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Characterization of Terminal Oxidase Expression in *Bacillus subtilis*

by

Pamela Susan David

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled "Characterization of Terminal Oxidase Expression in *Bacillus subtilis*" submitted by Pamela Susan David in partial fulfillment of the requirements for the degree of Master of Science.

Supervisor, Dr.B.C. Hill, Department of Biological Sciences

Dr.G.M. Gaucher, Department of Biological Sciences

Dr.K.J. Stevenson, Department of Biological Sciences

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Aug 17/93
Date

ABSTRACT

Bacillus subtilis, a strictly aerobic bacterium possesses a branched respiratory chain. When *Bacillus subtilis* is grown under standard aerobic conditions, three terminal oxidases are expressed. There are two oxidases which use heme A as a prosthetic group and one that uses heme B. Different culture conditions have been experimented with and their effect on the level of the three terminal oxidases has been determined. Cultures of *Bacillus subtilis* have been grown with either glucose or succinate as the carbon source and at two extremes of oxygenation. The most prominent effect is seen when the oxygen level is increased which results in a higher cell density and an increase in the B-type terminal oxidase. To further understand the role of these three terminal oxidases in the growth of *Bacillus subtilis*, an insertional inactivation experiment was done. This experiment results in a mutant of *B. subtilis* which is deficient in one of the heme-A linked oxidases, cytochrome caa_3. The mutant lacking a cytochrome *c* oxidizing ability, exhibits identical growth characteristics compared to wild-type when grown on succinate-containing, modified super rich medium. Growth on defined minimal medium under the two levels of aeration has opposite results in both mutant and wildtype to those obtained on rich medium, and membrane extracts show the presence of a fourth terminal oxidase, cytochrome *d*. These effect of the mutation and the levels of expression of the terminal oxidases present in *B. subtilis* will be discussed.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my family whose support of my chosen field has allowed me to accomplish what I have thus far in my life.

I would like to thank especially Dr. Sui-Lam Wong, for his unfailing assistance in my initial foray into the world of molecular biology of *Bacillus subtilis*.

Also I would like to acknowledge my fellow graduate students, Clare Gallagher, Christine Ellis, Nathan Roth, and Nat Vettakkorumakankav, for always making time to hear about a gripe and grievance on a bad day and jubilant results on a good day. Also for lending me their expertise and opinion of the world of molecular biology or whatever other query I might have.

To my supervisor, Dr. Bruce C. Hill, a warm thank you and a kind word for always being available for a serious discussion or a light hearted conversation on whatever topic was at hand. For being in touch with the ongoing experiments in the lab and lending instruction in all aspects of science and academia.

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To my supervisory committee, Dr. G.M. Gaucher, and Dr. K.J. Stevenson, for their time and comments about this work.

DEDICATION

This thesis is dedicated to my father

Michael J. David

(1931-1991)

The only member of my family who lacked the

opportunity to pose the question,

"So what is it that you are working on anyway?"

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ABBREVIATIONS

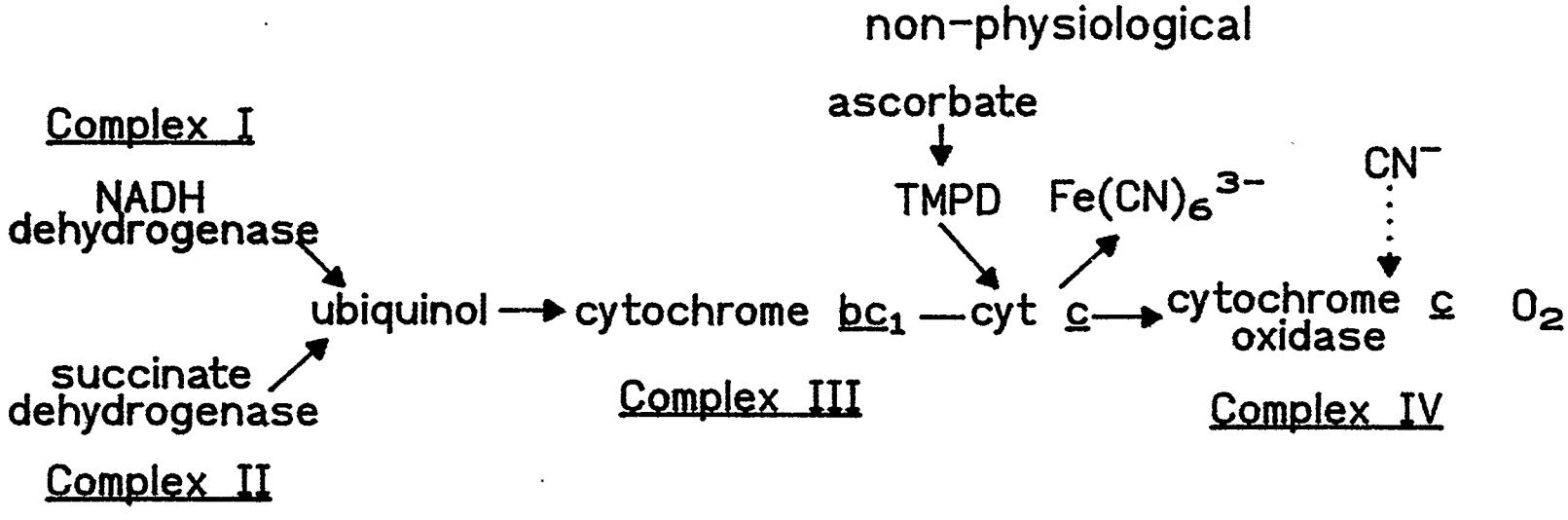
bp	base pairs
BSA	bovine serum albumin
cta	gene encoding cytochrome caa_3
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dGTP	deoxy guanosine triphosphate
dTTP	deoxy thymidine triphosphate
DOC	dissolved oxygen content
DT	sodium dithionite
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EGTA	ethyleneglycolbis-(aminoethylether) tetra acetic acid
kb	kilobase pairs
LB	Luria-Bertani medium
LMP	low melting point gel
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulphate
TAE	Tris-acetate EDTA
TMPD	N, N, N′, N′-tetramethyl-p-phenylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	ultra violet
VIS	visible

INTRODUCTION

The Respiratory Chain of Mitochondria

In eukaryotic cells, oxidative phosphorylation takes place in the inner mitochondrial membrane (Hatefi, 1985). Membrane-bound respiratory chains catalyze the transfer of reducing equivalents from a reduced substrate to an oxidant (Jones, 1987). This transfer is spontaneous and is based on differences in oxidation-reduction potential among the members of the chain. A release of free energy is associated with transfer of reducing equivalents and energy is conserved as a transmembrane electrochemical gradient of protons (Taber, 1993). In mammalian mitochondria the transfer of electrons from soluble and membrane-bound dehydrogenases to the final electron acceptor, catalyzes the conversion of oxygen to water. This process occurs down a free energy gradient composed of more than twenty discrete carriers of electrons which are grouped into four polypeptide complexes (Nicholls and Ferguson, 1992). The electrons flow from the dehydrogenases, to ubiquinol (ubiquinol:cytochrome *c* oxidoreductase) to the single terminal oxidase in the respiratory chain, cytochrome *c* oxidase, (ferrocytochrome *c*:oxygen oxidoreductase) (see Figure 1). Of the four polypeptide complexes, complex I, III and IV, have also been shown to possess proton pumping ability (Hatefi, 1985). The terminal oxidase, also known as cytochrome aa_3, is composed of

Figure 1: Schematic of the mammalian mitochondrial respiratory pathway. The components of the pathway are grouped into four respiratory complexes as indicated. The dotted arrow indicates the site of action of the inhibitor cyanide, solid arrows indicate electron flow. Adapted from Nicholls and Ferguson, 1992.


non-physiological
ascorbate
Complex I
NADH
dehydrogenase
TMPD
Fe(CN)6 3-
CN-
ubiquinol
cytochrome bc1
cyt c
cytochrome c
oxidase
O2
succinate
dehydrogenase
Complex III
Complex IV
Complex II

13 distinct subunits and contains 4 metal centers (Chan and Li, 1990). There are two copper ions, designated Cu_A and Cu_B, and two heme A moieties, designated as cytochrome *a* and cytochrome a_3 (Saraste, 1990). Cytochrome *c* oxidase catalyzes the four electron reduction of molecular oxygen to water with reducing equivalents derived from cytochrome *c*:

$$4\text{Cyt } c^{2+} + O_2 + 4H^+ \leftrightharpoons 4\text{Cyt } c^{3+} + 2H_2O$$

Equation 1

The three largest subunits of mammalian mitochondrial cytochrome *c* oxidase are encoded by mitochondrial DNA. There are bacterial oxidases that have been isolated (eg. *Paracoccus dentrificans*; Ludwig and Schatz, 1980) which contain only two of the three largest subunits of the mitochondrial oxidase. These two subunit enzymes are active in electron transfer and have the spectral characteristics of the mitochondrial oxidase. Therefore, it follows that the redox centers must be confined to subunits I and II (Nicholls and Ferguson, 1992). These two subunits form the structural and functional core of the mitochondrial cytochrome *c* oxidase.

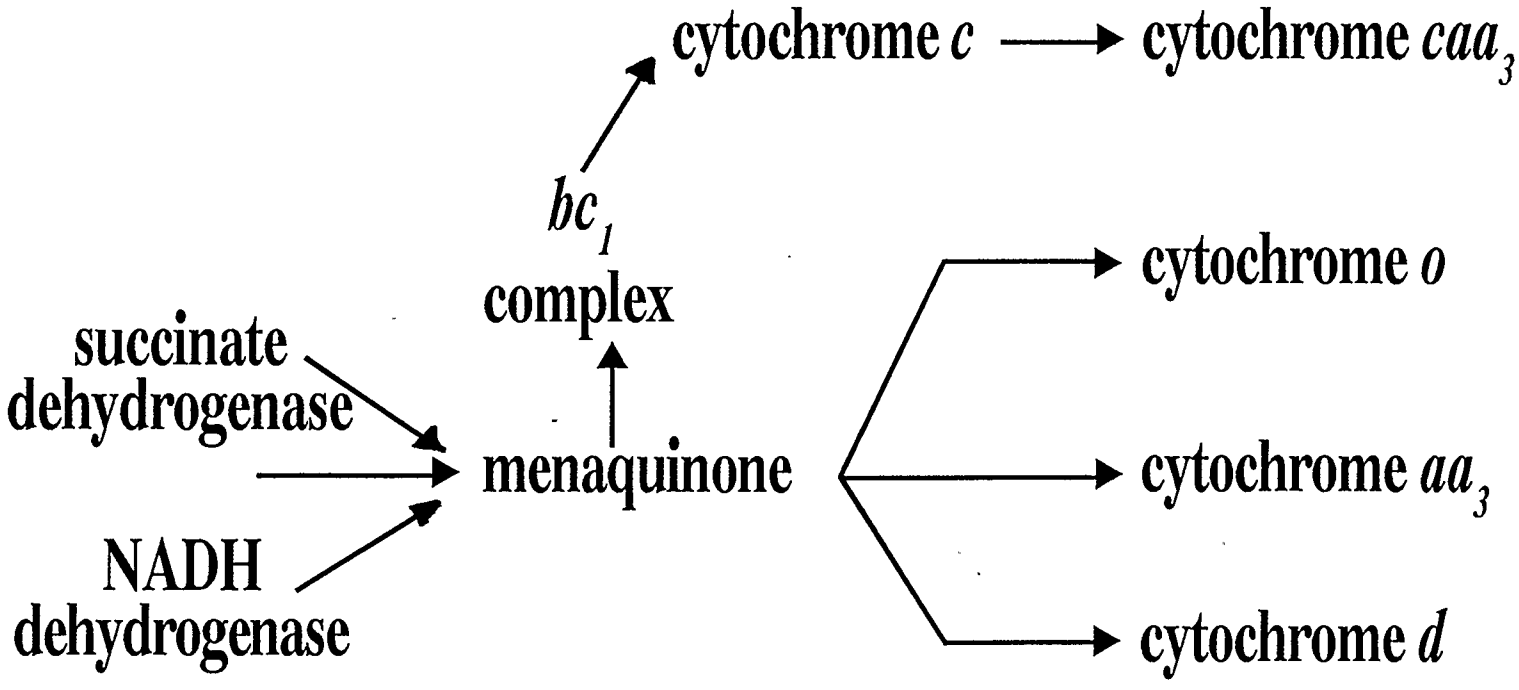
Bacillus subtilis is a gram positive, strictly aerobic bacterium and has been the longest known example of a bacterium containing the four characteristic cytochrome absorption bands observed in eucaryotic cells (Keilin, 1925). *Bacillus subtilis* was thought to possess a respiratory chain analogous to that of the mammalian mitochondria (Keilin, 1966). Recent research on *Bacillus subtilis* has shown that

although it possesses the spectral absoption bands due to the same heme moieties as that of mammalian mitochondria, it possesses quite a different arrangement of respiratory components. *Bacillus subtilis* is said to have a branched respiratory chain which ends in more than one terminal oxidase capable of reducing molecular oxygen to water. This branched respiratory chain differs from other known aerobic bacterial respiratory chains making its elucidation of interest in its own right.

The Respiratory Chain of *Bacillus subtilis*

Bacillus subtilis has a branched respiratory chain that ends in four distinct terminal oxidases all capable of reducing molecular oxygen to water (Taber, 1993). Figure 2 shows the sequence of electron flow in the respiratory pathway of *Bacillus subtilis* as it is understood today. Analogous to the mitochondrial pathway, electrons from the tri-carboxylic acid cycle are funnelled through a quinone moeity, which in *B. subtilis* is the lipid soluble menaquinone-7 (Downey, 1964). Here the pathway branches as electrons flow either through a cytochrome bc_1 complex (De Vrij *et al.*, 1987) to cytochrome *c*-550 (Von Wachenfeldt and Hederstedt, 1990) to cytochrome *c* oxidase(s) or from menaquinone-7 directly to the quinol oxidase(s). Of the four terminal oxidases of *B. subtilis*, cytochrome caa_3 is the only one in which cytochrome *c* oxidase activity has been demonstrated (Saraste *et al.*, 1991). The other three terminal oxidases are thought to be quinol

Figure 2: Schematic of the *Bacillus subtilis* respiratory pathway. Final electron acceptors are the terminal oxidases. Arrows indicate direction of electron flow. Adapted from Taber, 1993.


cytochrome c
cytochrome caa3
bc1
complex
succinate
dehydrogenase
menaquinone
cytochrome o
cytochrome aa3
NADH
dehydrogenase
cytochrome d

oxidases, but only cytochrome aa_3 has been verified as such (Lauraeus *et al.*, 1991; Santana *et al.*, 1992). Information on the other two terminal oxidases, cytochromes *o* and *d*, is minimal at present. Data on the four terminal oxidases of *Bacillus subtilis* is summarized in Table 1.

Table 1: Relevant data on the terminal oxidases of *Bacillus subtilis*

oxidase	heme moeity	subunit M_r[1]	# of ORF's[2]	electron donor	# of trans-membrane helices[3]
caa_3	C,A	69,40	6	cyto *c*	14,2
aa_3	A	72,37	4	MQ-7	15,2
d	D	ND	ND	ND	ND
o	B(?)[4]	ND	ND	ND	ND

1. Purified subunit molecular weights, for subunits I and II.
2. Open reading frames in operons.
3. Transmembrane helices for subunits I and II.
4. There is a possibility this heme moeity is heme O.

ND-not determined, cyto *c*-cytochrome *c*, MQ-7-menaquinone-7

The genetic sequences of the two heme A containing terminal oxidases, cytochromes caa_3 and aa_3, in *Bacillus subtilis* have been published (Saraste *et al.*, 1991;Santana *et al.*, 1992). The subunits that make up the cytochromes are organized in operon structures (Taber, 1993). The bacterial terminal oxidases have been shown from their genetic sequences to possess 6 and 4 open reading frames for cytochrome caa_3, and cytochrome aa_3, respectively. Two of the 6 open reading

frames in cytochrome caa_3 code for putative assembly or regulatory factors. However, the purified proteins that have been isolated are composed of only two subunits (Saraste *et al.*, 1991). These two main subunits, isolated from the aa_3 and caa_3 oxidases of *Bacillus subtilis* show sequence homology to the two largest mitochondrially encoded subunits in mitochondrial cytochrome *c* oxidase (Saraste, 1990).

Sequence alignment of subunit I of the heme A containing oxidases from eleven procaryotic and eucaryotic species show an overall homology of about 15% (Saraste, 1990). Ligands for the heme A moeities and Cu atoms in subunits I and II of these terminal oxidases are absolutely conserved in all genes sequenced. Quinol oxidases of the aa_3 type, or *bo* as in *E. coli*, not containing a Cu_A moeity no longer possess the conserved ligands for this atom.

Regulation of Expression of Terminal Oxidases of *Bacillus subtilis*

It has been known since the mid-1950s that expression of the terminal oxidases of *Bacillus spp.* are regulated (Chaix and Petit, 1956;1957; Smith, 1954). A number of studies have confirmed and expanded on earlier findings concerning the effect of carbon source, phase of growth, and aeration level on the expression of terminal oxidases, (Taber, 1974; McEnroe and Taber, 1984; Escamilla, 1987; van der Oost, 1991), but as of yet no study has determined the regulation mechanism for the production of one terminal oxidase over another. One

bacterial system which is well characterized is that of the facultative bacterium *Escherichia coli*.

E. coli is able to grow both aerobically and anaerobically. The presence of a branched respiratory chain in *E. coli* ending in two terminal oxidases follows its facultative nature where one of its terminal oxidases is expressed at either of its growth extremes. The level of oxygen available to the culture is one factor in the regulation of expression of its two terminal oxidases. Of these two terminal oxidases, cytochrome *bo* has a low affinity for oxygen and is expressed during aerobic growth while cytochrome *bd* has a high affinity for oxygen and is expressed during microaerobic growth (Spiro and Guest, 1991). Both *E. coli* terminal oxidases are quinol oxidases and lack heme A moieties. Despite this fact, the aerobically expressed cytochrome *bo* shows sequence similarity to the mitochondrial terminal oxidase cytochrome aa_3.

The *B. subtilis* system does not follow that of *E. coli* for the simple reason, perhaps, that *B. subtilis* is not a facultative organism. The necessity of four terminal oxidases in a strict aerobe and the role they play in the life cycle and growth of the bacterium has yet to be determined. One significant aspect of the *B. subtilis* life cycle that may reflect the complexity of terminal oxidase expression is the ability to sporulate. During sporulation the cell proceeds through seven characteristic phases ending with release of the

mature spore. The released spore is metabolically dormant carrying out no detectable metabolism or macromolecular synthesis (Nicholson and Setlow, 1990). Regulation of the cytochromes and other cellular products are required in the sporulation pathway, and particularly in the transition state. The transition state occurs between stationary phase and sporulation where the organism activates a large number of enzymes specific to this stage of the life cycle which allow the continued growth of the cell, albeit at a slower rate.

The presence of cytochromes caa_3, aa_3, *o* and *d*, has been shown in *B. subtilis* cells in various stages of growth and sporulation (Tochikubo, 1971). Due to the different levels of the cytochromes shown to be present, this study infers the necessity of certain terminal oxidases of *B. subtilis* at certain stages of the cell's life cycle. Elucidation of the number and role of terminal oxidases in the laboratory setting may not be complete as in its natural environment *B. subtilis* may spend more time in the transition state than in exponential growth (Hoch, 1993).

The respiratory chain of *Bacillus subtilis* is more complex than would have been thought for a strictly aerobic bacterium. The question addressed in this thesis was the response of the respiratory chain of *B. subtilis* to oxygen. That is the change in the levels of the relative components of the respiratory chain in response to an increased oxygen atmosphere. The response of *B. subtilis* in terms of the

respiratory chain to growth under high oxygenation causes an increase in the cell yield of the cultures and in the relative levels of the terminal oxidases. The heme A levels did not change in relation to each other under high oxygenation but the cytochrome *o* level increased. Another approach the complexity of the respiratory chain of *B. subtilis* and to the requirement of the terminal oxidases is a genetic one. Insertional inactivation of the *cta* gene of *B. subtilis* created a mutant strain, PD001, deficient in cytochrome caa_3 oxidase. Growth studies on this mutant strain show that this cytochrome caa_3 oxidase is not essential for growth of the bacterium on rich medium under two levels of oxygenation. Growth of both the wildtype and mutant strains on defined minimal medium causes production of cytochrome *d*, and shows impairment of growth of the mutant *B. subtilis* under oxygen. *Bacillus subtilis* growth under laboratory conditions on rich medium does not seem to be influenced by the compliment of terminal oxidases. Cytochrome caa_3 oxidase does not seem to be required for growth of the bacterium as the levels of the other terminal oxidases do not increase to compensate for its loss. This may indicate that the role of cytochrome caa_3 in the cell may not be a strictly bioenergetic one.

Materials and Methods:

Chemical suppliers: Acetic acid (glacial), ascorbic acid (AnalaR), boric acid, calcium nitrate, cobalt nitrate, dimethyl formamide, D-glucose, ethylenediaminetetra acetic acid (EDTA), [ethyleneglycolbis-(aminoethylether) tetra acetic acid] (EGTA), ferricyanide, H_2O_2, iron sulphate, KCl (AnalaR), K_2HPO_4, KH_2PO_4, $MgCl_2 \cdot 6H_2O$ (AnalaR), methanol, potassium hydroxide,sodium dodecyl sulphate (SDS), Na_2PO_4 (AnalaR), sodium citrate (AnalaR), sodium chloride, sodium dithionite, sodium hydroxide, sulphuric acid, $NiCl_2$ (AnalaR), Triton X-100, were obtained from BDH Chemical Co, Edmonton, Alberta.

Adenosine triphosphate (ATP), antifoam A, horse heart cytochrome *c* (type III), [Tris(hydroxymethyl)aminomethane] (Tris), L-histidine, L-tryptophan, lysozyme (grade 1 from chicken egg white), cupric sulphate, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, deoxyribonuclease I, L-glutamic acid, isoamyl alcohol, ribonuclease I, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, tetracycline, N, N, N′, N′-tetramethyl-*p*-phenylenediamine (TMPD), sorbitol, sodium hydroxide, succinic acid, bicinchoninic acid, antimouse IgG (whole molecule) peroxidase conjugate, 3, 3′-diaminobenzidine hydrochloride, and $ZnSO_4 \cdot 7H_2O$ were obtained from Sigma Chemical Company, St.Louis, Mo., USA.

Acrylamide, ammonium persulphate, bisacrylamide, bromophenol blue, Coomassie® brilliant blue R-250, N, N, N′, N′-tetramethylethylenediamine (TEMED), urea, all electrophoresis purity reagents, and Bio-Beads SM-2 were

obtained from BioRad Laboratories, Richmond, Ca., USA.

Isopropylthio-ß-D-galactoside (IPTG), 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal), agarose (ultra pure), low melting point agarose (ultra pure), buffer saturated phenol (ultra pure), lambda DNA, restriction endonucleases; Eco RI, Hind III, Bam HI, Sal I, Sma I, Bst EII, T_4 DNA ligase, T_4 DNA polymerase and T_4 polynucleotide kinase were obtained from GIBCO BRL, Life Technologies, Gaithersburg, Md., USA.

Ammonium sulphate, glycerol, chloroform, mineral oil and sodium acetate were obtained from Fisher Scientific, Nepean, Ontario. Ampicillin (sodium salt), dithiothreitol and proteinase K were obtained from Boehringer Mannheim Canada, Laval, Quebec. Tween 20 was obtained from Fluka, Ronkonkoma, NY., USA. Bovine albumin, fraction V powder, was obtained from Miles Laboratories, Inc., Kankakee, Il., USA. Yeast extract, agar, blood agar base, nutrient broth and tryptic digest of casein were obtained from Mikrobiologie, Edmonton, Alberta. Casamino Acids was obtained from Difco Laboratories, Detroit, Mi., USA. Oxygen and carbon monoxide were obtained from Linde Speciality Gases, Toronto, Ont. Restriction endonucleases Bcg I and Bcl I were obtained from New England Biolabs, Beverly, Ma., USA. Spectrapor membrane tubing was obtained from Spectrum Medical Industries, Inc., Los Angeles, Ca., USA.

Bacterial strains: *Bacillus subtilis* DB104 (his nprR2 nprE18 aprΔ3), *Bacillus subtilis* PD001 (his nprR2 nprE18 aprΔ3 ΔctaC) and *E. coli* DH5α. *B. subtilis* DB104 and *E. coli* DH5α were kindly provided by Dr. S.-L. Wong.

Maintenance of *Bacillus subtilis* cultures: Stocks of *Bacillus subtilis* were maintained as spore slants. Schaeffer's sporulation agar (Schaeffer *et al.*, 1965) contained per litre; 8g nutrient broth, 10 ml 10%(w/v) KCl, 10 ml 1.2%(w/v) $MgSO_4 \cdot 7H_2O$, 0.5 ml 1M NaOH, 1.5%(w/v) agar. The mixture is autoclaved and after cooling to 50°C; 1 ml 1M $Ca(NO_3)_2$, 1 ml 0.01M $MnCl_2$, and 1 ml 1mM $FeSO_4$ is added. Ten mls is poured into large sterile screw cap vials and tilted to cool, forming agar slants. The slants are streaked with bacterial cultures from fresh tryptose blood agar broth (TBAB) plates, and grown for 2 days at 37°C. Spore slants were stored at room temperature sealed with parafilm. Cultures were grown up overnight on TBAB plates, (without added blood), and stored at room temperature for up to one week.

***Bacillus subtilis* Growth in Liquid Medium:** One litre of culture medium was grown in a 2.8 litre Fernbach flask at 37°C with shaking at 250 rpm in an orbital shaker (Lab-Line, Orbit Environ-shaker), until the culture reached stationary phase, unless otherwise stated. Cultures were grown under two levels of oxygenation termed the "air" state and the "oxygen" state.

In the "air" state the flasks were sealed with a cotton plug during growth. In the "oxygen" state, a sintered glass sparger was immersed in the liquid culture medium. The 1 litre cultures were inoculated with 50 ml of starter culture prepared from freshly grown *B. subtilis* on tryptose blood agar base plates then grown overnight in air. Growth curves were determined spectrophotometrically via absorbance readings at 660 nm.

Composition of Culture Medium for *Bacillus subtilis*: Modified super rich medium, modified from Halling *et al.*, (1977), contained 3% carbon source, 2.5% tryptone, 2% yeast extract, and 0.3% K_2HPO_4, pH 7.5 with KOH. After autoclaving the medium was supplemented with 150μl/l micronutrient solution. The defined medium, (modified from that of Spizizen (1958) and C-Medium courtesy of Dr. S.-L. Wong) contained; 4.5% carbon source, 1.2% K_2HPO_4, 0.4% KH_2PO_4, 0.33% $(NH_4)_2SO_4$, 0.01% $MgSO_4$, pH 7.2. After autoclaving the medium was supplemented with 1.69mg/ml $MnSO_4$, 0.22mg/ml ferric ammonium citrate, 50mg/ml L-tryptophan, 50mg/ml L-histidine, 14.7mg/ml $CaCl_2$ and 150μl/l micronutrients. The carbon source used was either glucose or succinate in the modified super rich medium, or glucose and a 2:1 ratio of succinate:sorbitol in the defined medium. The micronutrient solution contained; 2.2% $MnCl_2$, 0.05% $ZnSO_4 \cdot H_2O$, 0.5% H_3BO_3, 0.016% $CuSO_4 \cdot 5H_2O$, 0.025% $Na_2MoO_4 \cdot 2H_2O$, 0.46% $Co(NO_3)_2 \cdot 6H_2O$, and 0.5 ml/100 ml concentrated H_2SO_4 (De Vrij *et*

al., 1983). Both types of media were supplemented as required with 15μg/ml tetracylcine, 10μg/ml kanamycin, unless otherwise specified.

Growth Media and Maintenance of *E. coli* Cultures: Luria-Bertani (LB) medium and agar was used for culturing *E. coli*. LB medium contains per litre; 10g tryptone, 5g yeast extract, 10g NaCl, pH 7.5. To solidify the medium for agar plates, add 1.5%(w/v) agar before autoclaving. The antibiotic ampicillin was added to 75μg/ml as required. Plates of *E. coli* were grown up overnight at 37°C and stored at 4°C for up to one month.

Dissolved Oxygen Content: The dissolved oxygen content of the culture medium during culture growth was monitored via a Clark-type electrode, (Yellow Springs Instruments) immersed in the culture medium. For these experiments, the cultures were stirred via a magnetic stirrer.

Harvesting *Bacillus subtilis* Cells: A 1 litre culture was harvested by centrifugation at 7000 rpm, 4°C for 10 minutes. The harvested cells were washed in 13 ml of 50mM K_2HPO_4 and the cells were recentrifuged at 7000 rpm, 4°C for 15 minutes.

Membrane Preparation and Characterization: To the washed cells was added 200ml/10g cell wet weight of lysing solution

containing 50mM Tris, 1mM EDTA, 0.375mg/ml lysozyme, 0.025mg/ml RNAase and 0.025mg/ml DNAase at pH 7.8. The cells were resuspended in lysing solution prior to addition of 0.3g of anhydrous $MgCl_2$ to 200 ml of lysing solution. The mixture was incubated at 37°C with shaking at 100 rpm for 45 minutes. Following this incubation 13 ml of 250mM EDTA, pH 7, was added per 200 ml lysing solution and incubated at room temperature for 3 minutes prior to addition of 1.7g of $MgSO_4$ per 200 ml lysing solution. This mixture was shaken for 15 minutes at 37°C, 100 rpm. The lysed cell mixture was centrifuged for 45 minutes at 4°C, 11,000 rpm. The resulting pellet was homogenized with 17.5 ml of 50mM Tris, 1mM EDTA, pH 7.8 and the suspension was centrifuged at 18,000 rpm, 4°C for 45 minutes. The pellet was retained as the membrane preparation.

Solubilization of Membrane Preparation: The membranes were resuspended and extracted in a solution containing 5%(v/v) Triton X-100, 50mM Tris, 1mM EDTA, pH 7.8 by incubation for 18h with stirring at 4°C. This procedure was carried out in 30 ml detergent buffer per 10g wet weight of cells. The following morning the mixture was centrifuged at 15,000 rpm for 15 minutes, 4°C to give a beige pellet and a reddish-brown supernatant. Subsequent extractions of the beige pellet under conditions as described above produced no further extraction of cytochromes and about 5% of the total protein that was extracted in the first incubation.

Membrane Protein Concentration Determination: Protein concentrations were determined using the Bicinchoninic Acid protein assay, modified from Smith *et al.*, (1985). Six protein standards were used to prepare a calibration curve for the determination of unknown protein concentrations. The protein standards contained 0, 20, 40, 60, 80, and 100μg of bovine serum albumin. The concentration of stock bovine serum albumin was 1mg/ml. Each protein standard was contained in a final volume of 100μl. The unknowns were similarly prepared by diluting a fixed volume of unknown protein solutions to a final volume of 100μl. Two ml of protein determination reagent, (50:1, bichinchoninic acid solution:$CuSO_4 \cdot 5H_2O$) was added to each tube, the tubes were vortexed and then incubated at 37°C for 45 min. The absorbance at 562 nm was determined. The initial tube in the protein standards containing no bovine serum albumin was used as a blank.

Absorbance Determination: Spectroscopic measurements were made on a Shimadzu UV-160 UV-VIS spectrophotometer using Shimadzu PC-160 software for instrument control and data handling.

Membrane Extract Heme Concentration: Cytochrome concentrations were calculated from spectra of membrane extracts using the following extinction coefficients; heme B reduced minus oxidized extinction coefficient of 20 $mM^{-1}cm^{-1}$ for the wavelength pair of 562-540 nm (Kita *et al.*, 1983). Heme A

reduced minus oxidized extinction coefficient of 25 $mM^{-1}cm^{-1}$ for the wavelength pair 600/602-630 nm (Saraste *et al.*, 1991), and heme A reduced plus CO bound minus reduced extinction coefficient of 15 $mM^{-1}cm^{-1}$ for the wavelength pair 590-605 nm (Greenwood *et al.*, 1974).

Enzymatic Assay: For the assay of cytochrome *c* oxidase activity the assay mixture contained 650μl 50mM Tris, 1mM EDTA, pH 7.8, plus 150μl of 150μM reduced horse heart cytochrome *c*. The reaction was initiated by the addition of 60μl membrane extract in 5% Triton X-100. Absorbance was recorded at 550 and 540 nm for the duration of the experiment at 4 second intervals using the Hewlett Packard 8452A Diode Array Spectrophotometer and the data manipulated with accompanying kinetic software.

Reduced Cytochrome *c*: Horse heart cytochrome *c* was reduced with excess sodium ascorbate and allowed to stand for five minutes. The excess ascorbate was removed by passing the mixture through a Sephadex G-25 column. The concentration of reduced cytochrome *c* was determined spectrophotometrically using an extinction coefficient of 21.2 $mM^{-1}cm^{-1}$ for reduced minus oxidized spectrum at 550 minus 540 nm (Margoliash and Frohwirt, 1959).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis: A 12% polyacrylamide slab gel with 4% SDS and 12% urea was used to determine the protein content of the membrane extracts. The amount of Triton X-100 in the membrane extracts was reduced either by dialysis or using BioBeads prior to electrophoresis. The gel, depending on its size, was run at 20 or 40 mA until the dye front came out of the gel. Sample volumes loaded were a maximum of 20 or 40μl protein sample, 1 or 2μl DTT and 1 or 2μl bromophenol blue (0.05% in 30% ethanol/H_2O) depending on the gel size. Molecular weight of the protein bands were determined using high and low molecular weight Rainbow Markers (Amersham). The gels were stained with Coomassie blue stain, 1g/l Coomassie blue R-250; 400ml/l methanol, 100ml/l glacial acetic acid, H_2O, for at least 15 min and then destained in 400ml/l methanol, 100ml/l glacial acetic acid, H_2O.

Dialysis of Membrane Extracts: A sample of membrane extract, (200μl) was put into dialysis tubing with 5%(v/v) SDS. The membrane extracts were dialyzed for two days at room temperature with stirring vs 4 changes of 500 ml dialysis buffer, (50mM Tris, 1% SDS, pH 6.8).

Bio-Beads: The amount of Triton X-100 in the membrane extract was reduced prior to SDS-PAGE via the batch method with Bio-Beads (SM-2 20-50 Mesh). The Bio-Beads, (3g dry Bio-Beads per

1 ml of membrane extract) were swollen according to the procedure of Holloway, (1973). The mixture of swollen Bio-Beads and membrane extract was incubated for one hour at room temperature with agitation at 100 rpm. After the sample was recovered by pipetting the liquid from the beads, SDS was added to a final concentration of 5%(v/v). This mixture was incubated at 40°C for one hour and is stored at room temperature prior to electrophoresis.

Plasmid Vectors: The *B. subtilis* plasmid pUB18, and the *E. coli* plasmid pBM$^+$ (pBluescribe M13$^+$), were courtesy of Dr. S.-L. Wong.

Restriction Enzyme Digestion: The restriction enzyme digests were carried out in a total volume of 10μl using 1μl of the appropriate restriction enzyme, 1μl of the supplied 10x restriction enzyme buffer, plasmid DNA, and autoclaved MilliQ water. The digests were incubated for a minimum of one hour at the appropriate temperature according to the specification of the restriction enzyme supplier.

Agarose Gel Electrophoresis: A 0.8% agarose gel in TAE buffer, (40mM Tris-acetate, 1mM EDTA) containing 0.5μg/ml ethidium bromide is heated to a boil. The agar is cooled to 50°C under running water with swirling, and then poured into the gel form, (HE33B minnie horizontal agarose submarine unit, Hoeffer

Scientific Instruments). DNA samples in 10μl plus loading buffer, (6x buffer; 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40%(w/v) sucrose in water) are run at 12 V/cm in TAE buffer with ethidium bromide until the dye front has progressed a sufficient distance down the gel. Molecular weights of the DNA bands were determined by comparison to a BstEII digest of lambda DNA. The DNA bands were visualized with UV light and polaroid photographs taken of the gel. Procedure as adapted from Maniantis, (1989).

Low Melting Point Agarose Gel Electrophoresis: A 0.9% low melting point agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA) containing 0.5μg/ml ethidium bromide is heated to a boil. The gel is cooled to 50°C under running water with swirling and poured into the gel form in the cold room. The gel is run at 6 V/cm in TAE buffer containing ethidium bromide in the gel tray which has been chilled at -20°C. Procedure as modified from Maniantis, (1989).

Miniscreen for *B. subtilis* Plasmid DNA: Two mls of LB broth containing required antibiotics were inoculated with one loopful of bacteria, grown for 2-3 hours at 37°C with shaking at 250 rpm. The 2 ml culture was centrifuged (Beckman Microfuge 12) for 2 minutes at 4°C, 10,000 rpm discarding the supernatant after each spin. The pellet was washed in 1 ml SET buffer (20% sucrose, 50mM Tris, 50mM EDTA, pH 7.6), vortexed

for 1 min and centrifuged for 2 min as before. The pellet was resuspended in 80μl SET, 20μl SET/lysozyme (SET buffer/20mg/ml lysozyme) and vortexed, then 5μl RNAase buffer (10mg/ml pancreatic RNAase in 0.1M NaOAc) was added and vortexed again. The mixture was incubated in a 37°C waterbath for 10 minutes. Two hundred μl fresh lytic mix (1% SDS, 0.2M NaOH) was added and mixed gently by inversion prior to sitting on ice for 5 minutes. Cold 1.5M KOAc, (150μl) was added to give a pH of 4.8, mixed by inversion and incubated on ice for 20 minutes. The mixture was centrifuged for 10 min at 13,000 rpm as above. The supernatant was transferred to a clean 1.5 ml tube and centrifuged again for 10 minutes at 13,000 rpm. The supernatant was transferred to a clean 1.5 ml tube and extracted 3 times with an equal volume of buffer saturated phenol. To the supernatant was added 2-2.5 times 95% ethanol and 1/10 volume 3M NaOAc. This was incubated at -20°C for a minimum of 30 min and centrifuged at 13,000 rpm for a minimum 30 min. The pellet was washed with 1 ml of 70% ethanol and spun for 5 min at 13,000 rpm. The DNA pellet was vacuumed dry for 10 minutes (Spin-Vac) and resuspended in 20μl of TE buffer, (10mM Tris, 1mM EDTA, pH 7.4) and stored at -20°C.

Miniscreen for *E. coli* Plasmid DNA: Two mls of LB broth containing required antibiotics were inoculated with one loopful of bacteria and grown overnight at 37°C with shaking at 250 rpm. The 2 ml culture was centrifuged in a 1.5 ml tube

for 5 min at 4°C, 10,000 rpm discarding the supernatant after each spin. The cells were resuspended in 100µl ice cold solution I, (50mM glucose, 25mM Tris, 10mM EDTA, pH 8) and incubated on ice for 5 min. Two hundred µl of freshly made solution II, (10% SDS, 1M NaOH), was added, inverted to mix and incubated on ice for 5 min. One hundred and fifty µl of ice cold solution III, (83.6% 5M KOAc, 16% glacial acetic acid) was added, inverted to mix, and incubated on ice for 5 min. The mixture was centrifuged for 5 min at 4°C, 13,000 rpm. The supernatant was transferred into a clean 1.5 ml tube and extracted once with an equal volume of buffer saturated phenol. The aqueous layer was removed and extracted once with chloroform:isoamyl alcohol, (24:1). To this layer was added 2-2.5 volumes ice cold 95% ethanol and 1/10 volume 3M sodium acetate, incubated at -20°C for a minimum 30 min and centrifuged at 4°C, 13,000 rpm for a minimum 30 min. The pellet was washed with 1 ml ice cold 70% ethanol and spun at 13,000 rpm for 5 min. The pellet was vacuumed dry for 10 minutes and resuspended in 20µl of TE buffer, with 1µl of RNAase buffer. Procedure as modified from Maniantis, (1989).

Ligation of Plasmid DNA in Low Melting Point Agarose Gel: The plasmid DNA bands were cut out of the LMP gel with a sterile razor blade under long wavelength UV light. The volume of the ligation reaction of the plasmid DNA fragments in LMP gel depended on the volume of the sample in the LMP gel. The

following is the protocol used for a 5μl volume of LMP gel where the DNA ratio is 1 volume vector DNA to 4 volumes insert DNA. The sample of DNA in LMP gel was melted for 3-4 min at 65°C. Seventeen μl of autoclaved MilliQ water was added and this mixture was incubated at 65°C for 10 min. After the tubes readjusted to room temperature, 6μl of 5x T_4 DNA ligase buffer and 1.3μl T_4 DNA ligase were added. The ligation reaction took place overnight at room temperature. Procedure as adapted from Maniantis, (1989).

Attachment of Synthetic BamHI DNA Linkers: For the linker phosphorylation reaction 1μl of 10x Linker-kinase buffer, (0.66M Tris-Cl, pH 7.6, 10mM spermidine, 0.1M $MgCl_2$, 150mM DTT, 2mg/ml BSA), 1μl T_4 polynucleotide kinase, 2μl 5mM ATP, 1μl linker and 5μl H_2O were added to a 1.5 ml tube and incubated at 37°C for one hour. The subsequent ligase reaction was carried out directly in the original mixture with the addition of 2μl 5x T_4 DNA ligase buffer, 1μl 5mM ATP, 2μl T_4 DNA ligase, 1μl BSA and 5μl DNA. Ligate overnight at room temperature. Procedure as modified from Maniantis, (1989).

Removal of Protruding 3′ DNA Termini: Plasmid DNA was digested with desired restriction enzymes in 10μl total volume. After digestion 1μl 2mM dNTPs, and 1-2 units of T_4 DNA polymerase was added and incubated for 15 min at 12°C. The reaction was terminated by heating the reaction mix to 75°C for 10 min.

Procedure as modified from Maniantis, (1989).

Competent Cell Preparation and Transformation of *B. subtilis*: From a freshly grown plate suspend 1 loopful of bacteria into 1.5 ml SPI media; (SPI salts, 0.2% $AmSO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.19% NaCitrate$\cdot 2H_2O$, 0.02% $MgSO_4 \cdot H_2O$) plus 1/100 volume 50% glucose and 1/100 volume casamino acids/yeast extract (2% casamino acids, 10% yeast extract) and grow for 3.5-4 hours at 37°C with shaking at 250 rpm. Transfer 0.5 ml of the culture in SPI medium to 4.5 ml SPII medium, (SPI medium plus 1/100 volume 50mM $CaCl_2$ and 1/100 volume 250mM $MgCl_2$) and grow for 1.5 hours at 37°C with shaking at 250 rpm. Add 50μl of 1mM room temperature EGTA, and let grow for 10 min. Aliquot 10μl of the plasmid DNA construct into the desired number of tubes and add 0.5 ml of bacterial culture to each tube, grow for 1.5 hours at 37°C with shaking at 250 rpm. Plate 100μl aliquots on room temperature TBAB plates plus any required antibiotic and incubate plates at 37°C. Protocol as modified from Gryczan *et al.*, 1978;Dubnau and Davidoff-Abelson, 1971.

Competent Cell Preparation of *E. coli*: Two mls of LB broth were inoculated with a loopful of bacteria and grown up overnight at 37°C with shaking at 250 rpm. The cells were pelleted in a sterile 1.5 ml tube with 2 spins of 5 min each at 4°C, 10,000 rpm. The cells were washed in 1 ml 10mM NaCl, vortexed to resuspend and centrifuged as above. The cell

pellet was resuspended in 1 ml of 30mM $CaCl_2$, incubated on ice for 20 min and centrifuged at 5,000 rpm for 10 min. The pellet was gently resuspended in 200μl of 30mM $CaCl_2$, for immediate use. To store frozen competent cells the pellet was resuspended in 30mM $CaCl_2$ plus 15% glycerol and frozen at -80°C for up to 3 months. Procedure as modified from Maniantis, (1989).

Transformation of *E. coli*: To a volume of plasmid DNA in a sterile 1.5 ml tube was added enough sterile water to make 100μl final volume. This mixture was centrifuged for 1 min at 4°C, 10,000 rpm. The top 90μl was removed and placed in a clean sterile tube and 10μl of 300mM $CaCl_2$ was added. This 100μl was added to 200μl of competent cells mixed gently and incubated on ice for 1 hour. The cells were heat shocked at 42°C for 60-75 seconds, added to 2 ml LB broth and grown for 3-4 hours at 37°C with shaking at 250 rpm. The cells were plated in 75μl aliquots on the desired plates and grown overnight at 37°C. Procedure as modified from Maniantis, (1989).

Western Immunoblot: An SDS polyacrylamide gel of two identical sets of lanes was run. One half of the gel was stained as stated previously (see SDS-PAGE gel) and the other half blotted via wet electrophoretic transfer (Towbin *et at.*, 1979). The gel section to be blotted was equilibrated in the

immunoblot transfer buffer, (25mM Tris, 190mM glycine, 20% methanol), for 15 min with agitation to facilitate the removal of SDS and urea from the gel. All components of the blotting apparatus were soaked in the transfer buffer. The blotting membrane, polyvinylidene difluoride, (PVDF), was first wetted in 100% methanol and then in the transfer buffer. Blotting was carried out for 1 hour or more at 100 V as required. The blotted half of the gel was stained, as stated previously, to determine the completeness of protein transfer. The filter is blocked in BLOTTO, (5%(w/v) non-fat dry milk, 0.2% Tween 20 in phosphate buffered saline (PBS)), for 2 hours at room temperature with agitation. The filter was washed in two changes of PBS, (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2) with shaking for 5 min each and incubated with the primary antibody, (1:100 dilution, 3% BSA in PBS), with agitation for 1 hour. After four washes in PBS as before, the filter is incubated with secondary antibody complexed to horseradish peroxidase, (1:2000 dilution, 3% BSA in PBS), with agitation for one hour. The filter is again washed in four changes of PBS as before. Detection of the secondary antibody is by diaminobenzidine with metal ion enhancement, (6mg of 3,3'diaminobinzidine hydrochloride in 9 mls of 50mM Tris, pH 7.6, 1 ml 0.3% $NiCl_2$, and 10μl of 30% H_2O_2) added to the blot and developed with agitation, for 1-5 min. The reaction is stopped by rinsing the blot with PBS. Procedure as adapted from Harlow and Lane, (1988).

Results

Effect of Oxygenation on *Bacillus subtilis*

Bacillus subtilis was grown under two levels of aeration, termed oxygen and air, to determine the effect of aeration on the growth of cells and cytochromes expression. *B. subtilis* cells were cultured on modified super rich medium containing succinate as the carbon source. Growth of the cultures under pure oxygen caused a visible increase in the cell density of the culture as opposed to cells grown under air. Growth under oxygen also increased the amount of proteins present in the membrane extract, but did not effect the cell growth rate as reflected in the culture doubling time (see Table 2).

Table 2: Culture growth data for *B. subtilis* DB104. Wildtype *B. subtilis* grown on modified super rich media with succinate under oxygen and air.

Growth Conditions	Cell[1] Pellet (g/l)	TX-100[2] Membrane Extract (mg/ml)	Culture Doubling Time (min)
air	7.1	3.1	38
oxygen	20.7	8.29	38

All cultures were grown in 1L of medium to stationary phase before harvesting. Membrane extract prepared and protein concentration determined as described in *Materials and Methods*. The values in this table were obtained from one experiment. Doubling time determined from semi-logarithmic plot of growth curve.

1. Wet weight of cell pellet
2. Triton X-100 membrane protein extract concentration

Differential expression of the two heme A containing terminal oxidases, cytochromes *caa*$_3$ and *aa*$_3$, in *Bacillus subtilis* was observed when the bacterium was grown on different carbon sources to different growth stages (Laureaus *et al.*, 1991). Cultures grown to stationary phase on glucose showed an increase in the heme A containing terminal oxidase cytochrome *aa*$_3$, whereas cultures grown on succinate under maximal aeration and harvested before stationary phase showed an increase in the heme A terminal oxidase cytochrome *caa*$_3$. These results were duplicated on modified super rich medium with either glucose or succinate as the carbon source. Cultures grown on glucose yielded the largest cell pellet wet weight under both levels of aeration, but had comparable membrane protein concentrations, heme A concentrations and heme A to protein ratios with cultures grown on succinate under air, and lower levels when compared to growth on succinate under oxygen (see Table 3).

Table 3: Comparison of Growth of *Bacillus subtilis* on two carbon sources and under two levels of aeration.

Growth Conditions	Cell[1] Pellet (g/l)	TX-100[2] Membrane Extract (mg/ml)	Heme A[3] Concentration (μmol)	Heme A[3]: Protein Ratio (μmol/mg
Glucose/air	9.73	4.84	1.25	.258
Glucose/O_2	27.67	6.75	0.15	.022
Succinate/air	7.07	3.09	0.65	.210
Succinate/O_2	20.01	8.29	0.57	.069

All cultures were grown in 1L of modified super rich medium under the conditions stated. Glucose cultures were grown to stationary phase and succinate cultures to mid-log phase before harvesting. Membrane extracts prepared and protein concentration determined as described in *Materials and Methods*. The values in this table were obtained from different experiments.
1. Wet weight of cell pellet.
2. Triton X-100 membrane extract protein concentration
3. Heme A concentration from cytochromes caa_3 and aa_3.

B. subtilis cultures show an increase in cell density and yield, in grams per litre of culture, when grown on oxygen as opposed to air on modified super rich medium (see Table 1). This response is seen in the growth curve in Figure 3 as an extended length of time the culture remains in exponential phase when grown under oxygen as opposed to air. The effect of additional aeration on cultures grown to stationary phase is shown in Figure 4. The air-grown stationary phase culture re-entered the exponential phase of growth when exposed to pure oxygen. Since the oxygen supply to the culture appears to play a large role in culture growth, the level of oxygen in the culture medium during growth was monitored. In fermentation

Figure 3: Comparison of growth curves of *Bacillus subtilis* cultures under two levels of aeration. Growth curves and aeration levels as stated in *Materials and Methods*. Cultures grown on modified super rich medium containing succinate. (*) grown under air, (+) grown under oxygen.

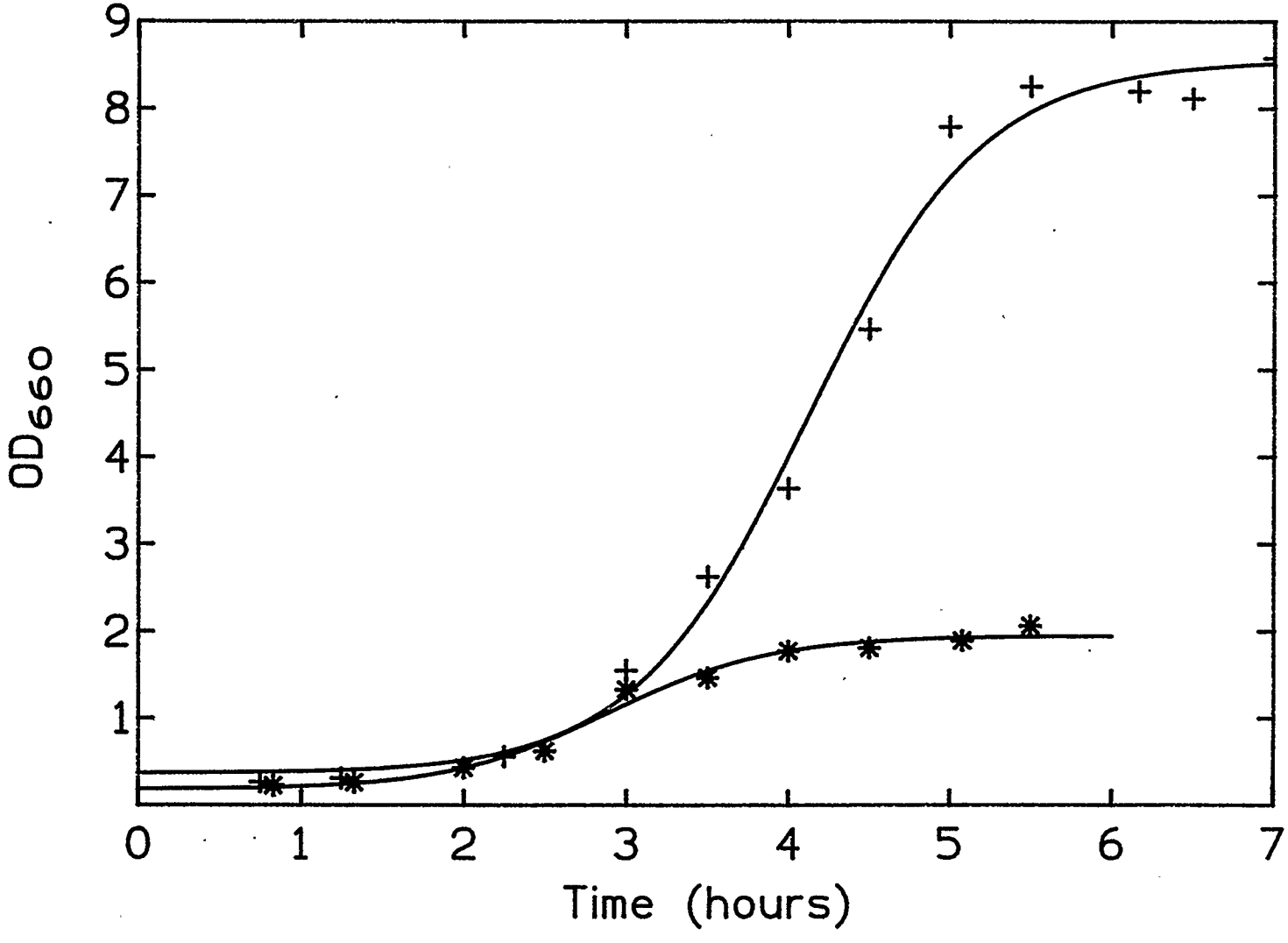

9
8
7
6
5
4
3
2
1
OD_{660}
0
1
2
3
4
5
6
7
Time (hours)

Figure 4: Growth curve of *Bacillus subtilis* culture grown under air then oxygen. Culture grown on modified super rich medium containing succinate until stationary phase under air. Half of the culture medium was removed at this "air" grown stationary phase and the culture growth continued under oxygen until a new "oxygen" grown stationary phase was reached. Arrow indicates initiation of oxygen into the culture media.

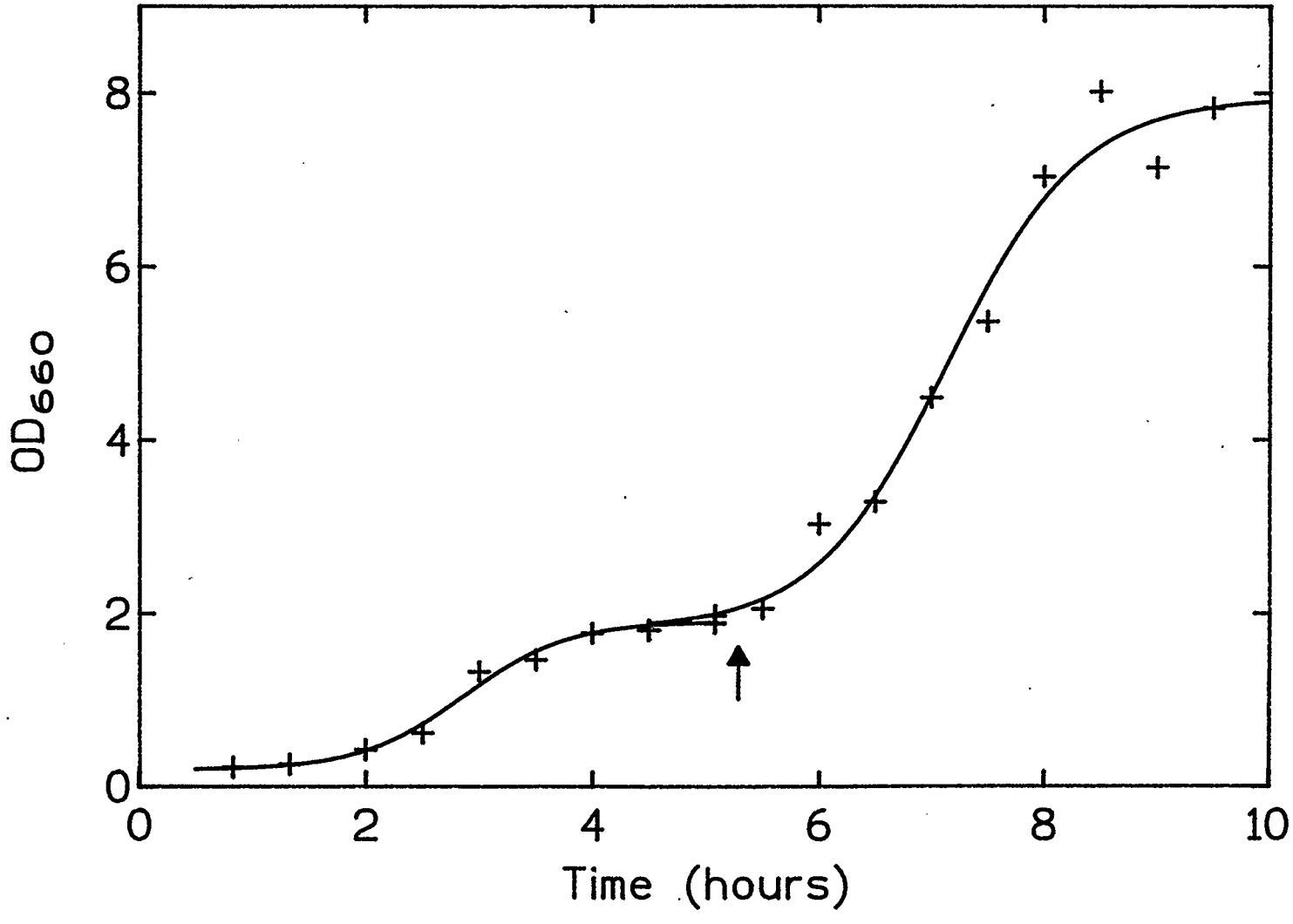
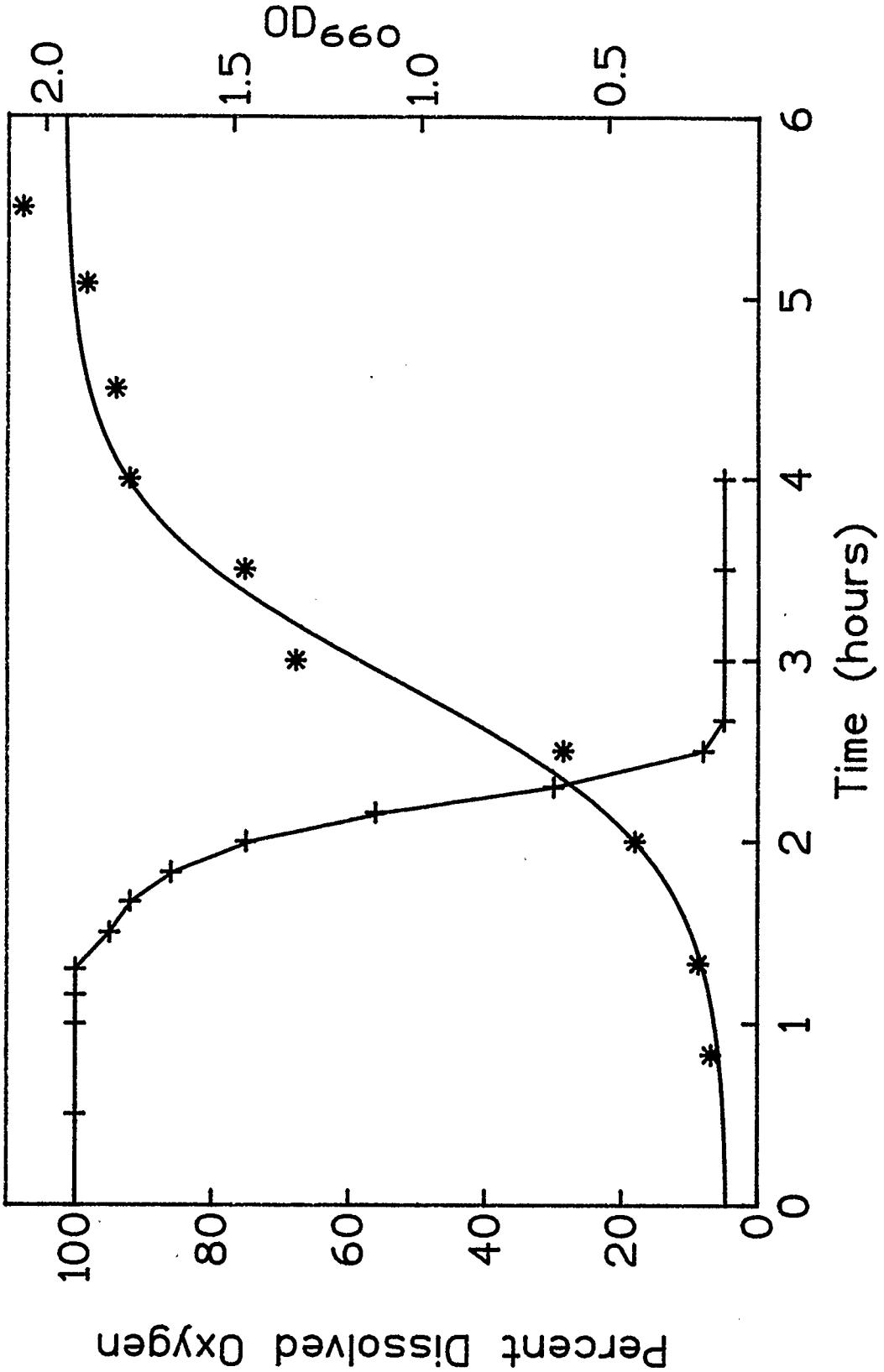

8
6
4
2
0
OD_{660}
0
2
4
6
8
10
Time (hours)

Figure 5: Growth curve and percent dissolved oxygen plot of *Bacillus subtilis*. Cultures were grown on succinate containing modified super rich medium under air. Dissolved air in the culture was monitored via an oxygen electrode in the culture medium. (*) growth curve. (+) percent dissolved oxygen.


Percent Dissolved Oxygen
100
80
60
40
20
0
OD660
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
Time (hours)

for successful bacterial culture growth it is recommended that the dissolved oxygen content (DOC) of the culture be maintained above 10% (Stanbury and Whitaker, 1984), as below this value the availability of oxygen to the culture is thought to effect culture growth. Figure 5 shows an arithmetic plot of a culture growth curve and the corresponding trace of DOC. This Figure shows that the level of dissolved oxygen begins to drop as the culture enters exponential growth. The dissolved oxygen content continues declining rapidly until it reaches a low steady level below 10% DOC. The level of oxygen reaches this low steady state level before the cell cultures reach stationary phase therefore it is the oxygen availability to the culture that is limiting growth. The same pattern of dissolved oxygen levels and cell growth were observed with cultures grown under pure oxygen. The oxygen concentration declined to very low levels, below 10%, when the culture entered exponential phase. As the oxygen cultures were aerated to a greater extent during growth resulting in a higher cell density, the low levels of dissolved oxygen in these cultures is a reflection of the level of transport of oxygen to the cells, rather than a lack of oxygen being supplied to the culture.

Cytochrome Content

One aim of this work was to characterize the levels of different cytochromes in the respiratory chain of *Bacillus subtilis* expressed under different conditions. An accepted

method of determining the cytochrome content of the *Bacillus subtilis* cell membrane, and the one used in this thesis, is spectrophotometric determination (Poole, 1988). Spectral data is used to determine the type and concentration of heme moieties present using the specific absorption spectrum of the heme group found in the particular cytochrome. Thus a particular spectral signature is easily assigned to the presence and amount of cytochromes *a*, *b*, *c* and *d*. (see Table 4).

Table 4: Characteristic absorption wavelengths for respiratory cytochromes.

cytochrome	α_{max} λ (nm)	reference λ (nm)	organism	ΔE $mM^{-1}cm^{-1}$
c	550	540	***B. subtilis***	21.2[2]
b	560	540	*B. subtilis*	16.9[3]
aa_3[1]	603	630	*B. subtilis*	25.2[4]
aa_3	600	630	*B. subtilis*	26.6[4]
d	628	650	*E. coli*	18.8[5]
o	560	580	*E. coli*	24.2[6]

1. aa_3 of cytochrome caa_3.
2. Margoliash and Frowirth, 1959.
3. Lauraeus and Wikstom, 1993.
4. Lauraeus *et al.*, 1991.
5. Kita *et al.*, 1986.
6. Puustinen *et al.*, 1991.

To enhance detection of specific absorbances of the membrane extracts over the visible range of wavelengths specific chemical reductants are used which selectively reduce

different cytochromes. This selective reduction is based on the redox potential or kinetic reactivity of the chemical reductant relative to a particular respiratory component. The reduced heme moieties absorb more strongly than oxidized hemes of the respiratory chain and by utilizing the selective nature of certain chemical reductants it is possible to separate the individual heme absorbances of the membrane extract. To determine an individual heme absorbance value, the spectrum of the oxidized extract is subtracted from that of the chemically reduced spectrum of the same membrane extract. This results in a reduced minus oxidized difference spectrum which gives specific signals for the different classes of cytochromes. The chemical reductants normally employed for the reduction of heme moieties are sodium dithionite, a strong reductant capable of reducing the entire respiratory chain, and the combination of ascorbate plus TMPD, (N, N, N′, N′-tetramethyl-*p*-phenylene diamine) a thermodynamically weaker reductant which is kinetically specific for cytochromes *c* and aa_3. Figures 6 to 9 are an example of the absorption spectra obtained from the membrane extract of a single culture of *B. subtilis* DB104. These spectra are from a culture grown on succinate containing modified super rich medium under oxygen.

Figure 6 shows a typical series of spectra obtained for a sample of membrane extract from *B. subtilis*. Figure 6A shows absolute spectra of an oxidized sample of membrane extract, trace A, and the same membrane extract reduced with

sodium dithionite, trace B. Figure 6B is a difference spectrum constructed from the absolute spectra in panel A by subtraction of the oxidized spectrum in Figure 6A, trace a, from the reduced spectrum, trace b. The membrane extract in this case is from wildtype *Bacillus subtilis* and is in buffer with 5% Triton X-100. An additional complication encountered when such high levels of Triton X-100 were used to solubilize the membrane, was that the respiratory chain is partially reduced in air-saturated buffer. Thus, to ensure that the air oxidized membrane sample used to obtain the initial spectrum is completely oxidized, the oxidant ferricyanide is added to the sample to create a ferricyanide-oxidized spectrum. Trace b in Figure 6A is the ferricyanide-oxidized state. The effect of ferricyanide is shown in Figure 6C which is the air-oxidized respiratory chain minus the ferricyanide-oxidized spectrum. The small peaks at 550 and 600 nm show the amount of cytochromes *c* and *a* that remain reduced in the air oxidized membrane extract.

In order to determine which of the heme containing proteins might be terminal oxidases, carbon monoxide is added to the reduced membrane extract. Terminal oxidases will form a stable adduct with carbon monoxide (CO) at the open coordination site on the heme which is there to react with the substrate oxygen. This CO-bound form of the protein is spectrally detectable by measuring the difference between the CO-bound form of the reduced cytochrome and the reduced

Figure 6: Absolute absorption spectra of *Bacillus subtilis* membrane extracts. Membrane extracts produced as described in *Materials and Methods*. (A) Absolute spectra of a sample of membrane extracts. Scan a is the ferricyanide oxidized membrane extract. Scan b is the membrane extract reduced with 25mM sodium dithionite. (B) Dithionite reduced minus oxidized difference spectrum of scan b minus scan a in (A). (C) Difference spectra of air oxidized minus ferricyanide oxidized membrane extracts.

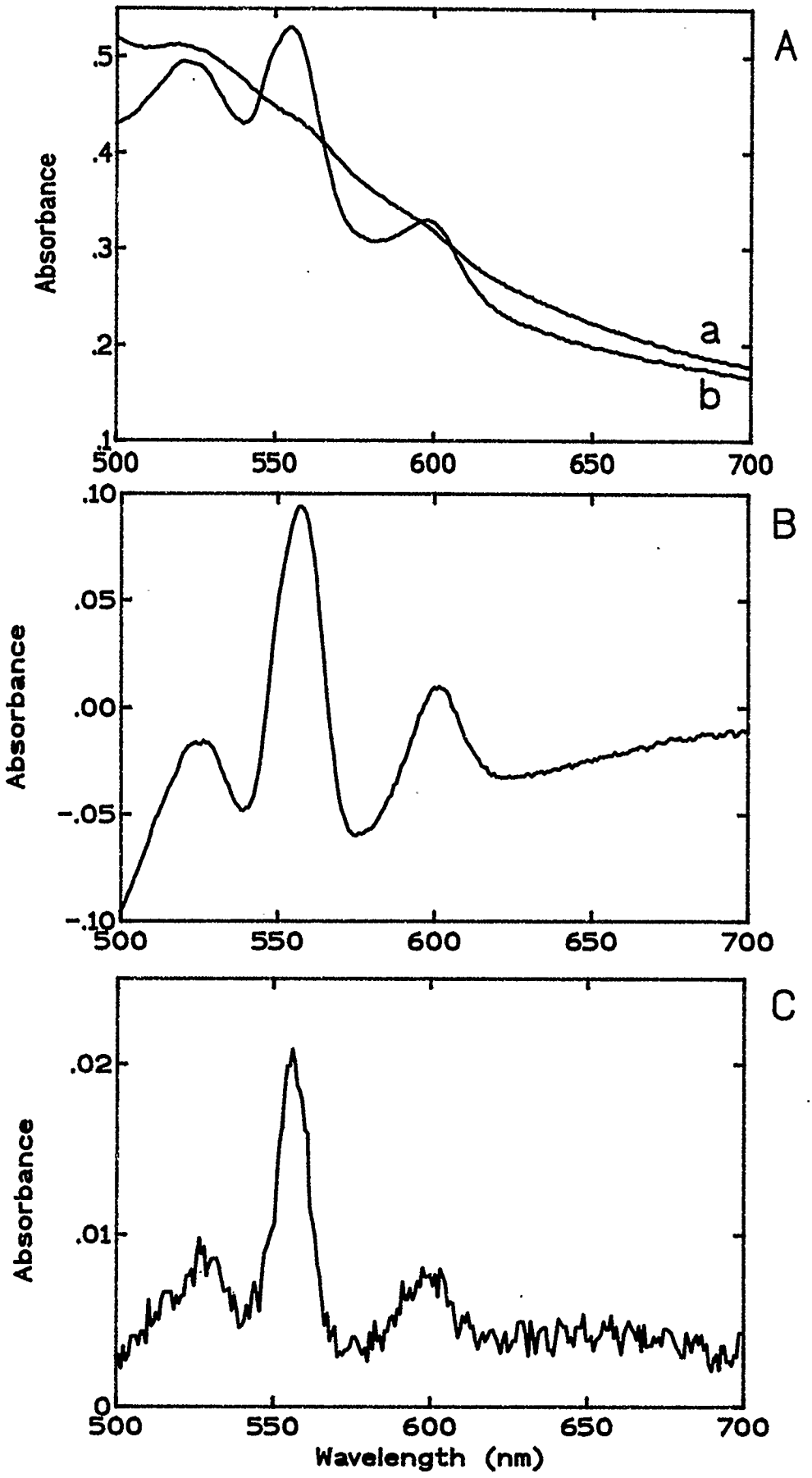

A
Absorbance
.5
.4
.3
.2
.1
a
b
500
550
600
650
700
B
Absorbance
.10
.05
.00
-.05
-.10
500
550
600
650
700
C
Absorbance
.02
.01
0
500
550
600
650
700
Wavelength (nm)

Figure 7: Dithionite reduced plus carbon monoxide bound minus dithionite reduced difference spectrum of *Bacillus subtilis* membrane extract. Membrane extract produced as described in *Materials and Methods*. A sample of membrane extract was reduced with 25mM dithionite and bubbled under a stream of CO for 10 minutes. This dithionite plus CO spectrum is subtracted from the original dithionite reduced spectrum to give this dithionite plus CO minus dithionite difference spectrum.

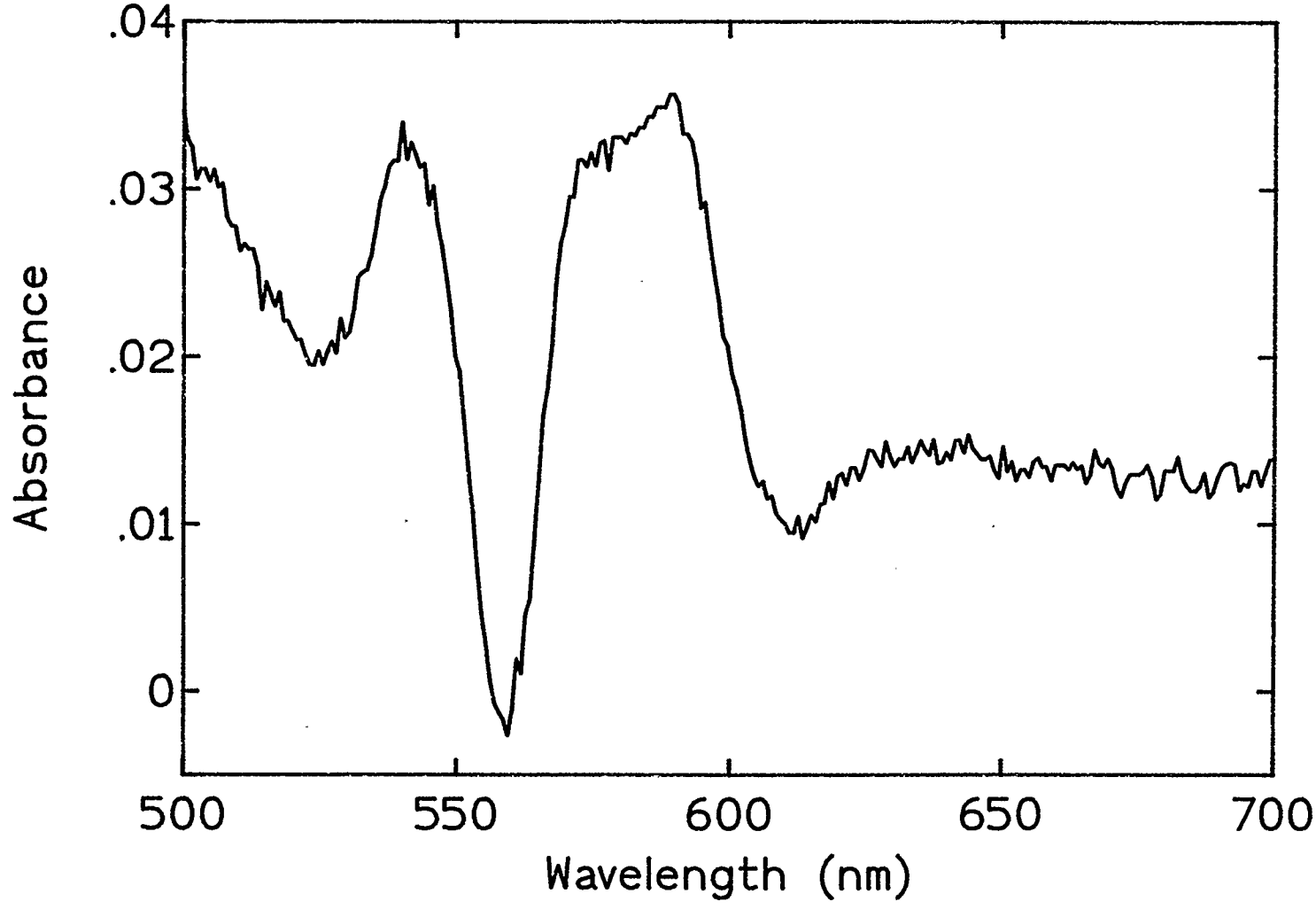

.04
.03
.02
.01
0
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 8: Ascorbate plus TMPD reduced minus oxidized difference spectrum of *Bacillus subtilis* membrane extract. Membrane extract produced as described in *Materials and Methods*. A sample of membrane extracts was reduced with 5mM ascorbate and 0.2mM TMPD. The ascorbate and TMPD reduced spectrum was subtracted from the ferricyanide oxidized spectrum giving the ascorbate plus TMPD minus ferricyanide oxidized difference spectrum.

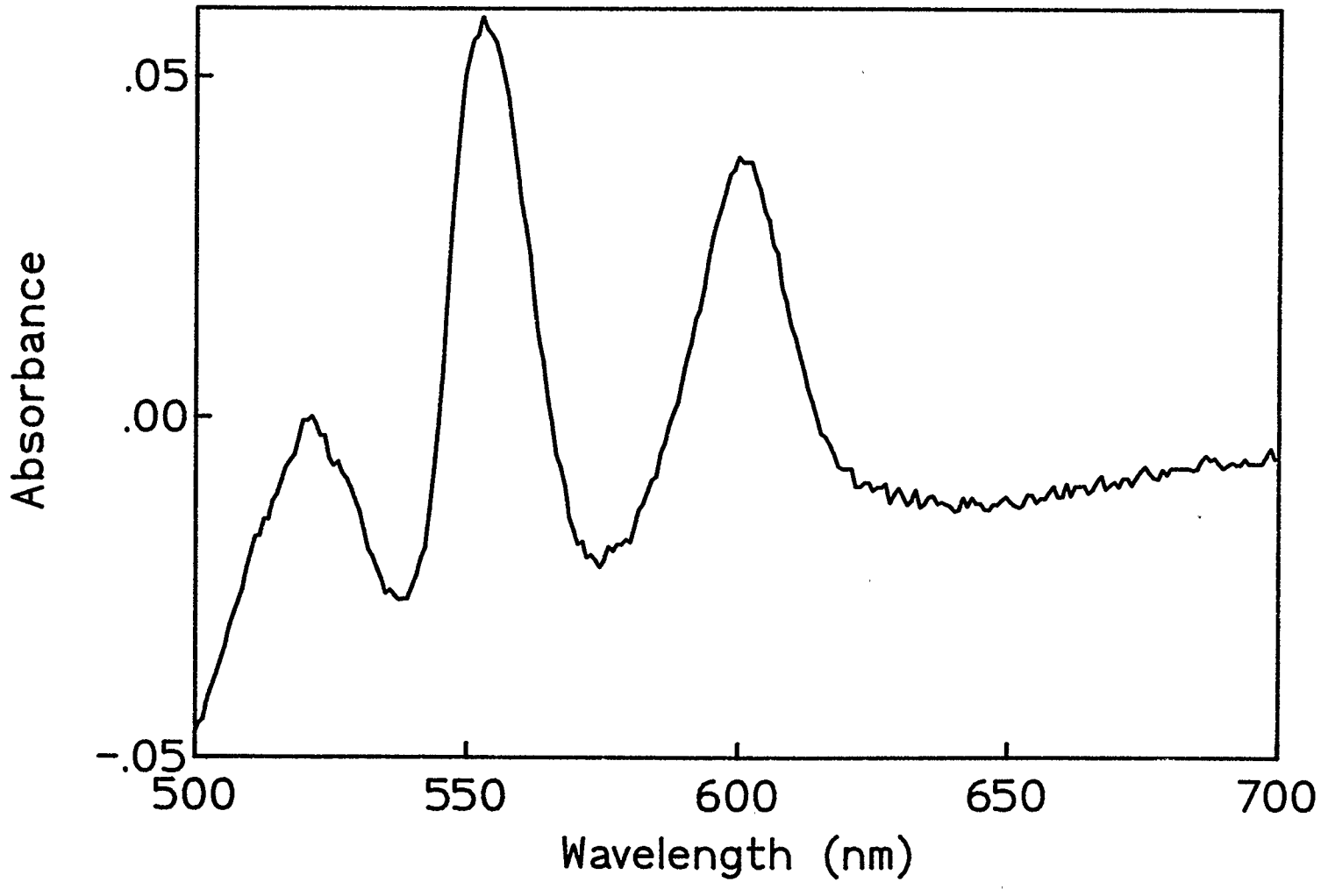
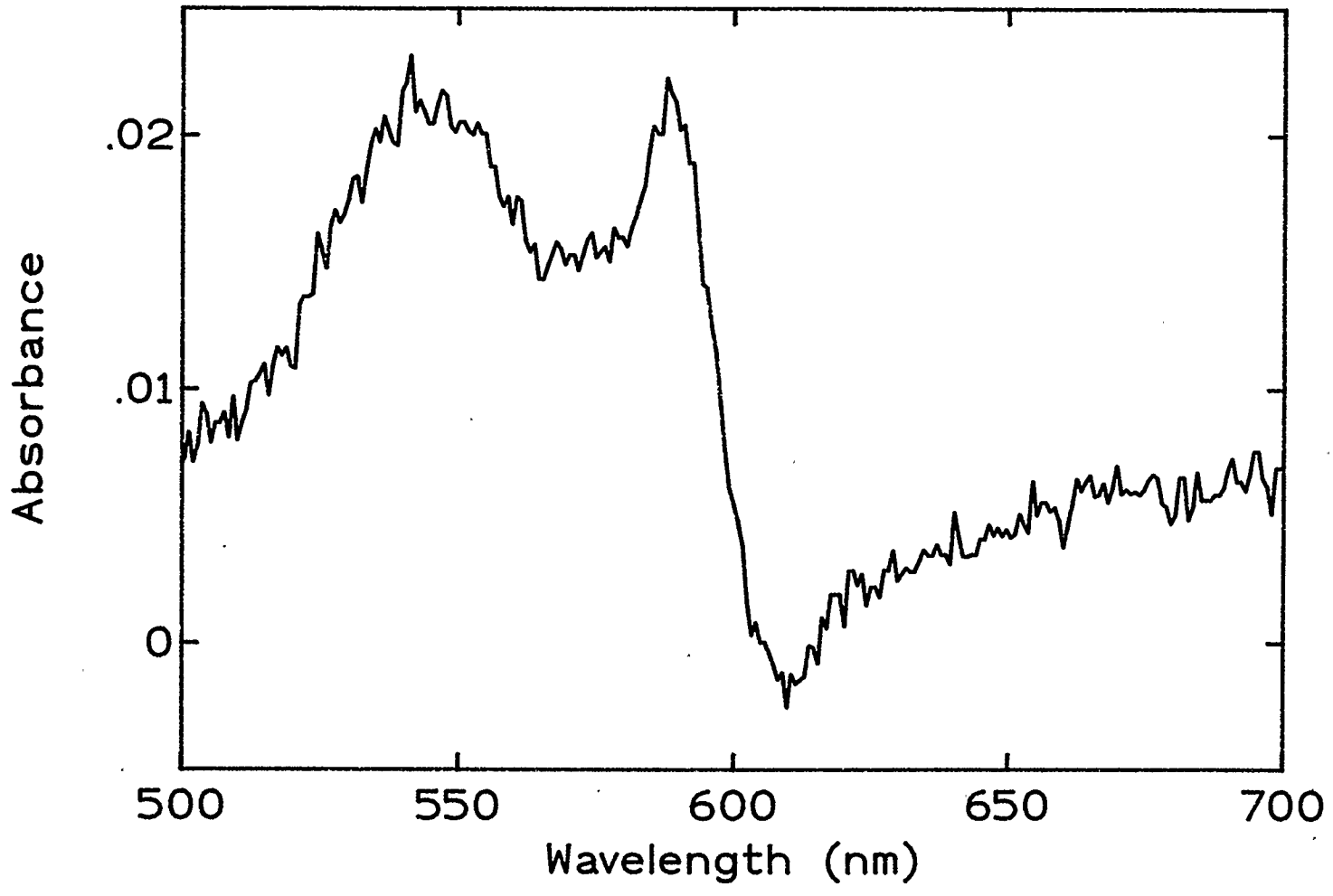

.05
.00
-.05
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 9: Ascorbate plus TMPD reduced and carbon monoxide bound minus reduced difference spectrum of *Bacillus subtilis* membrane extract. Membrane extract produced as described in *Materials and Methods*. A sample of membrane extracts was reduced with 5mM ascorbate and 0.2mM TMPD and bubbled under a stream of CO for 10 minutes. This spectrum was subtracted from the original ascorbate and TMPD reduced spectrum to give the ascorbate and TMPD reduced plus CO minus ascorbate and TMPD reduced difference spectrum.


.02
.01
0
Absorbance
500
550
600
650
700
Wavelength (nm)

cytochrome in the absence of CO. In this case the difference spectrum is referred to as the CO-bound reduced minus reduced spectrum. The CO-bound difference spectra for cytochrome *a* and *b* terminal oxidases are detectable as specific peak to trough absorbance patterns (Poole *et al.*, 1981;Poole *et al.*, 1982). Figure 7 shows the dithionite reduced, CO-bound minus dithionite reduced difference spectrum. In this Figure the spectral components of the cytochrome a_3-CO complex and the cytochrome *b*-CO complex are apparent. The cytochrome a_3-CO complex gives rise to the trough at 611 nm and the peak at 590 nm, whereas the cytochrome *b*-CO complex gives the trough at 558 nm and the peaks at 541 and 575 nm. Cytochrome *b* species which bind CO have conventionally been assigned as cytochromes *o*. Recently, a new type of heme, heme O, has been identified as a chemically distinct heme in the well-characterized cytochrome *o* oxidase of *E. coli* (Wu *et al.*, 1992). Whether or not the CO-binding cytochrome *b* of *B. subtilis* contains heme O has not been determined. Figure 8 shows the ascorbate plus TMPD reduced minus ferricyanide oxidized difference spectrum. Ascorbate plus TMPD acts to reduce the cytochrome caa_3 and cytochrome aa_3 oxidases of the *B. subtilis* membrane extract (Hill *et al.*, 1993). Comparing Figure 6B, the dithionite reduced membrane extract with Figure 8, the ascorbate plus TMPD reduced membrane extract one can see the different specificities of these chemical reductants. Specifically Figure 6B shows absoption peaks for all the cytochromes in the

membrane extract with peaks at 600, 560 and 550nm, and Figure 8 shows absoption peaks at 600 and 550nm for hemes A and C only.

When CO is added to the ascorbate plus TMPD reduced membrane and subtracted from the original ascorbate plus TMPD reduced spectrum, the CO-bound minus ascorbate plus TMPD reduced difference spectrum shows the signals of the cytochrome a_3-CO complex (see Figure 9).

Figure 10 shows the dithionite reduced minus ferricyanide oxidized difference spectrum of membrane extracts from *B. subtilis* grown on oxygen and air. An increase in the heme B levels due to cytochrome *o* over and above the increase in the other heme concentrations can be seen at 560nm in Figure 10. This is more clearly visible in the dithionite reduced plus CO bound minus dithionite reduced difference spectra of cultures grown on oxygen and air, shown in Figure 11. The cultures grown in either air or oxygen show about the same level of cytochrome a_3-CO complex when the membranes are reduced with ascorbate plus TMPD. However, in membranes reduced with dithionite then combined with CO to reveal the level of cytochrome *o* there is a distinct increase in the level of cytochrome *o* in the oxygen-grown culture.

Analysis of *B. subtilis* Plasma Membranes

The levels of membrane proteins in *B. subtilis* cultures grown on oxygen and air are detectable via SDS polyacrylamide gel electrophoresis. A SDS-PAGE gel of *B. subtilis* membrane

Figure 10: Dithionite reduced difference spectra of *B. subtilis* DB104 grown under oxygen and air. Cultures grown in succinate containing modified super rich medium under air and oxygen. Membrane extracts in 5% Triton X-100, produced as described in *Materials and Methods*. Membrane extracts reduced with 25mM dithionite. (——) cultures grown under oxygen. (---) cultures grown under air.

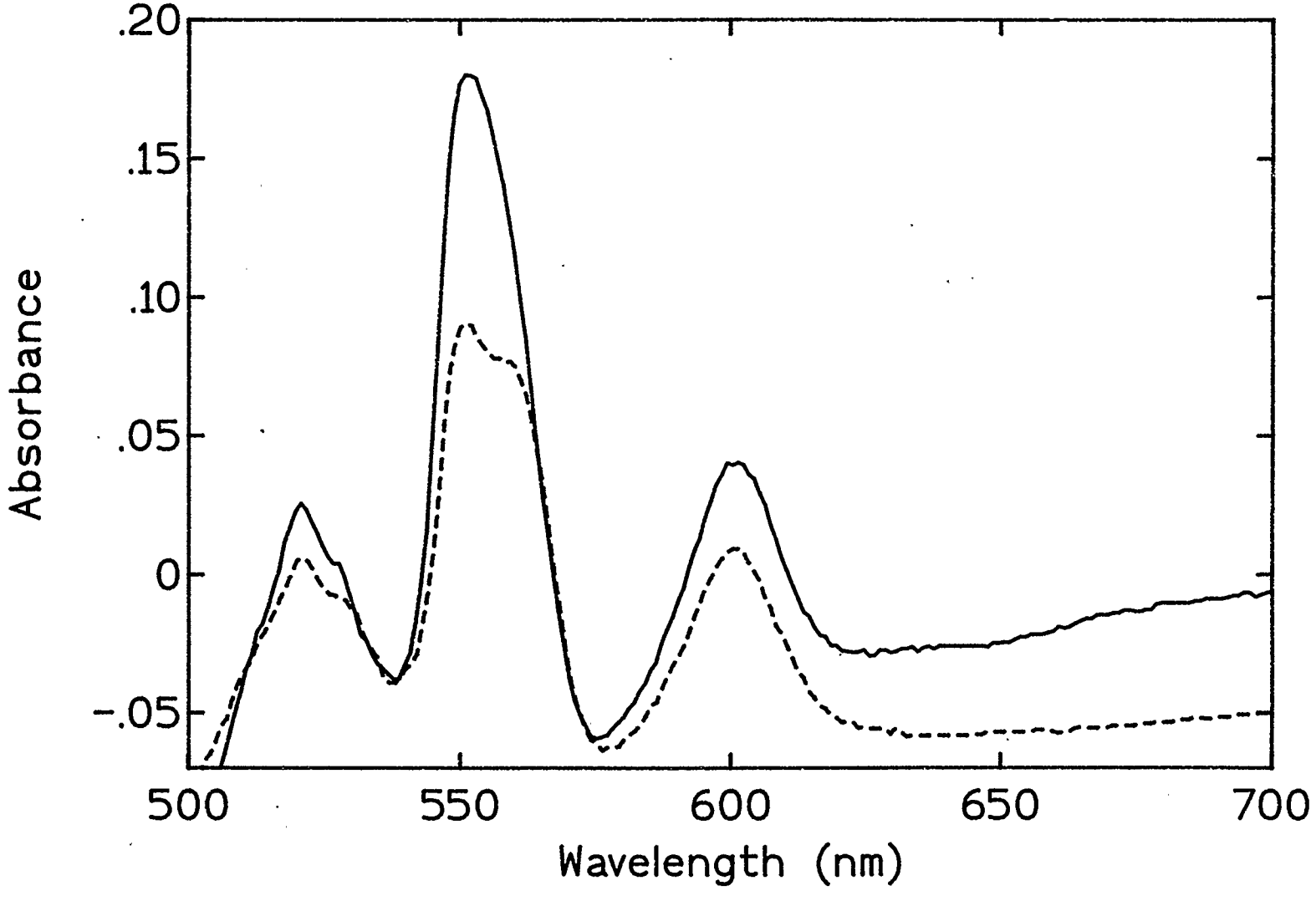
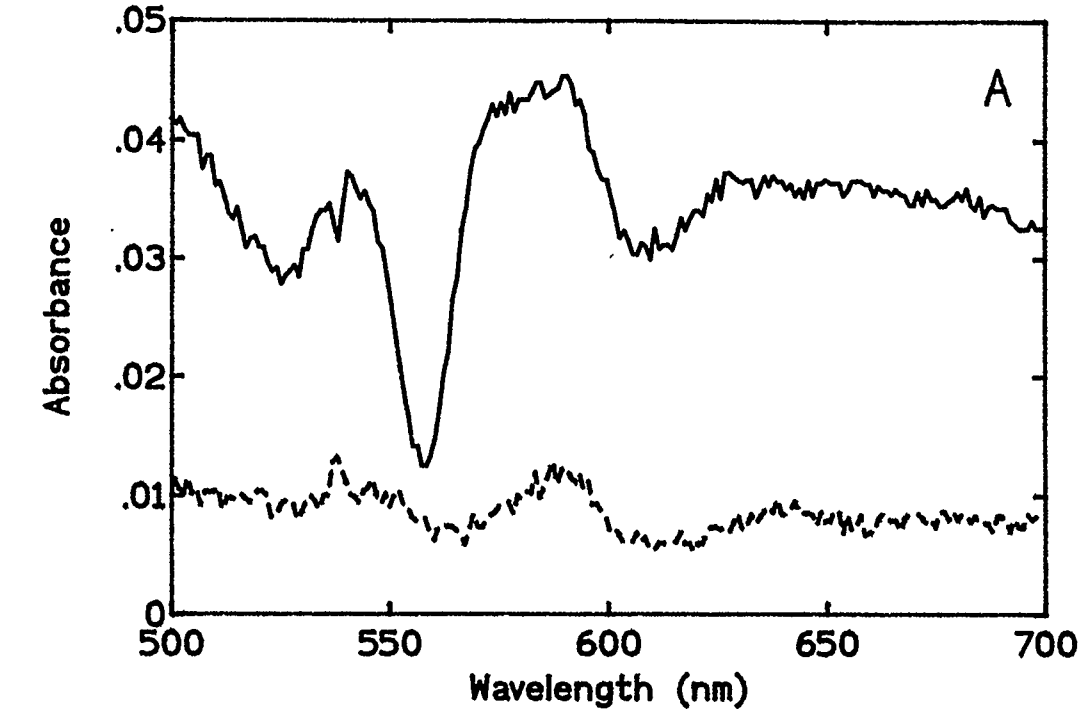

.20
.15
.10
.05
0
-.05
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 11: Reduced plus carbon monoxide bound minus reduced difference spectra of *Bacillus subtilis* membrane extracts grown under oxygen and air. Membrane extracts reduced with either 25mM dithionite (A) or 5mM ascorbate plus 0.2mM TMPD (B), bubbled under a stream of CO for 10 minutes, and subtracted from the original reduced spectrum. A) dithionite reduced difference spectrum of cultures grown under oxygen (——), cultures grown under air (---). B) ascorbate plus TMPD reduced difference spectrum of cultures grown under oxygen (——), cultures grown under air (---).


A
.05
.04
.03
.02
.01
0
Absorbance
500
550
600
650
700
Wavelength (nm)

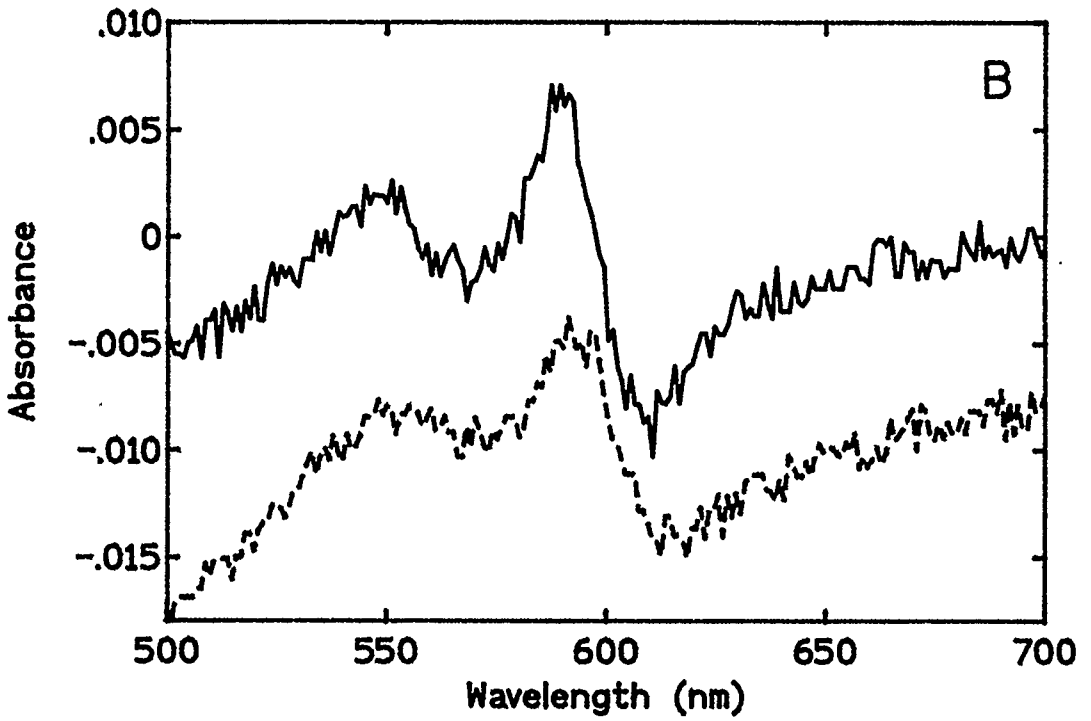
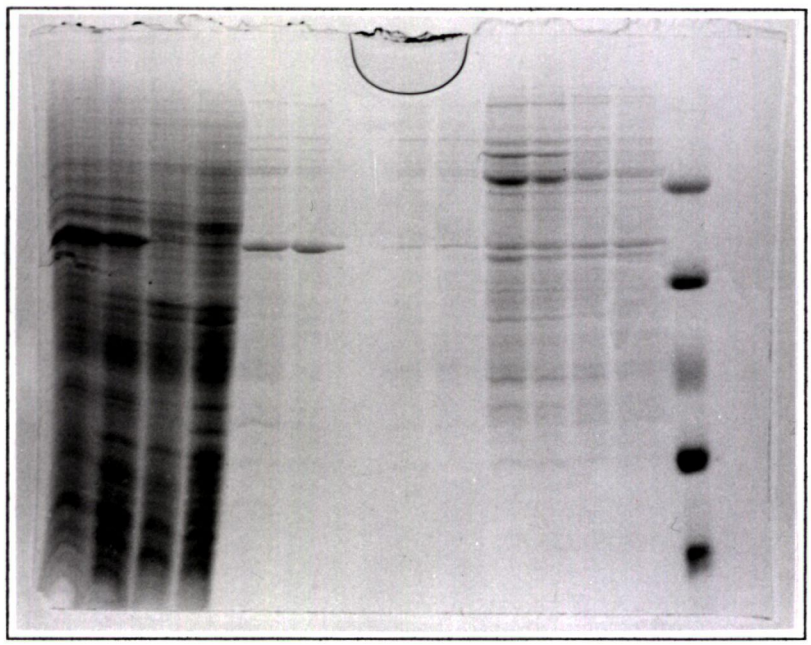

B
.010
.005
0
-.005
-.010
-.015
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 12: SDS polyacrylamide gel of a serial extraction of *Bacillus subtilis* wildtype and mutant membranes grown under oxygen and air. First and second extract as described in *Materials and Methods*. Final extract is solubilized in 1% SDS containing buffer, and treated by dialysis as the other samples. wt, wildtype, m, mutant.

Serial Extraction of *Bacillus subtilis* Cell Membranes
Grown Under Two Levels of Aeration

Final Extract				Second Extract				First Extract			
O_2		air		O_2		air		O_2		air	
wt	m	wt	m	wt	m	wt	m	wt	m	wt	m



extracts shows protein bands the entire length of the gel. There are detectable changes in the densities of certain of the protein bands when the membrane extracts of cultures grown on oxygen and air are compared. Figure 12 shows a SDS-PAGE gel of a serial extraction of *B. subtilis* membranes extracts grown under oxygen and air for both the wildtype and mutant strains. At this point in the results the figure is being used to illustrate the changes in the wildtype membrane extracts grown under oxygen and air. Two bands of molecular weight 51 and 44 kDa and two bands of molecular weight 34 and 33 kDa are expressed to a greater extent in the oxygen grown cultures as opposed to the air grown ones. One band at 34 kDa is not as readily extracted in 5% Triton X-100 as the other membrane proteins and this band can be seen carried through the second extract and also in the SDS-solubilization of the final pellet.

Preparation of a Mutant Strain of *Bacillus subtilis*

From the sequence data of heme A containing terminal oxidase from *Bacillus subtilis* it can be concluded that *B. subtilis* possesses two distinct heme A oxidases; caa_3 (Saraste *et al.*, 1991) and aa_3 (Santana *et al.*, 1992). Cytochrome caa_3 is a cytochrome *c* oxidase that is coded for by the *cta* operon which includes six open reading frames; *ctaA-F* (see Figure 13). Two of the subunits, coded for by the genes *ctaD* and *ctaC*, (subunits I and II, COI and COII) are analogous to the mitochondrially encoded subunits I and II of mammalian

cytochrome *c* oxidase (Saraste *et al.*, 1991). Cytochrome caa_3 differs from mammalian cytochrome *c* oxidase in that it possesses a covalently attached heme C domain incorporated into subunit II (Saraste *et al.*, 1991). This motif was seen previously only in the cytochrome oxidase from the thermophilic species *Bacillus* PS3 (Sone *et al.*, 1979). The publication of the sequence of cytochrome caa_3 genes has allowed for the possibility of creating a mutant using molecular biological methods. The method of insertional inactivation of a gene entails removal of a section of DNA from the gene to be mutagenized and replacement of this DNA with that coding for an antibiotic resistance marker (Maniantis, 1989). Using this procedure a 730bp section of subunit II, (COII, *ctaC*) of the operon structure encoding cytochrome caa_3 was removed and an antibiotic resistance marker was inserted in its place. Subunit II of cytochrome caa_3 oxidase contains an N-terminal extension, when the sequence is compared to mammalian cytochrome *c* oxidase (Saraste *et al.*, 1991). This extension is thought to form a covalently attached cytochrome *c* domain as it contains a conserved heme C binding motif and has been shown to contain a heme C (van der Oost *et al.*, 1991). With this sequence information subunit II was chosen to be the site of insertional inactivation. The *cta* gene encoding cytochrome caa_3 was obtained using the polymerase chain reaction from a *B. subtilis* chromosomal DNA template (L. Vo, unpublished

Figure 13: Schematic of the *cta* operon of cytochrome caa_3. from *Bacillus subtilis*. Arrows indicate the direction of transcription. Shaded areas are non-coding regions. *ctaC-F* encodes the functional subunits of cytochrome caa_3, also known as subunits II, I, III, IVB respectively. *ctaA* and *ctaB* are regulatory or assembly factor genes. *pycA* encodes pyruvate carboxylase. Subunit II was insertionally inactivated to form the mutant *B. subtilis* PD001. Adapted from Taber, 1993.

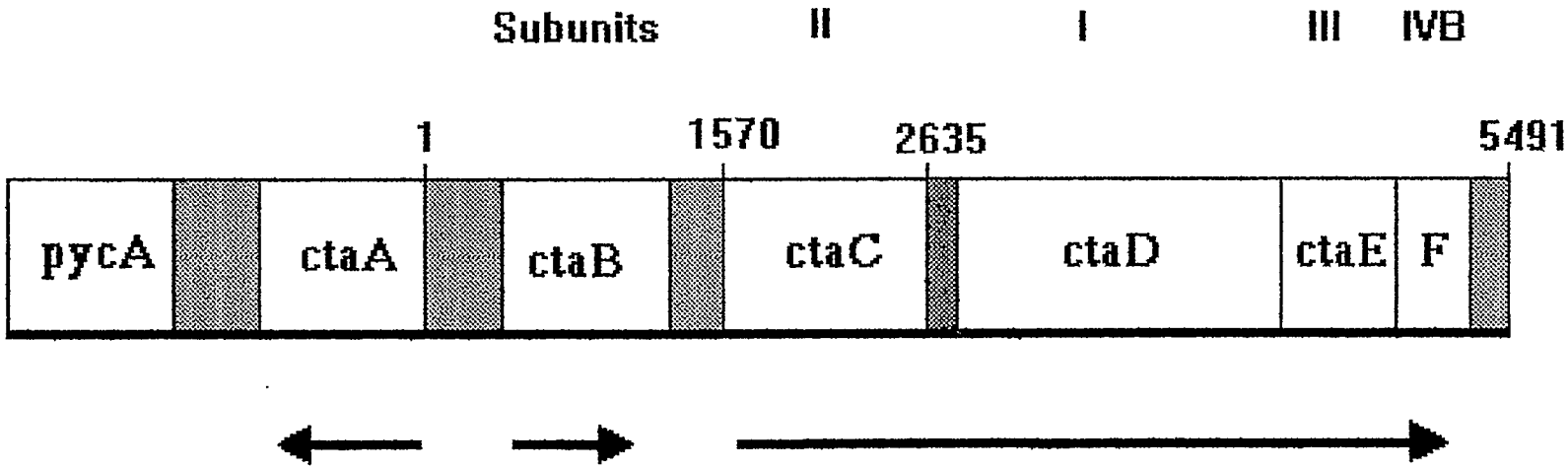
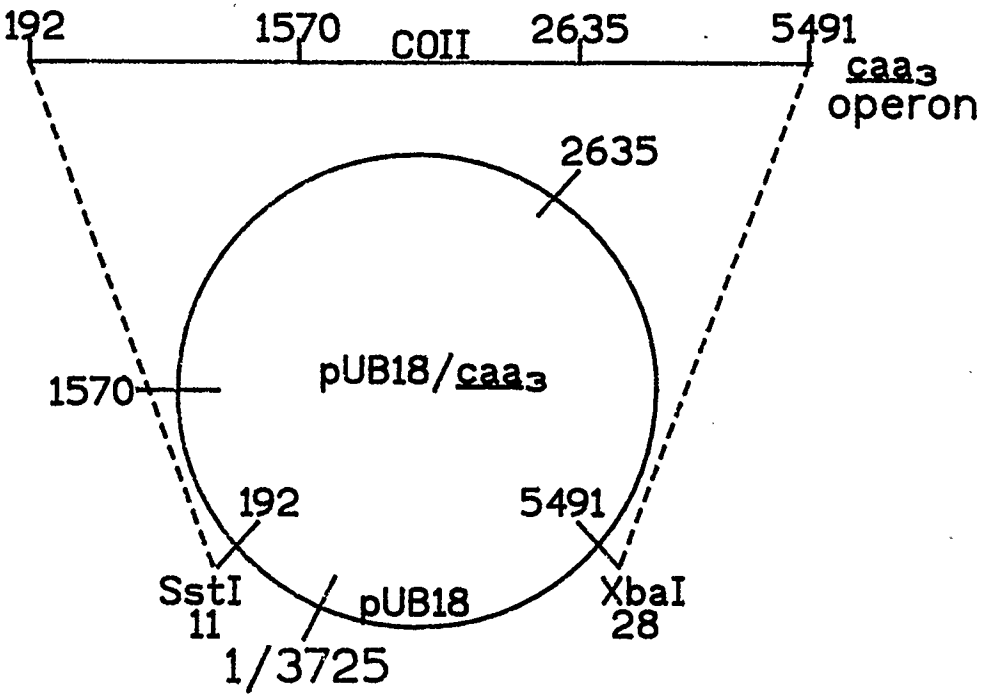

Subunits
II
I
III
IVB
1
1570
2635
5491
pycA
ctaA
ctaB
ctaC
ctaD
ctaE
F

Figure 14: *B. subtilis* and *E. coli* plasmid constructs pUB18/caa_3 and pSD2. These are the first two plasmid constructs for the insertional inactivation protocol of subunit II of cytochrome caa_3 of *Bacillus subtilis* DB104. (A) *Bacillus subtilis* plasmid construct pUB18/caa_3. The *B. subtilis* plasmid pUB18 was digested with Sst I and Xba I in the multiple cloning region. The 5.491kb *cta* operon coding for cytochrome caa_3 was generated by PCR. The 5.491kb Sst I-Xba I fragment of *cta* was inserted into the digested pUB18 giving a 9kb final construct. (B) *E. coli* plasmid pSD2. A 3.277kb Bcl I fragment of *cta* containing subunit II from the pUB18/caa_3 construct was inserted into the Bam HI digested *E. coli* plasmid pBM^+ forming the final 6.481 pSD2 construct.

A


192
1570
COII
2635
5491
caa3
operon
2635
pUB18/caa3
1570
192
5491
SstI
11
pUB18
XbaI
28
1/3725

B

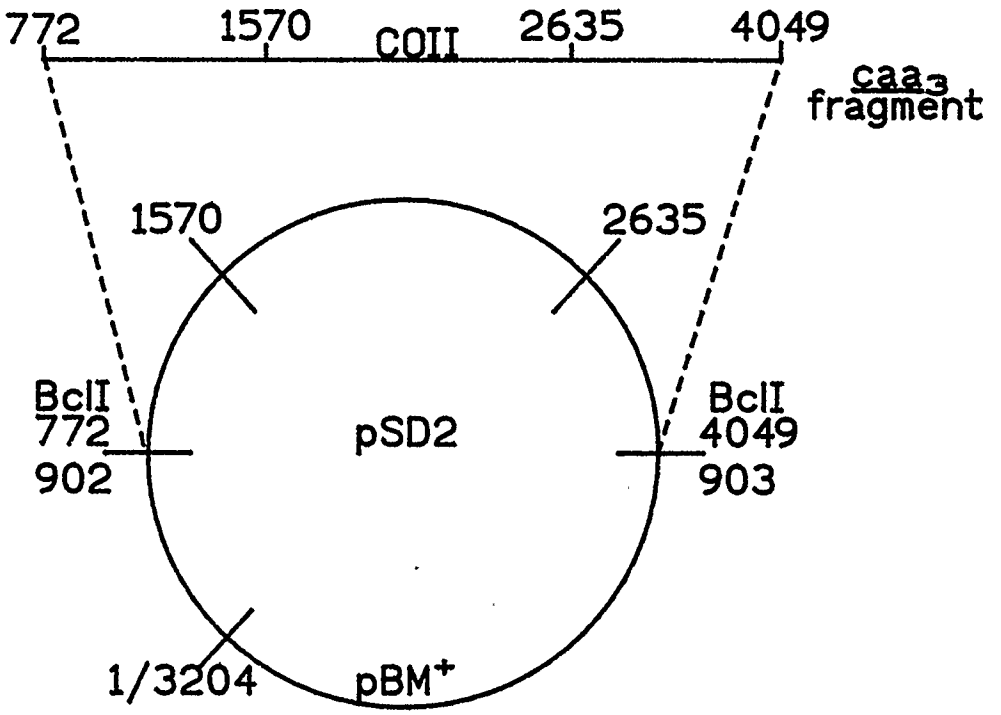
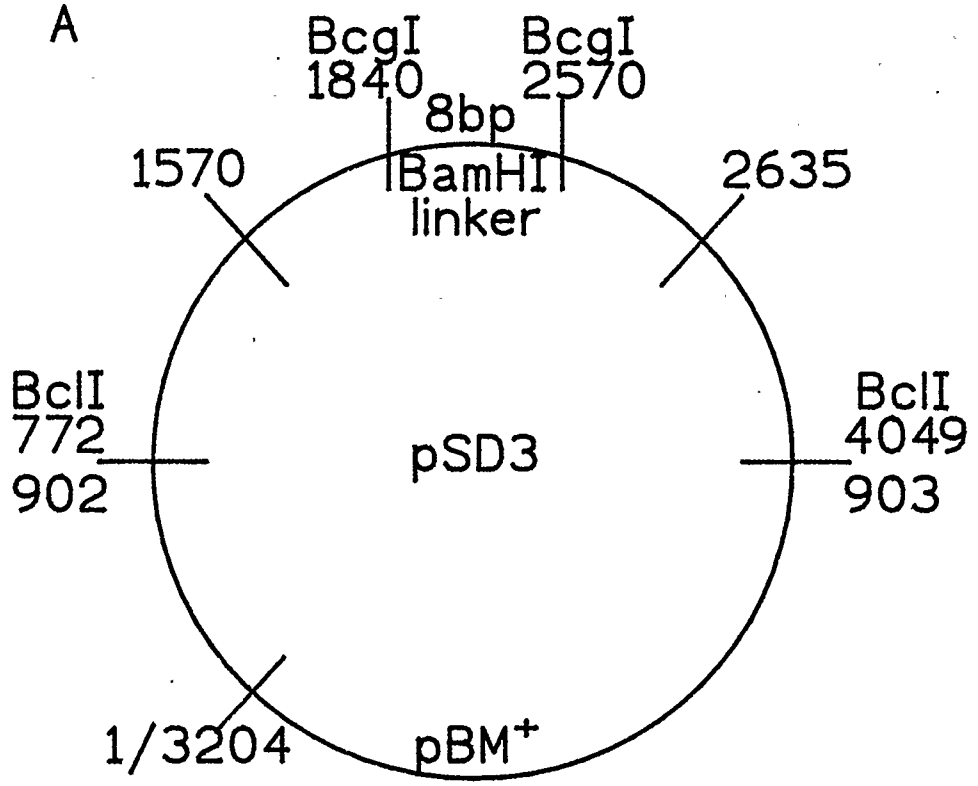
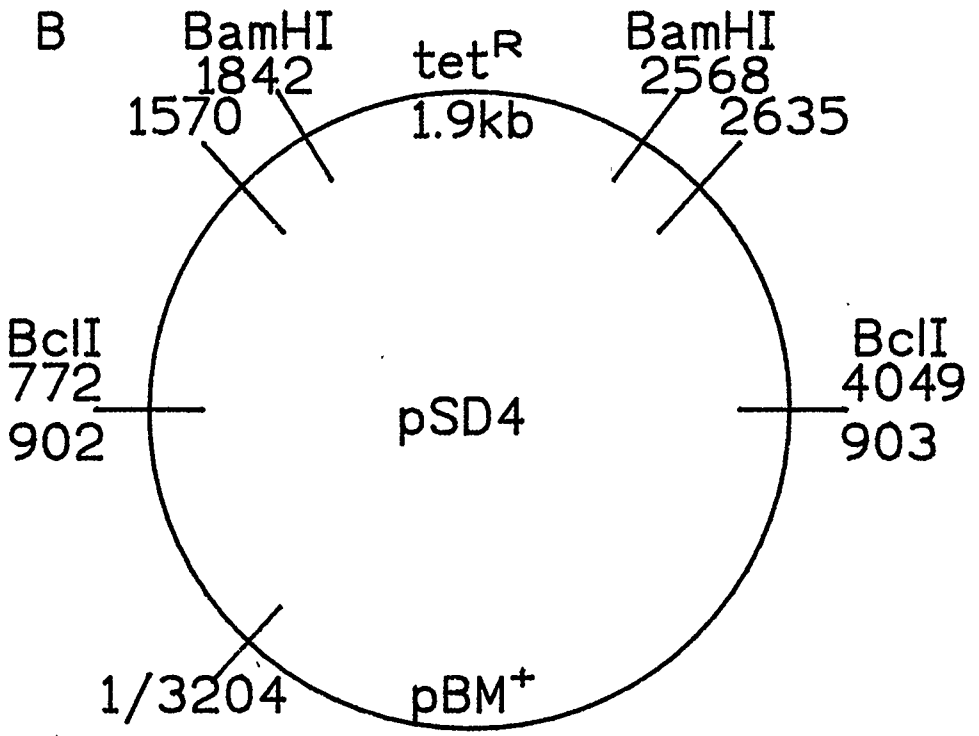

772
1570
COII
2635
4049
caa3
fragment
1570
2635
BclI
772
902
pSD2
BclI
4049
903
1/3204
pBM+

Figure 15: *E. coli* plasmid constructs pSD3 and pSD4. The second two plasmid constructs for the insertional inactivation protocol of subunit II of cytochrome caa_3 of *Bacillus subtilis* DB104. (A) *E. coli* plasmid pDS3. A 730bp Bcg I fragment was excised from the centre of subunit II and replaced with an 8bp Bam HI linker giving the 5.759kb pSD3 construct. (B) *E. coli* plasmid pSD4. A 1.9kb Bam HI ended tetracycline cassette (tet^R) was inserted into the Bam HI digested linker in pSD3 forming the 7.659kb construct pSD4.

A


BcgI
1840
BcgI
2570
8bp
BamHI
linker
1570
2635
BclI
772
902
pSD3
BclI
4049
903
1/3204
pBM+

B


BamHI
1842
tetR
1.9kb
BamHI
2568
1570
2635
BclI
772
902
pSD4
BclI
4049
903
1/3204
pBM+

data). This 5.491kb PCR fragment was inserted into a 3.725kb *Bacillus* plasmid, pUB18, forming the 9.216kb plasmid pUB18/caa_3 (see Figure 14A). A 3.277kb Bcl I fragment (772-4049 bp) of *cta* containing subunit II (1570-2635 bp) was removed by restriction enzyme digestion from this construct. This 3.277bp Bcl I DNA fragment was ligated to a Bam HI linearized *E. coli* vector pBM^+ (pBluescribe $M13^+$) forming the 6.481kb plasmid pSD2 (see Figure 14B). This construct, pSD2, no longer possessed the Bam HI or Bcl I sites. A 730bp Bcg I fragment (1840-2570 bp) was then removed from pSD2. A 8bp Bam HI linker was ligated to the blunt ended Bcg I digested pSD2 to form the 5.758kb plasmid, pSD3 (see Figure 15A). A 1.9kb tetracyline cassette, tet^R, (Itaya, 1992) was ligated into the Bam HI site in the 8bp Bam HI linker forming the 7.649kb plasmid pSD4 (see Figure 15B). This final construct pSD4 was linearized, transformed into *Bacillus subtilis* and grown under antibiotic resistance conditions (for tetracycline) to select for the formation of a mutant strain of *B. subtilis* via a double reciprocal crossover of the plasmid and chromosomal DNA.

Growth of Mutant *Bacillus subtilis*

Growth of the mutant strain of *Bacillus subtilis* on modified super rich medium does not differ greatly from wildtype, in cell yield per litre culture or doubling time (see Table 5). However, studies on defined minimal medium show a reversal in the response of both wildtype and mutant

cultures to growth under high and low aeration, as compared to those grown on modified super rich medium. The mutant *B. subtilis* strain grown on defined minimal medium under oxygen shows a distinct increase in the culture doubling time compared to wildtype.

Table 5: Culture growth data for wildtype and mutant *B. subtilis* grown on two different media under two levels of aeration.

Bacillus strain	Medium	Growth Condition	Cell[1] Pellet (g/l)	TX-100[2] Protein Extract (mg/ml)	Culture Doubling Time (min)
mutant	defined	air	3.60	6.08	156
mutant	defined	oxygen	2.40	4.30	232
wildtype	defined	air	5.30	7.72	150
wildtype	defined	oxygen	3.00	6.35	132
mutant	super rich	air	4.70	3.14	47
mutant	super rich	oxygen	13.00	5.47	43
wildtype	super rich	air	7.10	3.10	38
wildtype	super rich	oxygen	20.70	8.29	38

All culture were grown in 1L of medium on modified super rich medium containing succinate and defined minimal medium containing succinate and sorbitol. All cultures were grown to stationary phase before harvesting. Data in this table is from different experiments. Culture doubling time determined from semi-logarithmic plot of growth curve.
1. Wet weight of cell pellet
2. Triton X-100 membrane extract protein concentration.

Cytochrome Content of Mutant *Bacillus subtilis* PD001

Determination of the cytochrome content of the mutagenized strain was done spectrophotometrically. Figure 16 shows dithionite reduced minus oxidized difference spectra of membranes from wildtype and mutant *Bacillus subtilis* grown on modified super rich medium. The spectrum of the mutant shows losses of absorbance at 600 nm due to heme A and at 550 nm due to heme C relative to the spectrum of membrane from wildtype. Complete loss of heme A absorbance at 600 nm is not expected as in the wildtype this absorbance is contributed to by heme A moieties from both the heme A containing terminal oxidases, cytochrome aa_3 and cytochrome caa_3 (Saraste *et al.*, 1991). A similar difference can also be seen in the ascorbate plus TMPD reduced difference spectra in Figure 17. However in these spectra the contributions from cytochrome *o* are not observed. The mutant strain of *B. subtilis* also shows an increase in the heme B content when grown under oxygen. In cultures grown on defined medium, the cytochrome content of the mutant and wildtype is similar to that seen during growth on modified super rich medium, with the addition of detectable levels of cytochrome *d*. Figure 18 shows reduced minus oxidized difference spectra for both wildtype and mutant membrane extracts grown on defined medium under air. In Figure 18A the wildtype and mutant membrane extracts are reduced with dithionite and in Figure 18B the membrane extracts are reduced with ascorbate plus TMPD. Cytochrome *d* is now produced at

Figure 16: Dithionite reduced minus oxidized difference spectra of *B. subtilis* DB104 and PD001. Membrane extracts produced as described in *Materials and Methods*. Cultures grown on succinate super rich medium under oxygen. Membrane extracts reduced with 25mM dithionite. (——) Wildtype *Bacillus subtilis* DB104. (---) Mutant *Bacillus subtilis* PD001.

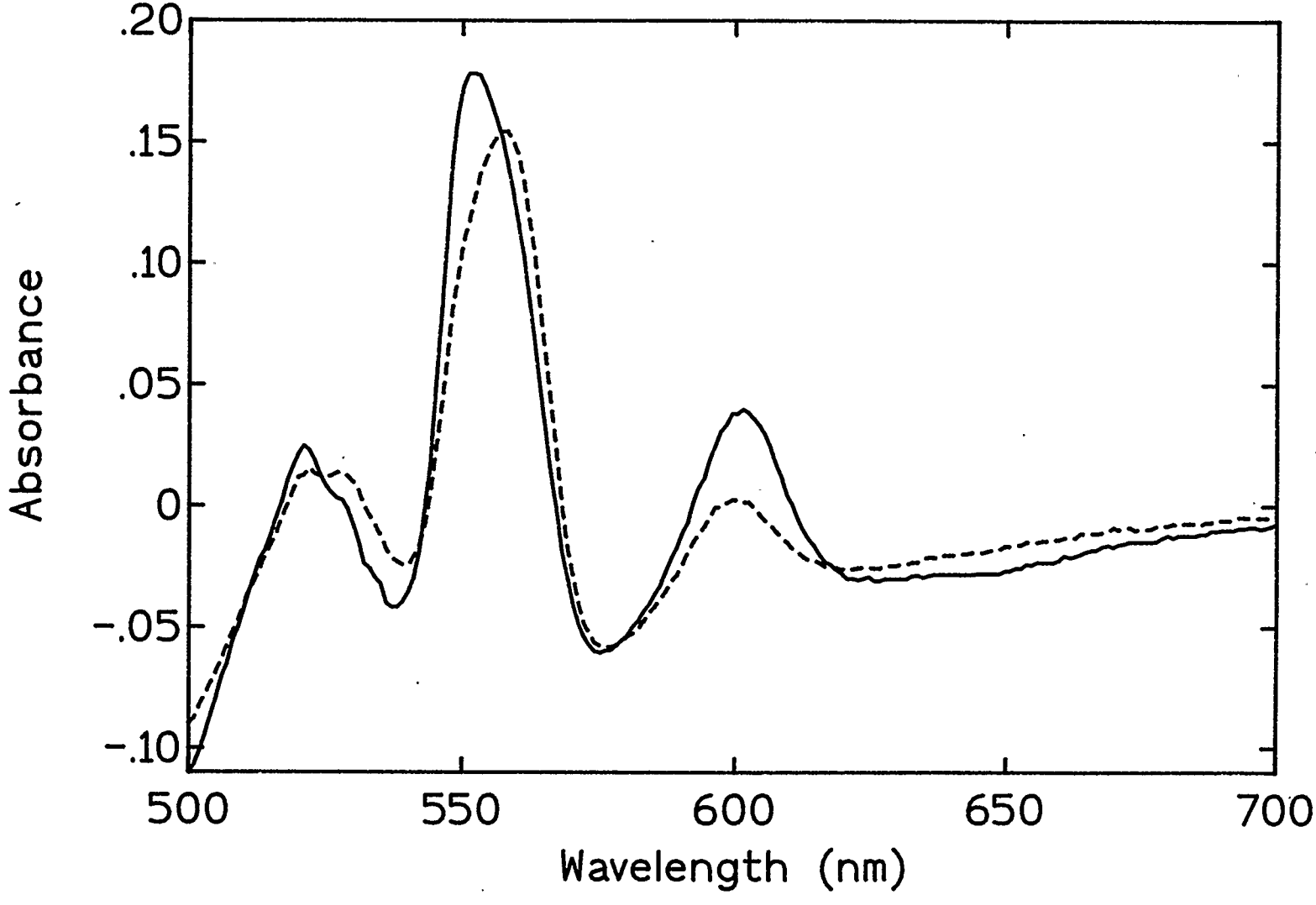

.20
.15
.10
.05
0
-.05
-.10
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 17: Ascorbate plus TMPD reduced minus oxidized difference spectra of *B. subtilis* DB104 and PD001. Cultures grown on succinate containing modified super rich medium under oxygen. Membrane extracts reduced with 5mM ascorbate plus 0.2mM TMPD. (—) Wildtype *Bacillus subtilis* DB104. (--) Mutant *Bacillus subtilis* PD001.

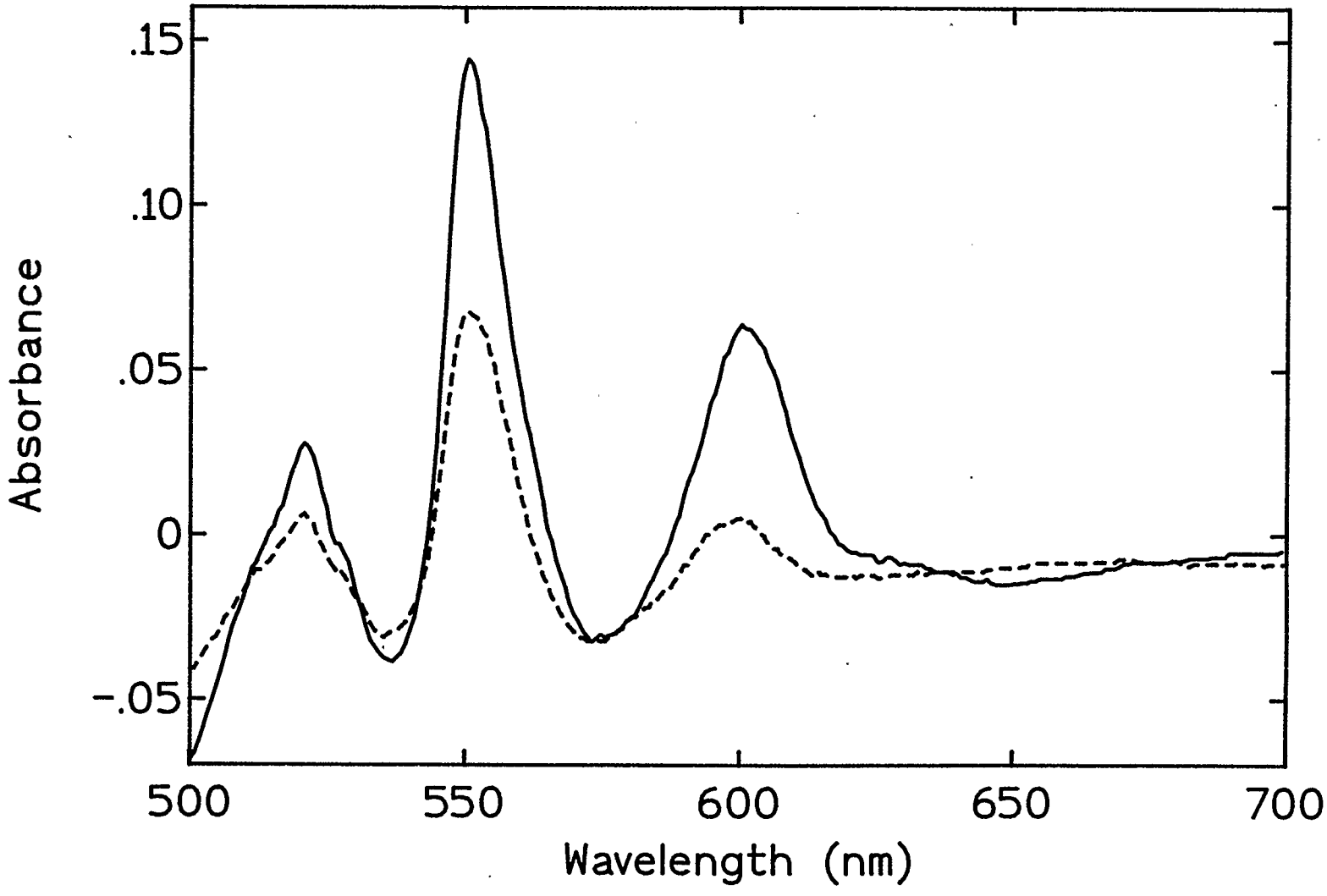
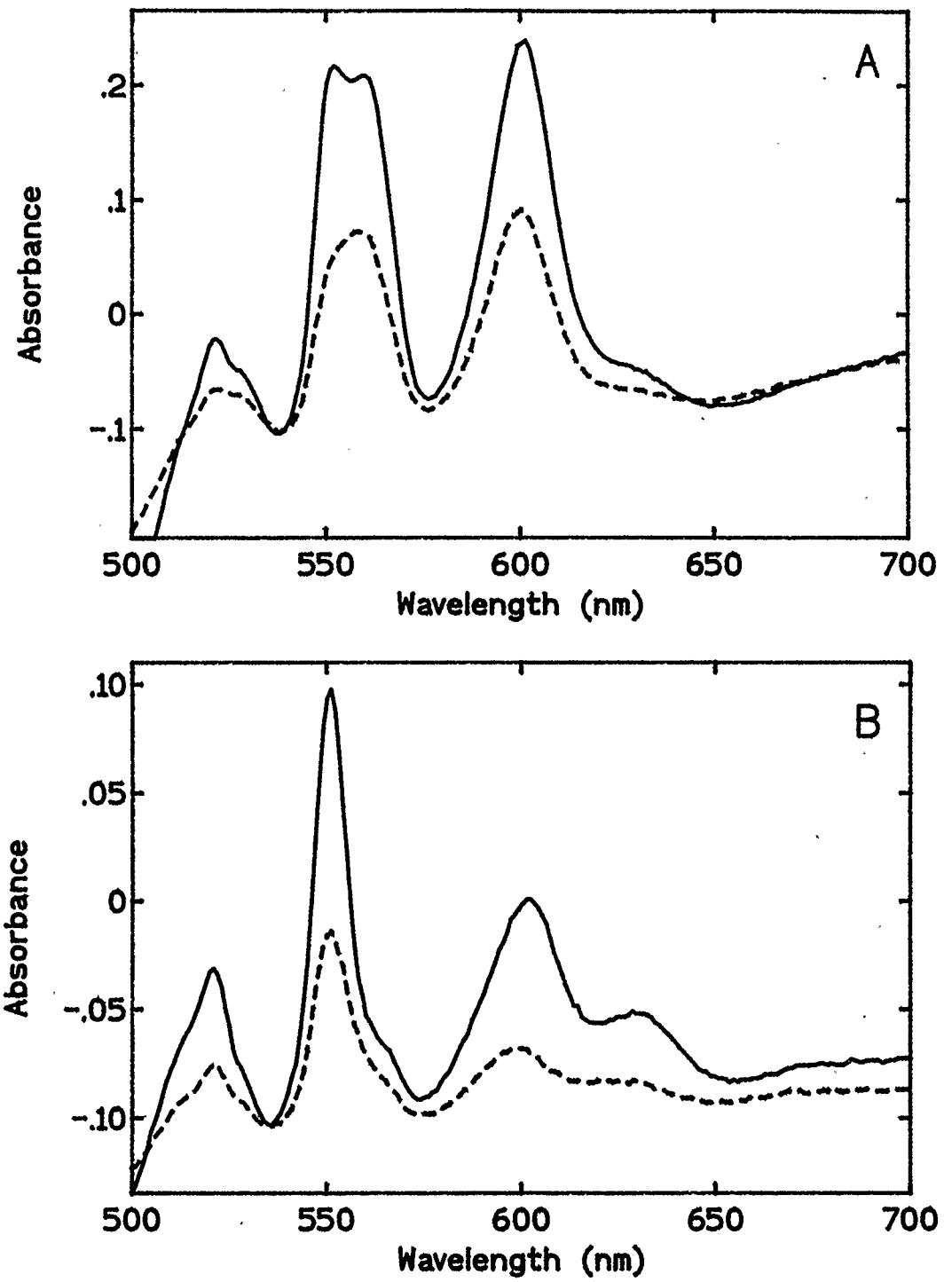

.15
.10
.05
0
-.05
Absorbance
500
550
600
650
700
Wavelength (nm)

Figure 18: Reduced minus oxidized difference spectra of *B. subtilis* DB104 and PD001 grown on defined media. Cultures grown on succinate and sorbitol containing defined medium under air, as described in *Material and Methods*. Membrane extracts reduced with either 25mM dithionite (A) or 5mM ascorbate plus 0.2mM TMPD (B). A) dithionite reduced difference spectrum of wildtype membrane extracts (———), mutant (---). B) ascorbate plus TMPD reduced difference spectrum of wildtype membrane extracts (——), mutant (---).


A
.2
.1
0
-.1
Absorbance
500
550
600
650
700
Wavelength (nm)
B
.10
.05
0
-.05
-.10
Absorbance
500
550
600
650
700
Wavelength (nm)

detectable levels and shows the characteristic absorption peak at 628 nm (Miller and Gennis, 1983).

Enzymatic Assay of Mutant and Wildtype *Bacillus subtilis* Membrane Extracts

The cytochrome *c* oxidizing ability of mutant and wildtype membrane extracts in 5% Triton X-100 was determined by an enzymatic assay using reduced horse heart cytochrome *c* as the substrate (Smith and Conrad, 1956). Figure 19 shows the 550-540 nm difference absorbance as a function of the following addition of aliquots of membrane extracts from mutant and wildtype *B. subtilis* grown under oxygen and air. In the two mutant membrane extracts, no oxidation of the reduced cytochrome *c* is detectable, whereas both the wildtype membrane extracts show activity. In addition, there is greater activity in the wildtype extract that was from cells grown under oxygen, reflecting the increase in cytochrome levels under these conditions. Since the insertional inactivation of the caa_3 oxidase gives a membrane extract with no cytochrome *c* oxidizing capability we conclude that the caa_3 is the sole cytochrome *c* oxidase in *B. subtilis*.

Western Immunoblot of Wildtype and Mutant Membrane Extracts

A difference in the levels of membrane proteins in the membrane extracts of the mutant *B. subtilis* strain are not easily discernable from the wildtype via SDS-polyacrylamide gel electrophoresis. It is difficult to determine the loss of the protein bands that correspond to the subunits of

Figure 19: Enzymatic assay of wildtype (DB104) and mutant (PD001) *Bacillus subtilis* membrane extracts to determine cytochrome *c* oxidizing ability. Oxidation of 25μM reduced cytochrome *c* in 1ml buffer (50mM Tris, 1mM EDTA, pH 7.4). Membrane extracts and reduced cytochrome *c* produced as described in *Materials and Methods*. Absorbance was monitored at 550-540 nm, at room temperature. (—·—) *B. subtilis* PD001, 455μg, grown under oxygen. (— — —) *B. subtilis* PD001, 188μg, grown under air. (---) *B. subtilis* DB104, 185μg, grown under air. (——) *B. subtilis* DB104, 440μg, grown under oxygen. Arrow indicates addition of 60μl of membrane extracts.

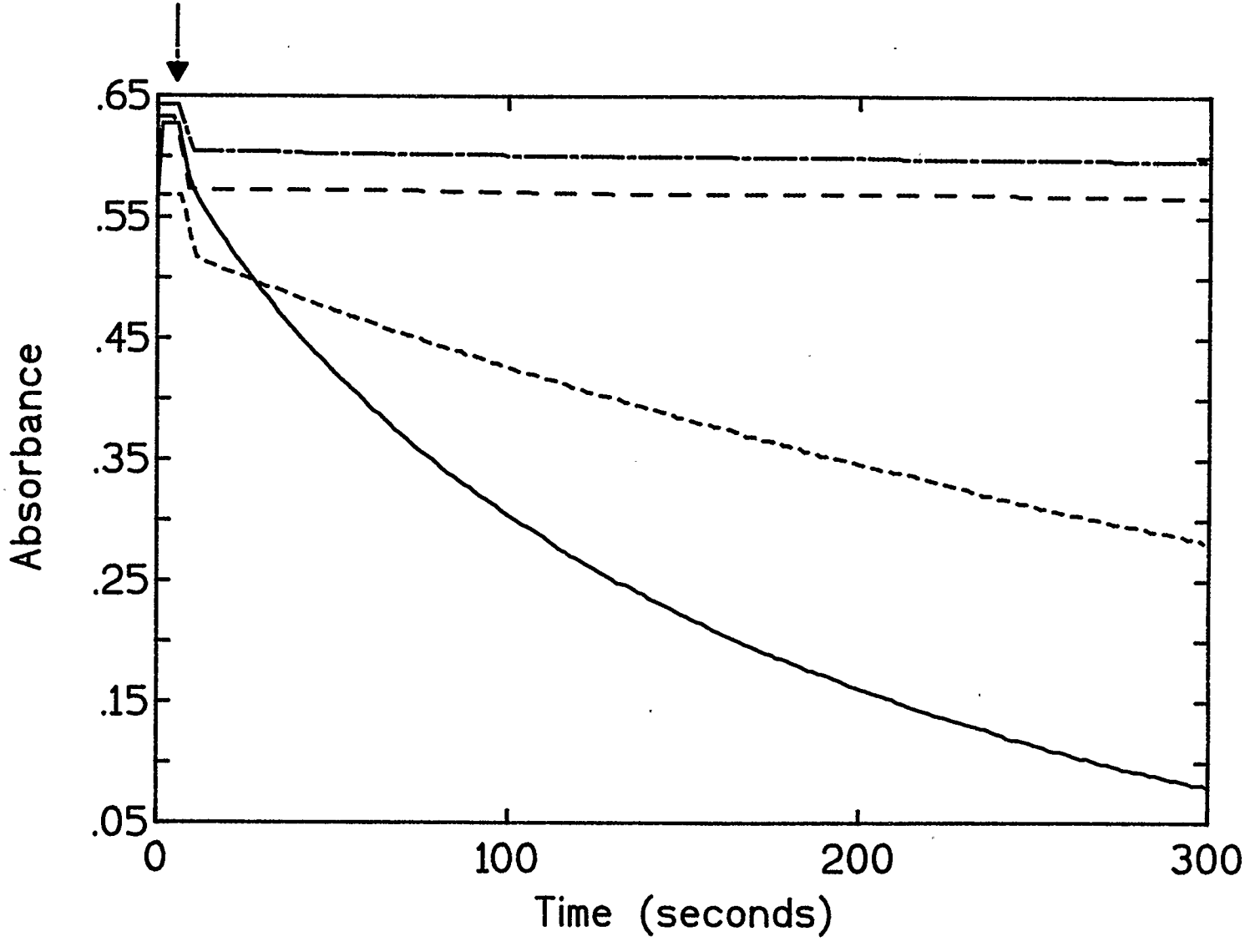
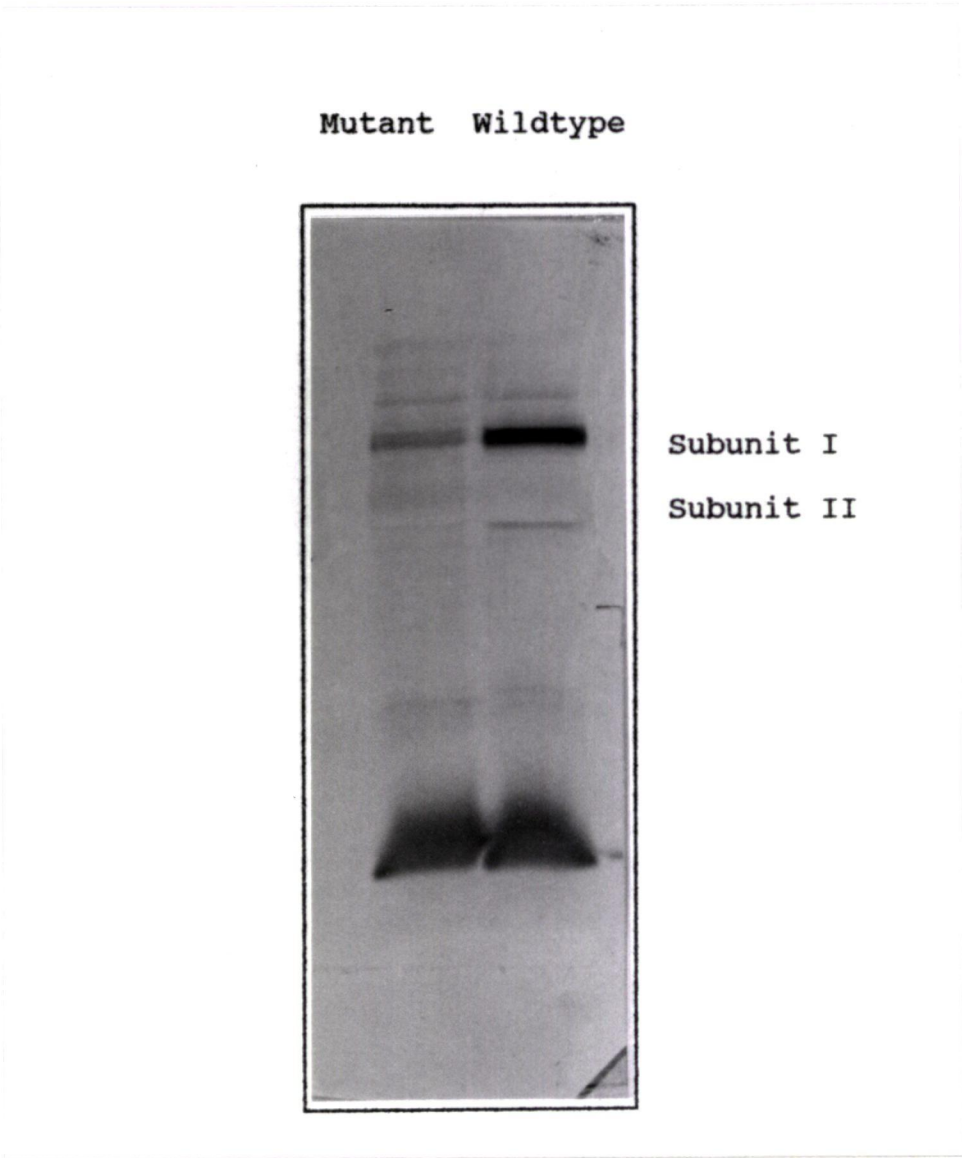

.65
.55
.45
.35
.25
.15
.05
Absorbance
0
100
200
300
Time (seconds)

Figure 20: Western immunoblot of membrane extracts of *B. subtilis* DB104 and PD001. Detergent levels in the membrane extracts were reduced using BioBeads as stated in *Materials and Methods*. Membrane extracts in SDS prior to polyacrylamide gel electrophoresis. Polyclonal antibodies were made to purified, SDS-denatured cytochrome caa_3.


Mutant
Wildtype
Subunit I
Subunit II

cytochrome *caa*$_3$ in the mutant due to the presence of other proteins that co-migrate with these subunits. However when comparing the mutant membrane protein extracts to wildtype, an increase in the density of one band is readily detectable (see Figure 12). Since it is not possible to tell definitively when looking at whole membrane extracts whether the proteins of *caa*$_3$ enzyme are present antibodies to the purified enzyme, consisting of subunits I and II, were made and used to probe the membrane extract. Figure 20 shows a Western blot of both wildtype and mutant membrane extracts probed with polyclonal antibodies to SDS-denatured, purified cytochrome *caa*$_3$ (prepared by W. Henning). Both subunits I and II of cytochrome *caa*$_3$ are detectable in the wildtype membrane extract and less intense bands corresponding to subunit I and II of cytochrome *caa*$_3$ are also detectable in the mutant.

Reconstitution of Subunit II in the Mutant *B. subtilis*

Two different experiments were performed in an attempt to reconstitute a functional cytochrome *caa*$_3$ in the mutant strain of *B. subtilis*. These two experiments entailed insertion of the first the entire 5.491kb *cta* gene into the *B. subtilis* plasmid pUB18 and secondly insertion of a 1.5kb section of the *cta* operon containing subunit II into pUB18. Neither of the transformed mutants carrying either of the plasmid constructs provided the mutant *B. subtilis* PD001 with a functional cytochrome *caa*$_3$. Growth of both of these transformed strains differed from that of the wildtype and mutant, indicating that

something other than the desired reconstitution of cytochrome caa_3 was occurring in these transformants.

Discussion

Bacillus subtilis is a strictly aerobic bacterium that possesses multiple terminal oxidases at the oxygen end of its respiratory chain. Originally knowledge of the *Bacillus subtilis* respiratory chain was based on information known from the mammalian mitochondrial system. The mammalian mitochondria possesses only one terminal oxidase in its respiratory chain, i.e. cytochrome aa_3, a cytochrome *c* oxidase. This terminal oxidase possesses 13 subunits, three of which are mitochondrially encoded and the remaining are coded for by the nucleus. The three mitochondrially encoded subunits are the subunits that are retained in the analogous but structurally simpler terminal oxidases found in some bacterial systems. These three subunits form the core of the enzyme in a structural and functional sense. Being that these three mitochondrially encoded subunits are the most important functionally, it is these subunits that show sequence similarity with other bacterial terminal oxidases, including those of *Bacillus subtilis*, with two hemes and a copper center in subunit I and a second copper (if present) in subunit II (Taber, 1993). In the case of *Bacillus subtilis* there are two terminal oxidases with sequence homology to mitochondrial cytochrome oxidase core subunits. The differing respiratory pathways of the mitochondria and *B. subtilis* have allowed structurally similar enzymes to receive electrons from

different sources. In the mitochondrial respiratory pathway, the quinol moiety, ubiquinone, is an electron carrier that receives electrons from dehydrogenases, such as NADH and succinate dehydrogenase, and passes them on to the cytochrome bc_1 complex. Ubiquinol does not act as a reductive substrate for the terminal oxidase in mitochondria. In the *B. subtilis* respiratory pathway, the chain branches at the quinol moiety, menaquinone, which either gives its electrons to the bc_1 complex, or directly to a terminal oxidase, in this case cytochrome aa_3.

The other heme A containing terminal oxidase in *B. subtilis*, cytochrome caa_3, is a cytochrome *c* oxidase (Saraste *et al.*, 1991). Functionally, this terminal oxidase is similar to the mitochondrial cytochrome *c* oxidase, but it also has structural similarity to the oxidase from the thermophilic organisms *Thermus thermophilus* (Buse *et al.*, 1989) and *Bacillus* PS3 (Ishizuka *et al*,. 1990). The similarity of the *B. subtilis* cytochrome *c* oxidase to the thermophilic bacteria is through the possession of a covalently attached cytochrome *c* domain in subunit II. The *B. subtilis* and thermophilic cytochrome *c* oxidase systems differ in the fact that the thermophilic bacteria do not contain a free cytochrome *c* whereas *B. subtilis* does (von Wachenfeldt and Hederstedt, 1990). Besides possessing a heme C binding site in the N-terminal domain of subunit II, the *B. subtilis* cytochrome *c* oxidase retains the four invariant carboxylic amino acids

found in other cytochrome *c* oxidases (Saraste, 1990). These carboxylic residues have been shown to be involved in the binding site for external cytochrome *c* (Millet *et al.*, 1983).

As for the other two terminal oxidases known to be present in *B. subtilis*, cytochrome *o* and *d*, information on them is preliminary at present. The presence of cytochrome *o* in *B. subtilis* has been reported in the literature in the past (Tochikubo, 1971; de Vrij *et al.*, 1987). Cytochrome *o* is thought to contain a B-type heme, a protoheme, as in the *E. coli* cytochrome *bo* oxidase. Recent work on the chemical structure of the heme from *E. coli* has shown the presence of a new heme moiety, heme O, which is spectrally indistinguishable from heme B but chemically unique (Wu *et al*,. 1992), and determined to be that possessed by *E. coli*. By analogy with the *E. coli* system, the heme moiety of *B. subtilis* cytochrome *o* might contain a heme O as well. To date no literature has reported whether cytochrome *o* is a quinol oxidase or a cytochrome *c* oxidase, but results from the enzymatic assay in this thesis indicate that it is not a cytochrome *c* oxidase.

The first report of the presence of cytochrome *d* in *Bacillus subtilis* was by Takahashi and Ogura (1982). More recently cytochrome *d* had been reported as a terminal oxidase in the facultatively alkaliphilic *Bacillus firmus* (Hicks *et al.*, 1991). Cytochrome *d* as a terminal oxidase seems to be expressed during stressful growth of the bacterium. In this

thesis, results show the presence of cytochrome *d* in wildtype and mutant strains of *B. subtilis* during growth in a defined minimal medium.

Oxygen Levels and its Affect on *Bacillus subtilis*

Oxygen concentration during growth affects a number of aspects of cell division and protein production in *Bacillus subtilis*. Increase in the level of oxygenation of the culture media during growth of *B. subtilis* increases the yield in wet weight of cells per litre culture whereas the culture doubling time remains unchanged. These results indicate that the total number of cells the culture media is able to support increases with increasing oxygenation of the culture, whereas the individual doubling time of each bacterium remains unchanged. Since the respiratory chain and the processes of oxidative phosphorylation and transmembrane solute uptake are anticipated to be key elements in any increased growth in response to oxygen we were interested to observe the effects on the level of the respiratory chain components. The levels of all the cytochromes present in the cell membrane increase under increased oxygenation. In particular the level of one of the putative terminal oxidases, cytochrome *o*, increases to a greater extent than that of the other terminal oxidases present. Results of the response of a *B. subtilis* culture to the level of oxygenation during growth were reported by Downey in 1964. Downey also reported increases in the levels of certain cytochromes in cells grown under these conditions but

the increased cytochromes he reported were those of cytochromes a_3 and *c* not cytochrome *o*.

Not much is known about the cytochrome *o* of *B. subtilis* but there is a cytochrome *o* in *E. coli*. In *E. coli* it is known that the bacteria express a cytochrome *bd* oxidase at low oxygen levels and a cytochrome *bo* oxidase at high oxygen levels so by analogy with *E. coli* the result of an increase in the cytochrome *o* levels in *B. subtilis* grown in high oxygen may not be surprising. Cytochrome *o* may also be a quinol oxidase as in *E. coli* but there has been no literature reports as to which membrane bound moiety passes electrons to cytochrome *o* in *B. subtilis*. The only confirmed terminal oxidases in *B. subtilis* are the heme A containing ones.

Results of the enzymatic assay, show that the mutant *Bacillus subtilis* membrane extract does not possess any cytochrome *c* oxidizing ability, indicating that cytochrome caa_3 is the only cytochrome *c* oxidase in *B. subtilis*. Due to the conserved structures of cytochromes *c* from various sources as determined by their cross reactivity in antibody binding and reactivity with mitochondrial cytochrome *c* oxidase (Pettigrew and Moore, 1987) the use of horse heart cytochrome *c* in this assay should not play a role in determining the presence or absence of a cytochrome *c* oxidizing ability of the membrane extracts. The rate of reaction of the cytochrome *c* oxidation of the wildtype DB104 strain of *B. subtilis* with its own oxidase may differ from that with the horse heart

cytochrome *c* but as the *B. subtilis* cytochrome *c* has not been isolated, other cytochromes *c* are utilized in assays of this type. Horse heart cytochrome *c* has previously been shown to be utilized as a substrate for bacterial terminal oxidases of the aa_3-type, for example *Rhodopseudomonas sphaeroides*, Azzi and Gennis (1986).

Effects of Mutation on *Bacillus subtilis*

Of the mutant *Bacillus subtilis* species lacking some terminal oxidases known to date, most are classical mutants whose deletions have been mapped via cotransduction of known genetic markers (James *et al.*, 1987; Taber 1974) or mutants involving more than one of the subunits of cytochrome caa_3 (van der Oost *et al.*, 1991). The classical mutants being deleted in unspecified areas of the respiratory pathway showed various affects of the mutations on growth, spectral data, incorporation of different antibiotics etc. Insertional inactivation of a single subunit of *Bacillus subtilis* using molecular biological methods produced a specific mutant strain of *B. subtilis* deficient in the terminal oxidase cytochrome caa_3. Determination of the loss of this terminal oxidase was carried out in a number of ways. Spectral data showed the loss of absorbance of heme A at 600 nm and that of heme C at 550 nm when compared to wildtype, indicative of the loss of cytochrome caa_3. The enzymatic assay showed the loss of any cytochrome *c* oxidizing ability in the mutant membrane extract when compared to wildtype, also indicative of the loss of

cytochrome *caa*$_3$. SDS-polyacrylamide gel electrophoresis of the mutant membrane extract did not show any detectable loss of the protein subunits corresponding to the molecular weights of those of cytochrome *caa*$_3$. This is most likely due to the large number of protein subunits in the membrane extract and the difficulty of identifying specific proteins in the gel. There is a detectable increase in one of the bands in the mutant membrane extract compared to the wildtype. This band has been tentatively assigned to that of cytochrome *o*, which is expressed in great quantity in the mutant *B. subtilis* PD001. All previously mentioned experiments carried out on the mutant strain of *B. subtilis* indicate the loss of cytochrome *caa*$_3$ both functionally and spectrally. The Western Immunoblot, unlike the SDS-PAGE gel, determines the presence of specific protein in the membrane extract. Using polyclonal antibodies specific to cytochrome *caa*$_3$, the Western blot can detect specifically the presence or absence of the subunits of cytochrome *caa*$_3$. The Western blot of the mutant membrane extracts showed the presence of subunit I of cytochrome *caa*$_3$, remaining in the membrane, although its level in the membrane was greatly decreased. Also barely detectable in the mutant membrane extract is a band corresponding to subunit II but shifted to a higher molecular weight. This small amount of subunit II is present due to readthrough of the mutated subunit from its intact promoter and subsequent insertion into the membrane. The increase in size of subunit II from the

PD001 mutant membrane extract corresponds to the increased size of this mutated subunit II due to the inserted 1.9kb tetracycline cassette. Readthrough from the promoter producing subunit II is plausible and explains the presence of subunit I in the blot of the mutant membrane extract. Due to the nomenclature of the *cta* operon, subunit II precedes subunit I but as the promoter of the polycistronic message is upstream of subunit II readthrough of this promoter must be occurring to account for the presence of subunit I in the mutant membrane extract.

Summary and Concluding Remarks

B. subtilis cultures respond to increased oxygenation by an increase in cell density and in the relative levels of a cytochrome *o*-like oxidase. Also of interest are the levels of other cellular proteins and their response to growth in the high oxygen environment which could be determined by two-dimensional gel electrophoresis.

Insertional inactivation of the cytochrome caa_3 oxidase impairs growth on defined minimal medium, but the mutant strain grows like wildtype on modified super rich medium. Sporulation studies can determine the necessity of cytochrome caa_3 for this pathway, and deletion of subsequent terminal oxidases will determine their importance in cell growth.

The enzymatic assay of the cytochrome caa_3 minus mutant shows that this oxidase is the only one of the four oxidases present in *B. subtilis* which is a cytochrome *c* oxidase.

The cytochrome caa_3 inactive mutant is defective in subunit II synthesis and should allow us to complement this mutation and generate site specific changes in the subunit II protein to determine which conserved residues are necessary for structure/function of cytochrome caa_3.

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