Filamentous organism bulking in nutrient removal activated sludge systems
Paper 9: Review of biochemistry of heterotrophic respiratory metabolism

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Abstract

Biochemical mechanisms for respiration by facultative organisms are reviewed in two sequential parts. In Part I, the biochemical reactions involved in the utilisation of substrate under aerobic and under anoxic conditions are outlined. In Part II, the mechanisms which initiate, regulate, and terminate respiration under each of aerobic and anoxic conditions are described. A consequence of Part II is identification of a series of conditions and mechanisms in which one of the intermediates of denitrification (nitrite, or nitric oxide) interacts with the aerobic respiratory enzymes (the cytochrome oxidases), resulting in inhibited aerobic respiration when organisms are alternately exposed to anoxic and aerobic conditions. This mechanism is an important aspect of a conceptual biochemical model for facultative heterotrophic organisms developed in Paper 10 (Casey et al., 1999a).

List of symbols

AA	=	anoxic-aerobic
CoA	=	coenzyme A
ADP	=	adenosine diphosphate
aq	=	aqueous
ATP	=	adenosine triphosphate
ATPase	=	adenosine triphosphatase
cs	=	cysteine
cyt	=	cytochrome
DO	=	dissolved oxygen
ETP	=	electron transport pathway
FAD	=	flavin adenine dinucleotide - oxidised
$FADH_2$	=	flavin adenine dinucleotide - reduced
FMN	=	flavin mononucleotide - oxidised
$FMNH_2$	=	flavin mononucleotide - reduced
Fp	=	flavoprotein
g	=	gaseous
GTP	=	guanosine triphosphate
hs	=	histidine
NAD^+	=	nicotinamide adenine dinucleotide - oxidised
NADH	=	nicotinamide adenine dinucleotide - reduced
NaR	=	nitrate reductase
NiR	=	nitrite reductase
NOR	=	nitric oxide reductase
N_2OR	=	nitrous oxide reductase
NO	=	nitric oxide
NO_2^-	=	nitrite
NO_3^-	=	nitrate
N_2	=	dinitrogen
N_2O	=	nitrous oxide
OUR	=	oxygen utilisation rate
Q	=	ubiquinone
QH_2	=	ubiquinol (reduced ubiquinone)
TCA	=	tricarboxylic acid (cycle)

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Introduction

The intention of this review is to outline the pertinent information and research which contributes to an understanding of and provides a basis for development of the conceptual respiratory model for facultative heterotrophic organisms described in Paper 10 (Casey et al., 1999a) of this series and the conceptual microbiological model for bulking by AA filaments (Casey et al., 1999b, Paper 11).

A considerable portion of the information set out in this paper is well accepted and widely documented in the microbiology and biochemistry literature and consequently, where general microbiology and biochemistry is described, it is not referenced. However, where information important to the bulking hypothesis is described or where research in an area is still being extensively conducted, references are cited to support the statements.

In the previous paper (Musvoto et al., 1999, Paper 8) of this series it was concluded that a more fundamental understanding is required of the biochemical mechanisms involved in respiration by aerobic facultative organisms. This is a consequence of the finding that AA filamentous organisms proliferate in activated sludge when this is cycled between anoxic and aerobic conditions. This cycling requires the organisms to utilise different electron transport pathways under each condition.

To examine this, a literature review is conducted, the objective of which is to identify the principal ETPs employed by facultative heterotrophic organisms under aerobic, anoxic, and alternating anoxic-aerobic conditions. This review is limited to facultative heterotrophic organisms since these are the organisms likely to be present under the cyclic anoxic-aerobic conditions found in activated sludge plants.

Background

Metabolism can be broadly described as the manner by which facultative heterotrophic organisms derive energy and matter for growth. It consists of two processes: the enzymatic biosynthesis of the complex molecular components of the organism itself (anabolism); and the enzymatic bio-reactions which generate energy to perform this biosynthesis (catabolism). The process of catabolism,

but not anabolism is described in this paper and is referred to as respiratory metabolism. In catabolism, complex organic compounds (substrate) are enzymatically degraded such that some of the electrons originally present in the organic compound (called the electron donor) are removed (oxidation) and transferred through a sequence of controlled biochemical reactions to a final compound (called a terminal electron acceptor). In this sequence of biochemical reactions there is a release of chemical energy, some of which is conserved through the formation of the energy-rich molecule ATP. The principal objective of the catabolic process is the formation of ATP and without electron donors and acceptors, this is not possible. The ATP produced by the catabolic process is utilised by the organism in the anabolic process for the synthesis of new cell material and in maintenance of cell function and structure. Facultative heterotrophic organisms could more appropriately be named chemo-heterotrophs as a consequence of their derivation of energy (catabolism) from the breakdown of organic compounds and their derivation of the principal source of carbon for cell synthesis (anabolism) from the same organic compounds (heterotrophic). The term facultative refers to their ability to switch between available terminal electron acceptors in response to the environmental conditions; oxygen (O_2) under aerobic conditions, and nitrate (NO_3^-) and/or nitrite (NO_2^-) under anoxic conditions.[*Note: In the field of sanitary engineering, anoxic is defined as a condition in which DO is absent or maintained zero by biological action and nitrate and/or nitrite is present or added in significant quantities. The condition in which no DO, nitrate or nitrite are present is called anaerobic. In the bacteriological disciplines, conditions of respiration with nitrate/nitrite present and DO absent are referred to as anaerobic respiration with nitrate/nitrite present. The condition termed anaerobic by sanitary engineers is referred to as fermentation by bacteriologists*]. The major catabolic biochemical pathways of facultative heterotrophic organisms under aerobic and anoxic conditions are described in **Part I** below and the mechanisms which regulate respiration are described in the succeeding **Part II**.

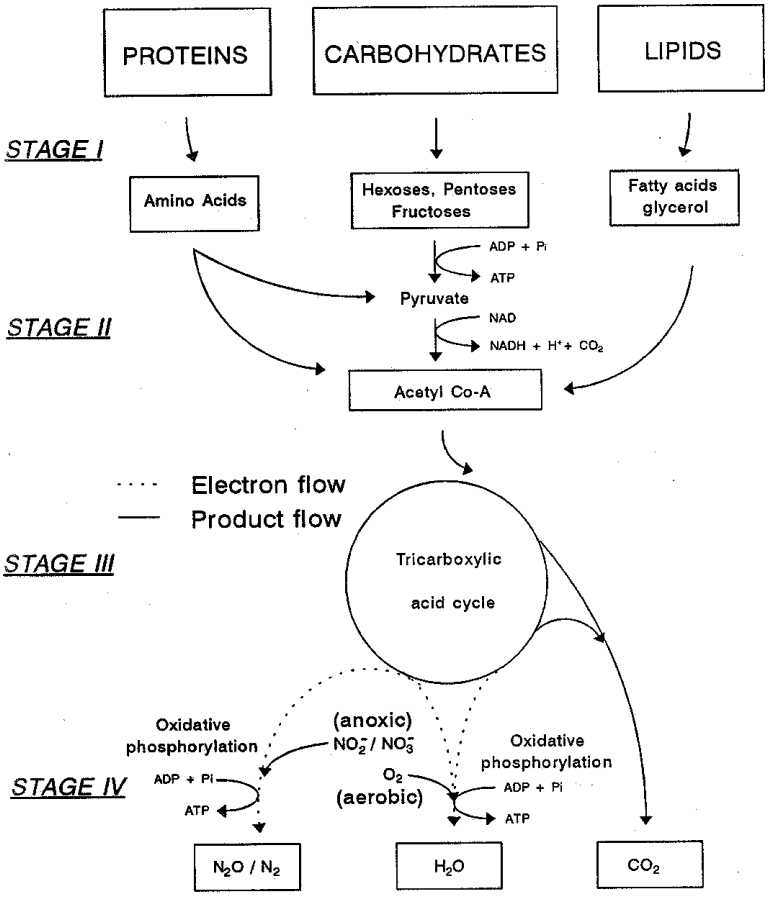

Figure 1
Stagewise division of substrate breakdown by facultative heterotrophic organisms, indicating the major metabolic pathway, reactants, and products (from Lehninger, 1975)

Part I: Aerobic and anoxic respiration in facultative heterotrophic organisms

Stages of respiratory metabolism (catabolism)

The processes of respiratory metabolism by heterotrophic organisms wherein organic substrates, such as carbohydrate, protein and lipids, are oxidised to the endproducts of H_2O and CO_2 can be divided into 4 stages and conceptualised as illustrated in Fig. 1. In Stage I, large complex organic molecules are enzymatically degraded (hydrolysed) to simpler ones; carbohydrates to hexoses and pentoses, proteins to amino acids, and lipids to fatty acids and glycerol.

In Stage II, the endproducts of Stage I are degraded further, resulting in the formation of acetyl-coenzyme A (acetyl-CoA) and carbon dioxide. For the degradation of each group of Stage I endproducts, i.e. amino acids, hexoses/pentoses, and fatty acids/glycerol, different biochemical pathways are employed.

In Stage III, the endproduct from Stage II, acetyl-CoA, enters the TCA cycle, a cyclic sequence of reactions catalysed by a series of enzymes. During the cycle, the acetyl group of acetyl-CoA is oxidised to form two molecules of CO_2, eight protons (H^+), four pairs of electrons (e^-) and one molecule of GTP which is energetically equivalent to one ATP; Coenzyme A is recovered.[*Note: The terminology "pairs of electrons" is used to indicate that electrons (e^-) are transported in pairs, unlike protons (H^+) which can be transported individually*]. The TCA cycle is the final catabolic pathway common to all aerobic and facultative organisms. It can be conceptualised as the mechanism by which all the foregoing substrates (in the form of acetyl-CoA) are converted to common products, i.e. electrons and protons, in the form of reduced NADH and reduced $FADH_2$.

In Stage IV, the electrons and protons associated with NADH and $FADH_2$ are removed and transferred along a pathway of electron and proton carrier enzymes of successively lower energy - the ETP - to a final (terminal) electron acceptor. During the process, much of the free energy of the electrons is conserved in the form of the energy-rich molecule ATP in a process termed oxidative phosphorylation. Under aerobic conditions, the electrons and protons are transferred to the final electron acceptor O_2 (with the formation of H_2O) and under anoxic conditions, electrons and protons are transferred to nitrate (NO_3^-) and/or nitrite (NO_2^-) (with the formation of H_2O and N_2). With NO_3^- or NO_2^- as external

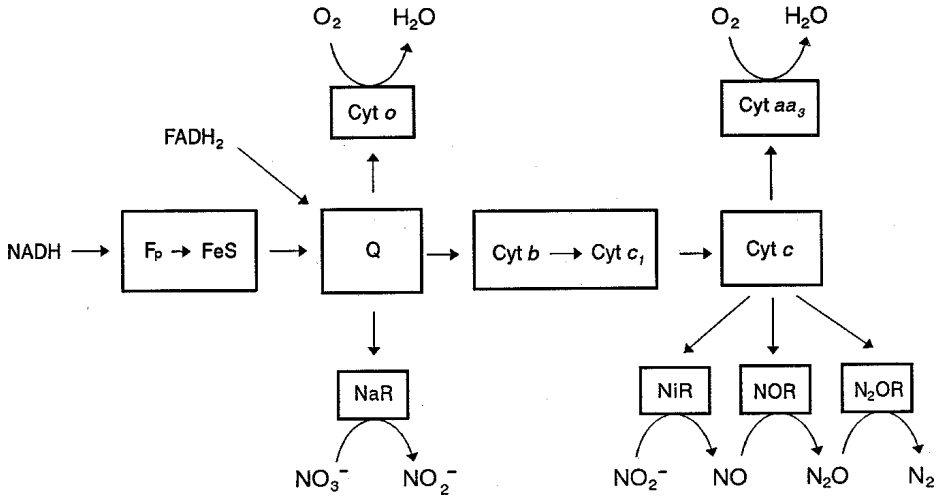

Figure 2
ETP for a typical facultative aerobic heterotrophic organism indicating the points of transfer of electrons to the terminal oxidases and reductases. (Adapted from Ferguson, 1982).

terminal electron acceptors, the gaseous products nitric oxide (NO) and nitrous oxide (N_2O) are produced intracellularly; these gaseous nitrogen oxides act as intermediate electron acceptors during the formation of dinitrogen (N_2).

The ETPs for aerobic and anoxic respiration have some common electron transferring complexes, i.e. some complexes which are utilised in both aerobic and anoxic respiration; other complexes are specific to aerobic or anoxic respiration. Figure 2 (an amalgamation of the ETPs proposed by Ferguson, 1982; Knowles, 1982; Stouthamer, 1980 and Payne, 1973) illustrates the complexes present in both the aerobic and anoxic ETPs of a typical facultative organism. A complete description of the significant developments in the elucidation of this pathway is given by Casey et al. (1993). The representation of the respiratory ETP as a linear arrangement of electron carriers is somewhat misleading in that no recognition is given to the location of each of the complexes with respect to the cytoplasmic membrane, or to the manner by which the location affects the movement of electrons, protons, and intermediates of the aerobic and anoxic pathways. In reality, the complexes are arranged within the membrane in a manner similar to that shown in Fig. 3. In this depiction, all of the respiratory complexes are included, irrespective of whether they are synthesised under aerobic or anoxic conditions.

The remainder of this review is devoted to description of the mechanisms of the aerobic and anoxic respiratory processes that occur in Stage IV. The mechanisms associated with aerobic respiration are reviewed first and then those associated with anoxic respiration.

Aerobic respiration

The respiratory enzyme complexes involved in the transfer of electrons and protons from NADH and $FADH_2$ to the terminal electron acceptor oxygen are shown in Fig. 4 which is an adaptation of Fig. 3 in that only the complexes synthesised under aerobic conditions are illustrated. These are, NADH dehydrogenase, ubiquinone (Q), cytochrome bc_1, cytochrome *c* and the two oxidases, cytochromes aa_3 and *o*. (Synthesis of the complexes shown in dotted outline is repressed under aerobic conditions, an aspect discussed in greater detail in Part II of this review.) The complexes, NADH dehydrogenase, ubiquinone, and cytochrome aa_3 are considered as three proton-pumping, energy-conserving sites, i.e. sites at which protons are pumped from the cytoplasmic (inner) to the periplasmic (outer) side of the membrane, this being the first step of the mechanism which links the processes of respiration (transfer of electrons to oxygen) and oxidative phosphorylation (production of the high energy molecule, ATP, from the low energy molecule, ADP). Usually the ETPs to cytochrome *o* and cytochrome aa_3 operate simultaneously, with the major portion of electron flow passing to cytochrome aa_3. Cytochrome aa_3 and cytochrome *o* are referred to as the primary and alternative oxidases, respectively. The series of reactions catalysed by each of the complexes of the aerobic ETP with respect to the cytoplasmic membrane are discussed below.

Although the level of microbiological and biochemical detail presented below may seem unnecessarily fundamental in the sanitary engineering context, it is considered necessary for the formulation and proper understanding of the AA filament bulking hypothesis.

NADH dehydrogenase enzyme complex

The NADH dehydrogenase enzyme complex consists of polypeptide chains collectively referred to as flavoprotein (Fp) and a series of iron-sulphur protein complexes (designated FeS). Flavoprotein contains a prosthetic (attached) group called flavin mononucleotide (FMN) or flavin which acts as the electron and proton transferring site of the complex.

The NADH dehydrogenase complex catalyses the transfer of two protons and a pair of electrons from NADH to ubiquinone in three steps.

In the first step, the prosthetic group, FMN of the flavoprotein (Fp) gains protons and electrons to give the reduced form $FMNH_2$ as follows:

$$NADH + H^+ + FMN \rightarrow FMNH_2 + NAD^+ \quad (1)$$

In the second step, a pair of electrons are transferred from $FMNH_2$ to a series of iron-sulphur protein complexes (FeS) on the periplasmic side of the membrane at which point two protons are released to the periplasm. The point at which protons are extruded through the membrane to the periplasm is conventionally regarded as the first of three proton-pumping or energy-conserving positions along the pathway and is referred to as Site I (see Fig. 4).

The reaction is as follows:

$$FMNH_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + FMN + 2H^+ \quad (2)$$

In the third step, a pair of electrons are transferred between the FeS complexes, and each reduced FeS complex donates one electron to the next carrier in the ETP, ubiquinone (Q).

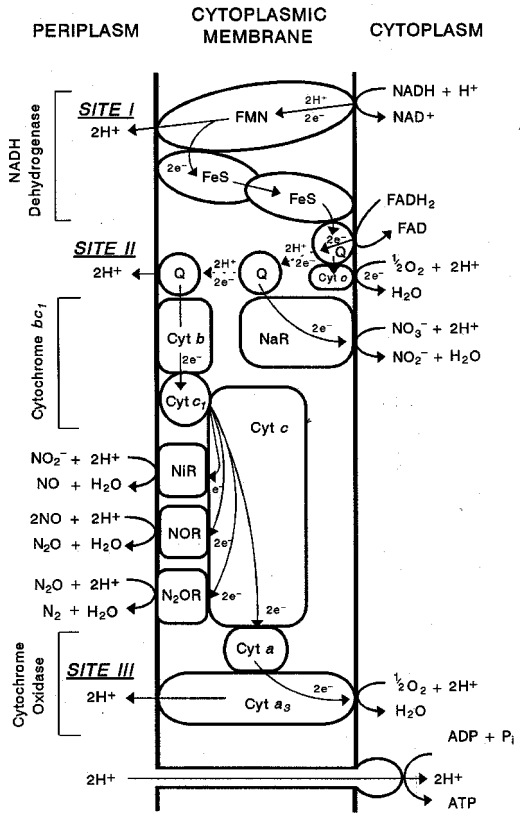

Figure 3

ETP for a typical facultative aerobic heterotrophic organism indicating the active site of both the aerobic and anoxic electron transferring complexes with respect to the cytoplasmic membrane and the three energy conserving (proton-pumping) sites.

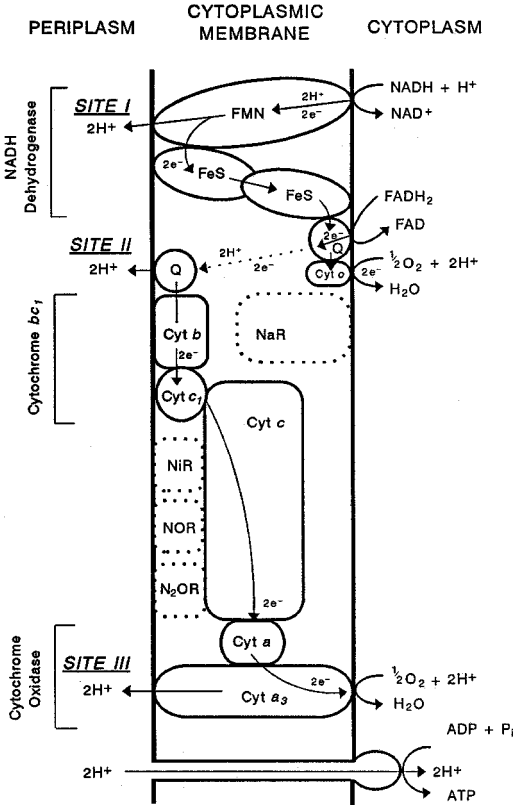

Figure 4

ETP for a typical facultative aerobic heterotrophic organism grown under aerobic conditions, indicating the presence of the oxidases, and synthesis at a low level of the nitrogen oxide reductases.

Ubiquinone

Ubiquinone is a mobile molecule which transports electrons and protons from the cytoplasmic to the periplasmic side of the membrane. [*Note: In the discussion of electron transfer to and from ubiquinone, when the molecule is in the oxidised state it is referred to as ubiquinone, and when it is in the reduced state it is referred to as ubiquinol].* As illustrated in Fig. 4, either of NADH and $FADH_2$ can act as the initial electron donor. For NADH as the initial donor, the transfer of electrons and protons is as follows:

$$Q\,(\text{ubiquinone}) + 2Fe^{2+} + 2H^+ \rightarrow QH_2\,(\text{ubiquinol}) + 2\,Fe^{3+} \quad (3)$$

For $FADH_2$ as the donor, the transfer of electrons and protons is as follows:

$$Q + FADH_2 \rightarrow QH_2 + FAD \quad (4)$$

Ubiquinone transports electrons and protons to the periplasmic side of the membrane where it extrudes two protons to the periplasm and transfers a pair of electrons to cytochrome *b* contained in the second of the main respiratory complexes, the cytochrome bc_1 complex as follows:

$$QH_2 + 2\ \text{cyt}\ b_{ox}(Fe^{3+}) \rightarrow 2\ \text{cyt}\ b_{red}(Fe^{2+}) + 2H^+ + Q \quad (5)$$

The point at which two protons are transferred to the periplasmic side of the membrane is conventionally regarded as the second of the three energy-conserving sites, i.e. Site II.

Cytochrome bc_1 complex

An important characteristic of cytochromes in general is that they are able to transfer electrons only, unlike NADH, NADH dehydrogenase, $FADH_2$ and ubiquinone discussed above, which transport both electrons and protons.

The cytochrome bc_1 complex contains two sequential electron transporting proteins, cytochrome *b* and cytochrome c_1. The cytochromes of the cyt bc_1 complex are intermediates in the transfer of electrons from ubiquinol to cytochrome *c*, the next complex in the pathway. Cytochromes are electron transporting proteins which contain iron in a prosthetic group and are referred to as haem proteins. The transfer sequence is from ubiquinol to cyt *b* to cyt c_1 to cyt *c*.

As with the iron atom of the FeS complexes, the iron atom of the haem group of the cytochromes alternates between an oxidised ferric (+3) state and a reduced ferrous (+2) state during electron transport. Regarding electron transport, it should be noted that the haem groups of the cytochromes are able to transfer only one electron at a time, unlike NADH, $FMNH_2$, and ubiquinol which transfer two electrons. Thus, even though it is not shown as such in Fig. 4, for each molecule of ubiquinol transporting two electrons and two protons, two complexes of cytochrome *b*, cytochrome c_1 and cytochrome *c* are required.

Cytochrome c complex

The cytochrome c complex accepts electrons from cytochrome c_1 of the cytochrome bc_1 complex and transfers them to the terminal electron transferring complex for aerobic respiration, the cytochrome oxidase complex, cytochrome aa_3.

Cytochrome oxidase complexes

The terminal cytochrome complex in the aerobic ETP, the cytochrome oxidase complexes (cytochromes aa_3 and *o*), transfer electrons to the terminal electron acceptor, oxygen. For obligate aerobic heterotrophic organisms, the aerobic cytochromes in the ETP to oxygen are contained in the cytochrome aa_3 complex (Poole, 1982). For facultative denitrifying organisms, an additional cytochrome oxidase complex, cytochrome *o* is present (Scholes and Smith, 1968; Schulp and Stouthamer, 1970; Lam and Nicholas, 1969a; Sapshead and Wimpenny, 1972; Willison and John, 1979). It has been found that for facultative organisms maintained under anoxic conditions, three to four times more cytochrome *o* is synthesised than when the same facultative organisms are maintained under aerobic conditions (Sapshead and Wimpenny, 1972). In facultative organisms undergoing aerobic respiration, both of cytochrome aa_3 and cytochrome *o* may be operative. The roles of cytochrome *o* and cytochrome aa_3 in facultative organism respiration are described in the biochemical respiratory model for facultative organisms in Paper 10 (Casey et al., 1999a). For electron transport mediated by the cytochrome aa_3 complex, cytochromes *a* and a_3 transfer electrons to oxygen in a complex mechanism which is not completely understood (Babcock and Wikström, 1992). The cytochrome aa_3 complex contains four electron transferring metal centres; 2 iron and 2 copper. Iron atoms are contained in each of cytochrome *a* and cytochrome a_3 and alternate between a reduced ferrous (+2) state and an oxidised ferric (+3) state during electron transport. Copper atoms are also contained in each of cytochromes *a* and a_3 and also mediate electron transport and alternate between a reduced (+1) state and an oxidised (+2) state (Poole, 1982). Cytochrome a_3 lies in the membrane, the active site located on the cytoplasmic side. At this site, 4 protons from the cytoplasm combine with an oxygen molecule and 4 electrons from the four metal centres of the cytochrome aa_3 complex to form water as follows:

$$\text{cyt } a\ (Fe^{2+},Cu^{+}) + \text{cyt } a_3\ (Fe^{2+},Cu^{+}) + O_2 + 4H^{+} \rightarrow 2H_2O + \text{cyt } a\ (Fe^{3+},Cu^{2+}) + \text{cyt } a_3\ (Fe^{3+}, Cu^{2+}) \quad (6)$$

The cytochrome aa_3 complex is conventionally regarded as the third of the three energy-conserving sites, i.e. Site III, at which protons are pumped across the cytoplasmic membrane to the periplasm. The distinguishing characteristic between the cytochrome aa_3 complex (Site III) and the other two proton-pumping sites, i.e. the NADH dehydrogenase complex (Site I) and the cytochrome bc_1 complex (Site II), is the manner by which protons are transferred across the membrane. At Sites I and II, two protons associated with the pair of electrons being transferred are ejected to the periplasm by the proton transferring molecules, $FMNH_2$ and ubiquinol respectively, whereas at Site III, proton transferring molecules are not present. The mechanism for removing protons from the cytoplasm to the periplasm at cytochrome aa_3 is thought to be a consequence of electron transport to and from the cytochrome, this transport inducing a change in the cytochrome a_3 protein conformation, causing part of the complex which spans the membrane to eject protons to the periplasm (Van Verseveld et al., 1981).

Under environmental conditions in which cytochrome *o* acts as oxidase, electrons flow directly from ubiquinol to cytochrome *o*; cytochromes bc_1 and *c* are not involved. Consequently, the electrons pass only two energy conserving sites, Sites I and II for NADH as electron donor but only one site, Site II for $FADH_2$ as electron donor. For NADH as electron donor, electrons are transferred to NADH dehydrogenase, then to ubiquinone and for $FADH_2$ as electron donor, to ubiquinone directly. For cytochrome *o* as oxidase, 4 protons from the cytoplasm combine with 1oxygen molecule and 2 pairs of electrons (transferred from ubiquinol to cytochrome *o*) to form water as follows:

$$2\text{cyt } o\ (Fe^{2+},Cu^{+}) + O_2 + 4H^{+} \rightarrow 2H_2O + 2\text{cyt } o\ (Fe^{3+}, Cu^{2+}) \quad (7)$$

The environmental conditions that determine which of cytochrome *o* or cytochrome aa_3 acts as terminal electron acceptor are described in Part II of this review.

A significant physical difference between cytochrome *o* and cytochrome aa_3 is in the metal electron-transferring centres contained in each cytochrome. Cytochrome *o* contains two iron-histidine (Fe-hs) centres and one copper-histidine (Cu-hs) centre, whereas cytochrome aa_3 contains both of these, and additionally a copper-histidine-cysteine (Cu-hs-cs) centre (Babcock and Wikström, 1992). The implications of this difference between the oxidases will become apparent in Part II of this review where the mechanisms of regulation of aerobic respiration are investigated. This in turn has implications with regard to differences in respiration between filamentous and floc-forming organisms, described fully in Paper 11 (Casey et al., 1999b), these differences forming the basis of the AA filament bulking hypothesis.

Anoxic respiration

The description above of aerobic respiration is for conditions where dissolved oxygen (DO) is in adequate supply. When DO becomes limiting, facultative heterotrophs switch from oxygen to nitrate/nitrite as terminal electron acceptor and respire anoxically. For nitrate/nitrite as electron acceptor, the first three stages illustrated in Fig. 1 and the greater part of the fourth stage of the ETP continue functioning unchanged; a difference in electron transport between aerobic and anoxic respiration becomes apparent only after the ubiquinone complex, i.e. in the electron and proton transferring complexes in Stage IV of Fig. 1. Under anoxic conditions, not all of the electron-transferring complexes shown in Fig. 3 are synthesised. Figure 5 illustrates the respiratory complexes present in a typical facultative aerobic organism grown under anoxic conditions. Synthesis of the complexes shown in dotted outline is repressed under anoxic conditions, an aspect discussed in greater detail in Part II of this review.

Definition of terms

Organisms can utilise nitrate through two processes, assimilatory nitrogen removal and dissimilatory nitrogen removal.

Assimilatory nitrogen removal is the reduction of nitrate to ammonium (NH_4^+), the ammonium being used for synthesis of cellular material. This is an anabolic process, is energy consuming, is not associated with the respiratory ETP and can occur under both aerobic and anoxic conditions. Because the process is not associated with the respiratory ETP, it is not considered further.

Dissimilatory nitrogen reduction is the reduction of nitrate to nitrite, or nitrite to one of the more reduced gaseous nitrogen oxide compounds, nitric oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2). The processes whereby nitrate and nitrite act as terminal electron acceptors during utilisation of organic substrate are referred to as nitrate and nitrite respiration respectively. The term nitrate reduction applies to the reduction of nitrate to nitrite only. The term denitrification is applied to the reduction of one of the ionic nitrogen oxides (nitrate or nitrite), to one of the gaseous nitrogen compounds, nitric oxide, nitrous oxide, or dinitrogen. The pathway for denitrification is composed of a number of sequential

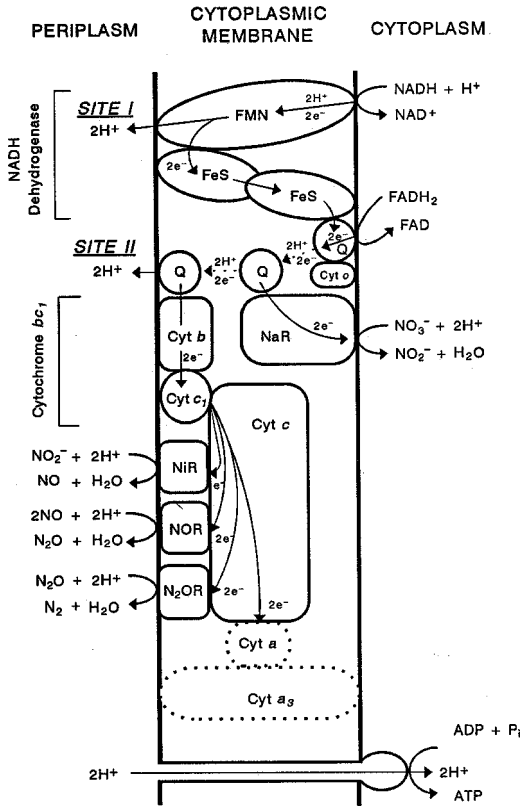

Figure 5

ETP for a typical facultative aerobic organism grown under anoxic conditions, indicating the presence of the reductases and cytochrome o, and the synthesis at a low level of the oxidase, cytochrome aa_3.

steps by which electrons are passed via the ETP to one of the nitrogen oxides, to produce another more reduced nitrogen oxide. Although opinions differ as to the intermediates produced in the denitrification pathway, an overwhelming body of evidence has accumulated that supports the sequential production of the intermediates in the manner proposed originally by Payne (1973):

$$\text{Nitrate}\,(NO_{3\,aq}^{-})^{(+5)} \rightarrow \text{Nitrite}\,(NO_{2\,aq}^{-})^{(+3)} \rightarrow \text{Nitric Oxide}\,(NO_g)^{(+2)} \rightarrow \text{Nitrous Oxide}\,(N_2O_g)^{(+1)} \rightarrow \text{Dinitrogen}\,(N_{2g})^{(0)} \quad (8)$$

[*Note: The values in brackets are the oxidation states of the nitrogen atom for each nitrogen oxide compound. The subscripts* $_{aq}$ *and* $_g$ *denote aqueous and gaseous states respectively*]. A review of the research which contributes to the general acceptance of this as the standard denitrification pathway is given by Casey et al. (1993).

The nitrogen oxides of the pathway, which comprise the more reduced species, i.e. nitrite, nitric oxide and nitrous oxide, are referred to as the intermediates of the denitrification pathway and dinitrogen as the end-product of denitrification. Reduction of one nitrogen oxide intermediate to another is accompanied by the transfer of electrons along the pathway to the specific nitrogen oxide. Reduction of each specific nitrogen oxide is catalysed by a specific enzyme; these enzymes belong to a group which generally is referred to as the nitrogen oxide reductases. Specifically, these are the nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases. The pathways for electron flow under anoxic conditions differ, depending on the nitrogen oxide available as terminal electron acceptor. With NO_3^- as terminal electron acceptor, electrons are transferred from NADH to NADH dehydrogenase to ubiquinone and then directly to nitrate reductase, i.e. the electrons do not pass through cytochromes *b*, c_1 and *c* (Fig. 5). With NO_2^-, NO and N_2O as electron acceptors, electrons pass from NADH to NADH dehydrogenase, to ubiquinone, to cytochrome bc_1 complex, to cytochrome *c* and then to nitrite-, nitric oxide-, or nitrous oxide reductase, respectively.

Not all organisms classified as denitrifiers can execute the entire pathway from nitrate to dinitrogen. Some organisms are capable of executing only part of the pathway, from one of the ionic nitrogen compounds (i.e. NO_3^- or NO_2^-) to one of the gaseous nitrogen compounds (i.e. NO or N_2O or N_2) and these organisms are referred to as partial denitrifiers. As mentioned above, some organisms reduce nitrate to nitrite only and these are referred to as nitrate reducers.

Electron transferring complexes and energetic yield associated with anoxic respiration

With one of the nitrogen oxides as terminal electron acceptor, electrons pass directly from ubiquinol to nitrate reductase for nitrate as electron acceptor and from ubiquinol via the cytochrome bc_1 and cytochrome *c* complexes for the other nitrogen oxides as electron acceptor. In contrast, with oxygen as terminal electron acceptor, electrons pass from the cytochrome bc_1 complex, through cytochrome *c* to either cytochrome aa_3 or cytochrome *o*. These differences in electron transport between aerobic and anoxic respiration have significant implications for energy conservation by the organism. For aerobic respiration, during electron transfer from NADH to cytochrome aa_3, electrons pass all three energy-conserving sites whereas for anoxic respiration, during electron transfer from NADH to the nitrogen oxide reductases, electrons pass only two energy-conserving sites. The consequences of these differences in the aerobic and anoxic biochemical pathways for organisms switching between aerobic and anoxic respiration in activated sludge processes are discussed in the biochemical model for facultative organisms described in Paper 10 (Casey et al., 1999a).

It will be noted that in this section of the review, considerable attention is devoted to nitrate reductase, reflecting not so much the particular importance of that complex in anoxic respiration, but more so the greater volume of research devoted to it. This situation has developed because nitrate is the first choice nitrogen oxide for reduction, being the most oxidised of all the nitrogen oxides (+5), as indicated in Eq. 8.

For the complete reduction of nitrate to dinitrogen, electrons are transferred through each of the nitrogen oxide reductases to the specific nitrogen oxides which become reduced. The complexes NADH dehydrogenase, ubiquinone, cytochrome bc_1, and cytochrome *c* are common to the aerobic and anoxic ETPs. Their roles in mediating electron/proton transfer have been discussed in detail under aerobic respiration above. Therefore, only their role in final electron transfer to the nitrogen oxide reductases is discussed here. To illustrate the mechanisms by which each reductase accepts and transfers electrons, the structure, function and location with respect to the cytoplasmic membrane of each reductase is described in turn.

Nitrate reductase

Nitrate is reduced to nitrite at nitrate reductase, the catalytic site of which is situated on the cytoplasmic side of the membrane (John, 1977; Kristjansson et al., 1978; Alefounder and Ferguson, 1980; Boogerd et al., 1983b). In transfer of electrons from NADH to nitrate reductase, electrons pass sequentially through the complexes of NADH dehydrogenase to ubiquinone as described for

aerobic respiration. In the process, the first of the energy conserving (proton-pumping) sites (Site I) is passed. The transfer of electrons through the NADH dehydrogenase complex is common to reduction of each of the nitrogen oxide reductases. The electrons then pass from ubiquinol to nitrate reductase.

At the catalytic site of nitrate reductase, 2 electrons are passed to nitrate which in the process takes up 2 protons from the periplasm to form nitrite and water as follows:

$$2H^+ + 2e^- + NO_3^- \rightarrow NO_2^- + H_2O \quad (9)$$

In the process of transferring 2 electrons to nitrate reductase, ubiquinol ejects 2 protons to the periplasm and establishes the site of NO_3^- reduction as the second (i.e. Site II) of the energy-conserving sites of the anoxic respiratory pathway as indicated in Fig. 5. This site corresponds to Site II of aerobic respiration at which ubiquinol ejects 2 protons to the periplasm in the process of transferring electrons to the cytochrome bc_1 complex. Thus, for each molecule of nitrate reduced, 2 electrons originating from NADH pass 2 proton-pumping sites, Sites I and II, at each of which 2 protons are translocated to the periplasm. Electrons originating from $FADH_2$ and transferred to nitrate, pass only one proton-pumping site, Site II. Thus a lower energy yield results for electrons originating from $FADH_2$ compared with electrons originating from NADH. Either of NADH or $FADH_2$ can serve as electron donor to each of the intermediates (nitrite, nitric oxide and nitrous oxide), but for simplification, the role of NADH only and not $FADH_2$ is described during discussion of the mechanism of reduction of each of the intermediates.

Given the cytoplasmic placement of the active site of nitrate reductase, nitrate has to be translocated across the cytoplasmic membrane, from the periplasmic (outer) to the cytoplasmic (inner) side in order to be reduced. The literature is not clear on the mechanism of this translocation. John (1977) proposed a transmembrane ionic carrier specific to nitrate. Boogerd et al. (1983a) proposed two uptake systems for nitrate which operate in sequence. The first system is an H^+-NO_3^- symport mechanism which initiates nitrate uptake and is dependent on the proton motive force (pmf) established by the translocation of protons across the cytoplasmic membrane. Nitrate crosses the membrane together with two or more protons during production of ATP, and is reduced to nitrite on the cytoplasmic side. The second proposed nitrate uptake system is an NO_3^-/NO_2^- antiport, the function of which is to take over NO_3^- uptake from the first system. For each nitrate molecule crossing the membrane to the cytoplasm, one nitrite molecule would pass back to the periplasm. However, because no physical evidence exists for the presence of such systems, Craske and Ferguson (1986) suggest that the nitrate reductase complex itself incorporates a nitrate-specific channel which provides access for nitrate to the active site of its reductase.

Nitrite reductase

The function of nitrite reductase is to reduce nitrite, originating either from the bulk solution or from the reduction of nitrate, to nitric oxide. Nitrite reductase, situated on the periplasmic side of the membrane (Meijer et al., 1979; Alefounder and Ferguson, 1980; Boogerd et al., 1981) accepts 1 electron from cytochrome *c* and at the catalytic site of the reductase the electron is passed to nitrite, which in the process also takes up 2 protons from the periplasm to form nitric oxide (NO) and water as follows:

$$2H^+ + e^- + NO_2^- \rightarrow NO + H_2O \quad (10)$$

If the electron from cytochrome *c* originated from NADH, then it must have passed through each of FMN, FeS, ubiquinone and cytochrome bc_1, and in its transfer passed two energy conserving sites, Sites I and II. Thus, for each pair of electrons which pass to nitrite, four protons are pumped across the membrane, which is equal to the number of protons pumped across the membrane for each pair of electrons which pass to nitrate. Therefore the organism has about the same energetic yield for nitrite as for nitrate (see note on p. 416, bottom of 2nd column).

The reduction of NO_2^- on the periplasmic side of the membrane necessitates the transport of NO_2^- from the cytoplasm where it is formed, to the periplasm where it is reduced, this transport apparently occurring as part of the NO_3^-/NO_2^- antiport mechanism described for the movement of nitrate across the membrane.

Two different types of nitrite reductase have been identified: a cytochrome complex containing haems of the *c*- and *d*-type, referred to as cytochrome cd_1 and a copper-containing cytochrome complex, both of which apparently perform the same function but are present in different species of denitrifying organisms (Shapleigh and Payne, 1985a).

Whereas it is indicated by Eqs. 8 and 10 that nitric oxide is the product of nitrite reduction, the production or not of nitric oxide through denitrification has been the cause of some dispute, because usually N_2O, but not NO is detected in denitrifying cultures (Averill and Tiedje, 1982; Firestone et al., 1979; Garber and Hollocher, 1981). This anomaly has been resolved with the finding that under normal denitrifying conditions, NO is maintained at constant low concentrations intracelluarly and is absent extracelluarly (Carr and Ferguson, 1990a; Goretski and Hollocher, 1990), but under adverse situations (e.g. high concentrations of NO_2^-, the presence of toxins, or in physiologically old cells), NO can be excreted extracelluarly (Verhoeven, 1956; Voßwinkel and Bothe, 1990). Proof of the presence of NO as an intermediate in the denitrification pathway has important implications for the conceptual model for facultative heterotrophic respiration formulated in Paper 10 (Casey et al., 1999a).

Nitric oxide reductase

The function of nitric oxide reductase is to reduce nitric oxide to nitrous oxide. Considerable uncertainty exists concerning the position of the active site of nitric oxide reductase. It has been tentatively placed on the periplasmic side of the membrane on the basis of proton uptake experiments (Ferguson, 1987; Stouthamer, 1988; Zumft, 1993), but there is still some uncertainty (Bell et al., 1992).

A feature which distinguishes nitric oxide reductase from the other nitrogen oxide reductases is that it is the site at which the N-N bond of N_2O is formed and 2 molecules of NO are required for the formation of 1 molecule of N_2O. Nitric oxide reductase receives a pair of electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to 2 molecules of nitric oxide which in the process take up 2 protons to form one molecule each of nitrous oxide and water as follows:

$$2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O \quad (11)$$

The pair of electrons gained by nitric oxide are transferred between the same complexes as electrons gained by nitrite, i.e. electrons pass the same two proton-pumping sites, Sites I and II, for electrons originating from NADH, but pass Site II only if the electrons originate from $FADH_2$. Thus for each pair of electrons originating from NADH and gained by nitric oxide, 4 protons are pumped across the membrane per pair of electrons transferred, which is the

same ratio for the reduction of nitrite and nitrate. Thus for either nitric oxide, nitrite or nitrate as electron acceptor, the organism has a similar energetic yield (see note at bottom of 2nd column).

Transfer of electrons from nitric oxide reductase to nitric oxide occurs via the reactive centre of the reductase, a *bc*-type haem (Carr and Ferguson, 1990b). Although no transport mechanism has been identified for movement of nitric oxide across the membrane, in all probability, the gaseous state of the molecule allows it to diffuse across the membrane. This potential for movement across the membrane has implications with regard to interaction of nitric oxide with cytochrome oxidase, an important aspect in the regulation of aerobic respiration described in Part II below.

Nitrous oxide reductase

The function of nitrous oxide reductase is to reduce nitrous oxide to dinitrogen. Nitrous oxide reductase situated on the periplasmic side of the membrane (Boogerd et al., 1981) receives a pair of electrons from cytochrome *c* and at the catalytic site of the reductase the electrons are passed to nitrous oxide which in the process takes up 2 protons from the periplasm as follows:

$$2H^+ + 2e^- + N_2O \rightarrow N_2 + H_2O \qquad (12)$$

The endproduct N_2 is released from the cell. The pair of electrons gained by nitrous oxide are transferred between the same complexes as electrons gained by each of nitrite and nitric oxide, i.e. electrons pass the same proton pumping sites as for nitrate, nitrite and nitric oxide.

Energetic aspects of respiration

The primary role of catabolic respiration, irrespective of whether it occurs under aerobic or anoxic conditions, is the production of energy for the purposes of biosynthesis of the complex molecular components of the organism anabolism and for cell maintenance. In the aerobic and anoxic respiratory pathways, the electrons and protons gained from organic substrate oxidation are transported to successively lower redox potentials during their passage between complexes. In this transport pathway, free energy associated with the electrons and protons is released; a portion (about 60%) of this free energy is conserved by the organism in the form of ATP.

In the foregoing discussion, mention was made of the three energy-conserving sites in the ETP, alternatively referred to as proton-pumping sites (Figs. 4 and 5). At these sites, free energy released by the electrons is used to transport protons across the cytoplasmic membrane. In a subsequent mechanism, termed oxidative phosphorylation, the protons flow back across the membrane through a protein called adenosine triphosphatase (ATPase) and energy is captured in the form of high energy phosphate bonds during the formation of ATP from ADP. The coupling of respiration and oxidative phosphorylation was proposed in 1961 by Mitchell and is known as the chemiosmotic theory. In short, to generate 1 molecule of ATP, 2 protons must cross the membrane from the periplasm to the cytoplasm via the ATPase enzyme. One ATP is generated at ATPase for each pair of protons pumped across the membrane at Sites I, II or III.

Establishing the position of the energy sites in the electron transport pathway

The chemiosmotic theory hinges on the definite identification of the position and function of the three proton-pumping sites described above. Since identification of the three sites as given in Fig. 3 forms an important part of the conceptual model for facultative heterotrophic organism respiration described in Paper 10 (Casey et al., 1999a), it is important that the literature is sufficiently well reviewed to identify the pertinent research in this area. The sites have been identified experimentally through three different approaches:

- Comparison of the ATP yield of different substrates from the known redox potentials of the complexes.
- Thermodynamic estimates of electron and proton flow between complexes which donate electrons at different points along the ETP.
- Inhibition of electron flow at specific sites on the electron transport pathway through the use of specific chemicals (Stryer, 1981).

Energetic yield associated with aerobic and anoxic growth

Considering Fig. 3 as representative of a typical facultative organism, the ATP yields can be compared for the use of oxygen under aerobic conditions and the nitrogen oxides under anoxic conditions.

Under aerobic conditions: Two cytochrome oxidases are available, cytochrome *o* and cytochrome aa_3. For cytochrome aa_3 as terminal oxidase and with NADH as electron donor, electrons pass all three of the proton-pumping sites with the concomitant energy conservation of 3 moles of ATP formed for the acceptance of 2 moles of electrons by 1 mole of oxygen. For the alternative oxidase cytochrome *o* as terminal oxidase, and with NADH as electron donor, electrons pass Sites I and II only, and the energetic yield associated with electron flow to oxygen mediated by this enzyme would be about $^2/_3$ of that associated with electron flow to oxygen via cytochrome aa_3.

Under anoxic conditions: With NADH as electron donor, electrons pass only the first two of the three proton-pumping sites with an associated energy conservation of 2 moles of ATP formed for the acceptance of 2 moles of electrons by 1 mole of nitrogen oxide. Experimental evidence of a cell yield with nitrogen oxides under anoxic conditions of approximately $^2/_3$ of the yield with oxygen under aerobic conditions has been presented by Koike and Hattori (1975a), Justin and Kelly (1978), Van Verseveld et al. (1981), Parsonage and Ferguson (1983), and Stouthamer (1988).

Although it can be concluded from the foregoing that less energy (ATP) is captured with nitrogen oxide reductases compared with oxygen reductases, comparing the different nitrogen oxides (NO_3^-, NO_2^-, NO, N_2O and N_2) as electron acceptors under anoxic conditions, from experimental determinations, approximately the same amount of energy is gained in the use of each (Hadjipetrou and Stouthamer, 1965; Koike and Hattori, 1975b; Van Verseveld et al., 1977; Kristjansson et al., 1978; Stouthamer et al., 1982; Garber et al., 1982; Shapleigh and Payne, 1985b). [*Note: Even though the literature demonstrates that the energy yield from the nitrate reduction (NO_3^- to NO_2^-) and NO_2^- or gaseous nitrogen oxides (NO, N_2O) reduction (NO_2^- to NO, NO to N_2O and N_2O to N_2) is similar, it would appear that from a proton balance across the cytoplasmic membrane this may not be so. With NO_3^- reduction, protons are transformed to water on the cytoplasmic side of the membrane, whereas with the reduction of the other nitrogen oxides, protons are transformed to water on the periplasmic side, thereby establishing a different proton motor force*].

Comparison of energetic yield of nitrate reducers and denitrifiers

For denitrifying (i.e. from ionic to gaseous N oxide forms) organisms, the reduction of two nitrate ions through each nitrogen oxide intermediate to form one dinitrogen molecule requires a total of 5 pairs of electrons as follows: 2 pairs of electrons to reduce 2 nitrate ions to 2 nitrite ions, 1 pair of electrons to reduce 2 nitrite ions to 2 nitric oxide molecules, 1 pair of electrons to reduce the 2 nitric oxide molecules to 2 nitrous oxide molecules and 1 pair of electrons to reduce 1 nitrous oxide molecule to 1 dinitrogen molecule. The 5 pairs of electrons each pass 2 energy-conserving sites, resulting in the transfer of 10 pairs of protons across the membrane and the concomitant generation of 10 ATP (Fig. 5). In comparison, for nitrate reducing organisms (NO_3^- to NO_2^- only), the reduction of 2 nitrate ions to 2 nitrite ions requires 2 pairs of electrons, which during transfer from NADH through the electron transferring complexes to nitrate reductase pass the first 2 energy-conserving sites, transferring 8 protons to the periplasmic side of the membrane, resulting in the generation of 4 ATP. On the basis of nitrate utilised, this is a considerably lower yield than that gained by the denitrifying organisms in reducing 2 nitrate ions through each nitrogen oxide intermediate to a molecule of dinitrogen. Thus, for conditions in which nitrate is limiting with respect to the electron supply, denitrifying organisms would gain an energetic advantage over the nitrate reducers because they gain 10 ATPs per 2 moles of nitrate utilised instead of 4. However, for conditions in which nitrate is in excess with respect to the electron supply, the capacity of the denitrifiers to reduce nitrate through each intermediate does not endow them with an energetic advantage because their energy acquisition is not limited by the supply of nitrate. In activated sludge systems subjected to anoxic/aerobic conditions, growth of facultative organisms in the anoxic zone is limited most often by substrate (electron donor) availability, not by nitrate/nitrite limitations. Thus, it is unlikely that denitrifiers would gain an advantage under such conditions.

Part II: Mechanisms of regulation of aerobic and anoxic respiration in facultative heterotrophic organisms

Introduction

In Part I, the biochemical pathways and enzyme complexes present in facultative organisms under steady-state aerobic and steady-state anoxic conditions were described. By definition, facultative organisms have the capability of respiring under either anoxic or aerobic conditions. In order to determine the effect of changes between aerobic and anoxic conditions on substrate utilisation by facultative organisms in activated sludge, it is important to understand how such organisms regulate the distribution of electrons between the aerobic enzymes (oxidases) and the anoxic enzymes (reductases) when the organisms are exposed to changes between anoxic and aerobic conditions and additionally how the synthesis and activity of the enzymes are affected by these changes.

In this section - Part II - it is the intention to examine the factors associated with initiation, regulation, and termination of aerobic and anoxic respiration. Each of these three phenomenon involve changes in both synthesis and activity of specific enzyme complexes.

To investigate the synthesis and activity of the denitrifying complexes of facultative organisms in pure culture, generally, research workers conducted experiments with two sets of growth conditions:

- Aerobically grown organisms (in which the denitrifying enzymes are absent and aerobic enzymes are present) were exposed to anoxic conditions to examine how denitrifying enzymes are synthesised and how aerobic enzymes are inactivated and degraded.
- Anoxically grown organisms (in which the denitrifying enzymes are present and aerobic enzymes are absent) were exposed to aerobic conditions to examine how aerobic conditions affect the activity of the synthesised denitrifying enzymes and synthesis of the aerobic enzymes.

This approach is fortuitous in that the two sets of laboratory experimental conditions are similar to conditions encountered in anoxic-aerobic activated sludge systems (i.e. cyclic changes from anoxic to aerobic, and from aerobic to anoxic). Because of this similarity, findings from the pure culture organism experiments have important implications for research into mixed culture facultative organism behaviour in activated sludge systems.

In this review, the ETP that illustrates the changes that occur when a facultative organism is subjected to changes between anoxic and aerobic conditions is illustrated in Fig. 3. This ETP has been developed from an accumulation of research findings with nitrate respiring (NO_3^- NO_2^-) and denitrifying (NO_3^-/NO_2^- N_2) organisms in pure culture; an extensive discussion of these research findings and their role in the conceptualisation of the ETP is given by Casey et al. (1993). Note that the above distinction between denitrification (ion to gas) and nitrate reduction (ion to ion) is retained throughout the review.

In this review and subsequent papers, a number of key terms are used to describe the effect of specific conditions on enzyme development; these terms are defined below. Enzymes are either constitutive, implying that the enzyme is formed irrespective of the environmental conditions, or inducible, indicating that the enzyme is produced in trace amounts under all conditions, and is synthesised in much larger amounts in the presence of a specific substrate for that enzyme. The synthesis of enzymes is controlled at the genetic level, and for inducible enzymes, synthesis is initiated by the process of induction as described above, and the halting of enzyme synthesis is a process referred to as repression. For an enzyme that has been induced, synthesised to a high level, and then repressed, repression can be overcome and synthesis again initiated by the onset of the conditions that originally induced the enzyme (or by other conditions), and this process is described as derepression. The rate of processes mediated by enzymes can be affected at the molecular level by the presence or not of the compound being acted on (reduced in the case of nitrogen oxide reduction) and this process is referred to as activation of the enzyme. The term inactivation then refers to the halting of the process mediated by the enzyme due to lower concentrations of the molecule to be acted on.

Aerobic conditions changed to anoxic conditions

Aerobic and denitrifying enzyme synthesis under aerobic conditions

Under aerobic conditions, the status of synthesis of the aerobic and anoxic reductases can be illustrated as shown in Fig. 4. Enzymes shown in dotted outline are not synthesised, or are synthesised at a low level only. The effect of oxygen on the development of the aerobic enzymes was measured for *Paracoccus (Pa.) denitrificans* grown at different DO concentrations; the level of cytochrome aa_3

in *Pa. denitrificans* grown at a low DO concentration was between the high aa_3 level developed under high DO concentrations and its absence under anoxic conditions (Sapshead and Wimpenny, 1972). In these experiments, cytochrome *o* was synthesised under both aerobic and anoxic/anaerobic conditions, but under aerobic conditions developed to only 70% of the level under anoxic conditions.

Under oxygen saturation conditions, the nitrate and nitrite reductases of *Pa. denitrificans* were measured at a low level (Lam and Nicholas, 1969a), and similar results were noted for the nitrate reductase of the nitrate reducing organism, *Escherichia coli* (Showe and De Moss, 1968).

Requirements for induction of denitrifying enzyme synthesis

Considerable variability exists between organisms in their requirements for synthesis of the denitrifying enzymes. These requirements can be summarised as follows:

- The minimum requirements for most facultative organisms for induction (initialisation) of synthesis of the nitrogen oxide reductases are low levels of oxygen or its absence (*Bacillus licheniformis*, Schulp and Stouthamer, 1970; *Proteus mirabilis*, De Groot and Stouthamer, 1970; *Pseudomonas* (*Ps.*) *stutzeri*, Kodama, 1970; *Ps. perfectomarinus*, Payne et al., 1971).
- Some facultative organisms require the absence of oxygen for derepression of the nitrogen oxide reductases and additionally, the presence of the specific nitrogen oxide which is the reactant for the reductase, for induction of synthesis of each nitrogen oxide reductase (*Pa. denitrificans*, Lam and Nicholas, 1969b,c; *Klebsiella aerogenes*, Pichinoty and d'Ornano, 1961; *Alcaligenes faecalis*, Kakutani et al., 1981; *Flavobacterium* sp., Firestone and Tiedje, 1979).
- For organisms that require only the absence of oxygen for induction of reductase synthesis (and not the presence of the reactant nitrate and/or nitrite), the reductases are synthesised to only about 40% of the level to which they are synthesised when the organisms are exposed to conditions under which oxygen is absent and nitrate/nitrite is present. For virtually all facultative organisms, maximum levels of the reductases are synthesised only in the presence of the specific nitrogen oxide which is the reactant for the reductase.

To examine the relative importance of oxygen and nitrate concentration in the synthesis of nitrate reductase, *Pa. denitrificans* was grown at different concentrations of oxygen with nitrate present (Sapshead and Wimpenny, 1972). At a high concentration of oxygen, synthesis of nitrate reductase was repressed, irrespective of the presence or absence of nitrate. However, as the concentration of oxygen was reduced in successive experiments, the relative importance of nitrate in the induction of synthesis of nitrate reductase increased, such that at a very low concentration of oxygen or under anoxic conditions, the presence or absence of nitrate was of primary importance in determining the levels to which nitrate reductase was synthesised.

Regulation of synthesis and activity of the denitrifying enzymes under anoxic conditions

Under anoxic conditions, denitrification is regulated by both control of enzyme synthesis and control of enzyme activity. Control of the synthesis of the reductases is exerted at the level of expression of the specific gene for synthesis of the reductases. More fundamental details of this mechanism are given by Casey et al. (1993). Control of the activity of the denitrifying enzymes under anoxic conditions involves complex interactions between the nitrogen oxides and the nitrogen oxide reductases. The interactions are virtually general for all facultative organisms in that the nitrogen oxides activate their own reductases and inactivate other reductases. No reports appear to have been published for nitric oxide with nitrate reductase and nitrate with nitrous oxide reductase. In the only conflicting research finding, the interaction of nitrate with nitrite reductase has been reported not as repressive but as activating. However, this result may have been due to the production of nitrite from nitrate reduction, the nitrite in turn activating the nitrite reductase. The nett effect of the interactions between each of the reductases and the oxides is that none of the nitrogen oxide intermediates (NO_2^-, NO, N_2O), increase to high concentrations intracellulary. The references for the experimental work conducted on the interactions between each of the nitrogen oxides and the nitrogen oxide reductases under anoxic conditions are too numerous to list here, but are given by Casey et al. (1993).

Anoxic conditions changed to aerobic conditions

Effect of aerobic growth conditions on the activity and synthesis of the denitrifying enzymes of organisms isolated from activated sludge

In the work reviewed thus far, the effects on aerobic and denitrifying enzymes of switching from aerobic to anoxic conditions have been examined for organisms in pure culture. Before examining the effect on pure culture of switching from anoxic to aerobic conditions (i.e. the reverse of the switch from aerobic to anoxic conditions discussed above), the effect of switching from anoxic to aerobic conditions on aerobic and denitrifying enzymes in facultative organisms isolated from activated sludge is discussed first.

For 24 facultative organisms isolated from anoxic-aerobic activated sludge systems, the effect of oxygen varied from total repression of nitrate reductase synthesis in some species to near non-repression in others (Krul and Veeningen, 1977). In a similar investigation (Simpkin and Boyle, 1988), the levels of synthesis of nitrate and nitrite reductase were analysed when activated sludge was subjected to various aerobic and anoxic conditions; the synthesis of these reductases was repressed by not more than 50% upon exposure to completely aerobic conditions, but this reduced synthesis could not account for the very low rates of nitrate and nitrite reduction measured following a change from anoxic to aerobic conditions. It was concluded that the DO has a significantly greater affect on the activity of the reductases than on their synthesis.

Inhibition of activity and repression of synthesis by oxygen of the denitrifying enzymes in pure culture organisms

It is generally accepted that the inhibitory effect of oxygen on denitrification results from two mechanisms: inactivation of the electron transferring mechanism of the reductases, and repression of synthesis of the reductases. Inactivation has an immediate effect on the rate of denitrification whereas repression is more long term, resulting in decreasing levels of the reductases with time. From work conducted with *Hyphomicrobium* X (Meiberg et al., 1980) and *Thiobacillus denitrificans* (Justin and Kelly, 1978), it was concluded that at low concentrations of DO, the activity of the nitrogen oxide reductases is inhibited and at higher concentrations of DO, synthesis of the enzymes is repressed.

Mechanisms of repression of synthesis by oxygen of the denitrifying enzymes

Korner and Zumft (1989) established that for *Ps. stutzeri* subjected to conditions of gradually decreasing DO concentration, the reductases were synthesised in the order nitrate-, nitrite-, and nitric oxide reductase (no result was reported for the synthesis of nitrous oxide reductase). Assuming that nitrous oxide reductase would be synthesised subsequent to the synthesis of nitric oxide reductase, it would be logical to assume that for an organism grown under anoxic conditions (all reductases fully developed), and exposed to increasing DO concentrations, synthesis of the reductases would be inhibited in the order, nitrous oxide-, nitric oxide-, nitrite-, and nitrate reductase.

Mechanisms of inactivation of the denitrifying enzymes by oxygen

Tiedje (1985) concluded that for a given oxygen concentration at which the activity of a specific reductase is affected, the reductases for the increasingly more reduced nitrogen oxides are affected at increasingly lower concentrations of oxygen, e.g. the activity of nitrous oxide reductase is inhibited at a lower concentration of oxygen than the activity of nitrite or nitrate reductase. In agreement with this result, Hochstein et al. (1984) demonstrated that for the activities of the reductases of *Pa. halodenitrificans*, the order for decreasing sensitivity to oxygen is nitrous oxide-, nitrite-, and nitrate reductase. [*Note:Because of its instability in aerobic environments, nitric oxide rarely appears as an external intermediate during inhibition of nitrogen oxide activity by oxygen. In experiments such as those described above, nitric oxide activity was not measured and consequently the workers did not consider nitric oxide as an obligatory intermediate in the denitrification pathway and thus did not consider the effect of oxygen on nitric oxide reductase*]. Under conditions of increasing concentration of DO, but sufficiently low so that enzyme synthesis was not repressed, the products and intermediates of denitrification appeared in the order dinitrogen, nitrous oxide, and then nitrite. The hierarchy for the effect of oxygen on the activity of the reductases is the same as the hierarchy for the effect of oxygen on their synthesis.

From these investigations, inactivation of the denitrifying enzymes under aerobic conditions was hypothesised to occur by one or more of the following three mechanisms. [*Note: A fourth mechanism by which oxygen could inactivate the nitrogen oxide reductases is through direct attachment to, or reaction with the reductases, in a manner which prevents the transfer of electrons to the nitrogen oxide electron acceptors. However, in research into this effect no such mechanism has yet been found*].

- ***Changes in the redox potential of the ETP results in electron flow to the reductases***

  Extracelluar oxygen ensures the presence of intracellular oxygen, which affects the intracellular redox potential of the ETP of the organism by acting as an electron acceptor at cytochrome oxidase, increasing the redox potential at the oxidase, thereby increasing electron flow to it, and reducing electron flow to other parts of the ETP (Payne, 1973; Stouthamer, 1988).

- ***Non-insertion of subunits of nitrate reductase into the cytoplasmic membrane prevents transport of electrons from electron transferring complexes to nitrate reductase***

  Nitrate reductase is composed of three subunits, labelled α, ß and γ (Craske and Ferguson, 1986), each of which has a specific role in the transfer of electrons to nitrate (Ingledew and Poole, 1984; Stouthamer, 1988; Stewart, 1988; Enoch and Lester, 1974; MacGregor, 1975a, b, 1976). Stewart (1988) suggests that the incorporation of nitrate reductase subunits α and ß into the membrane requires electron flow, and because oxygen prevents electron flow to nitrate reductase, the subunits cannot be incorporated in the presence of oxygen. This proposal was based on the finding of Hackett and MacGregor (1981) with *E. coli* in which synthesis of the γ-subunit occurs only in the absence of oxygen and presence of nitrate, and incorporation of the nitrate reductase subunits α and ß into the membrane is blocked following a shift from anoxic to aerobic conditions.

- ***Nitrate movement across the cytoplasmic membrane regulated through changes in its permeability***

  The two mechanisms above describe the manner by which oxygen could regulate nitrate reduction through control of the transfer of electrons to and from nitrate reductase. Attention has been directed also at the role of the cytoplasmic membrane in restricting nitrate movement to nitrate reductase under aerobic conditions (Alefounder and Ferguson, 1980). Four experiments can be viewed as instrumental in the promotion of this mechanism as a primary means of restricting nitrate reduction under aerobic conditions and these are listed below:

  (i) Intact cells [i.e. nitrate reductase on the cytoplasmic (inner) side, see Fig. 4], of denitrifying *Pa. denitrificans* did not reduce nitrate under aerobic conditions. However, inside-out membrane vesicles, i.e. part of *Pa. denitrificans*' cytoplasmic membranes were manipulated to function "inside-out" (so that access of nitrate to nitrate reductase was not prevented by the cytoplasmic membrane) did reduce nitrate under aerobic conditions (John, 1977).

  (ii) The cytoplasmic membrane of cells of *Pa. denitrificans* was made permeable to nitrate by the addition of the chemical Triton X-100, to allow passage of nitrate to its reductase - the simultaneous reduction of oxygen and nitrate was observed under aerobic conditions. In the absence of Triton X-100, nitrate was not reduced under aerobic conditions (Alefounder and Ferguson, 1980; Kucera et al., 1983b).

  (iii) The addition of transmembrane nitrate carriers such as benzyl or heptyl viologen radicals allow nitrate reduction under aerobic conditions with *E. coli*. Without such carriers nitrate reduction did not occur under aerobic conditions (Noji and Taniguchi, 1987).

  (iv) At low and high concentrations of oxygen, nitrate reduction did not occur in intact cells of *Ps. aeruginosa*, but in cell extracts of the same organism in which the cytoplasmic membrane was destroyed, nitrate reduction was observed (Hernandez and Rowe, 1987, 1988).

These experiments provide strong evidence that under aerobic conditions the membrane becomes impermeable to nitrate (i.e. restricts the movement of nitrate across the membrane), thereby limiting nitrate reduction. In a fifth experiment, in which a non-limiting supply of electrons (in the form of the chemical dihydroquinone) was supplied to the nitrate reductase of *Pa. denitrificans* under aerobic conditions, no nitrate reduction was observed (Alefounder et al., 1983). This indicates that restriction of electron flow to the reductases due to the presence of oxygen is not a significant mechanism for decreasing reduction of nitrate under aerobic conditions.

An additional finding in (iv) above was that, in intact cells, although nitrate reduction was prevented at both low and high DO concentrations, nitrite reduction was not inhibited at low DO concentrations, and was only partially inhibited at high DO concentrations (Hernandez and Rowe, 1987). This is due to differences in position between the active sites of nitrate and nitrite reductase; the active site of nitrate reductase is situated on the cytoplasmic (inner) side of the membrane and the active site of nitrite reductase on the periplasmic (outer) side (see Fig. 3). Reduction of nitrite from external sources is not affected by the impermeability of the membrane because it is not necessary for extracelluar nitrite to cross the membrane to the active site of its reductase. If the only source of nitrite is intracellular (i.e. from cytoplasmically reduced nitrate) then regulation of nitrate movement across the membrane by oxygen would represent a primary mechanism by which the whole denitrification pathway could be controlled. However, if an extracelluar source of nitrite is available under aerobic conditions (e.g. from oxidation of ammonium by nitrifiers in a mixed culture such as activated sludge, or through direct nitrite dosing in controlled laboratory conditions), then control by oxygen of membrane permeability to movement of nitrate will not regulate the reduction of nitrite or the nitrogen oxides which result from the reduction of nitrite (i.e. nitric oxide and nitrous oxide). This implies that under aerobic conditions the gaseous intermediates, nitric oxide and nitrous oxide are not restricted in their movement across the cytoplasmic membrane. Therefore, if there are no 'external' sources of nitrate, the whole of the denitrification pathway is controlled by the extent of the permeability of the cytoplasmic membrane to nitrate.

Mechanisms of regulation of aerobic respiration

An unusual finding in experiment (ii) described above, was that when the membranes of cells of *Pa. denitrificans* were made permeable with Triton X-100 under aerobic conditions, nitrate was reduced to nitrite intracellularly and when a certain nitrite concentration was attained, electron flow to cytochrome oxidase was reduced and electron flow to the nitrogen oxide reductases was increased (Kucera et al., 1983b). This finding has major implications with regard to aerobic respiration in the presence of nitrate and is of crucial importance to the biochemical model for aerobic facultative organism respiration developed in Paper 10 (Casey et al., 1999a) and is reviewed in greater detail below. Because the inhibition of oxidase activity was a consequence of an increase in nitrite concentration, the review focuses firstly on the interaction of cytochrome oxidase with nitrite and nitric oxide.

Inhibition of aerobic respiration by nitrite and nitric oxide

Rowe et al. (1979) observed that nitrite inhibited oxygen uptake in *Ps. aeruginosa*, but that nitrite did not inhibit oxygen uptake in specially prepared organisms which lacked nitrite reductase. No explanation was proposed by the authors. With *Pa. denitrificans*, Kucera and Dadák (1983) examined electron flow to cytochrome oxidase and nitrite reductase under aerobic conditions. An uncoupling chemical was added to reduce the transmembrane potential of the cytoplasmic membrane, allowing movement of nitrite across the membrane. Electron flow to cytochrome oxidase decreased and electron flow to nitrite reductase increased, the workers concluding that nitrite inhibits electron flow to cytochrome oxidase by interacting with that oxidase. With *Ps. aeruginosa*, Yang (1985) concluded that inhibition of aerobic respiration by nitrite was by direct inhibition at cytochrome oxidase. No inhibition was measured in experiments in which nitrate was added; the reasons for this will become apparent from the work reviewed below.

However, studies subsequent to those above indicated that inhibition may not be due to the presence of nitrite *per se*. From experiments with *Pa. denitrificans*, Kucera et al. (1986) concluded that a decrease in oxidase activity following an increase in electron flow to nitrite reductase was caused by the production of an inhibitory but unidentified intermediate resulting from the reduction of nitrite, possibly one of the denitrification intermediates, nitric oxide or nitrous oxide. To determine if either of these intermediates could inhibit oxidase activity, nitric oxide and nitrous oxide were added separately to membrane vesicles of *Pa. denitrificans;* nitric oxide severely inhibited oxidase activity but nitrous oxide had no effect. As a further indication that the inhibitory agent is a product of nitrite reduction, a culture of aerobically grown *Pa. denitrificans* (i.e. lacking nitrite reductase and therefore also not able to produce nitric oxide or nitrous oxide) demonstrated uninhibited oxidase activity in the presence of nitrite (Kucera et al., 1986). In experiments to determine whether nitrite could be reduced aerobically and to determine the identity of the inhibitory product, Kucera et al. (1987) demonstrated that nitrite can be reduced to nitric oxide under aerobic conditions. This finding forms a cornerstone of the biochemical model outlined in Paper 10 (Casey et al., 1999a) and establishes some of the fundamental background to the model for bulking by AA filaments outlined in Paper 11 (Casey et al., 1999b).

Results similar to those described above for pure culture denitrifying organisms have been found also for organisms isolated from activated sludge from a domestic sewage treatment plant. Krul (1976) examined the effect of nitrate, nitrite and nitric oxide on the OUR of an anoxically grown (with nitrate) *Alcaligenes* sp. isolated from activated sludge which had experienced anoxic conditions. Nitrate inhibited the OUR under aerobic conditions only when the DO concentration was less than 0.5 mgO/ℓ, nitrite inhibited the OUR only when the DO concentration was less than 2.0 mgO/ℓ, and nitric oxide inhibited the OUR even when the DO concentration was as high as 4.0 mgO/ℓ. Indeed, with nitric oxide addition, the OUR was suppressed to such an extent that the organism utilised nitrate and nitrite at rates similar to the rates of utilisation of nitrate and nitrite under anoxic conditions.

From these experiments, two important conclusions can be drawn concerning reduction of nitrate and nitrite under aerobic conditions:

- Inhibition of oxidase activity results from the interaction of nitric oxide with cytochrome oxidase and not from the interaction of nitrate, nitrite or nitrous oxide with cytochrome oxidase; inhibition of oxidase activity resulting from the addition of nitrate or nitrite is due to nitric oxide formation from the reduction of nitrate and nitrite, and not nitrate and nitrite *per se*.
- When nitric oxide inhibits the flow of electrons to cytochrome oxidase, a large proportion of electrons are diverted to the denitrification ETP, and nitrate and nitrite can be reduced.

Mechanism of inhibition of oxidase activity by nitric oxide

Although the experimental work described above demonstrated that the most likely inhibitory denitrification intermediate is nitric oxide, the work did not describe the mechanism of biochemical inhibition. Four possible mechanisms are outlined below and the evidence for and against each is briefly presented:

(1) ***Nitric oxide accepts electrons directly from cytochrome oxidase, thereby reducing the transfer of electrons from cytochrome oxidase to oxygen:*** While this is an obvious mechanism, there is little information available that supports it - indeed there is evidence against it, viz: Enzymes have a reactive site configuration specific to the molecule to be reduced (Robertis and Robertis, 1980) and it is unlikely that nitric oxide would by chance have the correct configuration to effect electron transfer from cytochrome oxidase.

(2) ***The extremely reactive nature of nitric oxide with dissolved intracellular molecular oxygen prevents oxygen attaining its site of reduction at cytochrome oxidase:*** For this mechanism also, there appears to be more evidence against it than for it: Carr and Ferguson (1990a) demonstrated that the nitric oxide reductase of *Pa. denitrificans* maintained the steady-state concentration of dissolved nitric oxide sufficiently low that reaction with oxygen was insignificant, but sufficiently high that reaction with the active site of cytochrome oxidase would inhibit oxygen reduction.

(3) ***Under aerobic conditions, electrons are directed away from cytochrome oxidase to the nitrogen oxide reductases by an intracellular redox effect due to the presence of nitric oxide:*** In support of this mechanism, Kucera et al. (1983a) proposed from experimental observation that under anoxic conditions, electron distribution between reductases is effected by redox control exerted by the concentration of the denitrification intermediates, and proposed that control of nitrate reduction by oxygen is effected by the same mechanism, thereby making it feasible that inhibition of oxidase activity by nitric oxide also occurs by the same mechanism.

(4) ***Nitric oxide interacts with cytochrome oxidase to form a complex which cannot then transfer electrons to oxygen:*** Nitric oxide has a particular affinity for proteins containing haem, iron-sulphur, and copper, all of which form metal-nitrosyl (NO-) complexes (Henry et al., 1991). A discussion of the reactivity of nitric oxide with these proteins is given by Zumft (1993). As described in Part I of this paper, facultative organisms have two oxidases (cytochrome *o* and cytochrome aa_3) and it is not apparent which of the oxidases is the subject of inhibition by nitric oxide. Work by Yang (1982) with *Pa. denitrificans* and Casella et al. (1986, 1988) with a *Rhizobium "hedysari"* strain concluded that inhibited aerobic respiration is a result of the inhibition of cytochrome *o*, this being the only oxidase synthesised under anoxic growth conditions and therefore the only oxidase immediately present under subsequent aerobic test conditions. Such experiments however do not necessarily exclude cytochrome aa_3 as also being subject to inhibition. Babcock and Wikström (1992) model cytochrome aa_3 as having one Fe and two Cu electron transferring centres, and cytochrome *o* as having one Fe and one Cu electron transferring centre, the inference from this work being that nitric oxide may not necessarily interact similarly with each of the two oxidases.

Considering the four mechanisms of inhibition: for (1) and (2) there is sufficient evidence to discard them. Mechanisms (3) and (4) have considerable supporting evidence and are important in the development of a conceptual biochemical model for inhibition of aerobic respiration in facultative organisms described in Paper 10 (Casey et al., 1999a).

Aerobic denitrification

From the review of inhibition of aerobic respiration it can be concluded that denitrification can occur under aerobic conditions, termed aerobic denitrification. This is a consequence of the finding that nitrite and nitric oxide, but not nitrate can be reduced under aerobic conditions: The active site of nitrate reductase is situated on the cytoplasmic side of the membrane and because oxygen makes the membrane impermeable to nitrate movement, the nitrate cannot reach the nitrate reductase under aerobic conditions. The active sites of nitrite reductase and nitric oxide reductase are on the periplasmic side of the membrane and externally produced nitrite is not required to cross the membrane to be reduced. Accordingly, nitrite can be reduced under aerobic conditions and the end-product nitric oxide can also be reduced on the periplasmic side of the membrane. The capacity of organisms for aerobic denitrification has been an area of debate for some time. The literature records claims for its occurrence among a great diversity of facultative organisms (Skerman et al., 1951; Mechsner and Wuhrmann, 1963; Hernandez and Rowe, 1987; Robertson and Kuenen, 1983, 1984a; b, 1990). The majority of literature is concerned with aerobic denitrification of nitrate, not nitrite, and it appears that the reports of aerobic denitrification of nitrate can be attributed to organism tolerance of reduced oxygen levels, not re-direction of electrons to nitrite reductase due to inhibition of cytochrome oxidase by nitric oxide as described above. However, aerobic denitrification of nitrite and nitric oxide by re-direction of electrons forms an integral part of the conceptual biochemical model for facultative organism respiration and inhibition postulated in Paper 10 (Casey et al., 1999a).

Conclusions

In this paper a review of literature concerned with the respiration of facultative organisms was conducted in two parts: Part I reviewed the biochemical mechanisms involved in utilisation of substrate for aerobic and anoxic respiratory processes, and the effect of the presence of oxygen, the absence of oxygen, and the presence of nitrogen oxides on synthesis of the aerobic and anoxic electron transferring complexes of the electron transport pathway (ETP); Part II reviewed the mechanisms which initiate, regulate, and terminate aerobic and anoxic respiration.

To summarise briefly, the major conclusions of the review are:

From Part I:

- An ETP can be described which serves as a general pathway for facultative aerobic organisms, representing the major electron transferring complexes and sequences.
- For facultative organisms respiring under aerobic conditions, cytochrome aa_3 is synthesised to a high level, and cytochrome *o* and the denitrifying electron transport complexes (the reductases) are at a low level. For facultative organisms respiring under anoxic conditions, the reductases are synthesised to a high level and oxidases, cytochromes aa_3 and *o*, are synthesised to a lower and higher level respectively than their levels under aerobic conditions.
- From the general ETP for facultative organisms, mechanisms resulting in lower yields under anoxic than aerobic condition are identifiable; a consequence of electrons passing only two, and not the three available proton-pumping (energy-conserving) sites.

From Part II:

- For facultative organisms initially respiring under aerobic conditions and then transferred to anoxic conditions, the oxidases are inactivated, and synthesis of the reductases is initiated.
- The nitrogen oxides regulate activity and synthesis of the reductases under anoxic conditions; the nitrogen oxides activate and promote synthesis of their own reductases and inactivate and inhibit synthesis of other reductases.
- For facultative organisms initially respiring under anoxic conditions, then transferred to aerobic conditions, three mechanisms have an effect on, or result in inactivation of nitrate reductase:
  - The presence of oxygen affects the intracellular potential of the organism, increasing electron flow to the oxidases, and reducing electron flow to the reductases.
  - Oxygen prevents insertion of nitrate reductase subunits into the cytoplasmic membrane, preventing electron transport to nitrate.
  - Oxygen reduces the permeability of the cytoplasmic membrane to nitrate, thereby reducing the accessibility of nitrate to its reductase.
- For facultative organisms subjected to the conditions described above, nitrite reduction is only partially inhibited, i.e. as a consequence of the reduced availability of electrons, but not as a consequence of the reduced access of nitrite to its reductase which is located on the periplasmic side of the membrane.
- For organisms subjected to the conditions described above, intracellular nitric oxide which accumulates under anoxic conditions, inhibits oxidase activity (aerobic respiration) and promotes denitrification of nitrite under subsequent aerobic conditions (aerobic denitrification).

The important characteristics of the ETP reviewed above are assembled into a biochemical model for anoxic-aerobic behaviour of facultative organisms which is described in Paper 10 (Casey et al., 1999a). This model forms the basis for formulating a hypothesis for anoxic-aerobic (AA) (formerly referred to as low F/M) filament bulking in anoxic-aerobic (N removal) and anaerobic-anoxic-aerobic (N&P removal) activated sludge systems. This hypothesis with supporting evidence is presented in Paper 11 (Casey et al., 1999b).

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