activity was determined by the following method: Cells were centrifuged, resuspended in assay buffer (100 mM Tirs-Cl pH 7.5, 50 mM NaCl), and sonicated. The soluble fraction was assaued for hemoglobin content using the CO difference spectrum method (Webster & Liu, 1974). β-lactamase assays were as per Sawai *et al* (1978). For isocitrate dehydrogenase assays, a Sigma kit was used. phoA activity was quantitated by a kinetic assay which measured the rate of hydrolysis of p-nitrophenyl phosphate (ΔA_{420}/min) at 30°C (performed on a Shimadzu UV260 Spectrophotometer).

Western blotting of VHb protein: Hemoglobin expressed in *E. coli* was purified from cell extracts (R. Hart and J.E. Bailey, manuscript in

preparation). This preparation was used to generate rabbit anti-globin antiserum (Cocalico Biologicals). Western blotting was done according to standard protocols (Winston *et al*, 1987). Using both purified hemoglobin as well as cell extracts from several *E. coli* strains that do not express VHb, the antiserum was found to predominantly cross-react with VHb.

4.4 Results.

Construction of a Ptac- hemoglobin fusion: The nucleotide sequence of the structural gene (Khosla & Bailey, 1988a) is in perfect agreement with the protein sequence determined independently (Wakabayashi *et al*, 1986). However, in order to rule out the possibility of an upstream codon initiating translation, or any other upstream signal affecting the localization of the protein, pRED4 was digested with *HindIII* (140 nt upstream of the start codon; Khosla & Bailey, 1989) and treated with exonuclease Bal31. The resulting mixture was digested with *SphI* (downstream of the native translation and transcription termination signals). The gene- containing fragments were gel- purified and cloned into the *HincII- SphI* sites of pUC19. A deletion fragment that mapped 2 bp upstream of the start codon was identified. A synthetic ribosome binding site (AGAGGA located 6 nt upstream of the start codon; synthesized at the Caltech Microchemical facility) with an upstream *BamHI* site was inserted at an appropriate spacing from the start codon. The resulting *BamHI- SphI* fragment was ligated

with a *HindIII- BamHI Ptac* fragment (Pharmacia Inc) and *HindIII-SphI* digested pBR322 to create a *Ptac-* hemoglobin fusion plasmid, pINT1. The plasmid was characterized by restriction mapping and a resulting clone, JM101/pINT1, was assayed for the ability to produce active hemoglobin in response to IPTG addition. An isogenic strain, JM101/pINT2, was constructed by subcloning the *HindIII- SphI* fragment from pRED4 into corresponding sites in pBR322. This strain expresses hemoglobin under the regulation of its native oxygen- regulated promoter.

Evidence of hemoglobin export to the periplasm: In order to quantify the relative distribution of VHb in the periplasm and the cytoplasm, cells expressing the protein were fractionated by the osmotic shock method. Since both hemoglobin activity as well as SDS-PAGE are relatively insensitive assays for the presence of the protein, in either case the protein had to be expressed to measurably high levels to be detected by either method. However, in case of the construct containing the native fragment (JM101/pINT2), care was taken to harvest the cells sufficiently before the stage when hemoglobin is produced as inclusion bodies, which are detectable under a light microscope. (Extreme oxygen limitation, typically encountered in early stationary phase, results in very high level induction of the native oxygen regulated promoter; discussed in Khosla & Bailey, 1989). This complication of inclusion body formation is not encountered in the case of the *Ptac-* hemoglobin fusion, even under maximal induction. The

absence of inclusion bodies in the samples used for fractionation was verified by sonication, followed by centrifugation at 16,000g for 20 min (Figure 1, lanes 2 and 6). Table 1 shows the relative hemoglobin activities in different fractions for each strain, as well as the relative activity of the control enzymes. The samples were also analyzed by SDS-PAGE (Figure 1). It can be seen that, at these expression levels, approximately one-third to one-half the active protein is exported to the periplasm in either strain. This suggests that localization signals are absent upstream of the presumed start codon.

N-terminal sequence of periplasmic and cytoplasmic VHb: An important result that was obtained from the fractionation experiments described above is that the molecular weights of cytoplasmic and the periplasmic hemoglobin appear to be identical as determined by SDS-PAGE. (The slight difference in VHb band positions in Fig. 1 may be an artifact of loading large amounts of protein which is required to verify the validity of the fractionation technique. The same VHb bands migrate at identical positions when more sensitive staining techniques such as silver staining are used (data not shown). This can also be verified from Western blots such as in Fig. 2.) The observed molecular weights also agree with the predicted value (MW 15,775). This suggests that VHb may lack a signal sequence that is cleaved during export of the protein. To verify this unambiguously, the N-terminal regions of periplasmic and cytoplasmic VHb from JM101/pINT2 were sequenced. The first 15 amino acids of both fractions were found to

be identical and were in agreement with the amino acid sequence determined previously (Wakabayashi *et al*, 1986) and the corresponding nucleotide sequence (Khosla & Bailey, 1988). This sequence is shown in Fig. 5.

Estimating periplasmic and cytoplasmic VHb levels at different gene doses: It could be argued that the inefficiency of VHb export as suggested above is an artifact of overexpression due to a multicopy plasmid. Furthermore, in an analysis of the possible *in vivo* role of globin (see below), an important parameter is the intracellular protein level and the distribution thereof in various cell fractions. An earlier study (Khosla & Bailey, 1988b) as well as subsequent investigations (unpublished results) on the effect of this protein on oxygen- limited aerobic growth and respiration in *E. coli* have been conducted with the gene on a multicopy plasmid. However, the extent to which the concentration and localization of active globin in such experiments are a consequence of gene dosage should be examined, since presumably the gene is present in the *Vitreoscilla* genome in a single copy. For this purpose two *E. coli* strains, GRO21 and GRO22, were also studied. Two independent integrants were used to eliminate the possibility of positional effects. These studies were facilitated due to the availability of anti-VHb antiserum. The results shown in Fig. 2 indicate that a considerable fraction, but not all, of the globin synthesized in these strains is exported to the periplasm. (The absence of any detectable cytoplasmic antigens other than VHb in the periplasmic fractions qualitatively

validates the fractionation technique.) Quantitation of globin activity by the CO- binding method revealed that the periplasmic fractions contain 35-45 % of total cellular globin activity. Furthermore, it was calculated that these single copy integrant strains produce roughly one-tenth the active globin synthesized by strains containing the *VHb* gene on a multicopy plasmid, when grown under identical conditions. Using typical dimensions of an *E. coli* cell (Glass, 1982), based on the molar quantities of globin that were determined from microsequencing of the periplasmic fraction, it was calculated that the periplasmic hemoglobin concentration is approximately 0.2 mM in the single copy case.

Evidence of protein export from proteinase K accessibility experiments: Additional biochemical evidence for the results of the fractionation experiments described above was obtained by the proteinase K accessibility technique. In this experiment GRO22 cells were spheroplasted by the lysozyme-EDTA method and aliquoted into two equal samples, one of which was lysed by sonication. Each sample was further split into two samples, one of which was treated with proteinase K (for 30 min on ice) and the other with an equal volume of distilled water. A whole cell control was also used. As can be seen in Fig. 3, the extent of degradation of VHb in the intact spheroplast sample (lanes 2,3) is comparable to that in the lysed spheroplast sample (lanes 4,5). This confirms that a significant fraction of synthesized VHb crosses

the inner membrane into the periplasm. In both lanes 2 and 4, no degradation product was detected.

Localization studies in Vitreoscilla: Osmotic shock techniques for fractionating *E. coli* could not be easily extended to *Vitreoscilla*. Limited success was achieved with the chloroform treatment method. Even though it was not possible to identify control periplasmic and cytoplasmic enzymes that could be assayed for with acceptable sensitivity, the absence of detectable cytoplasmic antigens in the periplasmic fraction can be considered qualitatively indicative of the purity of the fractions. The results of Fig. 4 demonstrate that approximately the same fraction of total cellular globin is exported into the periplasm in *Vitreoscilla* as is the case in GRO22.

Genetic evidence for protein export: Although some inner membrane proteins (Wolfe *et al*, 1983; Yazyu *et al*, 1984) and at least one outer membrane protein (de Geus *et al*, 1984) also lack a leader peptide that is cleaved during translocation, this is unusual for naturally occuring periplasmic proteins in Gram-negative bacteria. Hence, we undertook a genetic analysis, based on well-developed techniques of gene fusions (reviewed in Michaelis & Beckwith, 1982; Beckwith & Silhavy, 1983) to identify the location of the signal(s) within the protein that direct its export. Hoffman and Wright (1985) have demonstrated the utility of the *phoA* gene of *E. coli* for creating protein fusions in order to study the signals required for protein secretion. The screen for detecting a chimeric polypeptide that is capable of being exported

relies on two central properties of the *phoA* gene product, alkaline phosphatase: the N-terminal region of the enzyme is not essential for activity, and enzymatic activity is absent unless the protein is exported across the membrane barrier. This concept has been exploited by Manoil and Beckwith (1985) with the construction of a transposon-derived probe, Tn*phoA*, which can conveniently be used to generate *in vivo* fusion products with plasmid-encoded genes. Using this tool, a few hundred kanamycin hyperresistant colonies were screened. Two hemoglobin-phoA fusions were isolated as blue colonies that were capable of exporting the hybrid protein into the periplasm. Restriction mapping of these plasmids, pHbP1 and pHbP2, revealed that both fusion junctions mapped within the first 30 amino acids of the hemoglobin polypeptide, suggesting that the N-terminal domain of the protein contained at least one important determinant of the export process. Results from osmotic shock treatment of these cells (Table 2) are in qualitative agreement with this idea, even though the relative inefficiency of secretion of the fusion proteins may suggest a role for other regions of the globin polypeptide. (Periplasmic activity of exported β-lactamase- phoA fusion proteins produced from a pBR322- derived plasmid using Tn*phoA* in CC118 is typically about 150-200 fold higher than that in similar systems containing defective fusions (Manoil & Beckwith, 1985).) The purity of the fractions obtained were also checked by SDS-PAGE (data not shown). The locations of the fusion junctions are shown in Fig. 5. It should be pointed

out that while the above analysis, which relies on results from a limited number of fusions, does not exactly define the domain(s) that contain information for VHb export, it does provide strong evidence that the N-terminal region of the polypeptide is important in this process.

Analysis of the N-terminal signal sequence: Several unusual features of this localization signal were observed. First, the export signal is contained within the first 16 (or less) amino acids. This is somewhat smaller than naturally occurrng leader peptides so far identified in *E. coli* (compiled in Sjostrom *et al.*, 1987), which are 18-30 amino acids long. Secondly, although there is no homology between procaryotic signal sequences at the level of primary structure, all of them possess a characteristic positively charged N terminus, followed by a central stretch of hydrophobic residues (Duffaud *et al.*, 1985). The importance of both these motifs has been demonstrated through mutation/mutagenesis (Inouye *et al*, 1982; Vlasuk *et al*, 1983; Bedouelle *et al*, 1980). The hemoglobin signal sequence has a negative charge at its N-terminal end, although the first 16 amino acids as a whole have a net charge of zero. Hydrophobicity analysis of the sequence using the Kyte and Doolittle (1982) scale shows that the presence of the lysine residue at position 11 substantially reduces the overall hydrophobicity of the core domain.

It could be recalled that the N-terminal region of *Vitreoscilla* hemoglobin appears to have diverged the most from known eucaryotic

globin structures. Using empirical rules for secondary structure prediction (Chou & Fasman, 1974), the first 11 amino acids (i.e. the region before the putative B-helix) appear to have a strong likelihood to fold into a β- sheet. Again, this contrasts with previous observations that implicate the requirement for α- helical structure within a signal sequence (Emr & Silhavy, 1983).

An analytical investigation of globin function: In view of the above experimental results, it could be asked whether the ability of the cell to synthesize and export hemoglobin could increase the effective oxygen supply to the aerobic respiratory apparatus under oxygen-limiting conditions. This question is similar in spirit to the one addressed by the facilitated diffusion hypothesis, initially proposed by Wittenberg (1966), based on his experimental results which convincingly demonstrated for the first time that globins were capable of facilitating oxygen diffusion by reversible association with free oxygen and translational diffusion of the oxyglobin species. The molecular mechanism was rationalized by Wyman's (1966) mathematical formulation and analysis of the problem. Wyman's theoretical predictions agreed reasonably well with Wittenberg's data. In the bacterial case (schematically illustrated in Figure 6), the diffusion-reaction problem of aerobic respiration in the presence of hemoglobin can be viewed as a surface reaction on the inner face of the plasma membrane, bounded on one side by the plasma membrane, periplasmic space, outer membrane, and growth medium, and in the other direction by a semi-infinite

cytoplasmic domain. The bulk concentration of oxygen (O_b) in the growth medium can be assumed to be time-invariant. Under steady-state conditions, the equations and boundary conditions describing the model are listed in Table 3, where O denotes free oxygen, Hb denotes (deoxy)hemoglobin and HbO denotes oxyhemoglobin. k_1 and k_{-1} are the kinetic constants for the forward and reverse reaction in

$$Hb + O \rightleftharpoons HbO$$

Making the simplifying assumption that free oxygen is the only species that can diffuse across the inner membrane (i.e., there is no net translocation of Hb and HbO across the membrane at steady state), equation (4) in Table 4 implies that the flux of O at steady state at any point within the membrane is constant. Under this condition an order of magnitude analysis can be accomplished without specifying any transport-related biophysical parameters of the plasma membrane. This information would be necessary to solve (numerically) for the exact concentration profiles in the periplasm and cytoplasm; however, the objective here is assessment of the possibility that facilitated oxygen transport in the periplasm might increase the respiration rate. The order of magnitude analysis suffices for this purpose.

From equations 1 and 3 in Table 3 it follows that

$$D_{op}\frac{dO_p}{dx} + D_{Hbp}\frac{dHbO_p}{dx} = constant = -F_p$$

Similarly, equations 5 and 7 give

$$D_{oc}\frac{dO_c}{dx} + D_{Hbc}\frac{dHbO_c}{dx} = constant = -F_c$$

Here, the symbol D represents diffusivity. Subscripts p and c denote periplasm and cytoplasm, respectively. F_p and F_c represent total diffusive fluxes of free and bound oxygen. From boundary condition 6, $F_c \equiv 0$.

The assumption of instantaneous equilibrium of the reaction

$$Hb + O \rightleftharpoons HbO$$

at all points ($K_D = k_{-1}/k_1$) simplifies the problem greatly. Limitations of this assumption are discussed elsewhere (Wittenberg, 1970), although for an order of magnitude analysis such as this, they are not likely to be serious. The order of magnitude analysis begins by choosing appropriate scaling parameters:

$$\Theta_p = O_p/O_b$$

$$\Theta_c = O_c/O_b$$

$$X = x/x_p$$

$$Y_p = HbO_p/H_p = \frac{\Theta_p}{\frac{K_D}{O_b} + \Theta_p}$$

$$Y_c = HbO_c/H_c = \frac{\Theta_c}{\frac{K_D}{O_b} + \Theta_c}$$

where H_p and H_c denote the total hemoglobin concentrations (in both forms) in the periplasm and cytoplasm, respectively. Here, Y represents the fractional saturation of hemoglobin. The above differential equations finally reduce to

$$\frac{d\Theta_c}{dX} = 0$$

and

$$[1+(\frac{D_{Hbp}}{D_{op}}\frac{H_pK_D}{O_b^2})(\frac{1}{\frac{K_D}{O_b}+\Theta_p})^2]\frac{d\Theta_p}{dX}=\frac{-R}{\frac{D_{op}O_b}{x_p}}$$

where $\Theta_p = 1$ at $X = 0$. The motivation for a non-dimensional formulation is to identify combinations of parameters which determine the nature of the solution.

Two features become immediately obvious from the preceding two equations. First, there are no gradients of free or bound oxygen in the cytoplasm. Although this might be intuitively obvious given the above formulation of the problem, it must be emphasized that this result is a direct consequence of the assumption of instantaneous equilibrium everywhere, and need not be true if this is not the case.

Second and more importantly, we now have a relationship which directly equates the (non-dimensionalized) respiration rate with the (non-dimensionalized) total diffusive flux of free and bound oxygen through the periplasm. This allows us to address the question we had initially posed, namely, can the presence of hemoglobin in the periplasm significantly increase the observed respiratory rate of the bacterium which depends on transport of oxygen to the electron terminus? Under oxygen limitation, the rate of respiration may be approximated by a first order expression ($R = k(O_c)_{x=x_c}$). Using this, the last equation can be rewritten as follows

$$\frac{d\Theta_p}{dX}=(\frac{kx_p}{D_{eff}})(\Theta_c)_{X=\frac{x_c}{x_p}}$$

where $D_{eff} = D_{op}[1 + (\frac{D_{Hbp}}{D_{op}} \frac{H_p K_D}{O_b^2})(\frac{1}{\frac{K_D}{O_b} + \Theta_p})^2]$

Here, D_{eff} represents the effective diffusivity of oxygen in the periplasm, and it becomes immediately apparent that it increases in the presence of hemoglobin. Using experimental data obtained here as well as elsewhere, we can estimate the magnitude of this increase. The K_M value for oxygen consumption by cytochrome d in *E. coli* (i.e the higher affinity terminal electron acceptor) is 0.38 μM (Kita *et al*, 1984). This is in very good agreement with half- saturation constant for specific growth rates under oxygen- limited conditions (Chen *et al*, 1985), suggesting that respiration is indeed the growth rate-limiting reaction. Equilibrium binding studies indicate that the p_{50} for this globin is 0.18 mm Hg (Tyree & Webster, 1978; Orii & Webster, 1986), which corresponds to a K_D of approximately 0.25 μM. The diffusivity of globins in water is about 20- fold smaller than that of free oxygen (Wittenberg, 1970). Although the absolute magnitudes of the diffusivities in the periplasm are likely to be significantly lower, since diffusivity scales inversely with viscosity (Cantor & Schimmel), their ratio is unaffected. Since our interest is in oxygen limited growth, it follows that, under such conditions (say, $O_b = 0.5$ μM and a periplasmic hemoglobin concentration of 0.2 mM; see section d), the dimensionless term which represents the contribution of facilitated diffusion is significant ($\simeq 10$) compared to the free oxygen diffusion term ($= 1$). The actual extent of enhancement of respiratory activity in the presence of facilitated diffusion through the periplasm will also be

dependent on the permeability of the inner membrane to oxygen, as well as the maximal rate of respiration. This dependency is quantified by the (dimensionless) terms, $\frac{k}{P}$ and $\frac{kx_p}{D_{op}}$, where P is the membrane permeability. The marginal utility of hemoglobin in the periplasm will decrease as $\frac{kx_p}{D_{op}}$ becomes substantially smaller than $\frac{k}{P}$ or unity, whichever is greater. Physically, this stipulation can be interpreted as a requirement that the oxygen diffusion rate in the periplasm should not greatly exceed the transport rate through the membrane or the rate of respiration. The former is a reasonable assumption since the permeability of small molecules through membranes correlates with their solubility in a non-polar solvent (Stryer, 1981), and oxygen is more soluble in non-polar solvents than in water. Predicting an accurate value for $\frac{kx_p}{D_{op}}$, however, is not easy, since D_{op} is unknown in the periplasm, where the high macromolecular concentration is likely to affect viscosity, and consequently diffusivity (Richards, 1980). Using available data on respiratory rates in *E. coli* (Andersen & von Meyenberg, 1980), we estimate the magnitude of this dimensionless term to be of order 0.1. Using this value the presence of periplasmic globin will increase oxygen- limited respiratory rates in *E. coli* by about 10% according to this model. This analysis also implies that globin presence in the periplasm will have an even greater effect in gram-negative bacteria with higher intrinsic respiratory rates as are typically observed for many obligate aerobes. Perhaps *Vitreoscilla* is

one such bacterium. (A more detailed explanation of many steps in the above analysis can be found elsewhere (Carberry, 1976)).

4.5 Discussion.

Eucaryotic globins are well known as oxygen transporting molecules. Their extraordinary ability to do so is the result of two central properties. First, these heme proteins are localized so as to connect regions of oxygen availability with those where oxygen is utilized. In multicellular organisms, this property is due to an elaborate regulatory program which directs tissue-specific expression of these proteins during development. The equivalent property in a procaryote would involve environment- dependent synthesis and appropriate subcellular localization. Secondly, each globin has optimized its binding affinity (K_D) for oxygen so as to be most effective in its local environment. It could be argued that, despite their significant similarities at the secondary and tertiary structure level, at least some of the differences in primary structure of globins are the result of these evolutionary pressures.

The information within the *Vitreoscilla* hemoglobin gene appears to be in accordance with these ideas. Expression of the gene is induced only under conditions where oxygen availability is limiting to growth. The gene is expressed from a strong promoter, resulting in the synthesis of sufficiently high levels of the protein to engender physiological effects. The protein contains within it the necessary signal(s)

to be exported to the periplasm. These signals are localized in the N-terminal domain of the protein, which is not a crucial domain from the viewpoint of heme-binding, and hence could perhaps afford a greater degree of divergence from typical globin structures than the rest of the protein. It should also be noted that the dissociation constant (K_D) of this globin is reasonably close to the K_M value for oxygen consumption in *E. coli*. Qualitatively, this proximity is a measure of how well-tailored the globin is to the needs of cell respiration. If K_D is too large, then the relative contribution of the globin-mediated oxygen flux becomes insignificant. (If $K_D >> K_M$, then the magnitude of the diffusive flux is inversely proportional to K_D; see Results, section i). On the other hand, if K_D is too small, then, even at limiting oxygen concentrations, the protein remains largely saturated, and is less useful. (If $K_D << K_M$, then the magnitude of the diffusive flux is directly proportional to K_D; see Results; section i).

The absence of a cleaved leader peptide, and utilization instead of a signal sequence that is significantly different from typical procaryotic export signals may be due to several reasons. It could reflect some differences in properties of signal sequences between *E. coli* and *Vitreoscilla*, although the results of Figure 4 argue against this interpretation. Then again, it might have evolved in response to the specific requirements and constraints of the hemoglobin molecule. For one, the protein appears in both the soluble fractions of the cell. An inherently weak export signal might account for this. Also, the apoprotein

portions of other heme proteins such as cytochromes in *E. coli* as well as mitochondria are translocated prior to heme incorporation (Haddock, 1973; Zimmerman *et al*, 1979). Hence, if the generally accepted notion that protein translocation requires maintaining the nascent polypeptide transiently in an unfolded state holds in this case, then it would appear that folding of hemoglobin in the cytoplasm is kinetically competitive with translocation. Perhaps this unusual property of the signal sequence evolved specifically in order to enable it to be physiologically useful in the periplasm as well as the cytoplasm (see below). Then again, it could reflect an evolutionary constraint between correct folding and correct localization. In any event it is interesting to note a resemblance between the structure of this signal sequence and that of a mutant leader peptide of the maltose binding protein (Bedouelle *et al*, 1980). Mutant 16-1 has a Thr→Lys change in the middle of the hydrophobic core, which considerably reduces its hydrophobicity. The mutant protein was exported at approximately half the efficiency of the wild type.

An important issue that remains to be understood is the role of the cytoplasmic fraction of the protein. Although the analysis presented does not suggest any role for this fraction, this may be due to its oversimplification. For example, if the instantaneous equilibrium condition is not invoked, depending on the exact forms of the kinetics of binding and releasing oxygen, cytoplasmic gradients of free and bound oxygen might exist, and these could play some physiological

role. More importantly, if oxyhemoglobin can serve as an equivalent electron acceptor at some terminal oxidase, then the kinetics of respiration would be qualitatively altered (i.e. the reaction rate would be a function of the cytoplasmic hemoglobin concentration). As mentioned earlier, some evidence of such a possibility has been demonstrated in the case of myoglobin in cardiac myocytes (Wittenberg & Wittenberg, 1987). Since the osmotic shock method used in this work requires sonicating the spheroplasts, the possibility that part of the globin detected in the cytoplasmic fraction may be membrane-associated *in vivo* cannot be excluded. In fact, human hemoglobin has been shown to possess ability to associate reversibly with the erythrocyte membrane with a K_D of potential physiological significance (Fung, 1981). Since the aerobic respiratory chain of Gram-negative bacteria is bound to the inner membrane, a similar property in VHb might be consistent with the above function. Finally, the possibility that oxyhemoglobin interacts with an oxidoreductase, with effects on cytoplasmic chemistry, cannot be excluded.

The above experimental and theoretical results provide a basis to make important verificable predictions regarding the *in vivo* function of the protein and the role of its signal sequence. Since growth rate correlates with respirometric activity under oxygen-limiting conditions, comparative studies of the wild-type protein versus export mutants under conditons that vary total globin levels will be useful in evaluating the proposed model or alternative hypotheses.

4.6 Acknowledgements.

The authors wish to express their gratitude to Dr. George Weinstock for valuable advice and providing some of the strains, phages and plasmids used in this work. We also thank Dr. Scott Emr, Michael Kosinski, Elliott Altman, and John Puziss for fruitful discussions on different aspects of protein secretion, and Douglas Axe for useful comments on the mathematical formulation of the problem. Anti VHb antisera was generously provided by Dr. Dallas Hughes of Exogene Inc. The assistance of Dr. David Teplow and the staff of the Caltech Protein Sequencing Facility is also gratefully acknowledged. This work was supported by the Energy Conversion and Utilization Technologies (ECUT) program of the United States Department of Energy.

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TABLE 1: Determination of hemoglobin content in different cell fractions: Plasmids pINT1 and pINT2 carry the globin gene under the control of the *tac* promoter and its native oxygen- regulated promoter, respectively. Isocitrate dehydrogenase was assayed as a cytoplasmic control enzyme, whereas beta-lactamase was assayed as a periplasmic control enzyme. The *tac* promoter was induced from the time of inoculation with 0.5 mM IPTG. The oxygen- regulated promoter induces in response to microaerobic conditions, which are typically reached around an OD_{600} of 0.4-0.5 under the conditions grown. Cells were harvested at an OD_{600} of 0.75-0.8 and fractionated by osmotic shocking. Methods for activity assays are described in the Materials and Methods section.

	JM101/pINT1		JM101/pINT2	
	periplasm	cytoplasm +membrane	periplasm	cytoplasm +membrane
VHb concentration (A(419-436)/ mL cell sample)	1.5	3.3	2.6	2.7
beta- lactamase concentration (units/ mL cell sample)	674	84	670	75
isocitrate dehydrogenase (units/ mL cell sample X 1000)	<0.001	2.4	<0.001	2.3

TABLE 2: Periplasmic export of VHb-phoA fusion proteins: CC118 contains a deletion in *phoA*. Plasmid pOX1 carries the *VHb* gene under the control of its oxygen- regulated promoter, whereas pHbP1 and pHbP2 are derivatives of this plasmid carrying *VHb-phoA* in-frame fusions generated as described in the text. Other experimental conditions were similar to those described in the legend to Table 1. Activity measurements reported here were done as described in the Materials and Methods section. P represents the periplasmic fraction and C+M represents the cytoplasmic + membrane fraction.

	CC118/pOX1		CC118/pHbP1		CC118/pHbP2	
	P	C+M	P	C+M	P	C+M
alkaline phosphatase concentration (A_{420}/min. mL sample)	0.02	<0.001	1.53	8.60	0.52	5.02
beta-lactamase concentration (units/ mL sample)	235	17	298	9	235	17
isocitrate dehydrogenase concentration (units/ mL sample)	<0.001	0.50	<0.001	0.62	<0.001	0.22

TABLE 3: Steady state model for hemoglobin- facilitated oxygen diffusion and oxygen-limited bacterial respiration (subscripts p, m, and c denote periplasm, inner membrane, and cytoplasm, respectively)

$$D_{op}\frac{d^2O_p}{dx^2} = k_1(Hb_p)(O_p) - k_{-1}(HbO_p) \quad (1)$$

$$D_{Hbp}\frac{d^2Hb_p}{dx^2} = k_1(Hb_p)(O_p) - k_{-1}(HbO_p) \quad (2)$$

$$D_{Hbp}\frac{d^2HbO_p}{dx^2} = -k_1(Hb_p)(O_p) + k_{-1}(HbO_p) \quad (3)$$

$$\frac{d^2O_M}{dx^2} = 0 \quad (4)$$

$$D_{oc}\frac{d^2O_c}{dx^2} = k_1(Hb_c)(O_c) - k_{-1}(HbO_c) \quad (5)$$

$$D_{Hbc}\frac{d^2Hb_c}{dx^2} = k_1(Hb_c)(O_c) - k_{-1}(HbO_c) \quad (6)$$

$$D_{Hbc}\frac{d^2HbO_c}{dx^2} = -k_1(Hb_c)(O_c) + k_{-1}(HbO_c) \quad (7)$$

Boundary Conditions:

1. at $x = 0$, $O_p = O_b$
2. at $x = 0$, $\frac{dHb_p}{dx} = \frac{dHbO_p}{dx} = 0$
3. at $x = x_p$, $\frac{dHb_p}{dx} = \frac{dHbO_p}{dx} = 0$
4. at $x = x_c$, $\frac{dHb_c}{dx} = \frac{dHbO_c}{dx} = 0$
5. $(-D_{op}\frac{dO_p}{dx})_{x=x_p} = (-D_{oc}\frac{dO_c}{dx})_{x=x_c} + R(O_{x=x_c})$, where $R(O_{x=x_c})$ denotes the rate of reaction at the membrane-bound terminal oxidase.
6. O_c, Hb_c, HbO_c are bounded and position invariant far from the cytoplasmic membrane ($x \to \infty$).

4.9 Captions to Figures.

Figure 1: Cell fractionation of hemoglobin- expressing E. coli: **Lane 1 contains molecular weight standards. Osmotic shock fractionation results for strains JM101/pINT1 (lanes 6-9) and JM101/pINT2 (lanes 2-5) are presented. Lanes 2 and 6 contain samples obtained by centrifuging cell sonicates for 30 min at 16,000 g. Absence of any detectable VHb in these lanes is indicative of the absence of inclusion bodies in the cells. The cytoplasmic + membrane fractions (lanes 3 and 7), periplasmic fractions (lanes 4 and 8), and total cellular protein samples (lanes 5 and 9) contain protein from equal numbers of cells. Lane 10 contains protein from JM101 as a control. Samples were run on an 12.5% SDS-polyacrylamide gel and stained with Coomassie blue.**

Figure 2: Western blotting of periplasmic protein from GRO21 and GRO22: **Periplasmic proteins of harvested cells were released by chloroform treatment. Lanes 1 and 2 are samples from GRO21, and lanes 3 and 4 are samples from GRO22. Lanes 1 and 3 show the total cellular protein, whereas lanes 2 and 4 contain periplasmic proteins only. The indicated band was identified as VHb using appropriate controls (data not shown).**

Figure 3: Proteinase K accessibility experiments in intact and lysed spheroplasts of GRO22 cells: **5 mL culture was harvested and treated according to the protocol referenced in the text. Each lane contains protein sample from an equal number of cells and reveals the amount of intact VHb present after the designated treatment; (1) whole cells,**

no proteinase K treatment; (2) intact spheroplasts treated with proteinase K; (3) intact spheroplasts, no proteinase K treatment; (4) lysed spheroplasts treated with proteinase K; (5) lysed spheroplasts, no proteinase K treatment. Only the relevant portion of the Western blot is shown.

Figure 4: Fractionation of Vitreoscilla cells: Both GRO22 (lanes 1 and 2) and *Vitreoscilla* (lanes 3 and 4) were treated treated with chloroform to liberate periplasmic protein. In both cases, the same volume of a late log culture (OD_{600} = 0.7) was treated according to the same protocol. Lanes 1 and 3 contain periplasmic protein only, whereas lanes 2 and 4 contain total cellular protein. Samples were run on a 12.5% gel prior to Western blotting.

Figure 5: Primary structure of the N- terminal region of the Vitreoscilla hemoglobin protein: The nucleotide sequence as well as the amino acid translation of the N-terminal region of the protein is shown. The arrows indicate the junction points between *VHb* and the truncated *phoA* gene on plasmids pHbP1 and pHbP2 (see text).

Figure 6: Schematic representation of the E. coli cell envelope in the presence of hemoglobin: The cell wall and the outer membrane, being relatively more permeable, are not represented as diffusion barriers in the figure. The solid dot represents the terminal electron acceptor of the membrane-bound respiratory apparatus, which catalyzes the reduction of oxygen to water. The remaining symbols are explained in the text (see Results, section i).

Figure 1.

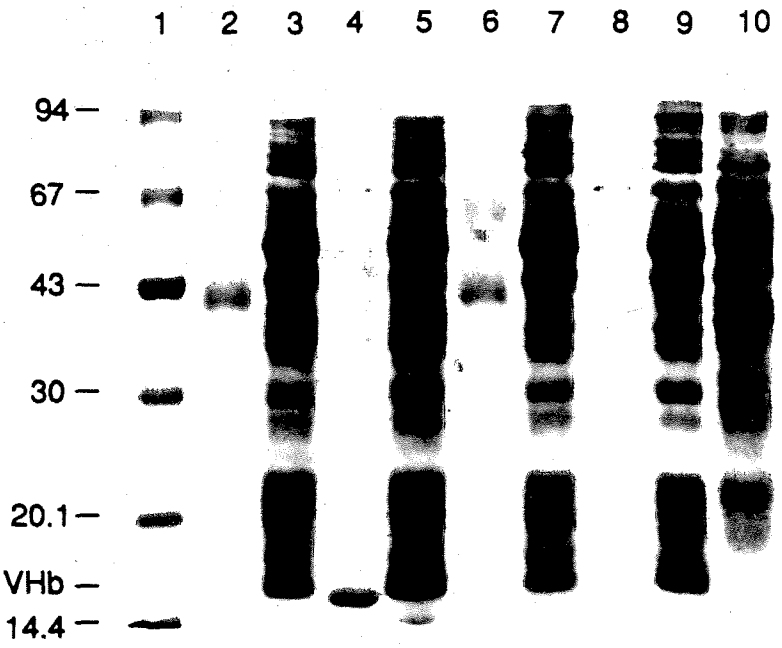


Figure 2.

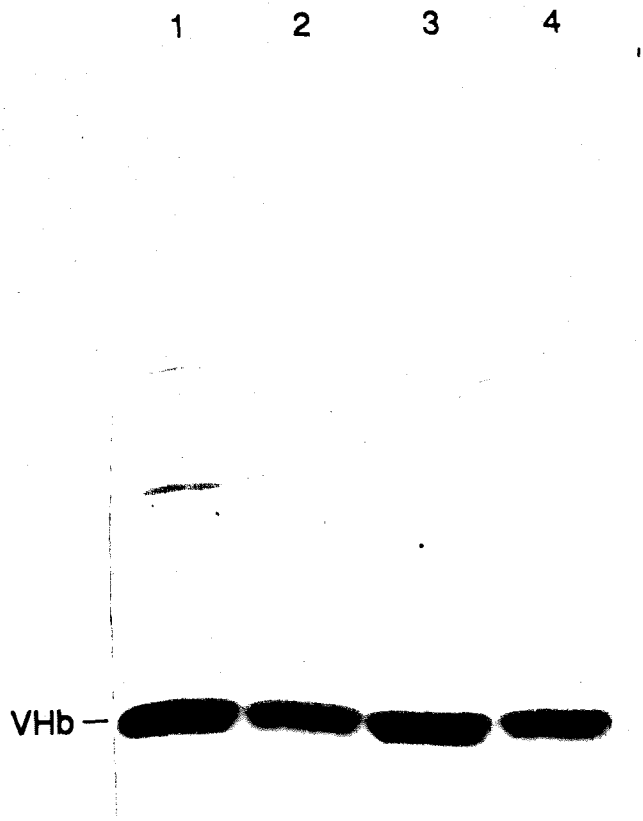


Figure 3.

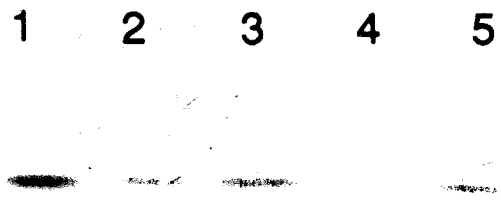


Figure 4.

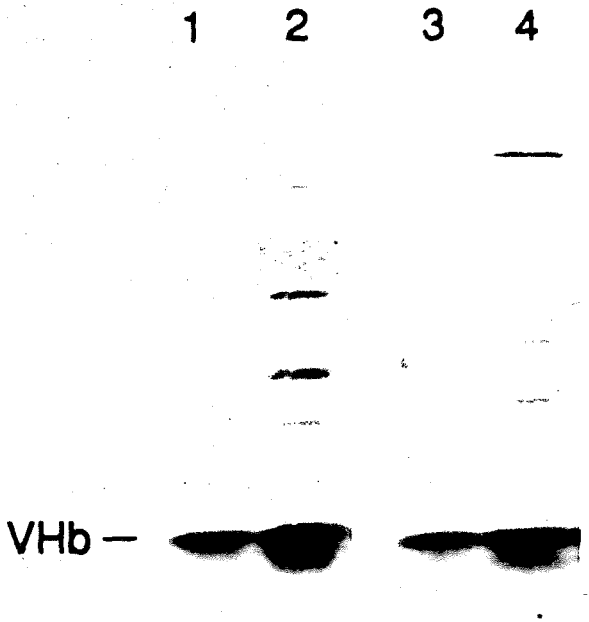


Figure 5.

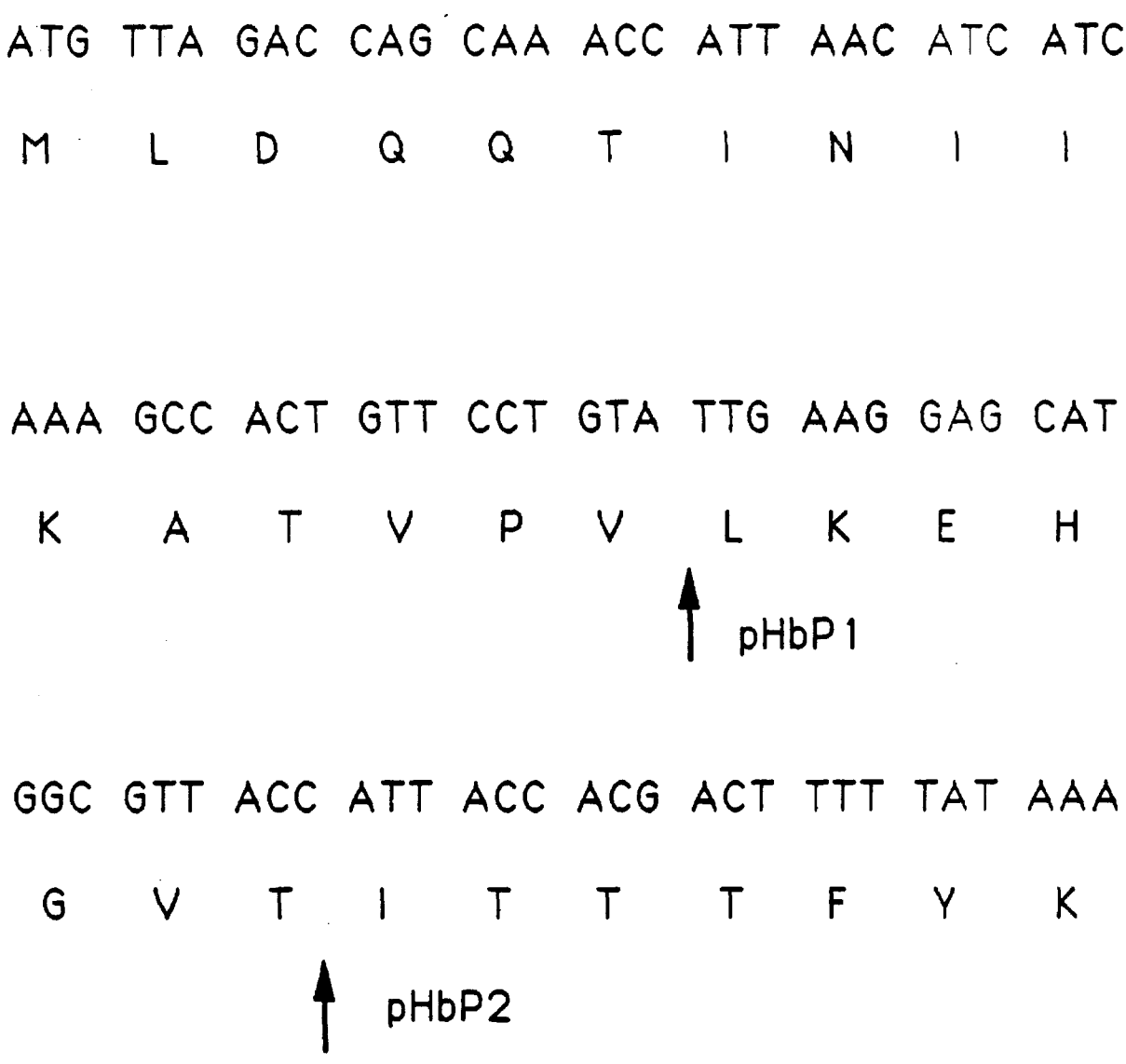
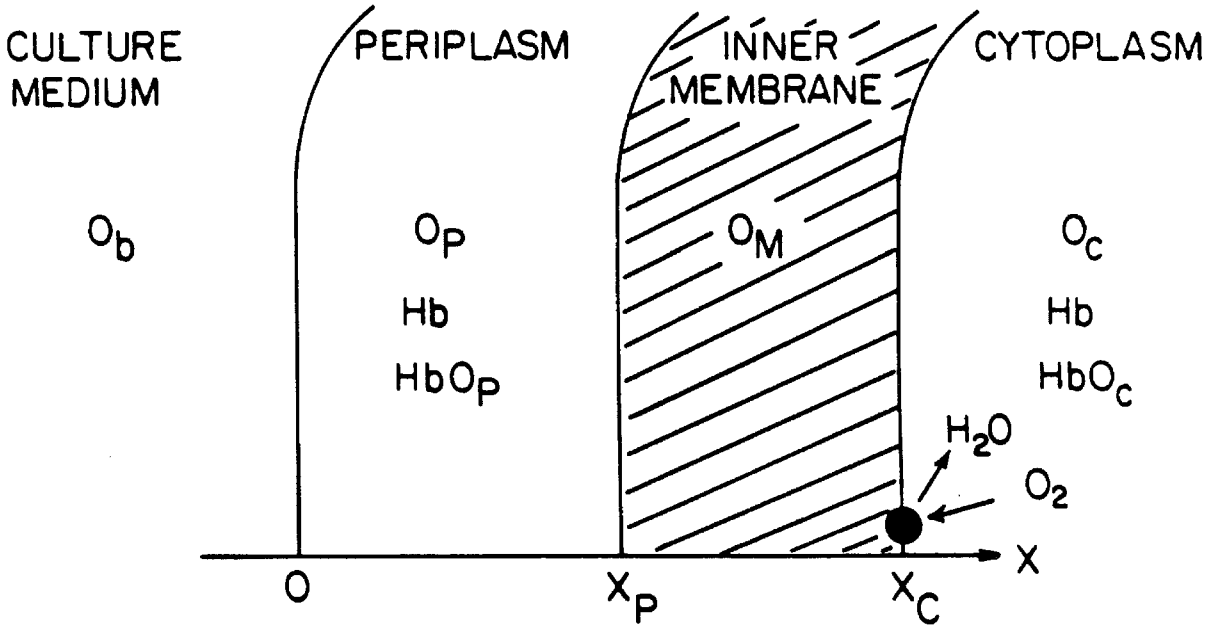

ATG TTA GAC CAG CAA ACC ATT AAC ATC ATC
M L D Q Q T I N I I
AAA GCC ACT GTT CCT GTA TTG AAG GAG CAT
K A T V P V L K E H
pHbP1
GGC GTT ACC ATT ACC ACG ACT TTT TAT AAA
G V T I T T T F Y K
pHbP2

Figure 6.


CULTURE MEDIUM
PERIPLASM
INNER MEMBRANE
CYTOPLASM
O_b
O_P
Hb
HbO_P
O_M
O_c
Hb
HbO_c
H_2O
O_2
X
0
X_P
X_C

CHAPTER 5

PHYSIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF THE FUNCTIONAL ROLE OF A BACTERIAL HEMOGLOBIN

Source: Khosla, C., Curtis, J.E., DeModena, J., Rinas, U., and Bailey, J.E. (1989) Bio/Technology. ***submitted.***

5.1 Summary.

The *Vitreoscilla* hemoglobin gene (*VHb*) has been cloned and the protein has been expressed in *E. coli* in its active form. Using *E. coli* strains containing *VHb* integrated into the chromosome in single copy, we have investigated the role of VHb on aerobic metabolism. Based upon protein synthesis as an important indicator of the energetic state of cells, the presence of VHb improves energy generation properties under oxygen- limiting conditions. Such effects are not observed under conditions of excess oxygen. Measurements of nitrogen utilization rates corroborate the observation of enhanced protein synthesis; however, the rates of carbon consumption and acid synthesis remain unchanged in strains expressing VHb. This suggests that the net effect of VHb in *E. coli* is to improve the efficiency, rather than the kinetics, of oxygen-limited aerobic metabolism. Given available information on the mechanisms of electron transfer to oxygen in *E. coli*, two broad classes of hypotheses could account for the mechanism of action of VHb. The facilitated diffusion hypothesis implies that VHb increases the net flux of oxygen to one or both of the terminal oxidases in the branched electron transfer chain. The intracellular redox effector hypothesis suggests that oxyVHb influences the activity of a key redox-sensitive molecule, which is capable of affecting the overall proton translocation efficiency of the cell in response to oxygen limitation. Using two dimensional gel electrophoresis it is shown that the intracellular concentrations of almost all proteins remains the same

in the presence of VHb under oxygen-limiting conditions. However, at least one protein is present in greater amounts in response to VHb expression. The presence of the *VHb* gene on a multicopy plasmid is also shown to enhance the protein synthesis rates in both *cyd* and *cyo* mutants.

5.2 Introduction.

Maintaining an adequate supply of oxygen to aerobically growing cell cultures is a central problem in a wide variety of bioprocesses. The problem becomes particularly acute at larger scales of operation, or under conditions employing high cell densities such as fed-batch fermentations and immobilized cell systems. Traditional approaches to this problem have focussed on improving the oxygen *transfer* properties of the *environment* in which cells are grown. Examples of this include optimization of aeration/ agitation configurations and improving the solubility of oxygen in the liquid phase by addition of chemicals such as perfluorocarbons. An alternative approach is genetic manipulation to improve the oxygen *utilization* properties of the *cells* themselves. Two possible strategies could be used to guide such an approach. On one hand the availability of adequate information on the genetics, enzymology, and *in vivo* kinetics of aerobic respiration, and how it is coupled to cell metabolism, could guide attempts at genetic manipulation by revealing the rate-limiting steps and mechanisms of their control. In a small number of model systems, such as the enteric bacterium, *Escherichia coli*, considerable knowledge along these lines has

accumulated over the past two decades (for review, see (1-3)); however, it may be optimistic to expect these results to provide a basis for anything beyond trial and error attempts at manipulating aerobic metabolism. As an alternative strategy it may be worth exploring appropriately chosen niches in our natural environment for potential solutions that have evolved in nature to this as well as other similar problems.

Recently we illustrated the potential applicability of one such solution to the problem of oxygen-limited growth (4). The bacterium *Vitreoscilla* sp. is a gram negative obligate aerobe. Species of this genus live in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. The above species synthesizes a soluble, hemoglobin-like molecule (VHb) in response to hypoxic environents (5). Although the mechanism of action of VHb is not known, its marked structural homology to eucaryotic globins has led to the hypothesis that VHb enables the bacterium to survive in oxygen-limited environments (5). Studies on the cytochrome composition of the respiratory chain in *Vitreoscilla* have revealed the presence of two types of terminal oxidases, cytochrome *o* and cytochrome *d* (6). Such a branched arrangement of two Class II terminal oxidases is very similar to the respiratory chain of *E. coli* (1-3), and raises the possibility of studying the influence of VHb on aerobic respiration using *E. coli* as a surrogate host. Equally important, it was argued that this example tests the feasibility of exploiting naturally evolved strategies

to ameliorate potential bottlenecks in an important but imperfectly understood metabolic function.

The *VHb* gene has been cloned from a *Vitreoscilla* genomic library (7,8) and its nucleotide sequence has been determined (8). The protein can be expressed in in *E. coli* in a form that is identical to VHb from *Vitreoscilla*, as judged by visible spectroscopy (7; C.K. and J.E.B., unpublished observations) and N-terminal sequencing (9). The upstream sequence adjacent to the *VHb* gene includes an oxygen-regulated promoter element (ORE) that is induced in *E. coli* under microaerobic conditions by a mechanism that does not involve autoregulation by the *VHb* gene product (10). This suggests that VHb may function in *Vitreoscilla* under conditions which have a close parallel in *E. coli*. Early studies demonstrated that the presence of the *VHb* gene under the control of its native promoter on a multicopy plasmid in *E. coli* enhances the growth characteristics of the host in oxygen limited fed-batch fermentations (4).

Two main hypotheses have been proposed to account for the functional role of a molecule such as VHb in a bacterium. On one hand, it has been suggested that VHb plays the role of an oxygen storage trap under conditions of oxygen starvation (5) (hereafter referred to as the *oxygen buffer hypothesis*). However, given typical intracellular VHb concentrations in *Vitreoscilla* and the rapid kinetics of bacterial respiration, the oxygen buffering capacity of oxyVHb would likely be limited to no more than a few seconds. While this argues against a

role for VHb under prolonged oxygen-limited conditions, the oxygen buffer hypothesis cannot be ruled out. For example, it may be that in its natural habitat the characteristic residence time for this bacterium in pockets of oxygen starvation is comparable to the time scale for which intracellular *VHb* can act as an oxygen buffer. The second hypothesis (5), called the *facilitated diffusion hypothesis*, was originally proposed by Wittenberg (11) to show that, at physiologically relevant concentrations of globin and oxygen, the diffusion of oxygenated globins can contribute significantly to the net oxygen flux that occurs in response to gradients in oxygen concentration. The hypothesis has been used with some success to explain the mechanism of action of myoglobin in muscle cells (11) and leghemoglobin in rhizobial-plant symbiotic systems (12); however not all properties of such globin-containing systems can be explained by this hypothesis (for example, see (13)). Since the respiratory chain of gram negative bacteria is localized in the inner membrane of the cell, important evidence for the validity of this hypothesis in the case of VHb could be obtained by determining the sub-cellular localization of the protein. Recently it has been shown using biochemical and genetic means that a significant fraction of the intracellular VHb content is indeed localized in the periplasmic space of *E. coli* and in *Vitreoscilla* (9). Furthermore, given available biochemical data, it was suggested on theoretical grounds that the facilitated diffusion hypothesis is a plausible mechanism of action of VHb.

We report here our results concerning the effects of VHb on *E. coli* meta-bolism. Specifically, using the assumption that net protein synthesis in growing cells is an important indicator of the energetic state of the cells, we have studied the influence of VHb on the generation of energy via aerobic catabolism of glucose under oxygen-limiting and oxygen-excess conditions. We also present an analysis of the overall protein composition under oxygen-excess and oxygen-limiting conditions, obtained via two-dimensional gel electrophoresis. The role of VHb in cytochrome d (*cyd*) and cytochrome o (*cyo*) mutants has been investigated. Based on an analysis of our results, we evaluate possible functions of VHb.

5.3 Results.

Although it has not been explicitly demonstrated, it is reasonable to assume that the *VHb* gene is present in a single or low copy number in the *Vitreoscilla* genome. Two lines of evidence support this assumption. First, in low stringency Southern blots the relative intensity of the band corresponding to the *VHb* gene is approximately similar to that of certain non-specific bands (C.K. and J.E.B., unpublished observations). Second, under microaerobic (i.e. induced) conditions the intracellular concentration of VHb in *Vitreoscilla* is approximately similar to that when the *VHb* gene is present in a single copy in the *E. coli* genome (9). For this reason we have focussed our *in vivo* studies on *E. coli* strains in which the *VHb* gene has been introduced in a single copy.

In the fed-batch fermentations reported here, both OD_{590} and total protein concentrations were monitored over the course of each fermentation. However, for several reasons the total protein concentration has been used as the most sensitive and reliable indicator of growth. First, anomalous correlations have been obtained under certain conditions in shake-flask cultivations between absorbance values (at several wavelengths) and biomass concentration (dry cell weight) when VHb is expressed in *E. coli* cells (C.K. and J.E.B., unpublished observations). Second, it has been observed that, amongst the major macromolecular constituents (DNA, RNA, protein) of an *E. coli* cell, the ratio of protein concentration to biomass remains relatively invariant over the range of growth rates considered here (14). Third, based on stoichiometric calculations it can be shown that more than half the ATP consumed by *E. coli* for growth-related functions is directed towards protein synthesis (15). Thus it can be argued that the net rate of protein synthesis in a growing culture is indicative of the energetic state of the cells. Since VHb has been hypothesized to enhance aerobic metabolism under oxygen-limiting conditions, protein synthesis rates can be used as a qualitative indicator of the role of VHb under hypoxia. Finally, protein synthesis may be considered as a model for a variety of energy- requiring biocatalytic functions which may be enhanced due to the presence of VHb. In this work net protein synthesis was monitored in two independent ways. On one hand, the total protein concentration in culture samples was monitored by

the Bradford assay. On the other hand, pBR322-based plasmids containing a reporter chloramphenicol acetyltransferase (*cat*) gene under the control of the *tac* promoter, or a *lacZ* gene under the control of the *recA* promoter, were transformed into different strains. As a result CAT or β-galactosidase is expressed constitutively in these cells at moderate levels (data not shown, but, for example, see data in Figure 1(f)). Due to the sensitivity and accuracy of the activity assays for these enzymes, this is taken as an independent measure of the relative protein synthetic capabilities of different cultures grown under identical conditions. These data also indicate the influence of globin on cloned protein synthesis under the conditions considered here.

VHb-mediated enhancement of oxygen-limited growth properties: E. coli strain GRO21 was constructed by integrating the *VHb* gene under the control of its native oxygen-regulated promoter into the *lac* operon of MG1655 (an unmutagenized K12 strain) in a single copy. Both MG1655 and GRO21 were transformed with plasmid pTCAT containing a *tac-cat* fusion. Figure 1 shows the results of fed-batch fermentations with the two strains under fixed aeration/ agitation conditions. Cells were grown in minimal medium with glucose as the carbon source and ammonium sulfate as the nitrogen source. The dissolved oxygen concentration dropped below detectable limits during the batch phase of growth (indicative of oxygen limitation) and did not increase thereafter. The synthesis of VHb in GRO21 is induced in response to oxygen limitation (Figure 1e). As can be seen

in Figures 1a, b, c, and d, the growth properties of the two strains are identical by a variety of criteria in the absence of oxygen limitation. However, under oxygen-limiting conditions GRO21 grows more rapidly, as indicated by total protein synthesis (Fig. 1b), CAT activity (Fig. 1b), and nitrogen utilization rates (Fig. 1c). Surprisingly, no differences are observed between the two strains with respect to carbon consumption or acetate synthesis (Fig. 1d). The values of net acid-base balance calculated over the course of the fermentations (data not shown) were in excellent agreement with the acetate profiles, suggesting that acetate synthesis is a reasonable measure of the kinetics of carbon fermentation. The protein composition of samples from the two fermentations are compared in Figure 1f.

In order to eliminate the possible influence of the position of integration of the *VHb* gene on the above results, a similar experiment was repeated with GRO22 as the VHb-expressing host. GRO22 was constructed in the same way as GRO21, except that the locus of integration is the *xyl* operon instead of the *lac* operon. In terms of both VHb expression levels and oxygen-dependent regulatory characteristics the two strains are comparable (10). Results comparing the growth properties of MG1655/pTCAT and GRO22/pTCAT are shown in Figure 2. Since here too the two strains grew identically in the presence of excess oxygen, only the results of the oxygen-limited regime of the experiment are shown. Again, GRO22 grows better in terms of total protein synthesis as well as CAT activity.

To investigate whether the above results could be an artifact of specific features of the *tac-cat* fusion construct, a different promoter/reporter gene construct was tested in a similar manner. Plasmid pGE245 contains a *recA-lacZ* protein fusion under the control of the *E. coli recA* promoter. Although the *lac* operon is intact in MG1655, and the exact position of integration of the *VHb* gene in GRO21 is not known (integration could have occured anywhere in the *lacPOZY* region), it is assumed that, in a medium containing glucose without any inducer of the *lac* operon, the contribution of β-galactosidase expressed from the chromosomal *lac* operon is a negligible fraction of that expressed from a strong promoter such as *recA* on a multicopy plasmid. This appears to be a reasonable assumption since the specific β- galactosidase activity in strains containing pGE245 is approximately 2000 Miller units, compared to approximately 1 Miller unit that is typically produced in an uninduced *E. coli* culture grown in the presence of glucose (16). Figure 3 compares the growth properties of MG1655/pGE245 and GRO21/pGE245 under oxygen-limited conditions. Again, expression of *VHb* enhances total protein and β-galactosidase synthesis properties of the cells.

Effect of VHb expression on cell growth and respiration in the absence of oxygen limitation: Synthesis of heme *b* in *Vitreoscilla* is strongly induced by the onset of oxygen limitation (17). This suggests that VHb (which is the major heme *b* - containing protein in *Vitreoscilla*) is beneficial to *Vitreoscilla* only in hypoxic environments, but not under conditions of

excess oxygen. The results presented above indicate that the presence of VHb in *E. coli* benefits the cells under oxygen-limiting conditions. Hence, it may be appropriate to investigate the effect of VHb on *E. coli* under conditions of excess oxygen. This would also serve as a test of the validity of studying the mechanism of VHb function in *E. coli*. For this purpose a fusion between the *tac* promoter and the *VHb* structural gene was integrated into the *lac* operon. The resulting strain, GRO13, was transformed with plasmid pTCAT. The growth properties of MG1655/pTCAT were compared with GRO13/pTCAT in fed-batch fermentations similar to the ones described above except that (i) IPTG was added to both fermentors to induce VHb synthesis, and (ii) the dissolved oxygen concentration was maintained above 50% air saturation throughout the course of the experiment. As can be seen in Figure 4a, expression of VHb in the absence of oxygen limitation does not enhance total protein or CAT synthesis. Furthermore, this negative result cannot be attributed to extremely low VHb concentrations (compare Figure 4b to Figure 1e).

Support for the above result also comes from *in vitro* experiments in which the effect of VHb on the rate of membrane- catalyzed aerobic oxidation of NADH was studied. VHb purified from an overproducing *E. coli* strain, which was determined to be identical to *Vitreoscilla* VHb as judged by visible spectroscopy, has no significant NADH oxidase activity (data not shown). However, on purification the protein is stable only in its oxidized, but not oxygenated, state. Upon re-

duction with dithionite, followed by exposure to air, purified VHb tends to auto-oxidize fairly rapidly. (A similar observation has also been made with VHb purified from *Vitreoscilla* (18), suggesting that this is an intrinsic property of VHb.) In contrast VHb in crude *E. coli* lysates is indefinitely stable in the oxyVHb state. The result indicates that, as is the case in *Vitreoscilla*, *E. coli* also possesses certain reductase(s) that can stably maintain VHb in its reduced form within the cell (7). Subsequent investigations have localized this reductase activity to the soluble fraction (i.e. cytoplasm + periplasm) of the *E. coli* cell (C.K. and J.E.B., unpublished observations). Hence, in order to investigate the effect of purified VHb on the NADH oxidation activity of the membrane fraction, it was necessary to add a minimum volume of membrane-free soluble fraction to an (air-saturated) reaction mixture containing membrane fraction and 0.5 mM NADH. Results of these experiments showed that the presence of physiologically relevant (and even excess) quantities of oxyVHb does not affect the kinetics of NADH oxidation *in vitro* under conditions of excess oxygen (data not shown).

Two-dimensional gel electrophoretic analysis of protein composition: Two-dimensional electrophoresis has been used previously to identify proteins induced in *E. coli* in response to aerobiosis (19) as well as anaerobiosis (20). In a more recent study (21), while the number of aerobically inducible proteins was in close agreement with the earlier work, the number of anaerobically induced polypeptides was greater, sug-

gesting that the exact growth conditions used for generating anaerobic samples may be important. In this work the protein patterns of samples from the fermentations described in Figure 1 were analyzed via two-dimensional gel electrophoresis. From each fermentation three samples were analyzed corresponding to conditions of excess oxygen (DO > 40% air saturation; sample collected 6h post-inoculation), shortly after onset of oxygen limitation (DO < 2% air saturation; sample collected 8h post-inoculation), and at the end of the run (DO < 2% air saturation; sample collected 26h post-inoculation). Only the data corresponding to the final time points is explicitly shown in Figure 5.

Several observations have been made from an analysis of these gels. First, under all conditions studied here the overall protein patterns of the two strains are similar. In particular this is true for most of the proteins whose levels increase or decrease in response to a change from well aerated to microaerobic conditions (indicated with circles or squares, respectively, in Figure 5). The most notable among these proteins is one that migrates with an apparent molecular weight of 31 kDa. This protein is virtually absent under well aerated conditions, but is a major component in oxygen-limited cells. The overall similarity in the patterns observed for the two strains suggests that the VHb- mediated enhancement is most likely assignable to the presence of VHb, and not to a secondary response of *E. coli*.

Despite the apparent similarity in the protein patterns over the course of the two fermentations, a significant difference is observed in the intracellular concentration of at least one polypeptide between the two strains (indicated by a triangle). This polypeptide, which is not visible in any of the earlier samples, has an apparent molecular weight of 48 kDa. Major changes have been observed in this spot in samples from various *E. coli* strains (including several VHb$^-$ strains) (U.R., unpublished observations). Such increases are coincidental with a reduction in growth rate under typical batch cultivation conditions with a complex medium.

VHb-mediated enhancement of growth properties of cyd and cyo mutants: A candidate mechanism of VHb action is that it specifically interacts with one of the two terminal oxidases present in *E. coli* (as well as in *Vitreoscilla*). A preliminary investigation into this possibility was conducted with *E. coli* strains carrying Tn5 transposon insertions into the *cyd* and the *cyo* loci (gifts of Dr. R.B. Gennis). Earlier studies have shown that strains mutated in either of the two terminal oxidase functions have similar growth properties to an unmutated isogenic strain; however cells lacking both functions are unable to grow aerobically on non-fermentable substrates (21, 23). For the purpose of these experiments, both *cyd*$^-$ and *cyo*$^-$ strains were transformed with pBR322 and pINT2 (a pBR322-based plasmid carrying the *VHb* gene under the control of its own promoter). The results of shake-flask experiments in complex medium with the above strains are shown in Figure

6. In both backgrounds the degree of enhancement of the rate of protein synthesis under oxygen limitation is approximately the same. The oxygen-dependent regulatory characteristics of the *VHb* promoter also remain unaffected in the two hosts compared to strains capable of expressing both cytochrome oxidases (data not shown). This suggests that the beneficial role of VHb is not restricted to the presence of a specific terminal oxidase activity.

5.4 Discussion.

A physiological analysis of the globin effect: The following argument attempts to identify the physiological function that is influenced by the presence of the *VHb* gene under oxygen- limiting conditions in *E. coli*. Table I shows a simplified representation of the major cellular functions that are involved in synthesis and utilization of energy. Here the reaction with the rate R_5 represents the maintenance energy requirements of the cell (24). Based on available data, one could ask which numerical values listed in Table I remain unchanged due to the presence of the *VHb* gene. The results of Figure 1d indicate that R_1 and R_2 do not change. One could also argue that, whatever be the mechanism of action of VHb, it is likely to be a result of the oxygen-binding properties of the hemeprotein. Hence, the stoichiometries of all the oxygen-independent reactions in Table I are unlikely to change (i.e. a, b, c, f, g, and n should remain constant). Furthermore, the necessicity of balancing the oxidation and reduction halves of the reaction involving oxygen consumption dictates that d remains unchanged. Lastly,

physiological studies with *E. coli* have demonstrated that the rate of acid synthesis (i.e. fermentation) is inversely related to the oxygen availability under carbon limitation (Pasteur effect, see ref 25). This is precisely the situation in our experiments during the latter period of the experiments described here (i.e. when the greatest difference is observed; for example, see Figures 1b-d). Thus, although we have not explicitly measured oxygen consumption rates in any experiment, independence of the rate of acid synthesis (R_1) to the presence of VHb indicates that the total rate of oxygen consumption (R_3) also does not change due to VHb presence. (It should, however, be remembered that R_3 is the sum of the rates of two parallel reactions at distinct terminal oxidases (see discussion later).)

Given this background we can identify which values listed in Table I change due to VHb expression. By definition,

$$R_4 = -\frac{1}{f}\frac{dNH_3}{dt} = \frac{1}{g}\frac{dP}{dt},$$

where the concentrations of ammonia and total cell protein (NH_3 and P, respectively) are expressed on a culture volume basis. From the data in Figures 1-3 it is clear that R_4 increases in the presence of VHb. The representation of protein synthesis in reaction 4 (Table I) incorporates the reasonable assumption that, under oxygen-limited growth, the rate of protein synthesis is proportional to the rate at which ATP is utilized for protein synthesis (the proportionality constant being $\frac{1}{g}$). Given an overall ATP balance, this rate is equal to the

net rate of ATP generation by reactions 1,3, and 5. This implies that the quantity $(bR_1 + eR_3 - R_5)$ increases due to VHb presence. All the terms in this expression, save for e and R_5, have been argued to remain unaffected by VHb. Thus, it follows that, under oxygen-limiting conditions, expression of VHb either increases the net stoichiometry of protons translocated (or ATP synthesized) from NADH oxidation, or reduces the net rate of proton leakage (or ATP consumed) for maintenance. Some hypotheses as to the mechanism of VHb action are discussed below.

It should be pointed out that true steady-state metabolic conditions are only approximated during certain sub-intervals in a fed-batch fermentation. For more quantitative analysis of the globin effect, analogous experiments in oxygen-limited chemostats would be required. However, fed-batch operations were the method of choice for this work for several reasons. These include the difficulty of attaining and monitoring microaerobic (oxygen-limited) steady-state conditions in chemostats, the problems of assuring genetic stability of (plasmid-carrying) strains over prolonged experiments in chemostats, the relative convenience of operating in a fed-batch mode, and the predominant role of fed-batch operations in most microbial bioprocesses.

Biochemical implications of the globin effect: In *E. coli* the rate of oxygen consumption does not seem to change due to VHb expression;

yet the efficiency of carbon utilization increases. The aerobic respiratory chain in *E. coli* includes several activities that are regulated in response to environmental changes. For example, cytochrome o (the low affinity terminal oxidase) is replaced by cytochrome d (the high affinity terminal oxidase) under oxygen limitation (26). Furthermore, although two distinct species of NADH dehydrogenases have been identified in *E. coli* membranes, only one of them can function as an energy coupling site (27). It has also been suggested that the number of protons translocated per ATP synthesized may differ under different growth conditions (28). Should the energy conversion efficiency of any of these (or other) activities be affected under oxygen limitation, VHb might ameliorate this effect.

Given available information on aerobic respiration in *E. coli*, as well as properties of VHb and the regulation of its gene, two general categories of hypotheses could be proposed to account for the mechanism of VHb action. The first one, called the facilitated diffusion hypothesis, implies that the presence of VHb enhances the oxygen flux to one or both terminal oxidases under hypoxic conditions. Should this be the case, the results presented here suggest that such an effect is not necessarily restricted to a specific type of terminal oxidase. Nevertheless, since the exact differences (if any) between the physiological roles of the two cytochrome oxidases in *E. coli* are not clear, it cannot be said whether the influence of VHb is limited to a single oxidase in a host that expresses both oxidases. In this context it may

be worth noting that the conditions under which the *VHb* promoter is induced (10) are remarkably similar to those under which cytochrome d is induced (26) in *Escherichia coli*. Furthermore, a significant degree of homology has been detected between the upstream regions of the two genes (10, 29, C.K. and J.E.B., unpublished observations). This is indicative of a common mode of regulation and may imply that VHb enhances the activity of cytochrome o and/or cytochrome d. Should the proton translocation efficiency of the two cytochromes differ under hypoxic conditions, such a mechanism could be consistent with the above suggestion of a net stoichiometric effect of VHb.

An alternative explanation of the mechanism of action of VHb, called the intracellular redox effector hypothesis, suggests that oxyVHb influences the activity of some key redox- sensitive molecule in the cell. This could be a sensor, a regulator, or even an allosteric site of a respiratory enzyme. Such an influence could in turn be transduced into an increase in the efficiency of energy conservation. Results obtained from two- dimensional gel electrophoresis have revealed a significant difference in the intracellular level of at least one *E. coli* protein in response to VHb synthesis. Whether this is a cause or an effect of VHb-mediated enhancement cannot be said.

An earlier observation may be relevant to either of the above hypotheses. From results of biochemical experiments we have estimated the VHb concentration in the integrant *E. coli* strains used here to be approximately 100 μM in the periplasm and 20 μM in the cytoplasm

(9). This is significantly higher than typical oxygen concentrations under microaerobic conditions (< 2 μM). In such a situation, should even a fraction of the intracellular VHb be oxygenated, it might provide a source of 'available oxygen' to one (or more) of the above oxygen-dependent reactions by an (as yet unknown) mechanism. The relatively high k_{off} values for this globin, calculated from flash photolysis experiments (30), support this argument. Further evidence for this hypothesis could perhaps be obtained by studying the effect of VHb on the conformation of a reporter molecule that is sensitive to the oxidative state of the intracellular environment (e.g., a molecule containing sulfhydryl groups).

Implications of these results on the role of VHb in Vitreoscilla. It might be appropriate to ask whether the limited increase observed in carbon consumption efficiencies ($Y_{P/S}$) in *E. coli* is sufficient to account for a selective advantage to *Vitreoscilla* due to the presence of VHb. In this context it is important to remember that *E. coli* is a facultative aerobe, whereas *Vitreoscilla* is an obligate aerobe. In other words, while carbon utilization and energy generation is exclusively dependent on aerobic respiration in *Vitreoscilla*, *E. coli* can consume carbon and synthesize ATP via fermentative catabolism under oxygen limitation. Thus, while the growth rate of an obligate aerobe is directly dependent on the available oxygen concentration, the same is not necessarily true of a facultative aerobe, and is further complicated by the eventual inhibition of growth by acidic byproducts of oxygen-

independent energy generation. Hence it may be that the same mechanism by which VHb produces a limited enhancement of microaerobic growth in *E. coli* could result in a far greater selective advantage to *Vitreoscilla* (and possibly other obligate aerobes which encounter a similar bottleneck in respiration). Then again, while the mechanism of action of VHb has been exquisitely tuned to the demands of respiratory function in *Vitreoscilla* by natural selection, minor differences between the two bacteria could result in a decrease in the magnitude of the VHb effect in *E. coli*.

5.5 Materials and Methods.

Bacterial strains and plasmids: MG1655 (λ^-, F^-) is an unmutagenized *E. coli* K12 strain obtained from Cold Spring Harbor Laboratory. The construction of strains, GRO21 and GRO22 (10), and of plasmids pTCAT (31) and pGE245 (32) has been described elsewhere. Strain, GRO13, was constructed in a manner identical to the construction of GRO21, except that a *tac-VHb* promoter fusion (9) was used instead of the *VHb* gene under the control of its native promoter. GO103 (F^-, *thi, gal, rpsL, cyd::kan*) and GO104 (F^-, *thi, gal, rpsL, cyo::kan*) were gifts from Dr. R.B. Gennis. The construction of plasmid pINT2 has been described elsewhere (9).

Fermentation and shake-flask conditions: Fermentations were carried out in a Bioflo III fermentor (New Brunswick Scientific) with a working volume of 2.5L. Cells were grown at 37°C and pH 7 under aeration/agitation conditions specified in the captions to the figures.

In all cases, the batch medium consisted of 4 g/L glucose, 0.4 g/L $(NH_4)_2SO_4$, 4.35 g/L K_2HPO_4, 1.5 g/L KH_2PO_4, 1 mL/L trace metal mix (8.3 mM Na_2MoO_4, 7.6 mM $CuSO_4$, 8 mM H_3BO_3), 1 mL/L vitamin mix (0.042% riboflavin, 0.54% pantothenic acid, 0.6% niacin, 0.14% pyridoxine, 0.006% biotin, 0.004% folic acid), 1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.2 mM $FeCl_3$, and 50 mg/L ampicillin. The feed medium consisted of 430 g/L glucose, 110 g/L $(NH_4)_2SO_4$, 8 g/L $MgSO_4$, 1 mL/L vitamin mix, 1 mL/L trace metal mix, 0.2 mM $FeCl_3$, 0.05 mM $CaCl_2$, and 100 mg/L ampicillin. Details of feeding protocols are described in the captions to figures.

Shake-flask cultivations were carried out at 37°C in a New Brunswick Scientific G24 gyratory shaker operating at medium setting. The broth contained 10 g/L bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L ampicillin (wherever required), and 30 mg/L kanamycin (pH 7).

Enzyme assays: Cell extracts used for assaying both β-galactosidase and CAT specific activity were obtained by sonication of fermentation samples. β-galactosidase was assayed at 30°C using o-nitrophenyl-β-D- galactopyranoside as the substrate. Change in A_{420} was monitored by a rate assay in a thermostatted spectrophotometer (Shimadzu UV260). Specific activity is expressed as Miller units (16). CAT activity was assayed with ^{14}C-labeled butyryl coenzyme A (New England Nuclear) according to recommended protocols (33). Specific activity

is expressed in units CAT / OD_{590}-mL. Standard CAT (Sigma) was used to calibrate the assay.

Gel electrophoresis and Western blotting: One dimensional gel electrophoresis of proteins was according to standard protocols (34). VHb was detected via Western blotting as described elsewhere (10). Two-dimensional gel electrophoresis was performed by a modification of the O'Farrell technique (35), as described elsewhere (36). Immediately prior to electrophoresis, samples were lysed in 2% sodium dodecyl sulfate and 30 mM dithiothreitol, sonicated for a few seconds, and boiled for 10 min. Isoelectric focussing was performed to equilibrium: 4h 200V, 4h 500V, and 8h 2000V. For this a model 175 chamber (Bio-Rad) was used. The ampholines used were 2% pH 3-10 (Serva), 2% pH 3-10 (Bio-Rad), and 1% pH 5-7 (Bio-Rad). The second dimension was run on a 9-16% SDS-polyacrylamide gradient gel at constant current (40 mA, 160 X 200 X 1.5 mm) in the Protean II Multi-Cell apparatus (Bio-Rad). Subsequently, the gels were stained according to the protocol of (36).

In vitro studies: VHb was purified from *E. coli* according to the method of Hart and Bailey (manuscript in preparation). *E. coli* membrane fractions and soluble fractions were obtained from sonicated cell samples by differential centrifugation (37). The kinetics of NADH oxidation were followed on a Shimadzu UV260 thermostatted spectrophotometer at a wavelength of 340 nm and a temperature of 30°C.

5.6 Acknowledgements.

The authors wish to thank Dr. R.B. Gennis for useful advice and for providing *cyo* and *cyd* mutants. We would also like to acknowledge the assistance of Nicole Tedeschi with the fermentations, and Dr. D.E. Hughes and Dr. J.L.Galazzo for helpful comments over the course of this work. One of us (U.R.) thanks the Deutsche Akademische Austauschdienst (DAAD) for the award of a post-doctoral fellowship. This research was supported in part by a grant from the Energy Conversion and Utilization Technologies (ECUT) program of the United States Department of Energy.

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Table 1:

A simplified representation of carbon metabolism and its coupling to energy synthesis and consumption

METABOLIC ACTIVITY			RATE
Glucose	$\longrightarrow$	a Acid + b ATP	R_1
Glucose	$\longrightarrow$	c NADH	R_2
NADH + d O_2	$\longrightarrow$	e ATP	R_3
ATP + f NH_3	$\longrightarrow$	g Protein	R_4
n H^+(in) + ATP	$\longrightarrow$	n H^+(out) + ADP	R_5

5.9 Captions to Figures.

Figure 1: Comparison of growth properties of MG1655/ pTCAT and GRO21/ pTCAT. Results of fed-batch fermentations with MG1655/ pTCAT (○) and GRO21/ pTCAT (●) are presented. In both cases the cells were grown under constant conditions of 0.2 L/min air supply and 300 rpm. The batch phase of growth lasted till an OD_{590} of 1.2. Thereafter the cells were fed with feed medium at 1.4 mL/h till the OD_{590} reached 2.5, following which the feed rate was kept constant at 2.8 mL/h. (a) The growth curves, as measured in terms of OD_{590}, are shown here for the two strains. The time corresponding to the onset of oxygen limitation (DO < 2%, 7h post-inoculation) is indicated by a vertical arrow. (b) The total protein concentration (- - - -) and CAT activity (——) profiles from the oxygen-limited phases of the two fermentations (beyond 7h post-inoculation) are presented. (c) The time course of the nitrogen source concentration in the two fermentors is shown. (d) Likewise, the time courses of the glucose concentrations (- - - -) and acetate concentrations (——→) are also shown. (e) Relative intracellular VHb levels in GRO21/pTCAT, as analyzed by Western blotting with anti-VHb antiserum, are shown over the time course of the fermentation. Each lane contains protein from approximately the same number of cells (10 μL of 5 OD_{590}). The samples are as follows (the numbers in parentheses represent the post-inoculation time of sampling): Lane 1, MG1655/pTCAT control (7h); lane 2, GRO21/pTCAT (5h); lane 3, GRO21/pTCAT (7h);

lane 4, GRO21/pTCAT (9h); lane 5, GRO21/pTCAT (16h); lane 6, GRO21/pTCAT (24h). (f) Samples over the course of both fermentations are analyzed by one-dimensional gel electrophoresis, followed by Coomassie blue staining. Again, each lane contains protein from approximately the same number of cells (15 μL of 10 OD_{590}). Lane 1 contains molecular weight standards (14.4, 20, 30, 43, 67, and 94 kDa). Lane 2 contains an MG1655 control sample grown in the same medium. Lanes 3-12 are (alternate) samples from MG1655/pTCAT and GRO21/pTCAT, respectively. They represent the following post-inoculation times: 7h (3,4), 9h (5,6), 12h (7,8), 16h (9,10), and 24h (11,12).

Figure 2: Comparison of protein synthesis in MG1655/pTCAT and GRO22/pTCAT under oxygen limitation. The time course of total protein concentrations (- - - -) and CAT activities (———) in fed-batch fermentations with MG1655/pTCAT (○) and GRO22/pTCAT (●) are plotted. Experimental conditions were identical to those described in Figure 1. Again, since the protein concentrations in the two cases were similar under oxygen- excess conditions, only the data from the oxygen limited phase of the experiment is shown.

Figure 3: Comparison of protein synthesis in MG1655/pGE245 and GRO21/pGE245 under oxygen limitation. The time course of total protein concentrations (- - -) and β-galactosidase activity (——) in fed-batch fermentations with MG1655/ pGE245 (○) and GRO21/ pGE245

(●) are plotted. Experimental conditions were identical to those described in Figure 1. Again, since the protein concentrations in the two cases were similar under oxygen-excess conditions, only the data from the oxygen- limited phase of the experiment is shown.

Figure 4: Comparison of protein synthesis in MG1655/pTCAT and GRO13/pTCAT under conditions of excess oxygen. (a) The time course of total protein concentrations (– – –) and CAT activities (——) in fed-batch fermentations with MG1655/pTCAT (○) and GRO13/pTCAT (●) are plotted. Cells were grown under conditions similar to those described in Figure 1, except that the DO was controlled above 50% air saturation throughout the course of the fermentations. IPTG was also added to both cultures to a final concentration of 0.5 mM. (b) Samples from the GRO13/pTCAT fermentation were checked for constitutive expression of VHb via Western blotting. Lane 1 contains a control sample from the MG1655/pTCAT fermentation. Lanes 2-5 contain samples spanning the entire period of the GRO13/pTCAT fermentation.

Figure 5: Protein compositional analysis of MG1655/pTCAT and GRO21/pTCAT. Samples of GRO21/pTCAT (A) and MG1655/pTCAT (B), taken over the course of the fermentations described in Figure 1 (see Results section), were analyzed by two-dimensional gel electrophoresis. Only the samples corresponding to the end of the fermentations (26h) are explicitly shown. Proteins that increase under mi-

croaerobiosis are marked with squares, while those that decrease are circled. A spot that is significantly more intense in GRO21/pTCAT is marked with a triangle. To provide a reference for comparing these patterns with known databases (38), the following spots are also marked: (1) elongation factor Tu, (2) elongation factor G, (3) elongation factor Ts, and (4) outer membrane protein OmpA. The right side of the gel corresponds to lower pH values.

Figure 6: Comparison of protein synthesis in cyd and cyo mutants. Cells were grown in 100 mL complex medium (see Materials and Methods) in a 250 mL shake-flask. (a) The time course of total protein concentrations in GO103 (×), GO103/ pBR322 (○), and GO103/ pINT2 (●) are shown. (b) The time course of total protein concentrations in GO104 (×), GO104/ pBR322 (○), and GO104/ pINT2 (●) are shown.

Figure 1a.

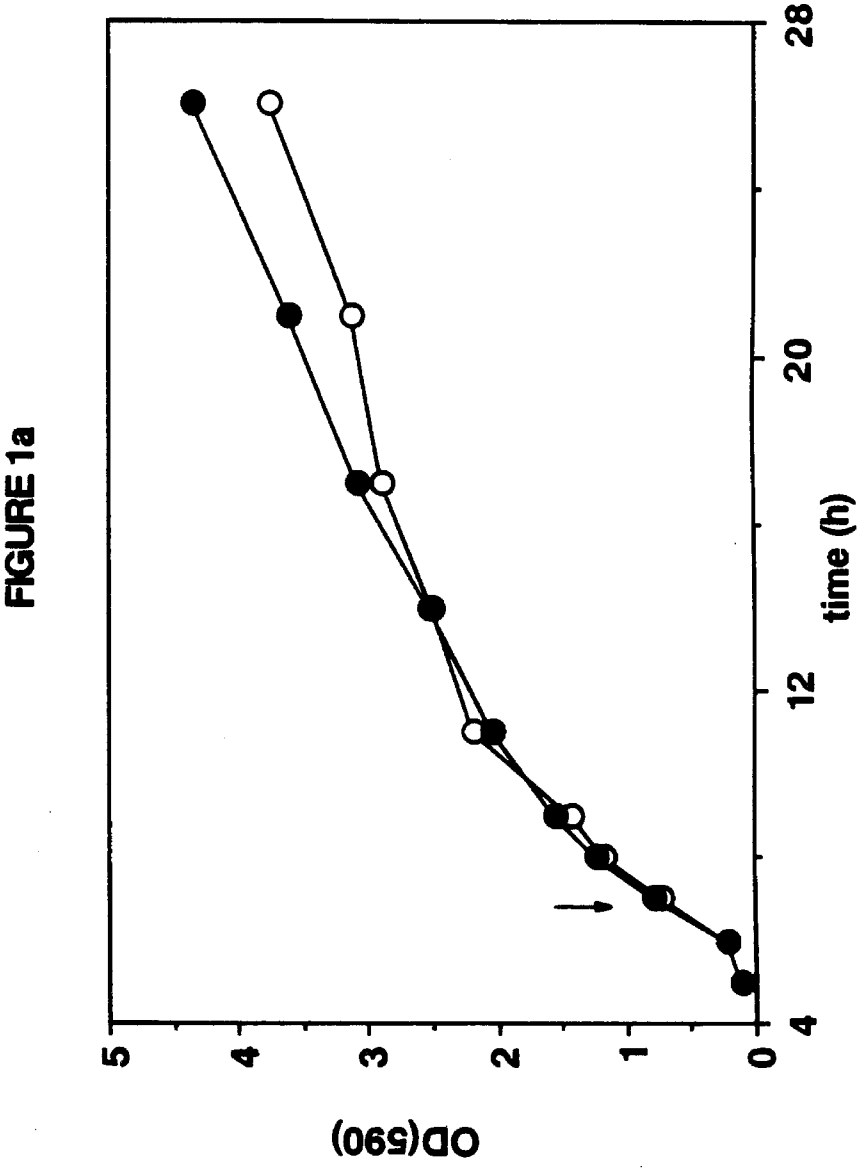


Figure 1b.

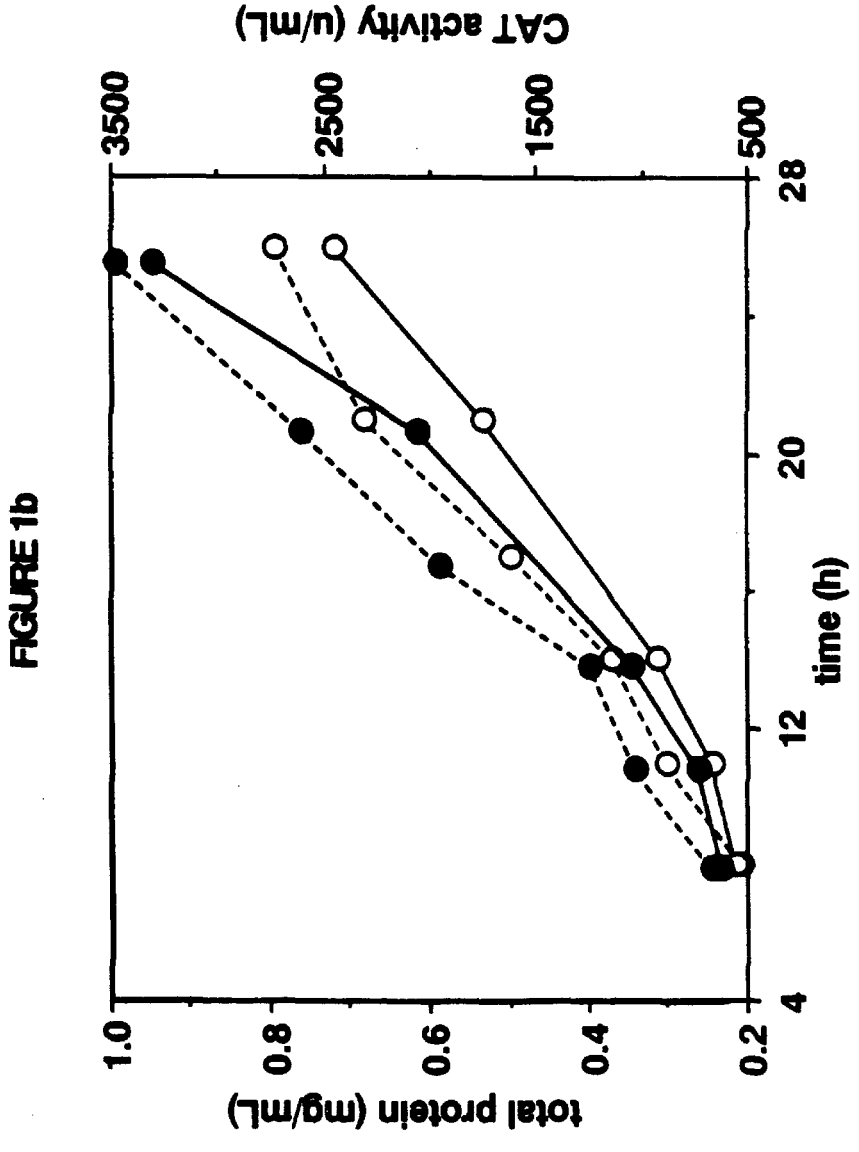


FIGURE 1b
CAT activity (u/mL)
3500
2500
1500
500
total protein (mg/mL)
1.0
0.8
0.6
0.4
0.2
4
12
20
28
time (h)

Figure 1c.

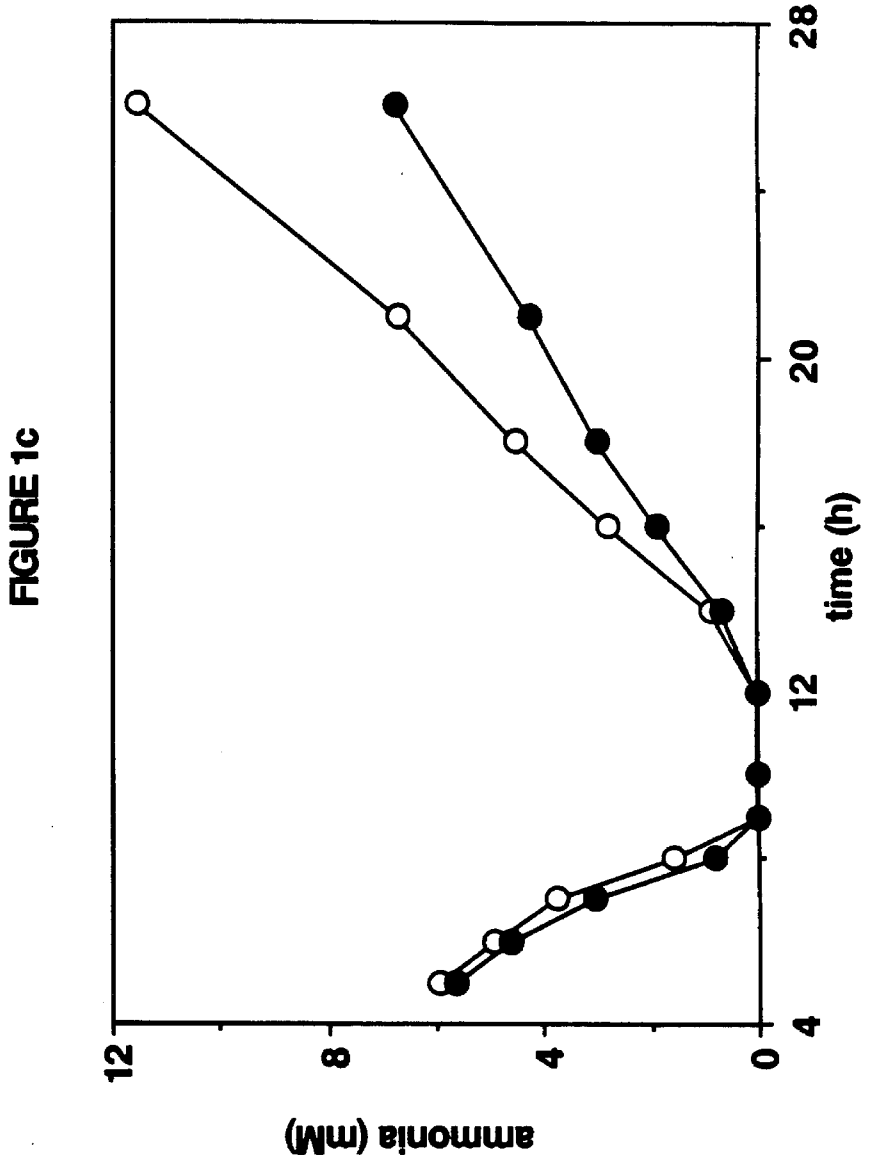


Figure 1d.

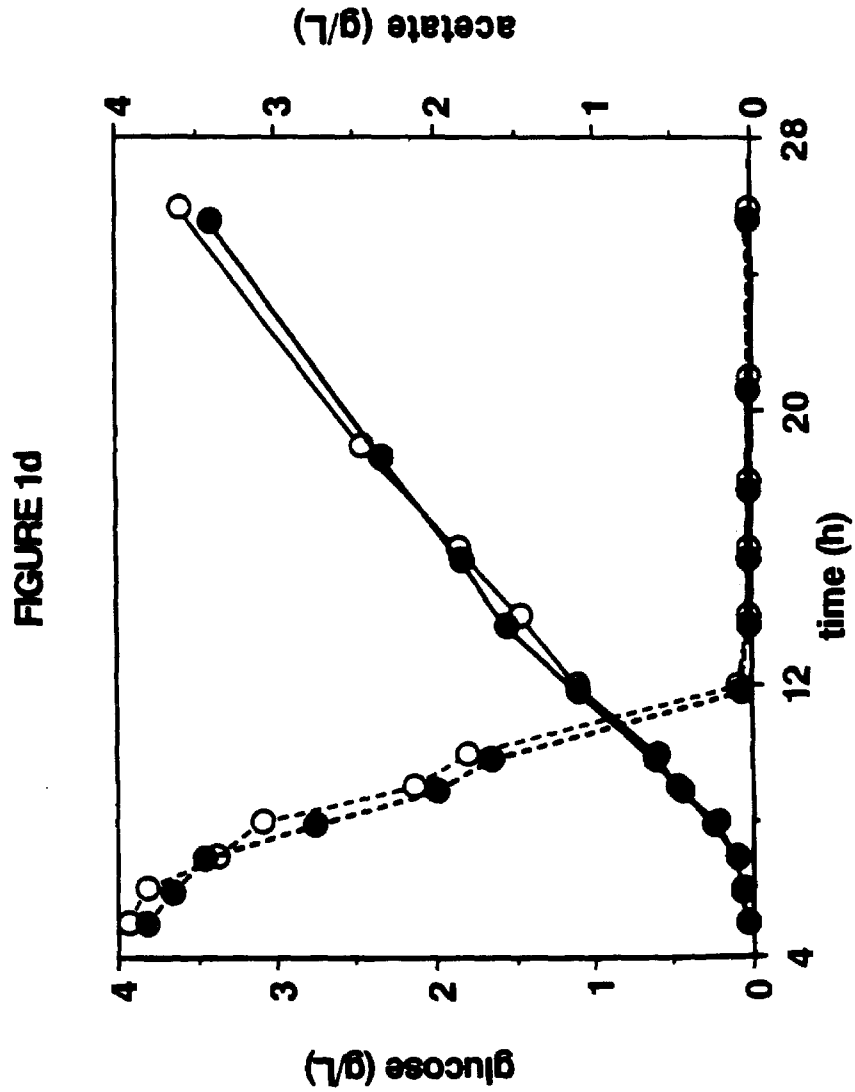


FIGURE 1d

Figure 1e.

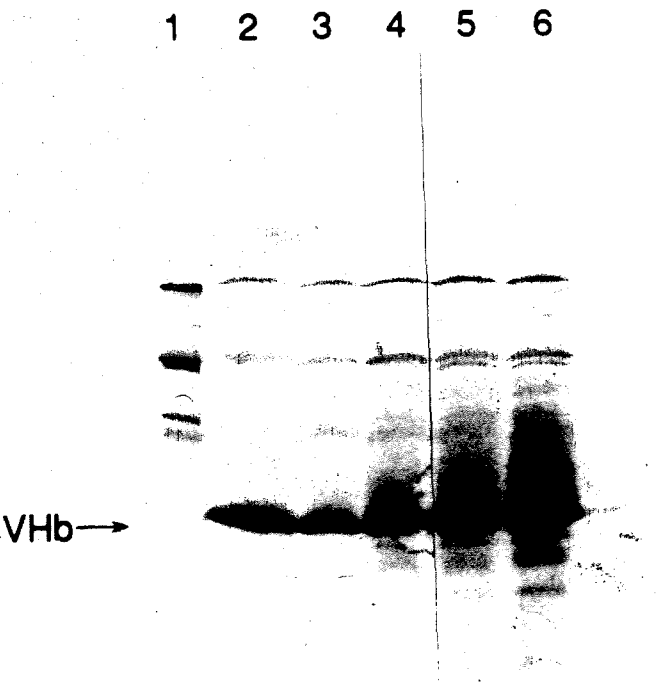


Figure 1f.

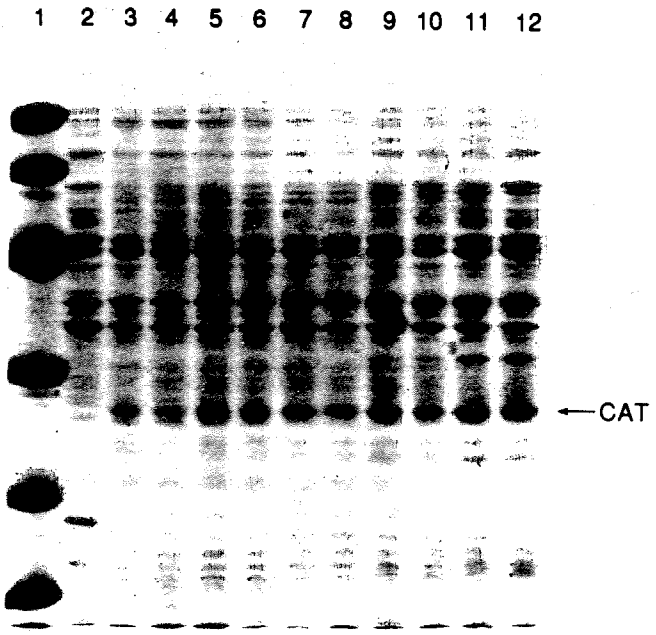


Figure 2.

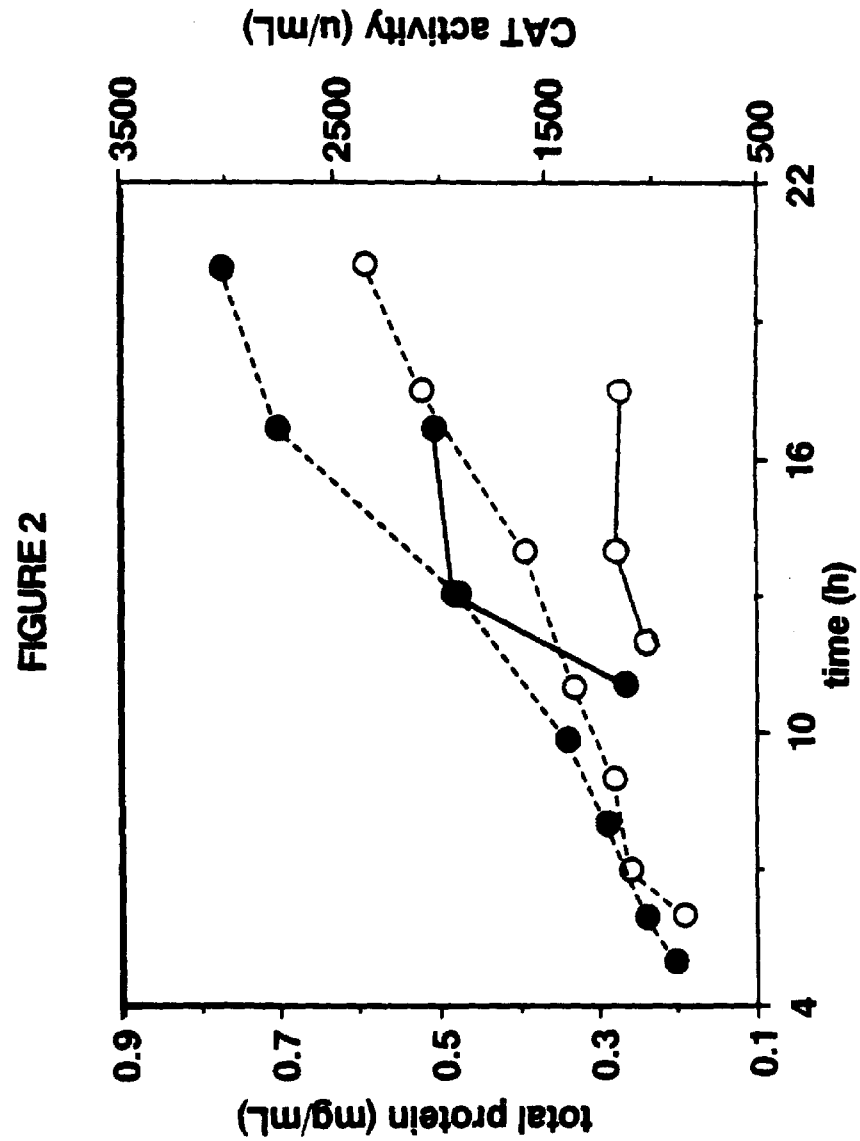

CAT activity (u/mL)
3500
2500
1500
500
22
16
10
4
time (h)
0.9
0.7
0.5
0.3
0.1
total protein (mg/mL)
FIGURE 2

Figure 3.

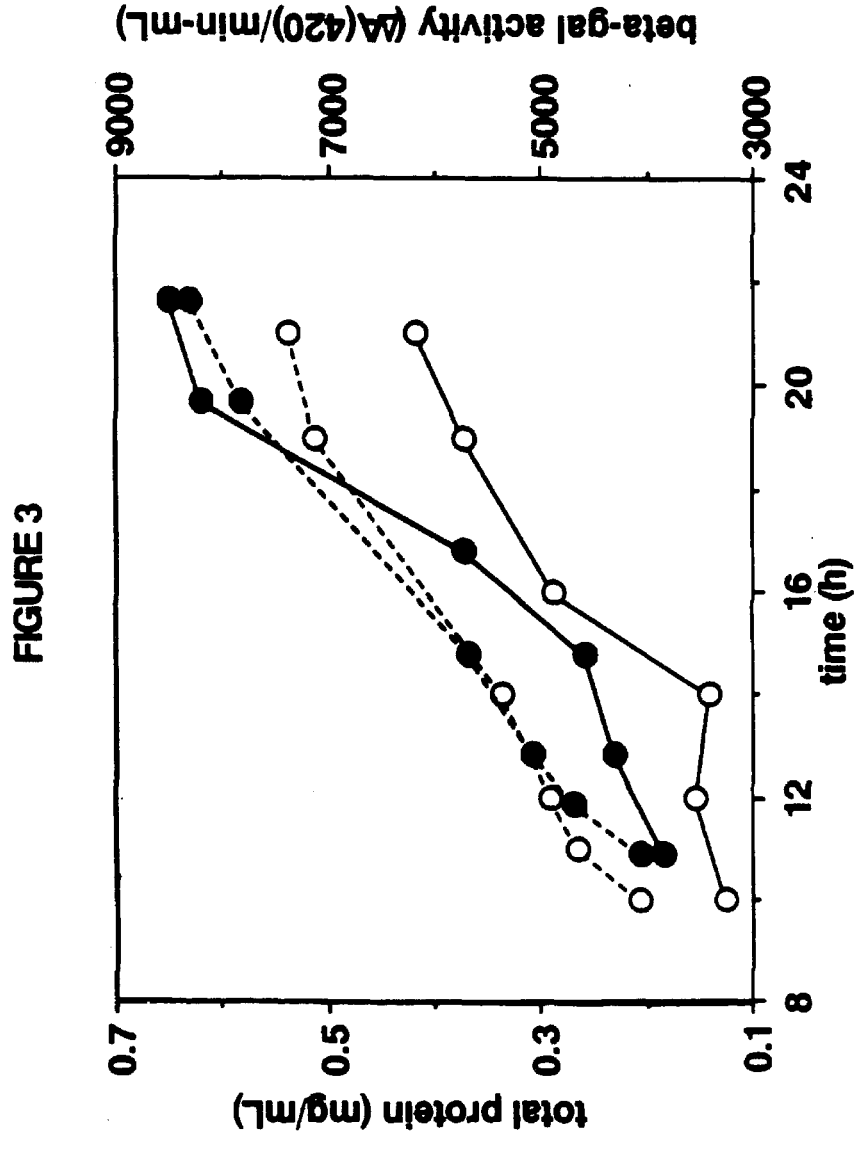


FIGURE 3
beta-gal activity (ΔA(420)/min-mL)
9000
7000
5000
3000
total protein (mg/mL)
0.7
0.5
0.3
0.1
8
12
16
20
24
time (h)

Figure 4a.

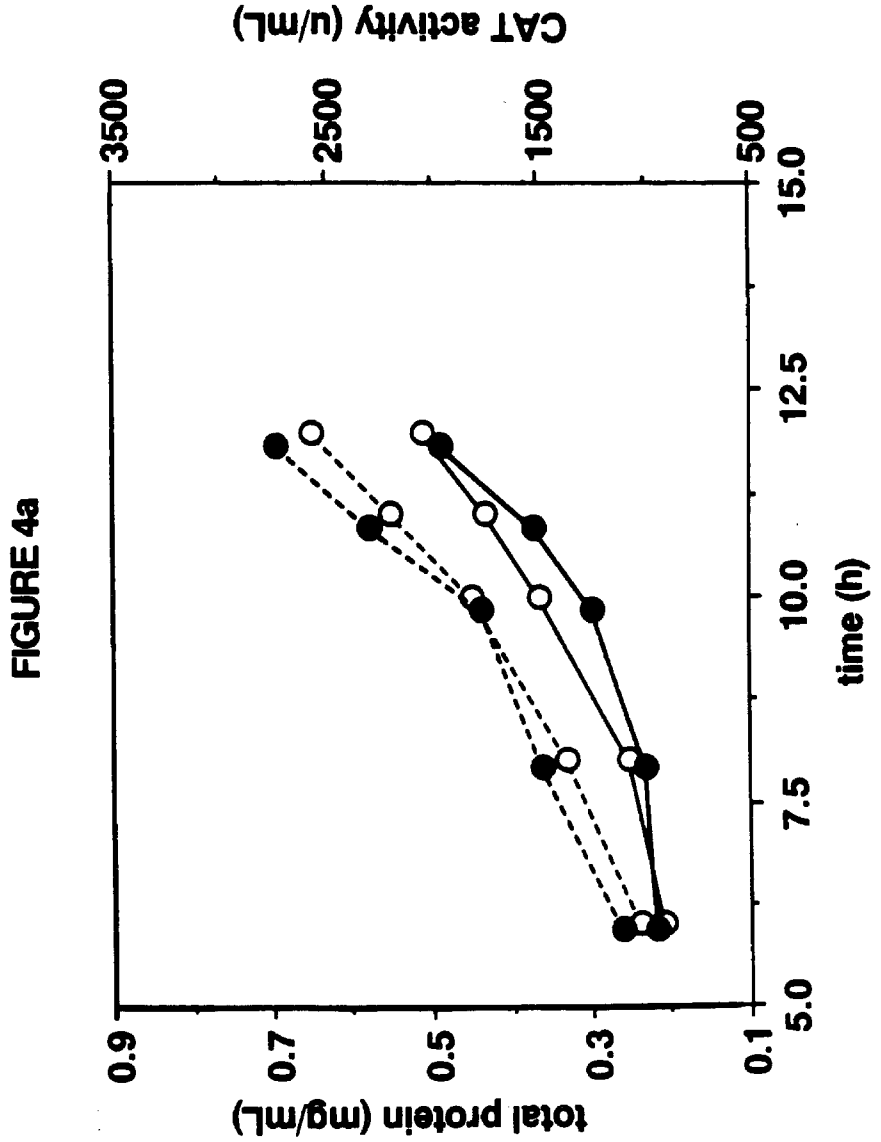


FIGURE 4a
CAT activity (u/mL)
3500
2500
1500
500
total protein (mg/mL)
0.9
0.7
0.5
0.3
0.1
time (h)
5.0
7.5
10.0
12.5
15.0

Figure 4b.

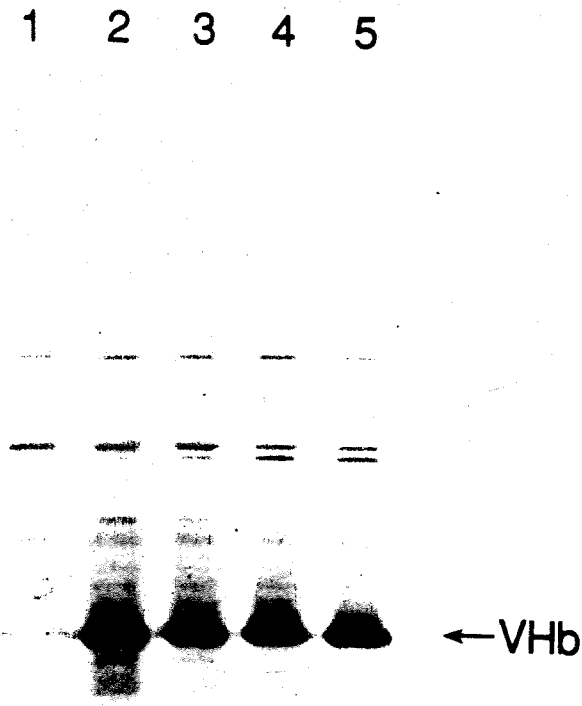


Figure 5a.

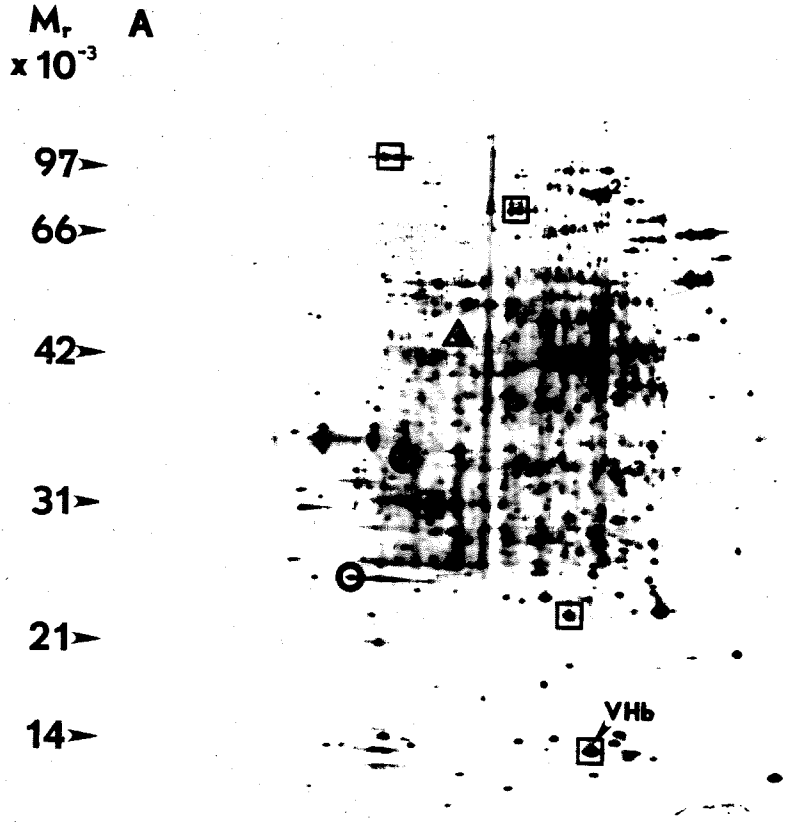


Figure 5b.

Figure 6a.

FIGURE 6a

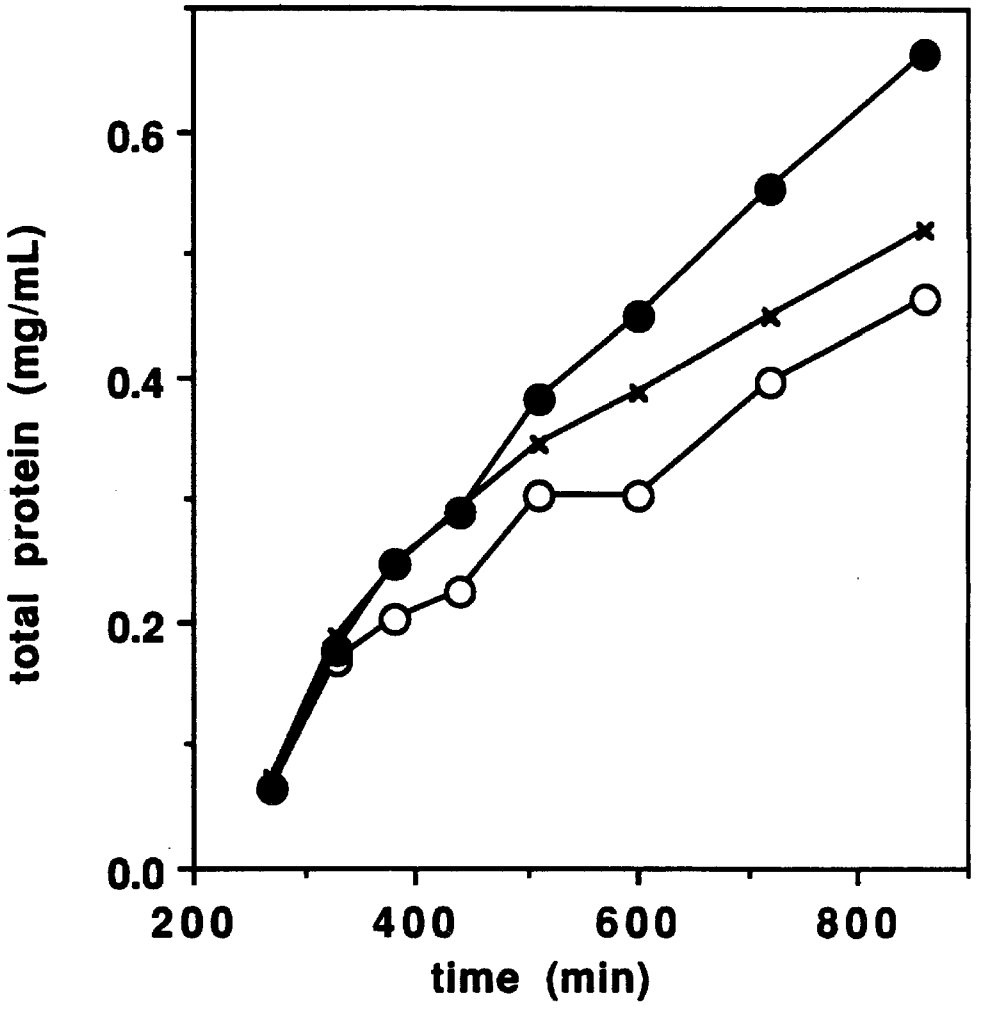
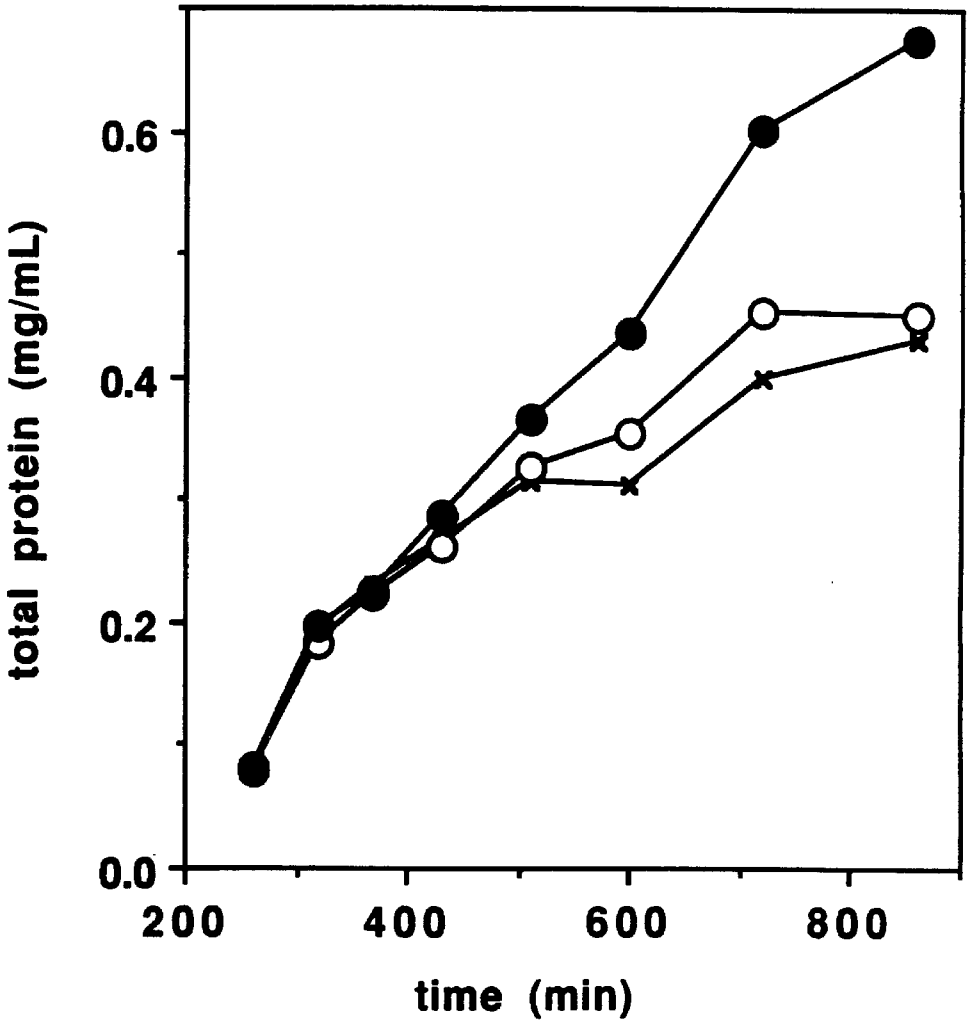


Figure 6b.

FIGURE 6b



CHAPTER 6

CHARACTERIZATION OF THE OXYGEN-DEPENDENT PROMOTER OF THE *VITREOSCILLA* HEMOGLOBIN GENE IN *ESCHERICHIA COLI*

Source: Khosla, C. and Bailey, J.E. (1989) J. Bacteriol. 171:5995-6004.

6.1 Summary.

The gene coding for the *Vitreoscilla* hemoglobin (VHb) molecule has been cloned and functionally expressed in *E. coli*. Using a plasmid-encoded gene as well as single copy integrants, the oxygen-dependent *VHb* promoter has been shown to be functional in *E. coli*. The promoter is maximally induced under microaerobic conditions (dissolved oxygen levels less than 2% air saturation). Direct analysis of mRNA levels as well as the use of gene fusions with *lacZ* has shown oxygen- dependent regulation occurs at the level of transcription. Transcriptional activity decreases substantially under anaerobic conditions, suggesting the presence of a regulatory mechanism that is maximally induced under hypoxic, but not completely anaerobic, conditions in *E. coli*. Primer extension analysis has been used to identify the existence of two overlapping promoters within a 150 bp region upstream of the structural *VHb* gene. The oxygen- dependent activity of both promoters is qualitatively similar, suggesting the existence of a common mechanism by which available oxygen concentrations influence expression from the two promoters. Analysis of promoter activity in *crp* and *cya* mutants has shown that both CAP and cAMP are required for full activity of the promoter. The *VHb* promoter contains a region of significant homology to the CAP- binding site near the *E. coli lac* promoter.

6.2 Introduction.

The response of bacteria at the level of gene regulation to profound environmental changes is a well-observed phenomenon. In some

of the better understood examples such as changes in temperature, osmolarity and nitrogen availability, this response involves sensing of the change by the cell and transmission of this information to a regulatory protein which, in turn, affects the expression of a multigene system (38). Another important environmental parameter for both obligate and facultative aerobes is oxygen availability. In *Escherichia coli* and *Salmonella typhimurium*, several genes have been shown to be differentially regulated by varying dissolved oxygen (DO) levels and, in the extreme case, by switching from aerobic to anaerobic conditions or vice versa (8,14,19,21,22,24,31,46). Multiple loci have been implicated in the regulation of these genes (1,10,19,20,22,40,46,52). As would be expected, mutants in many of these regulatory loci are pleiotropic (1,19,22,40). Likewise, the expression of some oxygen-dependent genes appears to be affected in more than one of these mutants (20,22,46), suggesting that these regulatory loci may function as components in one (or more) control cascades. Interestingly, many genes that are influenced by oxygen availability, such as those coding for some respiratory enzymes, are also influenced by other effectors. This has led to the proposal of the term "modulon" to describe a set of regulons that are subject to a common control mechanism (19). Oxygen-dependent control mechanisms revealed in some of the above studies include positive regulation by an activator protein (24), negative regulation by a repressor (20) and modulation of DNA superhelicity (10,52).

Recently, we reported the discovery of an unusual oxygen-regulated genetic switch that appears to be controlled by a well-conserved mechanism in obligate as well as facultative gram-negative aerobic bacteria. The bacterium *Vitreoscilla* is an obligate aerobe from the Beggiatoa family that synthesizes a hemoglobin-like molecule in response to growth in oxygen-poor environments (4,47). We have cloned and sequenced the gene coding for this protein and demonstrated functional expression of the protein in *E. coli* from its native promoter (25). Using this expression system on a multicopy vector, hemoglobin polypeptide levels as high as 15% total cellular protein are readily obtainable in *E. coli* under low oxygen conditions (25, Khosla and Bailey, unpublished observations). Under well-sparged conditions, however, hemoglobin levels were observed to decline sharply, leading to the hypothesis that the oxygen-dependent regulatory element(s) that control expression in *Vitreoscilla* are also recognized in *E. coli* (26).

The purposes of the present study were to characterize the response of the promoter to changes in oxygen availability in the environment, as well as to obtain initial insights with regard to the mechanism(s) by which the promoter is controlled.

6.3 Materials and Methods.

Bacteria, bacteriophages, and plasmids: Bacteria, bacteriophages, and plasmids used in this work are listed in Table 1.

Media and Growth Conditions: A buffered complex medium containing 10g/L Bactotryptone, 5g/L yeast extract, 5g/L NaCl, 3g/L K_2HPO_4, 1g/L KH_2PO_4 (pH 7) was used to grow cells. Culture tube and shake-flask growth were conducted in a New Brunswick G24 shaker incubator operating at medium setting. Batch fermentations were performed in a New Brunswick Bioflo II 2.5L fermentor under pH and temperature control. All growth was at 37°C. Plasmid-containing cells were grown in the presence of 100mg/L ampicillin, except in the fermentor (to minimize foaming problems).

Construction of single copy integrants of the VHb gene and its native promoter in E. coli: Plasmid pINT2 was constructed by subcloning the *HindIII- BamHI* fragment containing the *VHb* gene into corresponding sites in pBR322. With the intention of constructing a transposable element, a kanamycin resistance gene cartridge (Pharmacia) was inserted into the *SalI* site downstream of the *VHb* gene on pINT2 to create pINT4. An *EcoRI* fragment containing both genes was inserted between the Tn10 inverted repeats on pMM12, replacing the original Cam^R gene. The resulting plasmid, pINT6, was transformed into MG1655/ pMS421. This strain was grown in the presence of 0.5 mM IPTG to induce transposition. Cells were plated on lactose- MacConkey and xylose- MacConkey plates to select for events that had inactivated the *lac* and *xyl* operons, respectively. The resulting mutations were transduced into MG1655 using P1 phage. Two mutants, GRO21 and GRO22, were checked for the following phenotypes: lac^-,

xyl^+, kan^r, amp^s, spc^s and lac^+, xyl^-, kan^r, amp^s, spc^s, respectively (amp and spc are the plasmid markers). Although the assumption that the two strains contain single copies of the *VHb* gene was not confirmed by Southern hybridization, it is unlikely that both strains would contain more than one defective transposon, Tn10d(*VHb-Kan*). Parallel investigations on two such independent strains eliminate the possibility of positional effects of chromosomal integration.

DNA manipulations: In vitro deletions were constructed using the exonuclease Bal31 (36) as well as exonuclease III and S1 nuclease (18). For all other DNA manipulations, standard protocols were used (36).

DNA Sequencing: The endpoints of the deletions constructed as described above were determined by dideoxy sequencing of pUC19-derived plasmids into which the deletions were cloned. For this purpose, the Sequenase kit (US Biochemicals) and a modified protocol (29) were used. Both the universal and the reverse primers for pUC vectors were used (US Biochemicals). The standard sequencing reactions used in primer extension assays were performed with a custom-made oligonucleotide primer (see Results section).

Preparation and Analysis of RNA: 1.5 mL cells to be analysed for mRNA content were centrifuged for 1 min. in an Eppendorf centrifuge and resuspended in 0.3 mL 1.4% SDS, 4 mM EDTA preheated to 65°C. Following further incubation at 65° for 5-10 min., the samples were chilled and 0.15 mL saturated NaCl was added. The cell debris was spun out and nucleic acids were precipitated from

the supernatant using ethanol, and resuspended in dH_2O containing Inhibit-ACE (5 prime- 3 prime Inc.). The integrity of the RNA preparation was checked by observing intact ribosomal RNA bands in a 1% formaldehyde-agarose gel that was stained with ethidium bromide. All aqueous solutions involved were pretreated with diethyl pyrocarbonate.

Quantitation of hemoglobin mRNA content: Northern blot analysis of hemoglobin mRNA was done according to recommended protocols (12). Plasmid pRED4, labeled by the random hexanucleotide priming technique (New England Nuclear), was used as probe DNA. Hemoglobin mRNA content was quantitated by the method of Kornblum, *et al* (28). Counts thus obtained were normalized with plasmid DNA counts in order to obtain a measure of gene activity.

Primer extension analysis: Primer extension assays were done according to the protocol of Kingston (27). Total cellular RNA, prepared by the method described above, was used for this purpose. In each case the position of the transcription start point was determined by running sequencing reactions that used the same oligonucleotide as the sequencing primer.

Construction of lacZ operon and protein fusions: In order to construct *lacZ* fusions with the *VHb* promoter which would be useful for further genetic studies, derivatives of pMLB1010 and pMLB1034 which contained the IG region from the phage M13 were created. pGE245 is an example of such a plasmid which contains a *recA- lacZ* protein

fusion. The entire *recA* fragment was deleted from this vector by digestion with *BamHI* followed by religation and identification of a white colony on indicator X-Gal plates. This result in plasmid pCO1 which contains the IG region and is suitable for making protein fusions with *lacZ*. An analogous vector, pCO2, for making operon fusions was constructed by replacing the *BamHI- SstI* fragment in pCO1, which contains most of the truncated *lacZ* gene, with the corresponding fragment from pMLB1010, which includes a *trpA- lacZ* fusion. To construct *VHb-lacZ* fusions two characterized deletions, pRED503 and pRED509 (see above as well as the Results section), were used. The protein fusion plasmid, pOX11, was created by *BamHI* digestion and mung bean nuclease treatment of pCO1, followed by ligation with pRED503 that had been digested with *HindIII* and *XbaI* and treated with mung bean nuclease. This resulted in an in- frame fusion which was identified by transforming MC1061 with the ligation mixture and picking blue colonies. The recombinant plasmid was checked by restriction mapping. To construct an operon fusion plasmid, pOX21, plasmid pCO2 was digested with *SmaI* and *BamHI*, gel purified, and ligated with a mixture of two fragments produced by digestion of pRED509 with *HindIII* (end-filled with Klenow) and *BamHI*. Resulting transformants in MC1061 were screened for the presence of a correctly oriented insert by restriction mapping.

Construction of a VHb- CAT fusion: The vector pYEJ001 contains a promoterless CAT gene fragment flanked by *HindIII* sites. Plasmid

pRED509 was digested at the unique *XbaI* site downstream of the *VHb* promoter. The resulting ends were converted into *HindIII* cohesive ends with adaptor DNA (New England Biolabs). The vector was then ligated with the CAT insert, resulting in a loss of both *HindIII* and *XbaI* sites. However, a new *XmnI* site was introduced between the *VHb* promoter and the structural CAT gene. The entire fusion was then isolated by digestion with *HindIII* and *BamHI*, and cloned into the corresponding sites of pBR322, giving rise to plasmid pOX2.

Hemoglobin activity determination: Hemoglobin activity was determined by the following method: Approximately 20 mL cells were added to 10 mL ice-cold 1% NaCl containing 400 μg/mL chloramphenicol in order to inhibit further translation. Later, the cells were centrifuged, resuspended in assay buffer (100 mM Tris-Cl pH 7.5, 50 mM NaCl) and sonicated. The soluble fraction was assayed for hemoglobin content using the CO difference spectrum method (48). Total soluble protein content was assayed by the Bradford method (BioRad). Intracellular hemoglobin concentrations are reported as $\Delta Abs_{419-436}$/mg total soluble protein.

Western blotting of hemoglobin protein: Hemoglobin expressed in *E. coli* was purified from cell extracts (R. Hart and J.E. Bailey, manuscript in preparation). This preparation was used to generate rabbit anti-globin antiserum (Cocalico Biologicals). The globin content in samples of total cell protein was assayed according to standard protocols (51).

Enzyme assays: β-galactosidase assays were done by a slight modification of the Miller protocol (37). An appropriate volume of cells was diluted into 2 mL of Z-buffer plus β- mercaptol. Cells were lysed by adding 100 μL chloroform and 50 μL 0.1% SDS and vortexing well. The cell debris was removed by microfuging, and 1.5 mL supernatant was equilibrated at 30°C prior to the assay. For the assay 0.15 mL 4 mg/mL ONPG (pre- equilibrated at 30°C) was added as substrate and the change in A_{420} was followed by a rate assay in a thermostatted spectrophotometer (Shimadzu UV 260). It has been observed that following the time course of the change in absorbance improves the accuracy of the assay and also increases the dynamic range. Since the cell debris was spun down prior to the assay, measurement of A_{550} was unnecessary (37). Specific activity is expressed in Miller units (37).

Cells analysed for chloramphenicol acetyltransferase (CAT) activity were disrupted by sonication. The soluble fraction was assayed using ^{14}C labeled butyryl CoA (New England Nuclear) according to recommended protocols (39). CAT activity is expressed in units CAT/mg total soluble protein. Standard CAT (Sigma) was used to calibrate the assay.

6.3 Results.

Primary structure of the Vitreoscilla hemoglobin upstream region: The nucleotide sequence of the promoter region of the hemoglobin gene is

shown in Figure 1. Deletion analysis of the region 3' to the structural gene showed that the sequences downstream of the putative transcriptional terminator (25) were unessential for oxygen-dependent regulation (data not shown). This is corroborated by the results of the gene fusion experiments discussed below. Analysis of the sequence of the upstream region reveals a consensus Pribnow box and a ribosome binding site (25), as well as a perfect inverted repeat element downstream of the Pribnow box (Figure 1). Interestingly, despite the high strength of this promoter, it apparently lacks a consensus -35 region. However, approximately 20 bp upstream of the Pribnow box, there exists a region of strong homology with the CAP binding site near the *lac* promoter (15) (Figure 1). This led to the hypothesis that a functional cAMP-CAP complex is required to induce the promoter to its normal level of activity. This hypothesis is investigated below.

Oxygen dependent synthesis of hemoglobin mRNA and protein: Plasmid pOX1 was constructed by inserting the *Hind*III-*Sph*I fragment containing the hemoglobin gene from pRED4 into corresponding restriction sites in pBR322. In order to determine the response of the promoter to varying DO levels, strain HB101/pOX1 was grown to mid-log phase under well aerated conditions (DO greater than 60% air-saturation) and then subjected to a transient (initially approximately linear) decrease in DO level. The duration of this transient (ca. 40 minutes) is greater than the characteristic time (ca. 5 minutes) for response of mRNA levels in *E. coli*. During such a relatively slow change in DO,

one will observe at each time a VHb mRNA level which corresponds approximately to the steady state value for that instantaneous DO concentration. Thus, by using a slow ramp-shaped transient instead of a step change, the DO level at which induction occurs can be observed. More importantly, one can determine approximately the DO value at which induction is maximum.

As can be seen in Figure 2, regulation of the hemoglobin gene is transcriptional. When present on a multicopy plasmid, the gene appears to be induced when the DO falls below 40% air saturation; however this value may be different when only a single gene copy is present. Nevertheless, maximal gene expression occurs at DO levels around or below 2% air saturation (i.e. below the lower limit of sensitivity of the galvanic DO probe used to make these measurements). An interesting and potentially important observation is that the level of transcription decreases as the oxygen availability reduces still further and drops to almost the uninduced level when strict anaerobic conditions are obtained by sparging nitrogen into the fermentor. This result was checked by growing the cells in sealed bottles in deaerated medium that was pre-equilibrated with nitrogen. Cells thus grown were neither red in color (indicating the presence of functional hemoglobin) nor did they contain inclusion bodies. Reddish cells containing inclusion bodies are characteristic of cells grown under hypoxic, but not quite anaerobic conditions, such as in a culture tube (26).

Figure 2 illustrates a lag in active hemoglobin synthesis relative to message synthesis. This could be due to the requirement of elevated heme biosynthesis or it could indicate the presence of additional regulatory mechanism(s) operating at the post-transcriptional level (see below).

Two potential sources of artifacts may have contributed to the above results. First, the experiment was conducted with the gene present on a multicopy plasmid. This was necessary to obtain globin activity that could be quantitated with reasonable accuracy in a moderately sized sample. However, possible effects due to changes in copy number or plasmid DNA superhelicity cannot be ignored. Second, the relatively short time scale of the transient may have precluded some physiologically relevant event from occurring in the cell that could affect promoter response (e.g. de novo synthesis of a cascade of regulatory proteins). Both these issues were qualitatively addressed by constructing two single-copy integrant *E. coli* strains containing the globin gene under the regulation of its native promoter. The construction of two such strains, GRO21 and GRO22, is described in the Materials and Methods section. Examination of two such independent strains helped eliminate the possibility of positional effects of chromosome integration of a foreign gene in *E. coli*. GRO21 and GRO22 were grown in shake flask cultures, wherein the available oxygen concentration would be expected to decline monotonically with time (as the cell density increases) throughout the growth phase of

the experiment. Figure 3 shows the specific VHb concentration of cell samples as a function of increasing cell density (i.e. decreasing dissolved oxygen). As can be seen, VHb is virtually absent from early exponential cells that are well- aerated. In both cases intracellular VHb concentrations increase in response to oxygen limitation (as evidenced by a decrease in specific growth rate), and eventually decrease as the cell density increases beyond a certain point. Hence, even though the results in Fig. 3 do not provide any evidence for the level at which oxygen- dependent control occurs, they corroborate the conclusions drawn from the experiment with the *VHb* gene on a multicopy plasmid.

Mapping the transcriptional start site(s) of the promoter: Nucleotide sequencing of the promoter element revealed a putative Pribnow box that matched the consensus sequence (see above). However, the Northern blot in Figure 2a suggested a possible existence of a second (weaker) promoter located somewhere upstream of the major promoter element. To identify the promoter element(s) that are employed *in vivo* the 5' end(s) of *VHb* mRNA was mapped by primer extension analysis. For this purpose, a few representative samples of total mRNA from the experiment described in Figure 2 and the previous section were used. These corresponded to different oxygen concentrations. A 30-mer oligonucleotide probe corresponding to bases 142 to 171 (Fig. 1) was hybridized to the RNA preparations. The same oligonucleotide was used as a primer in standard sequencing reactions

with pOX1 as the template (Fig. 4). The products of the primer extension reaction are shown in Figure 4 (lanes 1-4). The major product was a polynucleotide that terminates at position 86 (Figure 1). A second less intense band corresponding to position 33 (Figure 1) was also observed. Some minor products were also observed which may be the result of premature termination of transcription by reverse transcriptase caused by secondary structure of mRNA in this region. Since DNA-RNA hybridization in primer extension analysis is carried out under conditions of excess oligonucleotide, the amount of extended product observed is indicative of the amount of *VHb* mRNA in the sample. Thus it can be seen that both the promoters are induced similarly in response to oxygen limitation. This also agrees with the coarse resolution that is obtained from the Northern blot (Fig. 2a) and therefore suggests that oxygen- dependent expression from the two promoters is controlled by the same mechanism. The major (downstream) transcriptional start site is located at the expected position relative to the consensus Pribnow box; this promoter is designated P1 (Fig. 1) and its structure is discussed in an earlier section. Located 6 bp upstream of the weaker promoter (designated P2, Fig. 1) is the sequence TTAAAA which may serve as a -10 region. However, since this hexamer is located only 20 bp downstream of the *HindIII* site, which corresponds to the 5' end of the globin gene fragment isolated from a *Vitreoscilla* genomic library (25) (numbered 1 in Figure 1), the physiological significance of this promoter may be

questionable. To address this issue the primer extension assay was repeated with total mRNA samples from MC1061/pOX11 and the same oligonucleotide primer. Plasmid pOX11 contains a translational fusion between the *VHb* gene and *lacZ* (see Materials and Methods section and also below). As shown in Figure 1 the nucleotide sequence upstream of the TTAAAA site is significantly different in this plasmid. The results of Figure 4 (lanes 5-6) indicate that not only is activity of P2 retained, but the strength of P2 relative to P1 remains similar. This suggests that the relevant domains involved in recognition of P2 by RNA polymerase are likely to be present on the *Vitreoscilla* genomic fragment. However, conclusive evidence with regard to this must await further genetic and biochemical experiments. Furthermore, the possibility that additional regulatory loci exist within the flanking sequence in the *Vitreoscilla* genome remains to be investigated.

Characterization of promoter function using lacZ fusions: In order to identify the regions in the upstream sequence that are important for oxygen-dependent regulatory activity and to study the promoter function via gene fusions, 5' and 3' deletion mapping experiments were conducted. The unique *Hin*dIII site at the 5' end and a unique *Bsu*36I site approximately 150 bp into the structural gene were used to generate 5' and 3' deletions, respectively. Several 3' deletions were isolated; however, the promoter region was extremely resistant to generating viable 5' deletions by both of the *in vitro* techniques that were tried. The smallest deletion that was isolated mapped 2 bp upstream of the

start codon. Whether this is an artifact of the deletion techniques used or is due to the potential lethality of an improperly regulated strong promoter is as yet unknown. The deletion endpoints of two 3' deletions, pRED503 and pRED509, are shown in Figure 1. These were used to create translational and transcriptional fusions, respectively, for further genetic analysis.

To facilitate further genetic analysis of the promoter function a *lacZ* protein fusion, pOX11, and a *lac* operon fusion, pOX21, were constructed using the promoter deletions in pRED503 and pRED509, respectively. The construction of these plasmids is described in the Materials and Methods section. The plasmids were transformed into MC1061. To test for oxygen-dependence of *VHb-lacZ* expression in the two strains, the specific activity of β-galactosidase was monitored over the time course of shake-flask cultivations. These results are shown in Figure 5 a and b. Since oxygen levels can be assumed to decrease monotonically over the course of growth, the specific activity can be alternatively plotted as a function of cell density for the two strains grown under identical conditions (Figure 5c). This provides a basis for evaluating the oxygen dependence of the promoter in a qualitative sense without making dissolved oxygen measurements. As can be seen in Fig. 5c, both the deletions retain the central features of oxygen-dependent expression: the promoter is induced in response to oxygen limitation, and there exists an optimum level of oxygen concentration (somewhere in the microaerobic regime) beyond which the

β-galactosidase activity falls. Furthermore, since the response of both the fusions to oxygen availability is similar, one can rule out the possibility of any oxygen-dependent control at the post-transcriptional level.

Effect of catabolite repression on promoter activity: Sequence homology had implicated a possible involvement of the catabolite activator protein (CAP) in the overall control of this promoter (see above). This is consistent with other observations that the presence of glucose reduced the amount of globin activity in *E. coli* cells. To address this question, plasmid pOX11 was transformed into MC4100, GE1050 (crp^-), and GE1051 (cya^-). As can be seen in Figure 6, promoter activity is substantially reduced in strains that are unable to synthesize CAP or cAMP. Furthermore, at comparable cell densities in a culture tube specific β-galactosidase activity in GE1051 was ten-fold higher in the presence of cAMP. (At an OD_{600} of 0.8-0.85, the specific activity of β-galactosidase in GE1051/pOX11 was 500 Miller units in the presence of 4 mM cAMP and 50 Miller units in the absence of cAMP). Taken together these results suggest that the cAMP-CAP complex is involved in modulating the activity of this promoter either directly or indirectly.

A strategy for oxygen-dependent induction of gene expression: The *VHb* promoter offers a convenient (and economical) method for the heterologous expression of foreign genes in *E. coli*, particularly at larger scales of growth. Figure 7 illustrates that high levels of expression of

the CAT gene product from plasmid pOX2 (described in the Materials and Methods section) can be induced in a well sparged fermentor by merely reducing the oxygen supply to the fermentor at the time of induction. These results are in agreement with the above observations which suggest that microaerobic conditions are required to achieve maximum activity of the promoter. In a log phase culture this state is achieved merely by allowing surface aeration.

6.5 Discussion.

Studies on regulatory responses to varying oxygen availability in diverse systems raise two key questions. First, what are the mechanisms that are responsible for sensing changes in oxygen availability in the environment, and how is this information transduced to the regulatory elements that control gene expression? For example, the presence of abundant oxygen in the environment prevents the expression of nitrogen fixation genes in *Klebsiella pneumoniae* (a facultative aerobe) (2), *Rhizobium meliloti* (an obligate aerobe) (11), and *Bradyrhizobium japonicum* (an obligate aerobe) (13). However the mechanism of oxygen influence is different in these bacteria. In *K. pneumoniae* the oxygen status affects the supercoiling of the *nifLA* promoter (10), and also causes nifL to modulate the activity of the nifA activator protein (2). In *R. meliloti* microaerobicity induces the expression of *nifA* (11), probably by influencing the activity of a transmembrane sensor/ transcriptional activator couple (9). In *B. japonicum* the presence

of oxygen irreversibly inactivates the nifA activator protein, which contains a potential redox sensitive/ metal binding center (30). In the yeast *Saccharomyces cerevisiae* at least two loci have been identified that are involved in the regulation of oxygen-dependent genes. Heme functions as an effector molecule in both these regulatory systems (34,50). In the case of HAP1, which activates the expression of the CYC1 and CYC7 genes, heme mediates induction by counteracting the masking of the DNA binding domain of the protein (41). Indirect evidence has also suggested that heme or a heme protein is involved in activation of erythropoetin in a hepatoma cell line in response to hypoxic conditions (16).

The second area of central questions concerns whether all these regulatory circuits respond in a monotonic fashion to increasing or decreasing DO levels. Do there exist mechanisms that, for example, are maximally induced under microaerobic conditions as compared to anaerobic or highly aerobic conditions? On biochemical grounds the presence of different oxygen-sensitive sensors as discussed above might argue for the existence of a variety of induction patterns. For example, optimal induction of gene expression under microaerobic conditions has been demonstrated in the case of nifA mediated activation of the nitrogenase promoter in *R. meliloti* (11).

The main conclusion that can be drawn from this study is that the regulation of the *Vitreoscilla* hemoglobin promoter in *E. coli* is transcriptional. Gene expression attains a maximum under microaerobic con-

ditions (DO less than 2% air saturation) and is substantially reduced under strict anaerobic conditions. This suggests that facultative aerobes like *E. coli* may possess multiple regulatory mechanisms which respond to changes in oxygen availability and that different mechanisms may have different maxima. For example, enzymes such as superoxide dismutase are required under high oxygen concentrations (23), whereas several proteins are expressed as part of the *fnr*-activated regulon under strictly anaerobic conditions (8,24). In addition, there also exist mechanisms that activate proteins such as cytochrome *d* in response to decreasing oxygen concentrations (14). Whether these regulatory circuits share any common intracellular sensor or transducer molecules remains unknown.

Several studies of gene activation (and chemotaxis) in procaryotes in response to environmental changes have led to the emergence of a common theme (9,43). Typically, a transmembrane protein detects an environmental change and transduces the signal to modulate the activity of a transcriptional activator. In the case of gene activation in response to decreasing oxygen availability, the inability of the cell membrane to function as an effective barrier against oxygen diffusion obviates the need for the sensing protein or metabolite to be membrane- anchored. It might be noteworthy, however, that the receptor for aerotaxis in *Salmonella typhimurium* is cytochrome *o* (32). It is not known whether the same protein is involved, directly or indirectly, in gene regulation. More recently two loci have been identified

that mediate repression of enzymes of aerobic pathways (eg. certain TCA cycle and aerobic respiratory enzymes)in *E. coli* (19,20). It has been suggested that the products of these two genes form a sensor-regulator couple. Should this be the case, it is conceivable that one or both of these products are also involved in controlling genes that are expressed in the opposite way.

Functional regulatory activity of the VHb promoter in *E. coli* suggests that this mechanism of regulation is fairly well conserved among both obligate and facultative aerobes. In another study, constitutive expression of the *Bradyrhizobium japonicum nifA* regulatory gene in *E. coli* activated a *nifA* dependent *B. japonicum* promoter under microaerobic conditions (12). Among other possibilities, this suggested that both *B. japonicum* and *E. coli* may possess a well-conserved mechanism for sensing oxygen changes in the environment and in turn altering the activity of regulatory proteins. The results of this study imply that both the sensing and regulatory mechanisms involved in this control circuit are conserved in a variety of gram-negative bacteria. Hence, genetic dissection of this promoter in *E. coli* might facilitate a similar investigation in other bacteria in which a homologous mechanism exists, particularly those in which genetic techniques are not as well developed (e.g., bacteria of the Beggiatoa family).

The presence of two promoters upstream of the *VHb* gene may have interesting implications. It suggests that multiple factors may be involved in controlling the overall level of globin expression. For

example, P1, which is the stronger promoter, may be influenced by catabolite repression. Thus, in the presence of a preferred carbon source, oxygen limiting conditions could lead to globin expression primarily from P2. It could also be possible that P2 is activated under conditions which lead to the presence of a different form of RNA polymerase holoenzyme. Isolation of *cis* and *trans* mutants that affect promoter function in *E. coli* may be helpful in addressing such questions. Since both P1 and P2 respond to oxygen changes similarly, they are likely to be under the control of a common mechanism. Given the arrangement of the two promoters, a negative regulatory mechanism is a likely candidate.

The observation of a lag in the synthesis of active VHb protein suggests that the availability of free heme may be a limiting factor. Feedback regulation of heme biosynthesis by an intermediate metabolite has recently been suggested (33). In this case, the lag could imply the presence of a regulatory mechanism preventing translation in the absence of heme or improper folding of the nascent polypeptide, leading to eventual degradation. It could also indicate the existence of a metastable folded state of the protein that is capable of incorporating heme, when available, to form active VHb.

The regulatory role of the cAMP-CAP complex has been well studied in the context of catabolism of preferred carbon sources (35). Addition of cAMP to cells growing in the presence of glucose has also been shown to elevate the synthesis of some TCA enzymes (19)

and cytochromes (5), as well as increase the efficiency of oxidative phosphorylation (17). Furthermore heme biosynthesis is also under the control of this mechanism (42). Hence, although it is shown in this work that both CAP and cAMP are required for the activity of the *VHb* promoter, it is not clear whether this is a direct or indirect effect. The presence of a region homologous to the CAP binding site of the *lac* promoter located around the -35 region of the major promoter argues for direct interaction between CAP and the promoter. However, alternative possibilities cannot be ruled out. For example, heme may be a component of an activator molecule in the overall regulatory circuit. If this is the case, heme levels decreased by absence of active CAP could account for lower activity of the *VHb* promoter. However, should *E. coli* CAP interact directly with the *VHb* promoter, investigation of an analogous activity in *Vitreoscilla* may be of interest.

The isolated promoter can be used to express high levels of a cloned gene product in an oxygen-dependent manner. Since high cell density batch cultures naturally progress towards an oxygen-limited regime of growth, this promoter provides an attractive alternative for heterologous expression of genes in *E. coli* without the requirement of a gratuitous inducer or temperature shifts which may have deleterious side-effects (44).

6.6 Acknowledgements.

The authors wish to acknowledge Dr. George Weinstock for providing several strains and plasmids used during the course of this

work. We also thank one of the reviewers of this manuscript for suggesting the possible existence of two distinct transcripts in the experiment described in Fig. 2. This work has been supported by the Energy Conversion and Utilization Technologies Program of the U.S. Department of Energy.

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Table I: Bacteria, bacteriophages, plasmids

Name	Description	Origin (reference)
Bacteria		
MG1655	*E. coli* **K12**	Cold Spring Harbor Lab.
JM101	*supE thi Δ(lac-proAB) [F' traD36 proAB lacI^q ZΔM15]*	**53**
HB101	*F$^-$ hsdS20 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1 supE44 λ$^-$*	**36**
GRO21	**MG1655** *lac::Tn10(dVHb-kan)*	This work
GRO22	**MG1655** *xyl::Tn10(dVHb-kan)*	This work
MC1061	*araD139 Δ(ara-leu)7697 ΔlacX74 galU galK r$^-$ m$^+$ strA*	**7**
MC4100	*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 ptsF25 deoC1*	**6**
GE1050	**MC4100** *Δcrp cam*	G.E. Weinsto
GE1051	**MC4100** *Δcya854 ilv::Tn10*	G.E. Weinsto
Bacteriophages		
P1vir	**P1vir**	**45**
Plasmids		
pRED4	**pUC19** *VHb*	**25**
pOX1	**pBR322** *Δtet VHb*	This work

pINT2	**pBR322** Δ*tet VHb*	This work
pINT4	**pBR322** Δ*tet VHb kan*	This work
pMM12	**pBR322** Δ*tet Tn10d(cam)*	Cold Spring
	tac'-'IS10Rtnp	Harbor Lab.
pINT6	**pBR322** Δ*tet Tn10d(VHb kan)*	This work
	tac'-'IS10Rtnp	
pMS421	**pSC101 spc**^{r} *laci*^{q}	Cold Spring
		Harbor Lab.
pRED503	**pUC19** *VHb'*	This work
pRED509	**pUC19** *VHb'*	This work
pYEJ001	**pBR322** Δ*tet cam*	Pharmacia
pOX2	**pBR322** Δ*tet VHb'-cam*	This work
pMLB1034	**pBR322** Δ*tet 'lacZ lacY'*	3
pMLB1010	**pBR322** Δ*tet* ϕ*(trpA-lacZ)(Hyb) lacY'*	3
pGE245	ϕ*(recA-lacZ)(Hyb)* **M13 IG region**	49
pCO1	**pBR322** Δ*tet 'lacZ lacY'*	This work
	M13 IG region	
pCO2	**pBR322** Δ*tet* ϕ*(trpA-lacZ)(Hyb)*	This work
	lacY' **M13 IG region**	
pOX11	**pCO1** ϕ*(VHb-lacZ)(Hyb)*	This work
pOX21	**pCO2** ϕ*(VHb-lacZ)*	This work

6.9 Captions to Figures.

Figure 1: Primary structure of the oxygen-regulated promoter element: The nucleotide sequence of the upstream region required for regulated expression of the *VHb* gene in *E. coli* is shown. The start codon of the structural gene is underlined. An inverted repeat element is shown with arrows. Bases marked as 503 and 509 correspond to the 3' ends of the truncated promoter in plasmid pRED503 and pRED509, respectively. The nucleotide designated 1 corresponds to the 5' end of the *VHb* gene containing fragment isolated from a *Vitreoscilla* genomic library (25). The sequence upstream of this base represents adjacent pBR322 DNA on plasmid pOX1. The sequence of the junction between the 5' end of the promoter fragment and vector DNA in plasmid pOX11 is shown immediately below this region. The origins of arrows designated P1 and P2 correspond to the transcriptional start sites of the two promoters. The putative Pribnow boxes of P1 and P2 are indicated with a double line and a dashed line, respectively. The sequence indicated below nucleotides 41-50 is a portion of the CAP binding site upstream of the *lac* promoter, which has previously been identified by footprinting studies (15). For details, see Results section.

Figure 2: Oxygen dependent regulation of the Vitreoscilla hemoglobin gene in E. coli: HB101/pOX1, grown in a fermentor (see materials and methods), was subjected to a transient decrease in dissolved oxygen (DO) concentration (see results), and hemoglobin mRNA and protein

activity were monitored. (a) Northern blot analysis of hemoglobin mRNA content. The numbers above the lanes indicate the times at which the samples were taken. The initial point corresponds to the sample taken before commencing the transient in DO. RNA from HB101/pBR322 was included as a control in the blot. The higher molecular weight bands correspond to plasmid DNA. (b) Normalized intracellular RNA content and protein activity (see materials and methods) are plotted against time. The DO trajectory is also shown. The vertical arrow on this curve corresponds to the point at which anaerobic conditions were introduced by sparging nitrogen into the fermentor. Since the experiment was not followed beyond 100 min, the significance of the decrease in *VHb* mRNA level at 90 min (see Fig. 2a) is unclear.

Figure 3: Oxygen dependent synthesis of globin in E. coli cells containing a single copy of the VHb gene: Western blot analysis of total cellular protein is shown. *E. coli* strains MG1655 (control), GRO21 (A), and GRO22 (B) were grown in 250 mL shake flasks containing 100 mL medium. Samples were taken at different points along the growth curve. The number above each lane represents the OD_{600} of the sample. Each lane contains total cell protein that corresponds to 5 OD_{600}-μL cells. Thus the relative intensities of the globin bands are indicative of the specific globin concentration in the samples. The faint bands that appear above the major band (VHb) are the result of cross-reaction of the antiserum with other antigens. This is probably due to the

presence of trace quantities of contaminant proteins in the purified VHb sample that was used to raise the antiserum.

Figure 4: Mapping the transcriptional start sites within the oxygen- dependent promoter element: The sequence ladders labeled pOX1 and pOX11 were generated by running the sequencing reaction products of the two plasmids using the same 30mer oligonucleotide primer (see text). Lanes 1-4 contain the primer extension products from RNA templates in the 21, 38, 70, and 100 min samples in Fig. 2a, respectively. Lanes 5 and 6 contain products from RNA templates of MC1061/pOX11 and MC1061/pCO1 (control), respectively. These total RNA samples were prepared from cells grown in culture tubes (5 mL) upto OD_{600}= 0.8. The two main reaction products in the above samples are indicated by arrows.

Figure 5: Oxygen- dependent expression of β-galactosidase from VHb-lacZ fusions: 250 mL shake flasks containing 100 mL liquid volume were inoculated with 0.1% overnight cultures and the specific activity of β-galactosidase was monitored through the growth phase. Cell density and specific activity are shown as a function of time for (a) MC1061/pOX11 (X) and (b) MC1061/pOX21 (O). The data from (a) and (b) is represented in (c) with the specific activity shown directly as a function of cell density. In this figure, the specific activity of MC1061/pOX21 is shown on double scale.

Figure 6: Effect of cAMP-CAP on the activity of the VHb promoter: 250 mL shake flasks containing 100 mL liquid volume were inoculated with

0.1% overnight cultures of MC4100/pOX11 (O), GE1050/pOX11 (Δ), and GE1051/pOX11 (X). The specific activity of β-galactosidase was monitored along the growth phase. Similar to Figure 5(c), specific activity is directly plotted as a function of cell density.

Figure 7: Oxygen dependent expression of CAT: HB101/pOX2 was grown in a fermentor under conditions described in the materials and methods section. At t=0, the air flow rate was decreased in order to reduce the DO level to the range 2-5% air saturation very rapidly. This condition was maintained until steady state was reached with respect to CAT activity. Thereafter, the sparging system was completely turned off (indicated by the vertical arrow), allowing oxygen uptake to occur only at the liquid surface. Another assay was conducted after allowing sufficient time for attaining a new steady state with greater CAT activity.

Figure 1.

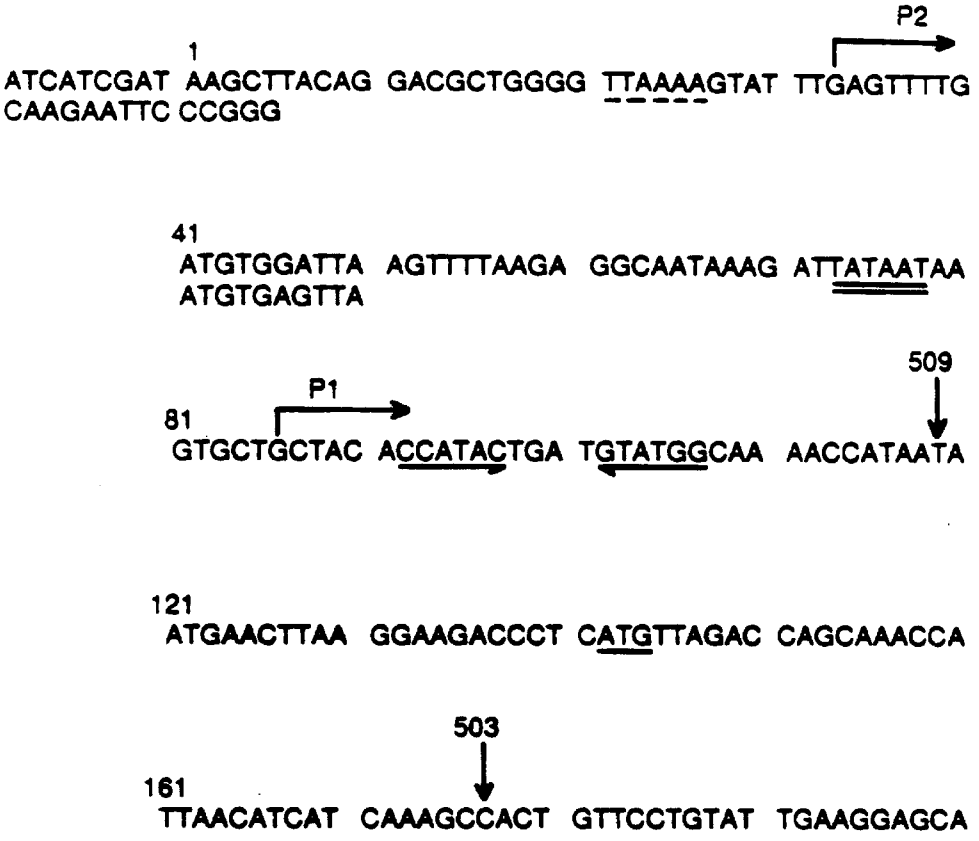

P2
1
ATCATCGAT AAGCTTACAG GACGCTGGGG TTAAAAGTAT TTGAGTTTTG
CAAGAATTC CCGGG
41
ATGTGGATTA AGTTTTAAGA GGCAATAAAG ATTATAATAA
ATGTGAGTTA
509
P1
81
GTGCTGCTAC ACCATACTGA TGTATGGCAA AACCATAATA
121
ATGAACTTAA GGAAGACCCT CATGTTAGAC CAGCAAACCA
503
161
TTAACATCAT CAAAGCCACT GTTCCTGTAT TGAAGGAGCA

Figure 2a.

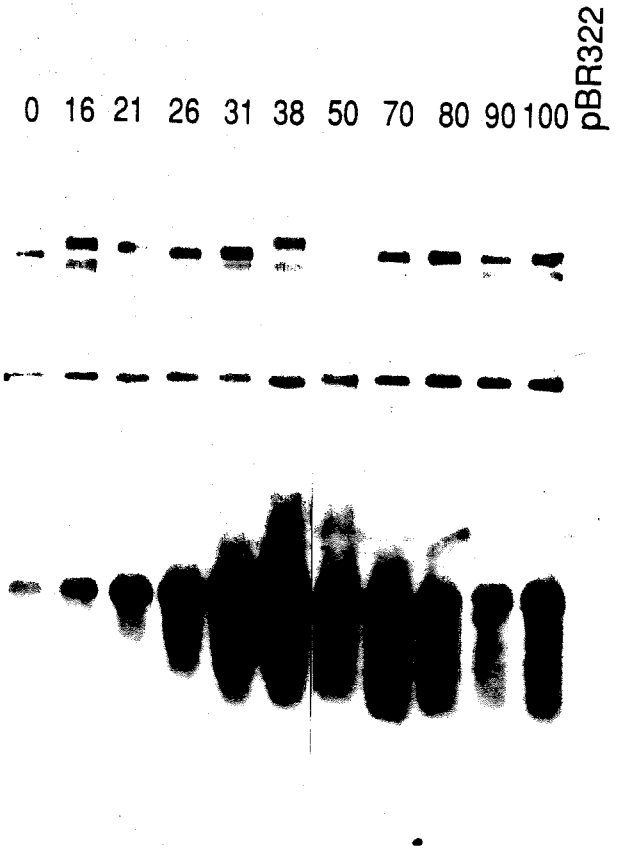


Figure 2b.

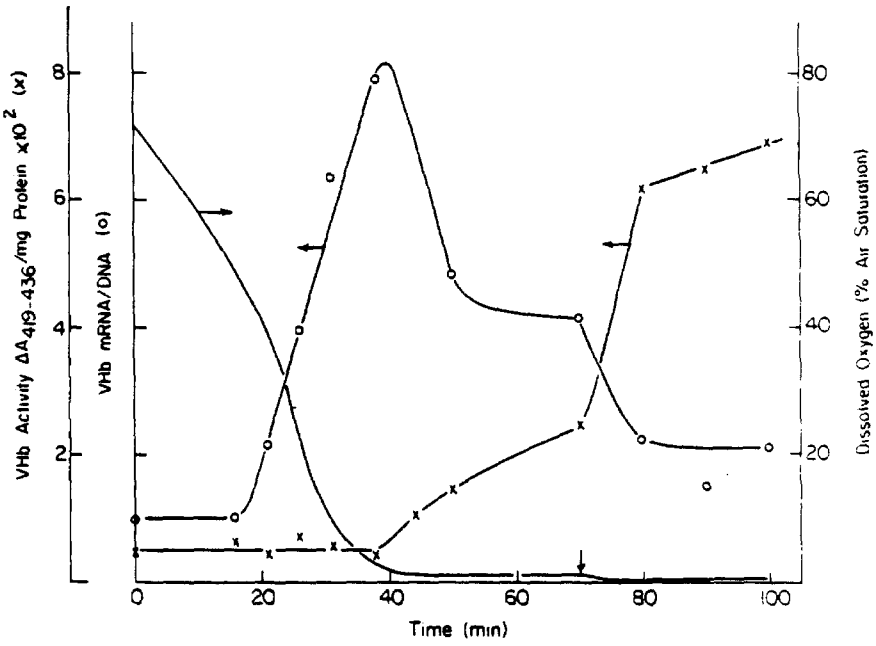

VHb Activity $\Delta A_{419-436}$/mg Protein x10^2 (x)
VHb mRNA/DNA (o)
Dissolved Oxygen (% Air Saturation)
Time (min)
0
20
40
60
80
100
2
4
6
8
20
40
60
80

Figure 3.

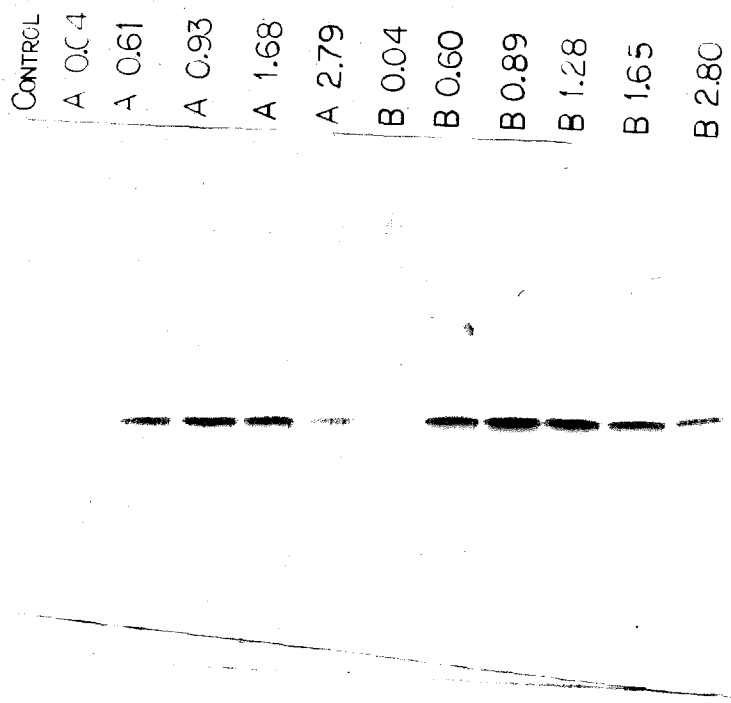

CONTROL
A 0.04
A 0.61
A 0.93
A 1.68
A 2.79
B 0.04
B 0.60
B 0.89
B 1.28
B 1.65
B 2.80

Figure 4.

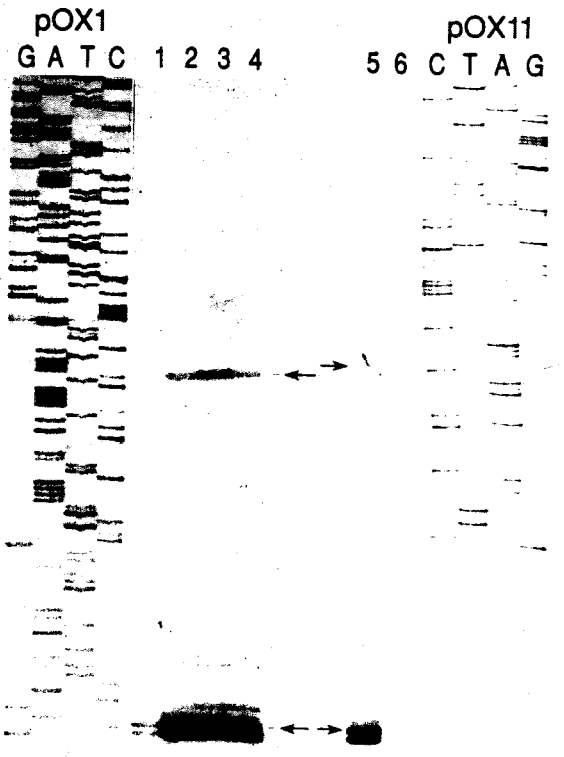


Figure 5a.

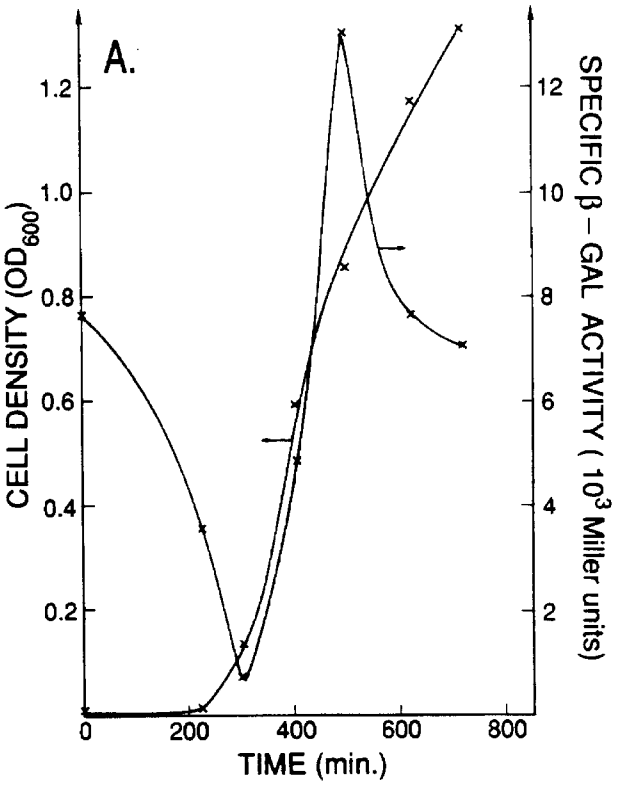

A.
CELL DENSITY (OD_{600})
1.2
1.0
0.8
0.6
0.4
0.2
0
200
400
600
800
TIME (min.)
SPECIFIC β – GAL ACTIVITY (10^3 Miller units)
12
10
8
6
4
2

Figure 5b.

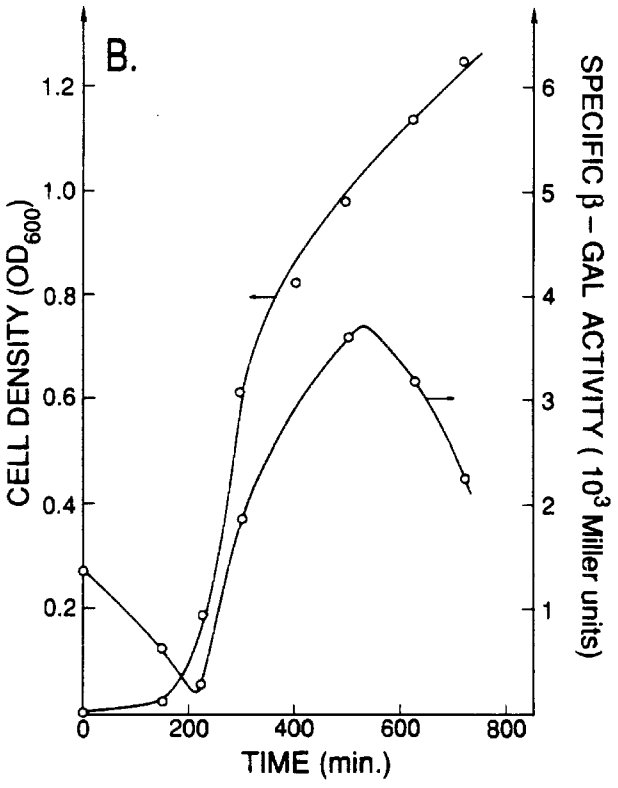

B.
CELL DENSITY (OD_{600})
SPECIFIC β – GAL ACTIVITY (10^3 Miller units)
TIME (min.)
0
200
400
600
800
0.2
0.4
0.6
0.8
1.0
1.2
1
2
3
4
5
6

Figure 5c.

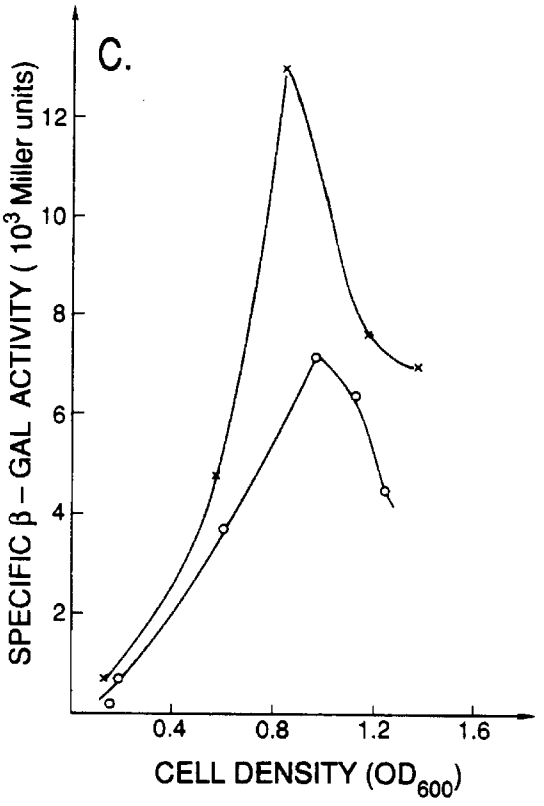

C.
SPECIFIC β – GAL ACTIVITY (10³ Miller units)
12
10
8
6
4
2
0.4
0.8
1.2
1.6
CELL DENSITY (OD600)

Figure 6.

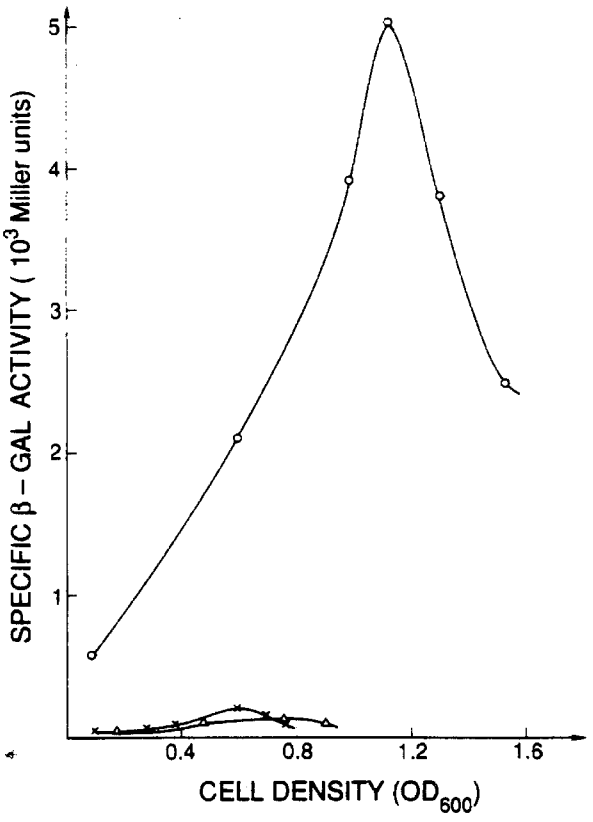
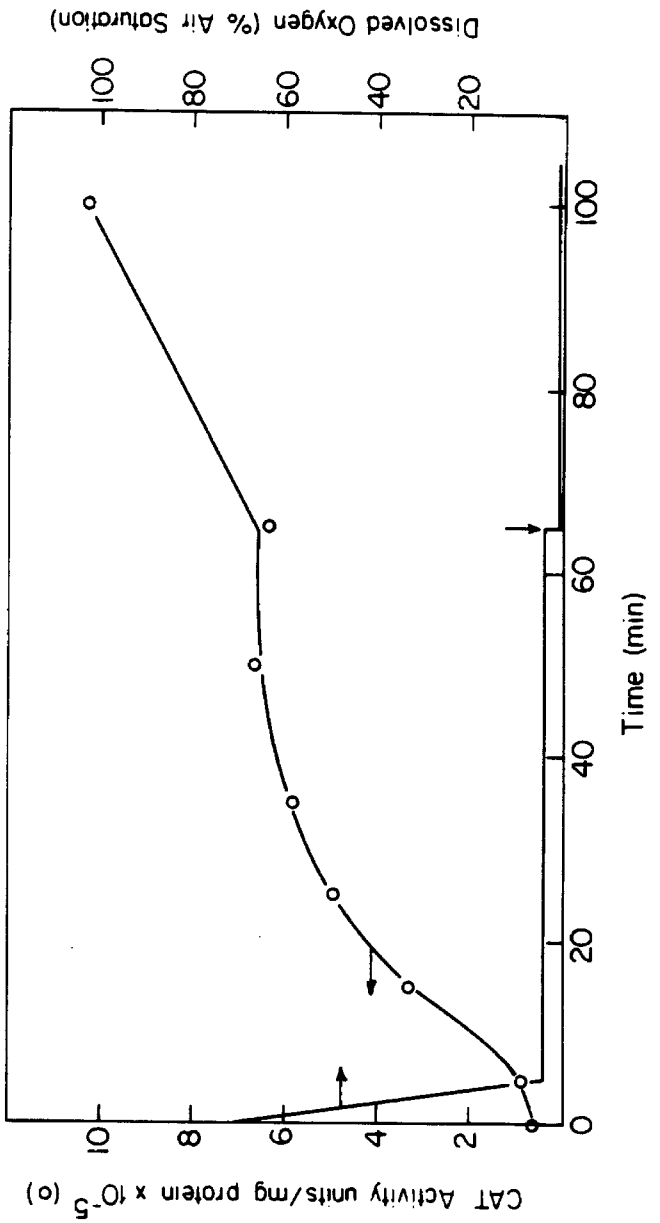

SPECIFIC β – GAL ACTIVITY (10^3 Miller units)
5
4
3
2
1
0.4
0.8
1.2
1.6
CELL DENSITY (OD_{600})

Figure 7.


CAT Activity units/mg protein x 10^{-5} (o)
Dissolved Oxygen (% Air Saturation)
Time (min)
0
2
4
6
8
10
20
40
60
80
100
20
40
60
80
100

CHAPTER 7

EXPRESSION OF RECOMBINANT PROTEINS IN *E. COLI* USING AN OXYGEN-RESPONSIVE PROMOTER

Source: Khosla, C., Curtis, J.E., and Bailey, J.E. (1989) Bio/Technology *submitted*

7.1 Summary.

The oxygen-dependent promoter of the *Vitreoscilla* hemoglobin (*VHb*) gene has been shown previously to be functional in *E. coli*. Earlier studies established that the promoter is maximally induced under microaerobic conditions and that its activity is also influenced by the cAMP-CAP complex. We demonstrate here that the promoter can be used for regulated, high-level expression of recombinant proteins in two-stage fed-batch fermentations. The promoter is maximally induced at dissolved oxygen levels lower than 5% air saturation. Despite the influence of catabolite repression, glucose and glycerol- containing media give comparable product levels under carbon-limited conditions such as those encountered in typical fed-batch fermentations. The possibility of a third level of control of promoter activity is also indicated. This mode of induction can be repressed by addition of a complex nitrogen source such as yeast extract to the medium. The observed promoter activity can be modulated at least 30-fold over the course of high-cell density fermentations producing either cloned β-galactosidase or cloned chloramphenicol acetyltransferase (CAT). Densitometer scanning of SDS-polyacrylamide gels revealed that β-galactosidase was expressed to a level of approximately 10% of total cellular protein.

7.2 Introduction.

Use of *E. coli* as a host of choice for high-level expression of recombinant proteins is widespread in modern biotechnological prac-

tice. Large-scale production of useful proteins is typically achieved via two-stage fed-batch fermentations. In the first stage of such a process, cells are grown to a high cell density under reasonably balanced growth conditions. This requires that the synthesis of the heterologous protein be minimized. In the second stage, high-level expression of the recombinant protein is activated. The properties of the promoter which regulates the expression of the recombinant protein are therefore crucial determinants of process productivity.

Several expression systems have been developed over the past decade for this purpose. The list of useful promoters includes *lac* (1), *trp* (2), *tac* (3), *pL* from bacteriophage lambda under the control of a thermosensitive *cI* repressor allele (4), *recA* (5), *phoA* (6), a promoter recognized by bacteriophage T7 RNA polymerase (7), and several others. The choice of a suitable promoter is usually based on the following three criteria:

1. The *strength* of the promoter, defined as the maximum attainable specific activity of the promoter (r_P). Operationally this can be defined as the specific rate of accumulation of a relatively stable reporter protein such as β-galactosidase or chloramphenicol acetyltransferase (CAT). Thus, $r_P = \frac{1}{X}\frac{dE}{dt}$, where E and X represent the volumetric reporter enzyme activity and cell density, respectively.
2. The *inducibility* of the promoter, defined as the ratio of the maximum to the minimum specific activity of the promoter.

3. The economy and the ease of utilization of the promoter.

It is also recognized that the relative performance of different promoters varies from protein to protein. This often prompts researchers to attempt heterologous expression of proteins of interest using more than one promoter.

During the course of molecular cloning of the *Vitreoscilla* hemoglobin gene (*VHb*), the native promoter of this gene was identified as a strong promoter in *E. coli* since VHb was expressed to a high level in the recombinant cells (8). Growth of the same cells under well-aerated conditions, however, resulted in a significant reduction of VHb concentration, leading to the hypothesis that cloned *Vitreoscilla* sequences upstream of the *VHb* gene served as an oxygen-regulated promoter in *E. coli* (9). More recently, genetic analysis has demonstrated that *VHb* expression in *E. coli* is regulated at the transcriptional level by a molecular mechanism involving recognition of an upstream oxygen-regulated promoter element (ORE) from *Vitreoscilla* by as yet unknown *trans*-acting factors (10). Furthermore, the promoter is maximally induced under microaerobic conditions, and also requires cAMP and the catabolite activator protein (CAP) for full activity (10). Results reported here show that the *VHb* promoter provides a convenient and economical method for regulated, high-level expression of recombinant proteins.

7.3 Results.

In previous shake-flask studies it was shown using *lacZ* fusions that the *VHb* promoter is induced when the cells encounter oxygen limitation over the course of batch growth (10). This results in considerable product accumulation by the time cells approach stationary phase. However, since promoter activity drops considerably as one approaches anaerobicity and since reliable on-line methods for measuring oxygen concentrations in the microaerobic regime do not exist, more quantitative information was required to scale up the performance of this promoter to a high-cell density fermentation process. In most of these studies a plasmid-borne gene fusion between the first 12 codons of *VHb* and a truncated *lacZ* gene under the control of ORE was used as a reporter system. However, to address questions that required a second reporter system, ORE fused to a promoterless *cat* cartridge was also used. The maps of the plasmids are shown in Figure 1.

Quantitation of oxygen-dependent induction during batch growth: Figure 2a shows how the activity of the promoter varies over the course of batch growth under different aeration conditions. From the profiles for growth and specific β-galactosidase activity at a constant aeration rate of 0.08 vvm it can be seen that promoter is activated when the dissolved oxygen level (DO) falls below 5% air saturation. (In our experience 5% can be regarded as the lower limit of accuracy of a standard polarographic probe under the above conditions of use.) However, the specific activity of the promoter is significantly reduced

under conditions of extreme oxygen starvation, which is the case when late log-phase cultures are grown in the absence of any sparging. Hence, although a mechanistic interpretation of these observations is not yet available, the results illustrate a need for development of an induction protocol for fed-batch fermentations which allows for aerated growth at dissolved oxygen levels below the detectable limit of standard DO probes. Figure 2b shows the results from one such protocol, developed using the following two guiding principles:

1. The promoter is maximally induced at DO equal to or below 1% air saturation (i.e., the approximate K_m value of *E. coli* cytochrome o (11).)
2. The $\frac{K_L a}{X}$ ratio corresponding to maximum promoter activity is an appropriate scaling parameter for ORE-based expression systems. This ratio is chosen because it should be proportional to the dissolved oxygen concentration under oxygen- limited conditions.

The results of Figure 2 also suggest that the activity of the promoter is not always fully repressed at high DO and is apparently regulated by some mechanism that is independent of DO. Since previous studies have indicated that catabolite repression affects promoter activity (10), the effect of the type and level of carbon source on this oxygen-independent activity was investigated. Furthermore, the effect of the concentration of the complex nitrogen source on promoter activity was also studied.

Effect of the carbon source on promoter activity: Glycerol was used as the carbon source in the batch experiments described above since it is not transported via the PTS uptake system and hence should allow for elevated cAMP levels compared to an equivalent glucose-containing medium (12). Results in Figure 3a corroborate this expectation: the specific promoter activity, represented in relative terms by the slopes of the profiles at a given cell density (see definition of specific promoter activity in the Introduction section), is lower in the presence of glucose than the activity in the presence of glycerol. (To allow cells to grow to a density comparable to that achieved at stationary phase in Figure 2 without encountering nutrient starvation, these experiments were conducted in a fed-batch mode.) This suggests that the use of glucose as the carbon source in two stage fed-batch fermentations is likely to reduce promoter activity during the growth phase prior to oxygen dependent induction.

Effect of the complex nitrogen source on promoter activity: Since yeast extract is also present in the batch medium used for the experiments of Figure 2, the influence of the concentration of yeast extract on the oxygen-independent activity of the promoter was also investigated. The results of Figure 3b show that increasing the concentration of yeast extract in the batch medium leads to a decrease in the specific activity of the promoter. This suggests that maintaining relatively high levels of yeast extract during the growth phase of a fed-batch operation may be useful in minimizing promoter activity.

In order to investigate the possibility that the observed oxygen-independent activity is peculiar to the host cell or the specific fusion used in the above experiments, similar investigations were conducted in which chloramphenicol acetyltransferase (CAT) was expressed from the *VHb* ORE in a pBR322-based plasmid (pOX2) contained in an unmutagenized *E. coli* K12 host (MG1655). The results of Figure 3c corroborate the presence of oxygen-independent activity, which can be modulated by the concentration of yeast extract. To determine whether the presence of excess yeast extract would adversely affect the oxygen-dependent activity of ORE under microaerobic conditions, these cultures were subjected to oxygen limitation in a manner similar to that described in Figure 2b. The resulting profiles of CAT specific activities showed that, under oxygen-limiting conditions, both the strength and the kinetics of induction of ORE are superior in the presence of higher concentrations of yeast extract (data not shown).

Regulated expression of β-galactosidase and CAT in fed-batch fermentations: The above results provide a basis for the development of a convenient protocol to use ORE-based expression systems in two-stage fed-batch fermentations. Figures 4 and 5 illustrate such processes using cloned CAT and β-galactosidase, respectively, as model products. In the absence of any nutrient limitation, the intrinsic mass transfer properties of the fermentors set limits on the maximum cell density that can be achieved prior to induction. The oxygen transfer capacity of the fermentor interacts with the feeding protocol to

dictate the achievable cell density at the end of the fermentations (12). In each case considered here, the final cell density achieved was approximately 20 g dry cell weight/L. The use of batch and feed media containing yeast extract successfully controls promoter activity during the growth phase of the cultivation, and also allows for rapid growth of cells. In both cases, the specific activity of the reporter enzyme increased at least 30-fold over the course of these fermentations, with at least 12-fold increase achieved by subjecting the culture to oxygen limitation. With both expression constructs, the inducibility of the promoter was better using glucose as the carbon source. Densitometry measurements indicate that β-galactosidase constitutes approximately 10% of the total cellular protein at the end of the fermentation (Figure 5b).

Two observations can be made from the above results. First, sparging with air alone limited the final cell density that could be achieved in these experiments. This maximum cell density can likely be increased through the use of pure oxygen and/or a reduction in the carbon source feeding rate. Second, both glucose and glycerol were used as carbon sources in these experiments. Surprisingly, despite the influence of catabolite repression on ORE, the use of glucose as a carbon source does not appear to adversely affect promoter strength under induction conditions. This could be due to the fact that, in glucose-limited protocols such as the one used here, the extracellular concentration of glucose is low enough to prevent inhibition of

adenylate cyclase activity (13).

7.4 Discussion.

Useful promoters for heterologous gene expression in *E. coli* are typically induced by addition of a gratuitous inducer molecule or by shifting an environmental parameter such as temperature. Each technique has advantages and disadvantages. Whereas the former approach tends to interfere less with host cell metabolism, gratuitous inducers are generally expensive and hence affect the economics of scale-up. On the other hand, environmental manipulation is more amenable to large scale application, but can have deleterious side-effects. For example, high temperatures are known to favor protein insolubilization and may lead to a lower yield of correctly folded product (14).

The microaerobic *VHb* promoter can be manipulated simply through process conditions. In addition to its strength and regulatory characteristics, the chief advantage of the *VHb* promoter is its strong induction at the onset of oxygen limitation, which is a regime of operation eventually approached by most aerobic bioprocesses. Hence the *VHb* promoter provides a convenient system for induction of product formation towards the end of the growth phase. Also, application of this promoter does not appear to be restricted to a specific host genotype. Based on high expression levels noted here and observed for other proteins expressed from the *VHb* promoter (data not shown),

microaerobic conditions are compatible with substantial accumulation of cloned gene product.

The presence of oxygen-independent modes of regulating the activity of the *VHb* promoter provides additional levels of control. For example, by growing cells in non-limiting concentrations of glucose, ORE activity can be reduced. Likewise, addition of yeast extract during the growth phase also decreases ORE activity. It should be pointed out, however, that the observed effects of yeast extract may not be attributable to a regulatory mechanism that involves a single component in yeast extract. The increase in curvature of the profiles in Figure 3b (which is indicative of increasing specific promoter activity) in response to decreasing yeast extract concentrations is suggestive of influence by multiple components, which get exhausted at different cell densities. Then again, yeast extract may merely increase the growth rate, which in turn alters the copy number and/or the superhelicity of the expression plasmid. Plasmid copy number is known to correlate inversely with growth rate (15), and oxygen-regulated gene expression can be influenced by DNA superhelicity (16).

7.5 Materials and Methods.

Bacterial strains and plasmids: MG1655 (λ^-, F^-) is an unmutagenized *E. coli* K12 strain obtained from Cold Spring Harbor Laboratory. 7C1 (IN(*rrnD-rrnE*), λ^-, F^-, *tonA*, *phoA*, *lacU169*) is a derivative of W3110. The construction of plasmids pOX11 and pOX2 is summarized in Figure 1 and described in detail elsewhere (10).

Fermentation conditions: Fermentations were carried out in a Bioflo III fermentor (New Brunswick Scientific) with a working volume of 2.5L. The inoculum was grown in 25 mL LB broth in a 250 mL flask for approximately 12 h. 4 mL of this culture was added to each fermentor. Cells were grown at 37°C and pH 7 under aeration/agitation conditions specified in the captions to the figures. In all cases, the batch medium consisted of 0.5% yeast extract, 0.5% carbon source, 0.4% $(NH_4)_2SO_4$, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.1 (vol)% trace metal mix (8.3 mM Na_2MoO_4, 7.6 mM $CuSO_4$, 8 mM H_3BO_3), 0.1 (vol)% vitamin mix (0.042% riboflavin, 0.54% pantothenic acid, 0.6% niacin, 0.14% pyridoxine, 0.006% biotin, 0.004% folic acid), 1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.2 mM $FeCl_3$, and 50 mg/L ampicillin, unless specified otherwise. The feed medium consisted of 43% carbon source, 11% $(NH_4)_2SO_4$, 0.8% $MgSO_4$, 1 (vol)% vitamin mix, 1 (vol)% trace metal mix, 0.2 mM $FeCl_3$, 0.05 mM $CaCl_2$, and 100 mg/L ampicillin, unless mentioned otherwise. Details of feeding protocols are described in the captions to figures.

Enzyme assays: Cell extracts used for assaying both β-galactosidase and CAT specific activity were obtained by sonication of fermentation samples. β-galactosidase was assayed at 30°C using o-nitrophenyl-β-D- galactopyranoside as the substrate. Change in A_{420} was monitored by a rate assay in a thermostatted spectrophotometer (Shimadzu UV260). Specific activity is expressed as Miller units (17). CAT activity was assayed with ^{14}C-labeled butyryl coenzyme A (New England

Nuclear) according to recommended protocols (18). Specific activity is expressed in units CAT / OD_{600}-mL. Standard CAT (Sigma) was used to calibrate the assay.

7.6 Acknowledgements.

The authors would like to thank Nicole Tedeschi for technical assistance with the fermentations. This research was supported in part by a grant from the Energy Conversion and Utilization Technologies (ECUT) program of the United States Department of Energy.

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7.8 Captions to Figures.

Figure 1: Construction of plasmids pOX2 and pOX11. The relevant features of the two plasmids are summarized in this figure. Plasmid pOX2 carries a promoter fusion between the *VHb* oxygen-regulated promoter element (ORE) and the *cat* gene. Plasmid pOX11 carries a protein fusion between the 5'end of the *VHb* gene and a truncated *lacZ* gene. Both are pBR322-based plasmids and confer ampicillin resistance on the host.

Figure 2: ORE activity in 7C1/pOX11 under oxygen-excess and oxygen-limited conditions. (a) Cell densities (————) and specific β-galactosidase activities (– – – – –) are shown over the course of batch growth with glycerol as the carbon source under different aeration conditions. In one case (○) the DO was controlled above 50% air saturation. In the second case (●) constant conditions of 400 RPM and 0.2 L/min (0.08 vvm) air supply were maintained throughout the course of the experiment. As a result, the activity of the promoter could be studied over wide ranges of DO and cell density. The single arrow denotes the point at which the DO falls below 5% air saturation. In the third case (×) growth conditions were identical to those in the second case, except that at the point indicated by the double arrow the air supply to the fermentor was turned off. (b) Cell density (————) and specific β-galactosidase activity (— — — —) are shown over the course of a fed-batch fermentation with glycerol as the carbon source. Feeding was commenced at 2.5 mL/h at an OD of 2.5. It was increased to 4 mL/h at an OD of 6. The DO was controlled

above 50% air saturation until the point indicated by the arrow, after which constant conditions of 400 RPM and 3 L/min air flow (which resulted in DO falling below the detectable limit within less than 5 minutes) were maintained.

Figure 3: Effect of the carbon source and yeast extract on ORE activity. Volumetric enzyme activities are plotted as a function of increasing cell density under fed-batch growth conditions. In all cases the DO was controlled above 50% air saturation throughout the experiment. Feeding was commenced at 2.5 mL/h at an OD of 2.5 in each case. (a) The effects of the type and amount of carbon source on β-galactosidase expression in 7C1/pOX11 were studied. In two cases cells were grown under carbon limitation with glucose (○) and glycerol (●) as carbon sources. In the third case (×) excess glycerol was maintained in the fermentor at all times by adding 5 g pulses of glycerol per OD increase in cell density. (b) The effect of the concentration of yeast extract on ORE activity in 7C1/pOX11 was studied. The concentrations of yeast extract in the batch medium were 1 g/L (○), 5 g/L (●) and 20 g/L (×). (c) In a similar experiment the specific CAT activity in MG1655/pOX2 is shown. Here the yeast extract concentrations were 5 g/L (●) and 20 g/L (×).

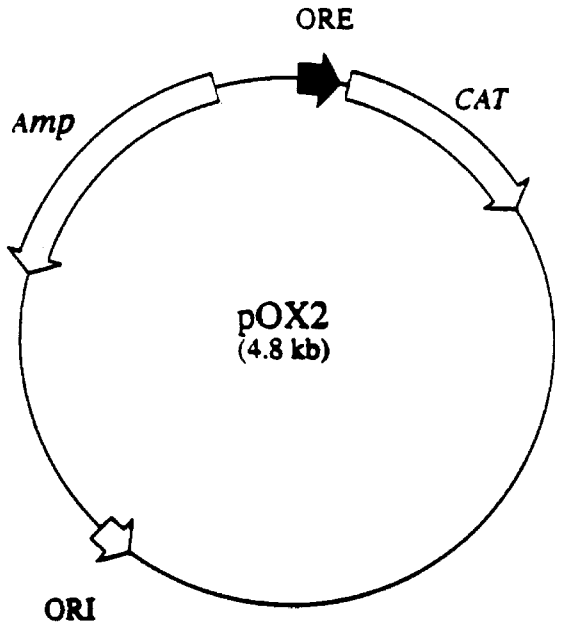
Figure 4: Oxygen-regulated expression of CAT in high cell density fermentations of MG1655/pOX2. Cell densities (————) and specific activities (– – – —) are plotted along the course of typical fed-batch fermentations using glucose (○) and glycerol (●) as carbon sources. In

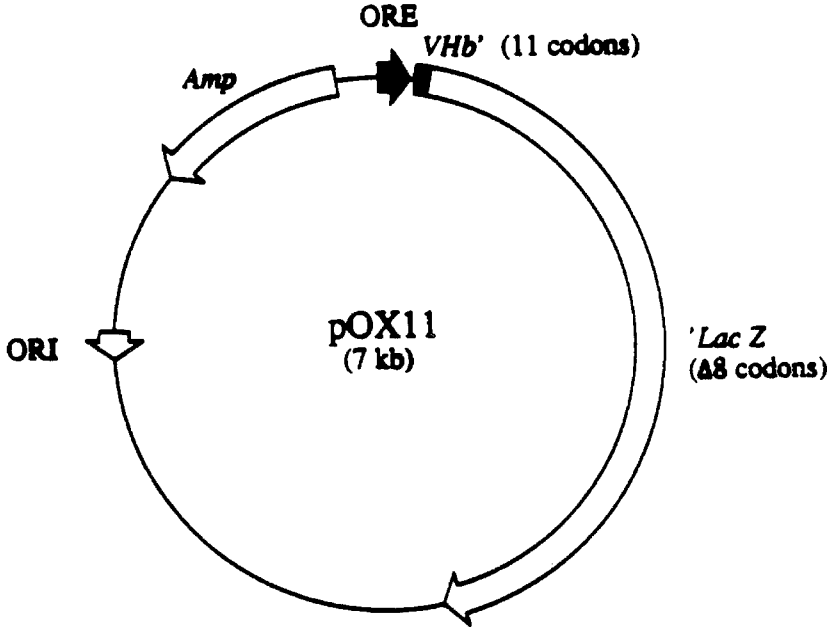
both cases cells were grown under carbon limited conditions with the DO controlled above 50% air saturation until the points indicated by vertical arrows (i.e. until the oxygen utilization rate exceeded the oxygen transfer rate limit of the fermentor, using air as the oxygen source). Thereafter the DO was rapidly dropped to a value below the detectable limit of the probe by maintaining constant conditions of 500 RPM and 3 L/min air flow rate. A dual port pump was used to provide an additional feed of 25% yeast extract at twice the flow rate as the nutrient feed. The following feeding rates were maintained: 3 mL/h between OD 2.5 and 5, 4.5 mL/h until OD 8, 7.5 mL/h until OD 10, and 12 mL/h thereafter.

Figure 5: Oxygen-regulated expression of β-galactosidase in high cell density fermentations of 7C1/pOX11. (a) The growth and induction conditions used in these experiments are identical to those described in the caption to Figure 4. Both glucose (○) and glycerol (●) were used as carbon sources. Cell densities (——) and specific activities (– – –) are plotted as a function of time over the course of the fermentations. (b) SDS-PAGE analysis (19) of samples from different stages of the fermentation described in (a) with glucose as the carbon source. Total cellular protein from the same cell mass was loaded in lanes 1-3. Lanes 1-3 are samples corresponding to 210 min (early log phase), 300 min (pre-induction), and 730 min (end of fermentation) cells (see Figure 5a). Lane 4 shows the total cellular protein from 7C1 cultures. Samples were run on a 7-15% gradient gel and stained with

Coomassie blue.

Figure 1.


ORE
CAT
Amp
pOX2
(4.8 kb)
ORI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VHb' (11 codons)
Amp
ORI
pOX11
(7 kb)
'Lac Z
(Δ8 codons)

Figure 2a.

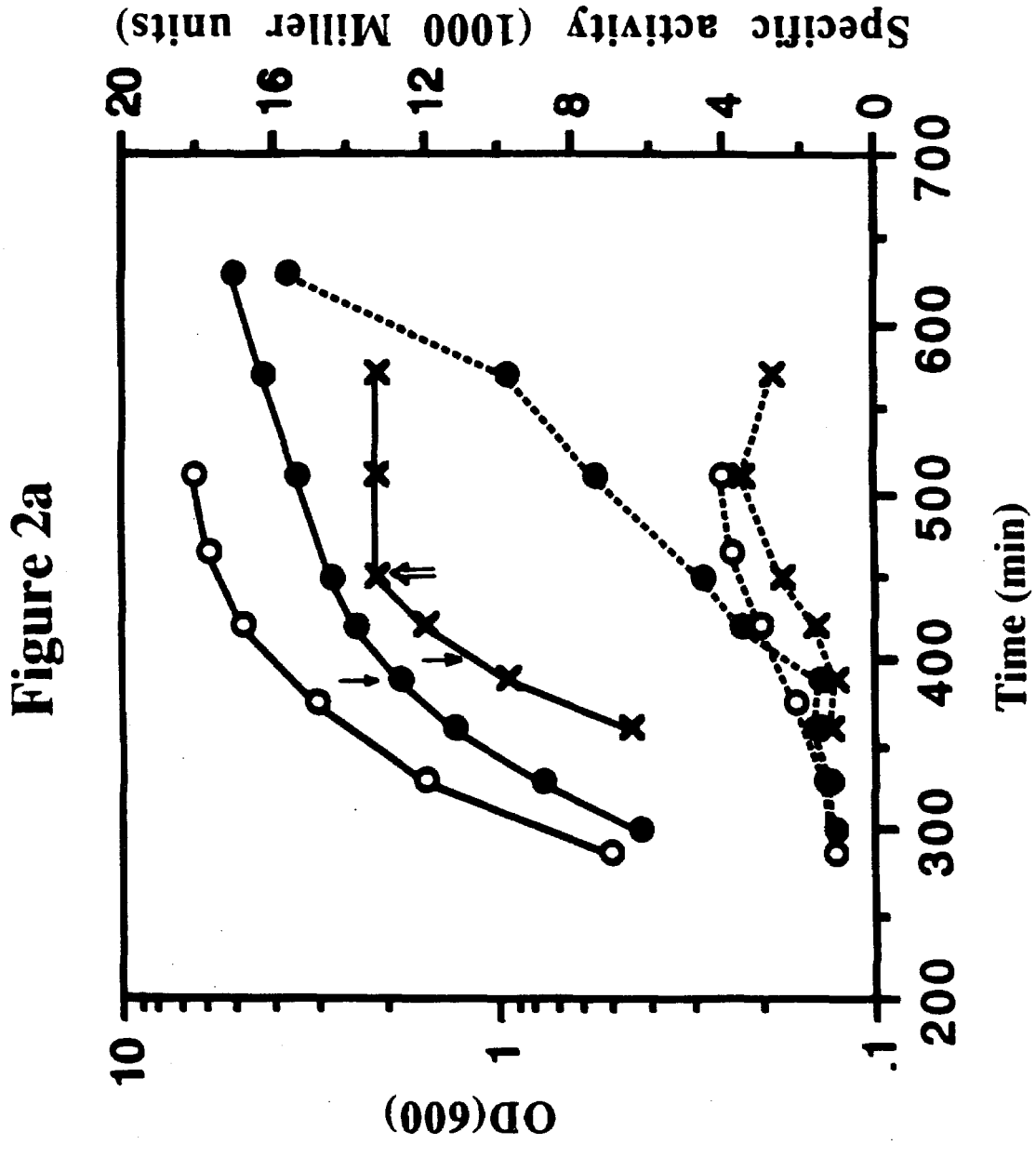


Figure 2a

Figure 2b.

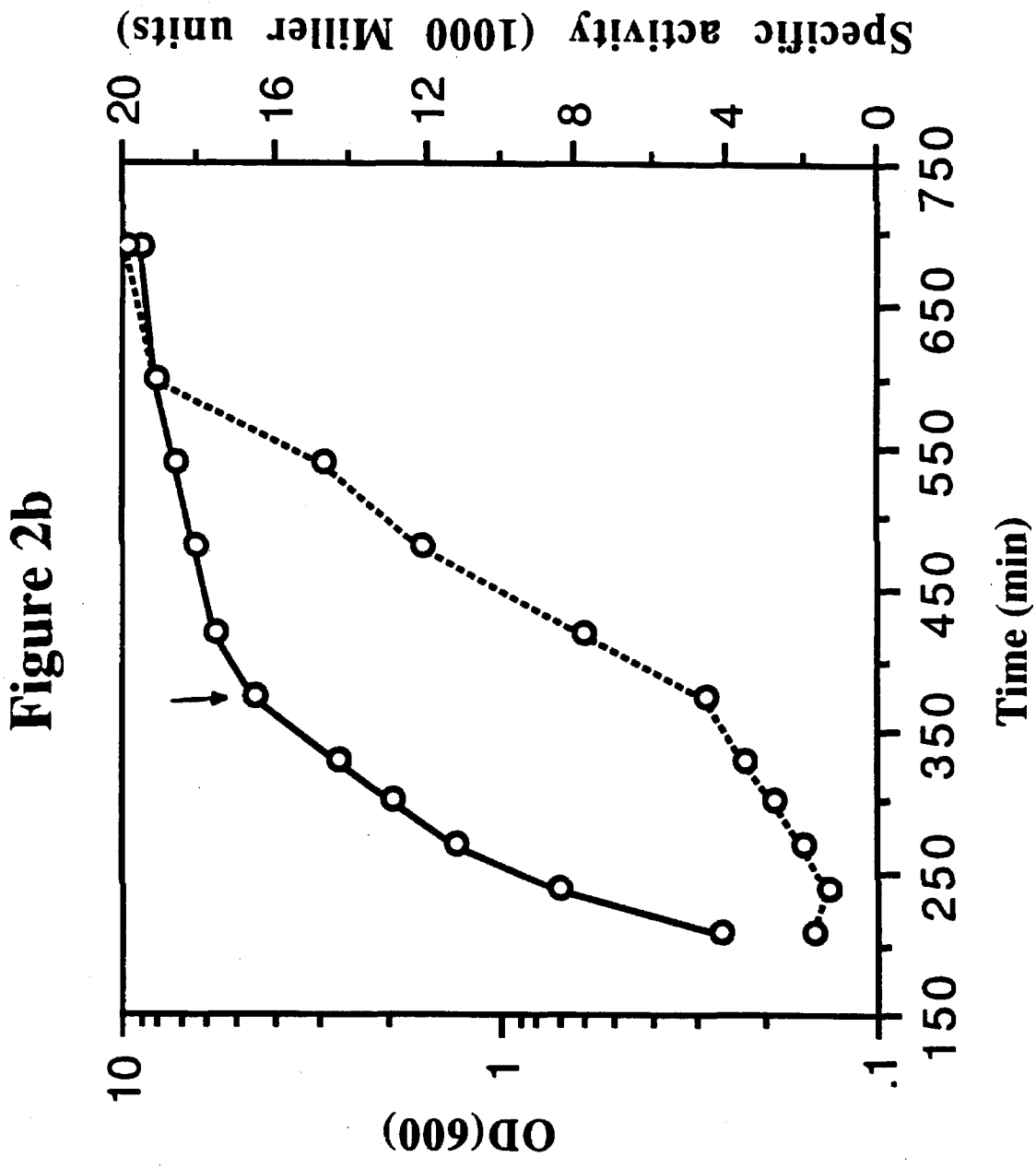


Figure 2b
Specific activity (1000 Miller units)
20
16
12
8
4
0
OD(600)
10
1
.1
150
250
350
450
550
650
750
Time (min)

Figure 3a.

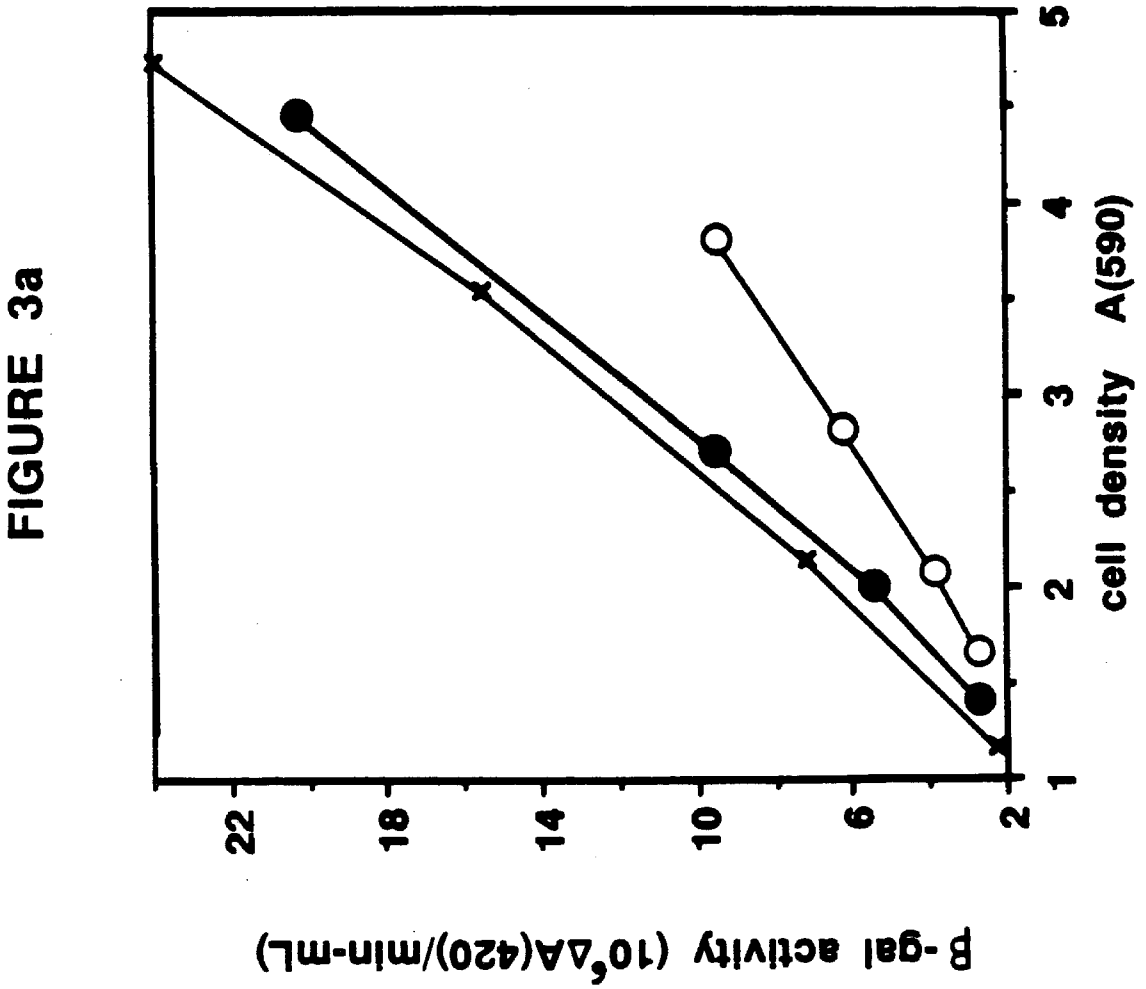


FIGURE 3a
β-gal activity (10^5ΔA(420)/min-mL)
22
18
14
10
6
2
cell density A(590)
1
2
3
4
5

Figure 3b.

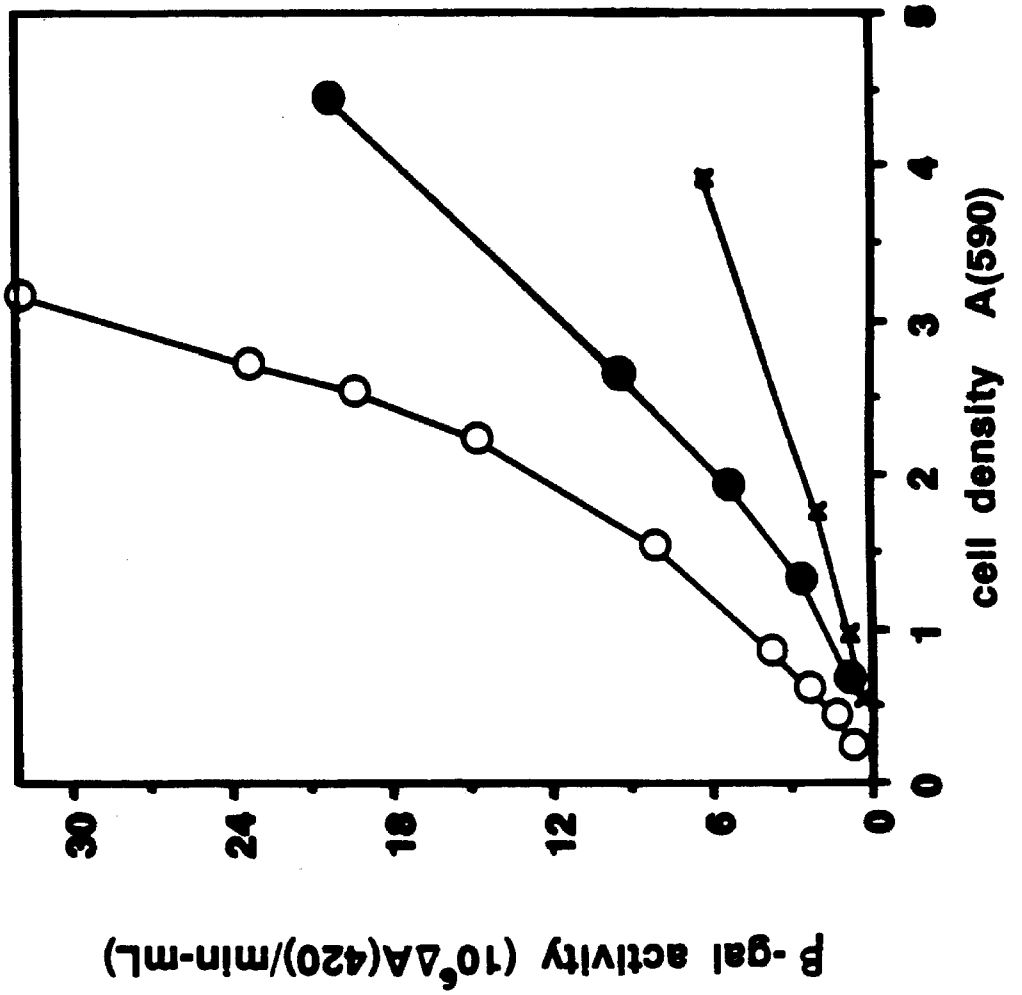


FIGURE 3b
cell density A(590)
0
1
2
3
4
5
β-gal activity (10^5ΔA(420)/min-mL)
0
6
12
18
24
30

Figure 3c.

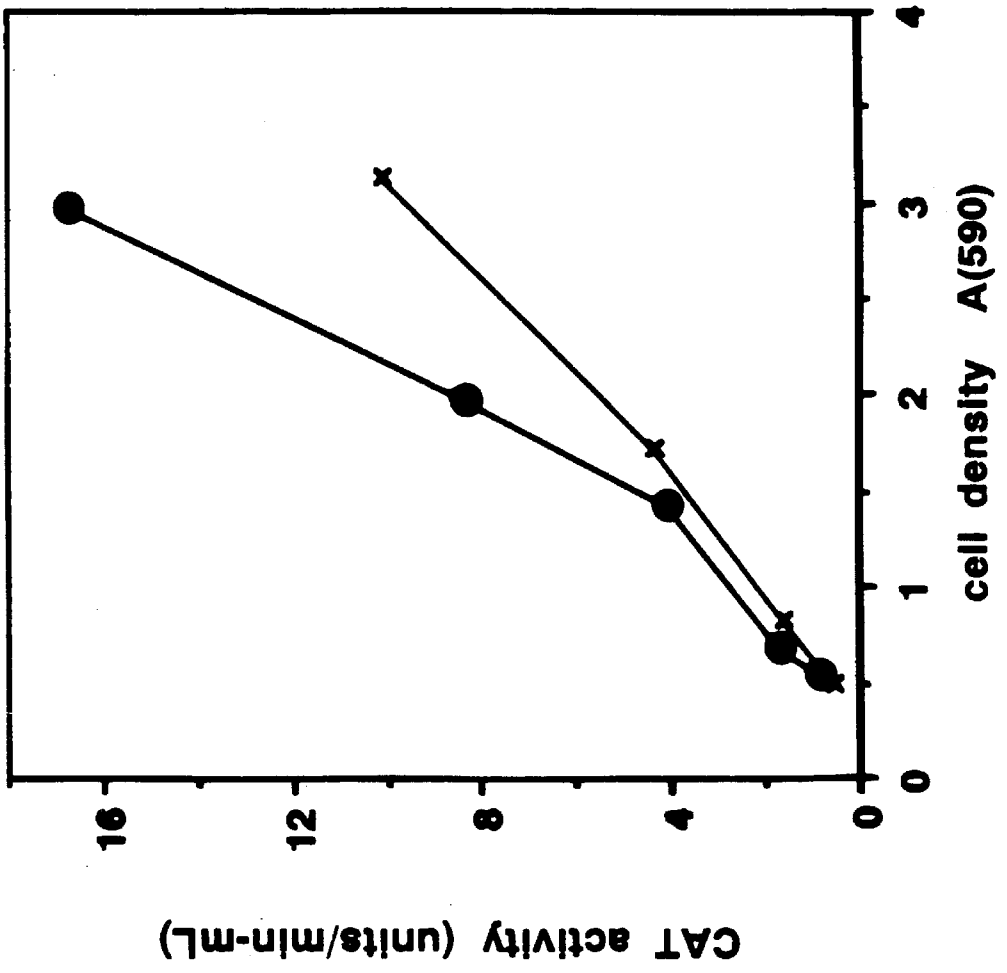


FIGURE 3c

Figure 4.

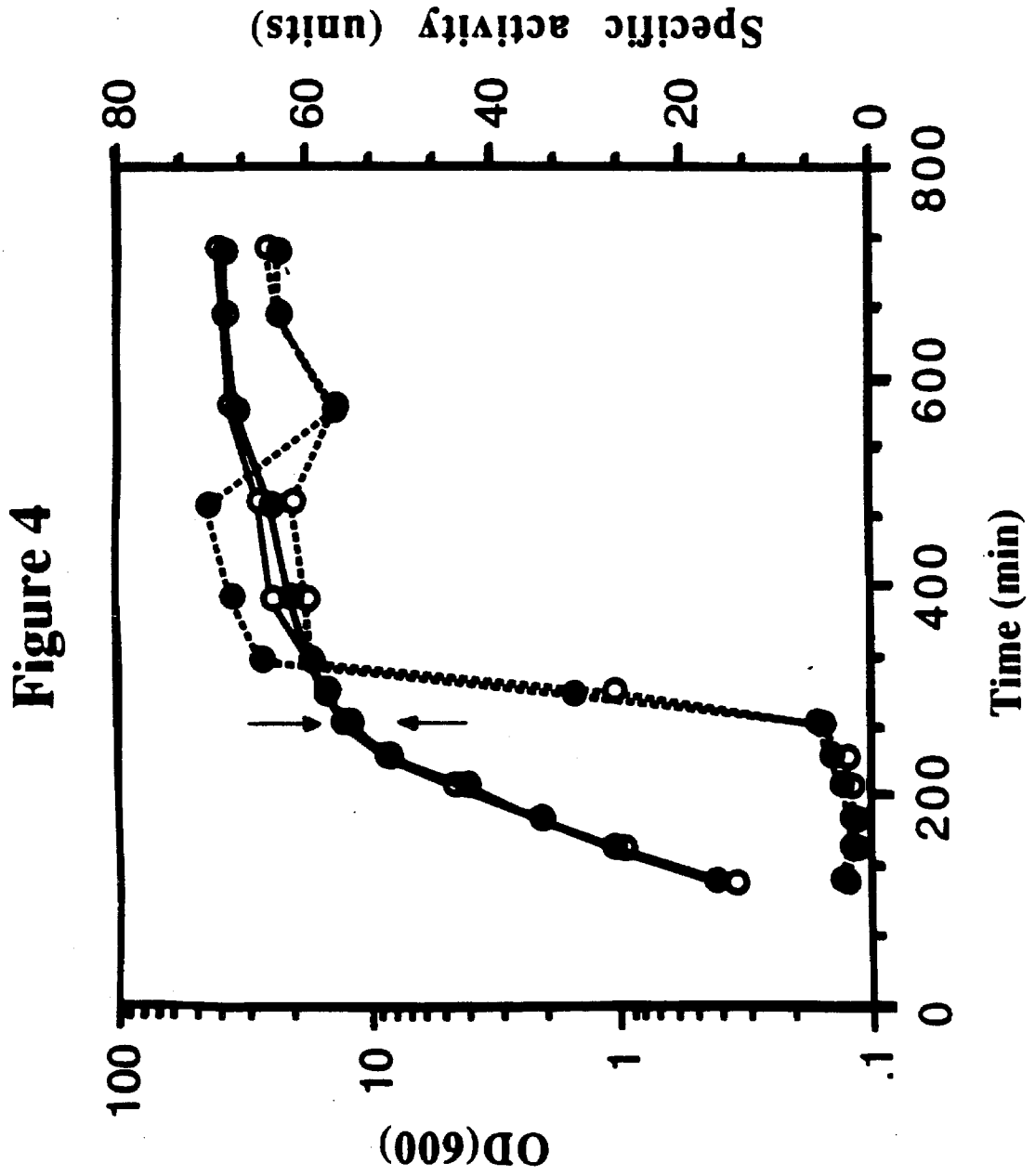


Figure 4

Figure 5a.

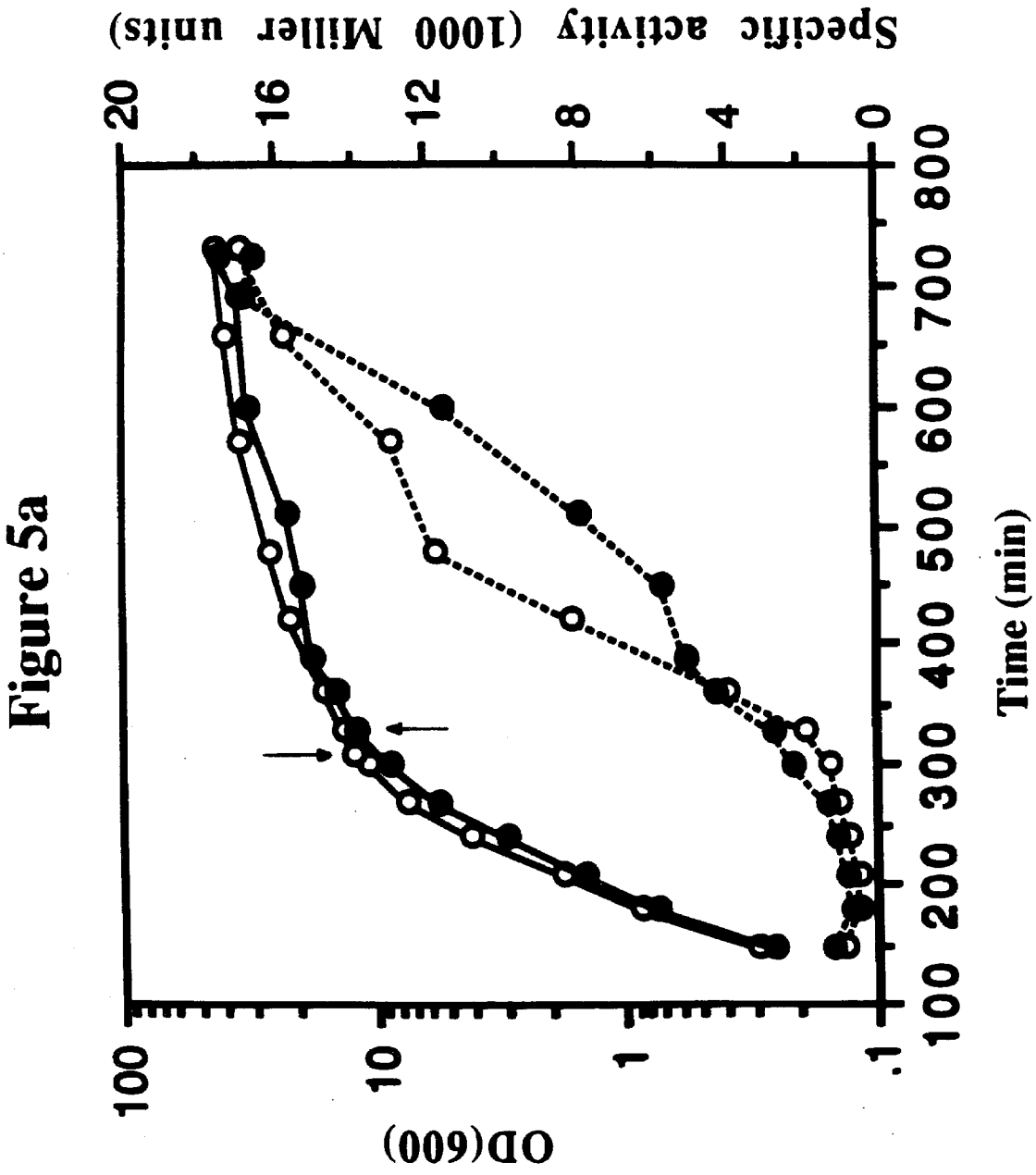
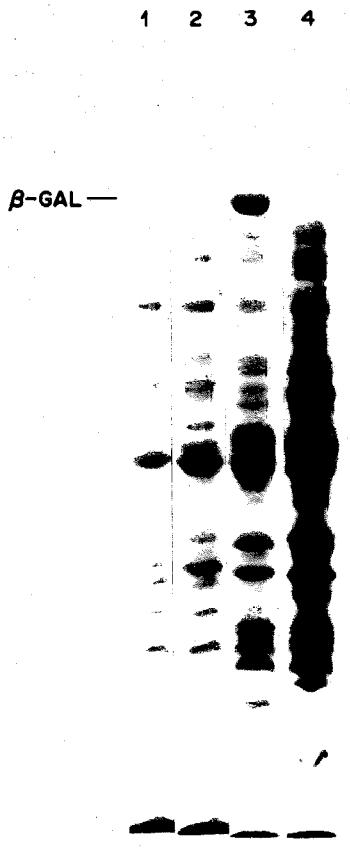


Figure 5a

Figure 5b.



CHAPTER 8

CONCLUSIONS

8.1 Tinkering with metabolism: what have we learned?

This work illustrates the use of genetic manipulation to transfer a metabolic function from an appropriately chosen environmental niche in order to address a problem of significance in bioprocesses. As explained in Chapter 1, it differs from earlier examples in that not only is the function of the cloned gene product unknown *a priori*, but the likely site(s) of influence in the recipient organism are also imperfectly understood. In that sense the success of such efforts depends heavily on the validity of the anthropomorphic argument which motivates the work. Nevertheless, a few important lessons can be learned from the studies presented here.

First, these results highlight the importance of studying microaerobic phenomena in aerobic cells. A lot of information is available in the literature on intracellular events that occur specifically in response to aerobiasis as well as anaerobiasis. (Operationally, aerobiasis can be defined as conditions where the oxygen concentration is sufficiently above the K_M for the lowest affinity terminal oxidase in the electron transport chain, whereas anaerobiasis can be defined as conditions where the oxygen concentration is sufficiently below the K_M for the highest affinity terminal oxidase.) However, the transition between the two regimes is by no means monotonic. It is likely that some regulatory events, which occur in response to changing oxygen availability, are specifically responsible for sustaining metabolism under microaerobic conditions, but may have limited utility under aerobic

or anaerobic conditions. A large number of bioprocesses operate in this regime, and the number increases as we progress towards larger scales of operation or systems employing dense cell cultures. Consequently, understanding mechanisms for regulating metabolism in response to microaerobiasis should be important for the improvement of such processes.

Second, with respect to genetic manipulation of metabolism, in a sense this example joins a growing list of examples in the literature, all of which indicate that it is very hard to predict *a priori* the complete ramifications of genetically perturbing metabolism. Consequently, in order to fully realize the potential of this approach, one invariably iterates through multiple attempts involving successive genetic changes, each of which sheds new light on the exact mechanisms of the metabolic processes of interest.

Third, these studies demonstrate the relative lack of information regarding physiological aspects of metabolism, as compared to the biochemistry of metabolism. For example, much data is available (and more is rapidly accumulating) from *in vitro* studies on the structure and function of various components involved in aerobic respiration in *E. coli*. However, apart from a few studies in the 1970's, *in vivo* stoichiometric and kinetic information on the individual enzymes, the pathway as a whole, its coupling to other metabolic functions, and above all, the effect of changing oxygen availability on these parameters, is sorely lacking. Two approaches are likely to be particularly

friutful in this regard. On one hand the development of new techniques and tools for on-line measurement could provide useful data non-invasively. On the other hand, modern molecular genetics could facilitate the generation of new mutants and analysis of existing mutants with the eventual aims of understanding *in vivo* phenomena, and setting the stage for further attempts at genetic manipulation.

Fourth, such attempts at cross-cloning metabolic functions underscore the need for versatile systems for gene transfer and manipulation that can be used to study phenomena in poorly understood bacteria. In their absence, while modern 'brute-force' genetic techniques can help us work around some disadvantages, a complete understanding of interesting metabolic phenomena in 'funny bugs' is not possible.

Finally, a comment could be made that is also relevant to many other areas of biological research. It appears that the most rapid success in borrowing poorly understood metabolic ideas from nature is likely to be achieved in those cases where another evolutionary concept can be exploited in the recipient organism: the power of genetic selection/ screening to maximize the benefits of perturbing cellular metabolism, and minimize its deleterious effects. This, together with biochemical and physiological investigations, will enhance our understanding of metabolism, as well as our ability to manipulate it in order to improve existing bioprocesses and develop new ones.

8.2 Suggestions for future work involving VHb and its relevance to aerobic bioprocesses.

The results presented here demonstrate the utility of *E. coli* as a surrogate host to study the mechanism of influence of VHb on oxygen metabolism. Several aspects of this work need to be pursued further in order to obtain a better understanding, that in turn would be helpful to identify future applications of VHb to bioprocesses. The most imperative question concerns the development of steady-state *in vivo* techniques (such as chemostats) to quantify the influence of VHb on growth and respiratory parameters under oxygen-excess and oxygen-limiting conditions. This may require the use of indirect measurements, such as the culture redox potential, to monitor oxygen availability at concentrations below the detectable limit of standard dissolved oxygen probes. Quantitative knowledge of parameters such as $Y_{X/S}$, $Y_{X/O}$, $Y_{H+/O}$, r_X, r_S, r_{O_2}, r_{CO_2}, and r_P will be needed to unequivocally identify the locus (loci) of VHb influence. In addition, the ability to estimate intracellular concentrations of NADH and ATP (i.e. the substrate and product of aerobic respiration, respectively) will be desirable. These physiological studies could then provide a basis for similar investigations with new and existing mutants that are defective in aerobic respiration. Likewise, parallel development of analogous *in vitro* techniques for making steady-state kinetic measurements with isolated cellular components is also likely to yield useful results. An important consequence of the above investigations will be the realization of important new knowledge on aerobic metabolism (in particular microaerobic metabolism) and its regulation in *E. coli*.

As mentioned earlier, the development of genetic tools to study *VHb* function in *Vitreoscilla* could also yield fruitful results. Some initial (unsuccessful) attempts in this direction are summarized in Appendix A. It should be pointed out that a native plasmid has been identified in *Vitreoscilla* (for reference, see Chapter 2). This could provide a useful replicon, and perhaps even a mobilization element for gene transfer into *Vitreoscilla*. Plasmids of other known incompatibility groups could also be tried. Finally, attempts to identify temperate phages in this species may be worthwhile.

Together with standard genetic methodologies that are available in *E. coli*, development of the techniques outlined above will also be useful for analyzing the mechanisms of regulation of the oxygen-responsive promoter element (ORE) of the *VHb* gene. In connection with oxygen-dependent regulation, two main questions need to be addressed. First, the *trans*-acting factor(s) responsible for sensing oxygen availability and accordingly influencing ORE activity are as yet unknown. (Based on available information these are likely to be conserved between *E. coli* and *Vitreoscilla*.) In this context, further knowledge on the mechanisms of regulation of the cytochrome d gene in *E. coli* may be helpful on account of the existence of homologous regions upstream of the two genes. Second, the exact biochemical mechanism by which ORE is maximally responsive to microaerobic environments needs to be identified. In addition, the versatility of ORE-based expression systems could be increased by developing genetic/ fermen-

tation strategies to further reduce the oxygen-independent promoter activity, particularly at high cell densities.

As more information on the mechanism of VHb action, as well as aerobic metabolism in general, accumulates, it may be possible to rationally predict the scope of VHb-mediated enhancement of bioprocesses involving other organisms. Some very significant potential applications are worth mentioning in this regard. For example, on account of their rapid kinetics of respiration and their mycelial morphology, the productivity of fermentations involving bacteria of the *Streptomyces* genus is seriously limited by oxygen availability. Since these bacteria are obligate aerobes, an enhancement in their respiratory properties could translate in improvement of productivity of many natural products of interest. In another case, it has been demonstrated that the productivity of amino acids by coryneform bacteria is optimal under microaerobic conditions. Citric acid synthesis by *Gluconobacter* is yet another example of a bioprocess whose productivity is sensitive to the redox state of the environment. While it may be somewhat early to predict the utility (if any) of VHb in these and other systems, initial investigations along these lines could perhaps be rationalized yet again with an anthropomorphic argument analogous to the one that started the work reported here.

APPENDIX A

ATTEMPTS AT DEVELOPING GENETIC TOOLS IN *VITREOSCILLA*

A.1 Introduction.

It is evident from earlier chapters that the availability of a variety of extremely versatile genetic tools in *E. coli* has greatly facilitated an analysis of the mechanisms of function of both VHb and ORE using *E. coli* as a surrogate host. In fact, the very premise with which this work was started (i.e. it may be possible to borrow genetic strategies from nature to manipulate metabolic functions of interest to bioprocesses) relied heavily on the availability of a number of genetic techniques in *E. coli*, which we now take for granted. While significant advances have been made, even at a fundamental level, with the above approach, it must be recognized that an ultimate appreciation of the evolutionary elegance of VHb and ORE will require an understanding of the role they play in their natural host, i.e. *Vitreoscilla*. To this end our ability to develop rudimentary genetic tools to manipulate this relatively unknown bacterium may prove invaluable.

Two minimum requirements must be satisfied to facilitate *in vivo* genetic studies with any organism.

1. A technique for introducing DNA into the cell (*gene transfer mechanism*) must be available. Since successful transfer events are relatively rare, they are selected for by virtue of enhanced survival (e.g. drug resistance). Consequently the gene transfer technique should have a transfer frequency that is at least an order of magnitude (preferably more) higher than the spontaneous reversion frequency of the organism to the screen.

2. A vehicle for delivering DNA into the organism (*cloning vector*). This permits the *in vivo* exchange as well as the *in vitro* analysis and manipulation of genetic loci of interest.

A central problem with the *ab initio* development of genetic techniques in new systems is that the diagnosis of failed attempts becomes a 'chicken-and-egg' situation. One cannot ascertain whether the gene-transfer mechanism, or the cloning vector (i.e., the replicon or the gene providing a selective advantage), or both are inappropriate.

In addition to the above two tools, the availability of mobile genetic elements (e.g. insertion elements, transposons, retroviruses), that can function in the organism of interest, is highly desirable. In summary, in the absence of limitations on the population size available for experimentation (which is not an issue in the case of most microbes), the versatility of the above tools directly influences the scope of questions that can be addressed in an organism using genetic approaches.

Over the course of this work, several attempts were made at developing the minimal tools required to conduct a genetic investigation on the mechanism of action of VHb and ORE in *Vitreoscilla*. The main results are summarized below.

A.2 Transformation.

The simplest approach attempted for gene transfer into *Vitreoscilla* was that of chemical transformation. The vector used was pRED2 (for

description, see Chapter 2). The aim was to isolate a recombinant *Vitreoscilla* colony that either permitted replication of this plasmid (colE1 replicon), or at the very least had integrated pRED2 into its chromosome via homologous recombination. A necessary prerequisite for success was that the β-lactamase gene should be expressed in *Vitreoscilla*. *Vitreoscilla* was grown at 30°C in PYA broth (1.5% peptone, 1.5% yeast extract, 0.02% sodium acetate, pH 8.0). For selection purposes 25 mg/L ampicillin was added to plates. Both the $CaCl_2$ (1) and the Hanahan (2) protocols were used. No decrease in viability was observed in either case; however no transformants were obtained either.

A.3 Conjugal mobilization.

Recent successes with this technique in a variety of Gram-negative bacteria (particularly purple bacteria, of which *Vitreoscilla* is a member) (3) motivated the studies described in this section. The experimental design used here was based on the following premise: through three-way crosses it may be possible to mobilize a plasmid (carrying the DNA of interest), which is normally not self-transmissible, from *E. coli* into *Vitreoscilla*, with the assistance of a helper plasmid possessing appropriate *tra* functions. The steps followed are outlined below.

1. A test plasmid pHC1, carrying a locus of interest, was constructed by inserting a (blunt-ended) 1.4 kb *BamH*I fragment carrying a *cat* gene (gift from Dr. P. Berget, Carnegie Mellon University) into

the (blunt-ended) *Mlu*I site in the *VHb* gene on plasmid pOX1 (Chapter 6). Successful transfer of pHC1 into *Vitreoscilla* could provide a vehicle for marker rescue mutagenesis of the *VHb* gene.

2. Two homologous plasmids, pRK2013 (4) and pRK2073 (5), of the IncP1 incompatibility group were selected as mobilizing plasmids. pRK2013 provides resistance to kanamycin, while pRK2073 provides resistance to streptomycin. The presence of *tra* genes on these plasmids could permit transfer of pHC1 via 3-way crosses.

3. In order to select against *E. coli* hosts in 3-way crosses, spontaneous revertants of VIT1 (the parent *Vitreoscilla* strain), which were resistant to rifamycin, kanamycin, or streptomycin were sought. Only 7 Rif^r mutants were isolated. Two of them, designated VIT11 and VIT12, were purified and used in further studies.

4. Prior to attempting 3-way crosses, the resistance of different strains to various drugs was checked. Since the presence of high concentrations of complex nitrogen sources was observed to increase the level of resistance of *Vitreoscilla*, as well as several *E. coli* strains, to rifamycin, modified PYA plates containing 0.5% peptone and 0.5% yeast extract were used hereafter. (A defined medium described earlier (6) was also tried; however, growth of *Vitreoscilla* on these plates was very poor.) On plates containing 80 mg/L rifamycin this resulted in an observed reversion frequency of approximately 1 in 10^{10} with all the *E. coli* strains.

5. 3-way crosses were conducted according to standard protocols (4,5) with acceptor:donor:mobilizer ratios ranging from 5:1:1 to 100:1:1. Cells grown on plates, as well as liquid cultures, were used for matings. Several hundred colonies that grew on selective plates were further screened for various drug resistance phenotypes. All of them appeared to be Rif^r revertant *E. coli* colonies. For 12 colonies from different crosses this was confirmed by SDS-PAGE (data not shown).

Taken together, these results suggested that the frequency of obtaining a marker-rescue mutant *Vitreoscilla* strain was lower than the background frequency of reversion in the experimental design described above.

A.4 Electroporation.

Recent development of high-voltage, low pulse-time instruments has led to dramatic breakthroughs with several procaryotes using this relatively new gene transfer technique (7). In order to test the feasibility of electroporating DNA into *Vitreoscilla*, two plasmids were chosen for these studies. Plasmid pRK310 is a relatively small (19 kb) broad host-range vector belonging to the IncP1 incompatibility group, which carries the *tet* gene (gift from Dr. M. Lidstrom, California Institute of Technology). Plasmid pHC1 (as well as the motivation for constructing it) has been described above. Both plasmids

were purified from two successive CsCl gradients and resuspended to a final concentration greater than 1 mg/mL. VIT1 cells were prepared for electroporation by growing to mid-log phase (OD_{600} = 0.5-0.6), followed by two washes with (equal volume) distilled, deionized water and finally two washes (1/50th volume) 272 mM sucrose. Cells were resuspended to a final volume of 1 mL in 272 mM sucrose and chilled on ice. 50 μL cells were used for each attempt at electroporation. Electroporation was performed on a Gene Pulser unit (Bio-Rad) (max output voltage 2.5 kV/cm) at a capacitance of 25 μF, using 0.2 cm cuvettes (Bio-Rad). Following this, the cells were immediately transferred into 1 mL PYA medium and incubated at 30°C for 4h. 0.25 mL of this culture was plated on selective plates (15 mg/L tetracycline, 20 mg/L chloramphenicol, 25 mg/L ampicillin); samples were also serially diluted to obtain viability counts.

Results obtained for both plasmids are summarized in Table I. The data for the two plasmids was obtained from two independent sample preparations. It is usually assumed that the window of maximum transformation efficiency via electroporation lies somewhere in the parameter range that leads to reduced viability. Results shown here suggest that this range corresponds to the upper limit of electric field strength with VIT1 under the conditions described above.

A.5 Suggestions for further work.

The above data provides a useful starting point for further research towards the development of useful genetic tools in *Vitreoscilla*. In particular, the techniques of conjugal mobilization and electroporation need to be explored further. With regard to mobilization, the primary problem appears to be the high reversion frequency of donor and mobilizer *E. coli* strains to rifamycin resistance. Consequently, the next logical step would be to attempt generating a spontaneous revertant of VIT1 that is resistant to a drug for which *E. coli* possesses a lower reversion frequency. Unfortunately, spontaneous reversion of VIT1 to streptomycin resistance (which satisfies the above requirement) appears to be a very rare event (< 1 in 10^{11}). Nevertheless, it may be useful to note that 10 Amp^r mutants of VIT1 were identified on one occasion (C.K. and J.E.B., unpublished results). The wide prevalance of *bla* genes as resistance markers on commonly used plasmids prevented further pursuit of these mutants as likely recepient candidates; however, in view of the above results this may be a promising alternative. Another option would be to attempt mild mutagenesis with the aim of isolating a Str^r mutant of VIT1.

A further possibility in connection with the development of conjugal transfer techniques concerns the use of broad host-range mob^+ plasmids of various incompatibility groups (3). The advantage here would be the improved likelihood of identifying a transfer event, without the need for homologous recombination.

With regard to electroporation, further investigations designed to identify the window of high-frequency transformation by manipulation of various parameters may prove fruitful. An examination of available literature suggests that the type of plasmid used may also have some influence on the frequency of successful transfers. Hence, again, the use of other plasmids (and drug resistance markers) would be desirable.

Finally, it should be noted that a native plasmid has been identified in VIT1 (8). Further studies on this plasmid could prove extremely useful. In particular, this plasmid, or derivative shuttle vectors, would serve as prime candidates for use in chemical transformation, conjugal mobilization, and electroporation experiments, since they could overcome the 'chicken-and-egg' problem outlined in A.1.

A.6 References.

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Table 1:

TABLE I

Results from electroporation

Plasmid	DNA (uL)	kV	ohms	time const (ms)	# survivors/mL	# transformants/mL
control	-	-	-	-	3.2×10^9	0
pRK310	1.1	1.25	200	4.7	2.8×10^9	0
pRK310	1.1	1.75	200	4.6	2.7×10^9	0
pRK310	1.1	2.50	200	4.5	1.2×10^9	0
pRK310	2.5	1.25	400	8.8	2.3×10^9	0
pRK310	2.5	1.25	200	4.4	3.2×10^9	0
control	-	-	-	-	1.0×10^{10}	0
pHC1	2.0	2.50	200	4.6	4.0×10^{10}	0
pHC1	3.0	2.50	200	4.5	4.0×10^{10}	0
pHC1	2.0	2.50	400	8.9	2.5×10^{10}	0