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Review

The respiratory chains of pathogenic pseudomonads

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Contents

I. Introduction

Pseudomonads are a wide and heterogeneous group of organisms. They can be found in soil and water, and are colonizers or pathogens of plants, animals or humans. Consequently, pseudomonads are also medically important, although they are isolated with varying frequency from clinical specimens. However, only species like *Pseudomonas mallei* and *Pseudomonas pseudomallei* are regarded mainly as pathogens for humans and animals. Others, such as *Pseudomonas aeruginosa* and *Pseudomonas putida* have an intermediate position; they can survive for extended periods outside the body but are nevertheless important medical pathogens due to their resistance to common antibiotics. The species *P. aeruginosa* has also been found to present a low grade of phytopathogenicity and thus represents a very rare case of a microorganism pathogenic to both plants and animals. More serious infectious plant diseases are caused by several members of this genus, such as *Pseudomonas solanacearum* and *Pseudomonas syringae*. The

Abbreviations: DAD, diaminodurene; DCIP, 2,6-dichlorophenolindophenol; EPR, electron paramagnetic resonance; ESR, electron spin resonance; HiPIP, high potential iron protein; MCD, magnetic circular dichroism; PMS, phenazine methosulphate; PQQ, pyrroloquinoline quinone; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TTFA, thenoyltrifluoroacetone; UHDBT, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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phytopathogenic pseudomonads cause an array of diseases in plants ranging from necrotic lesions and spot of fruit, stems, and leaves, to hyperplasias, tissue macerations, cankers and vascular infections.

Pseudomonads are normally described as strict aerobes [1]. This definition is not entirely correct for species such as *P. aeruginosa* which is capable of L-arginine-dependent anaerobic growth [2]. This latter species can also use nitrate (NO_3^-) as an alternative electron acceptor to oxygen, allowing growth to occur under oxygen-limited conditions. In this respect, it must be noticed that several characteristics used by microbiologists in identifying *Pseudomonas* species reflect features of their respiratory metabolism. Thus, the species is 'oxidase negative' in that it fails to catalyze the oxidation of di- or tetramethyl-*p*-phenylenediamine plus α-naphthol to indophenol blue. Conversely, a positive reaction generally reflects the presence of a membrane-bound high-potential cytochrome *c* linked to an active cytochrome *c* oxidase and is frequently also indicative of the presence of 'energy coupling site III' (see Section III).

This review will deal principally with the diverse respiratory electron-transport chains found in selected pathogenic *Pseudomonas* species growing in laboratory culture. This is an area that has surprisingly been neglected by the majority of biochemists and bioenergeticists during the last decade. It is the purpose of this article to update earlier accounts and to deal with the most recent advances in understanding the structure, function and membrane organization of the components of *Pseudomonas* respiratory chains. It is noteworthy that the *Pseudomonas* family is one of the most complex groups of Gram$^-$ bacteria with phenotypic similarities to many other genera. In order to appreciate better the context of this work and to guide the reader into the microbiology of pseudomonads, two paragraphs have been dedicated to some taxonomic, pathogenic and metabolic features of this bacterial group. As such they can be neither comprehensive nor exhaustive; for a wealth of current work on these topics the reader is referred to The Bacteria. A Treatise on Structure and Function (1986) (Sokatch, J.R., ed.), Vol. X (see Refs. 13 and 43). In addition, the volume Genetics and Biochemistry of Pseudomonas (1975) (Clarke, P.H. and Richmond, M.H., eds.) (see Ref. 29) is a valuable source of information.

IA. The genus Pseudomonas – general taxonomic considerations

Pseudomonas (pseudo (ψευδο) = false; monas (μονας) = unit) strains are typically polarly flagellated, Gram$^-$ rods usually less than 1 μm in diameter and not more than 4–5 μm in length, although some strains (fluorescent plant pathogens and saprophytes) may be considerably longer. The insertion and number of flagella are commonly used in species description [3]; however, the colony features, such as size, shape, color, edge and surface ornamentations can give in some cases even more important clues for species identification. For instance, *P. aeruginosa* strains normally have flat, creamy colonies, while very mucoid colonies originate from cells isolated from respiratory infections associated with cystic fibrosis. Useful properties for species determination can be the production of both alginate and pigments. In the genus, alginate, a polysaccharide composed of D-mannuronic acid and L-guluronic acid units [4], is produced only by *P. aeruginosa, Pseudomonas fluorescens, P. putida* and *Pseudomonas mendocina* [5]. Conversely, the production of pyocyanin and fluorescent pigments such as dehydroxy- and phenazine-1-carboxylic acids, oxy- and chlororaphine, aeruginosin A and B, and pyorubin, are characteristics of *P. aeruginosa* species [6–8]. These pigments are in general strong iron chelators and allow growth in media having a low iron content. Recently, fluorescent pigment production in the phytopathogen *P. syringae*, has been subjected to genetic analysis [9].

Of particular importance in the development of a system of classification of *Pseudomonas* species and species groups has been the study of nutritional properties of the strains. The nutritional spectrum of each species is characteristic and, in general, the variability among the strains of a given species is minor [10]. In contrast to many other bacterial groups, many pseudomonads do not excel in the utilization of sugars, which in many other microorganisms is the basis of useful determinative schemes. Organic compounds readily used by *Pseudomonas* species include hydrocarbons, carbohydrates, aliphatic acids, amines, amino acids, alcohols and aromatic compounds. This latter chemical family is of particular interest, since a taxonimic routine test is currently performed for determinative purposes involving the mode of cleavage of dehydroxylated intermediates (catechol, protocatechuic acid) which can be catalyzed by 1,2- and 2,3-dioxygenases, according to the group [11].

Genetic research in *pseudomonas* species has been truly massive and for a few species, *P. putida* and *P. aeruginosa*, a fairly detailed chromosome map is now available (Ref. 12, see also Ref. 13). Investigations performed with both DNA–DNA and ribosomal RNA–DNA systems have yielded very interesting information which can be used as a basis for the construction of a natural taxonomic system [14]. In particular, the results of experiments carried out with rRNA in *Pseudomonas* permit the grouping of the species of the genus into at least five sharply defined clusters, generally referred as 'RNA homology groups'. This system of classification has also received confirmation from the studies of Woese et al. [15] on the sequence analysis of

16S ribosomal RNA components. The different RNA homology groups can be clearly differentiated by the results of such analysis and, in addition, they have been found to be related to the different groups of purple sulphur and purple non-sulphur photosynthetic bacteria, thus confirming the hypothesis of the multigenic nature of *Pseudomonas* as presently defined [15–18].

Phytopathogenic fluorescent pseudomonads present particular taxonomic problems due to the fact that many strains isolated from various diseased plants have been named on the assumption that each organism was highly specific for its host and in the type of lesion that it produced. In 1966 a set of methods proposed by Lelliot, Billing and Hayward [19] under the name of LOPAT (levane formation, oxidase, potato rotting capacity, arginine hydrolase and tobacco hypersensitivity) provided a firm basis for clustering most phytopathogenic fluorescent species under two names: *P. syringae* and *Pseudomonas cichorii*, while *Pseudomonas marginalis* was found to resemble the saprophitic form of the *Pseudomonas fluorescens-P. putida* group. The results obtained by Sands et al. [20] allowed the grouping of most fluorescent plant pathogens in one cluster characterized by a negative arginine dehydrolase reaction, a positive hypersensitive reaction on tobacco leaves, and a slow growth rate as compared to that of saprophitic pseudomonads such as *P. putida* (see Table I). All organisms of this group are oxidase negative with the exception of *P. cichorii*; they are obligate aerobes and unable to grow at 37 °C, and their nutritional versatility is lower than that of the saprophytes.

At present, it is likely that two groups, the fluorescent species and *Pseudomallei-cepacia* group include practically all the pathogenic species of the genus. This fact is obviously important, since plant and animal pathogenicity may indicate that the subdivision based on nucleic acid hybridization in vitro probably approaches a natural classification of the genus.

TABLE I

Pseudomonas species classified into ribosomal RNA groups I and II (see Ref. 1 for RNA groups III, IV and V)

The data are from Ref. 1.

rRNA homology group and species	Synonymous	Comments
I		
P. aeruginosa	*P. pyocyanea*	Fluorescent pigments
	P. polycolor	produced by most species;
P. fluorescens	*P. marginalis*	saprophytic or opportunistic animal pathogens
	P. lemonnieri	
	P. geniculata	
P. chlororaphis		
P. aureofaciens		
P. putida	*P. ovalis*	
	P. convexa	
P. syringae [a]	at least 40 nome-species used as synonymous	Phytopathogenic
P. viridiflava [a]		
P. cichorii		
P. stutzeri	*P. stanieri*	Fluorescent pigments
P. mendocina		not produced
P. alcaligenes [b]		
P. pseudoalcaligenes [b]		
II		
P. mallei		Pathogenic
P. pseudomallei		
P. caryophilli		
P. cepacia [c]		
P. gladioli [c]		
P. solanacearum		
P. pickettii		

[a] Oxidase negative.
[b] Do not use glucose for growth.
[c] Do not denitrify.

IB. Pathogenicity for humans, animals and plants – metabolic aspects

Pseudomonas aeruginosa produces a variety of toxins and enzymes which contribute to the pathogenic properties of the bacterium [21,22]. These include two secreted proteins which are ADP-ribosyl transferase (toxin A and exoenzyme S) [23,24], several extracellular proteases (alkaline protease and elastase) [25], a cell-associated protein cytotoxin (leukocidin) [26], and two hemolysins, phospholipase C and rhamnolipid hemolysin [27,28]. *P. aeruginosa* is at present the most significant opportunistic human pathogen due to the widespread use of the antibiotics and other antibacterial agents to which this bacterium is notoriously resistant (see Ref. 29). The most likely explanation for this is either that the intact organism is resistant to antibiotics by destroying them or that its outer membrane layer prevents access of antibiotics to their site of action. Studies of antibiotics super-sensitive mutants of *P. aeruginosa* OAC1 suggest that their response to the same antibiotic may be correlated with specific changes in lipopolysaccharide structure (see Ref. 29).

Among the *Pseudomonas* species, the most serious animal pathogens are *P. mallei* and *P. pseudomallei* (see Table I). Strains of these two species are the agents of glanders and melioidosis, respectively, two related diseases that can be transmitted from animals to humans [30]. *P. mallei* produces an endotoxin called mallein, whereas *P. pseudomallei* produces a lethal factor with anticoagulant activity plus a skin-necrotizing proteolytic agent [31].

The various symptoms produced in plants by patho-

genic pseudomonads are likely to be caused by substances excreted by the pathogen. Among these substances there are enzymes capable of attacking various components of plant tissues, toxins and plant hormones. Some hydrolytic enzymes include β-glucosidases [32,33], xylosidases or xylanases [33,34], pectinases [35], of which the most common sort is polygalacturonic transeliminase [36] and cellulases [37]. Several fluorescent species described as pathovars of *P. syringae* produce toxigenic peptides, syringomycin and syringotoxin [38], that have been found to cause dramatic changes in oxidative metabolism of mitochondria isolated from etiolated shoots of susceptile maize lines [39]. Both toxins, in concentrations of the order of μmol per mg of mitochondrial protein, caused uncoupling of oxidative phosphorylation, stimulation of succinate, malate/glutamate and NADH-respiration, stimulation of ATP-ase activity, inhibition of ATP formation, increased swelling, and a decrease in mitochondrial membrane potential [39].

P. syringae and some members of the genera *Erwinia* and *Xantomonas* cause frost injury to plants. This phenomenon is associated to the ability of these phytopathogenic bacteria to nucleate ice formation in supercooled water [40,41]. It is thought that an ice nucleation site must bind water molecules in orderly array resembling an ice crystal. Recently, the sequence of an ice nucleation gene from *P. syringae* has been determined [42]. It predicts a translation product whose structure is consistent with the role of template, containing 122 imperfect repeats of the consensus octapeptide Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr. All the repeats are contiguous and higher order of periodicities are superimposed on the octapeptide pattern throughout.

The majority of pseudomonas species present an extraordinary catabolic potential. *P. cepacia*, for instance, is able to utilize as sole carbon and energy source a greater number of compounds than any other bacterium identified to date (see Ref. 43). Included among the many substrates reported to support growth of *P. cepacia* are the antibiotic penicillin, the herbicide 2,4,5-T and the unusual amino acid octopine [*N*-(D-1-carboxyethyl)-L-arginine]. Many of the compounds degraded by the saprophytic pseudomonas species are relatively inert, such as the normal and branched chain aliphatic hydrocarbons, aromatic structures such as toluene an *p*-cymene, and the alicyclic hydrocarbons, the premier example being camphor (see Ref. 44). Most metabolic pathways of hydrocarbon metabolism utilize the direct incorporation of molecular oxygen into the substrate via oxygenase chemistry and a major research effort has been expended in the study of the monooxygenases that are involved in the degradation of camphor by *P. putida*, strain C1B. General information about these degradation pathways is given in the book edited by Sokach (1986) (see Refs. 13 and 43).

II. Respiratory chain composition

IIA. Bacterial respiratory electron-transport chains

Bacteria generally have more than one terminal oxidase (also termed oxygen reductase): there are several dehydrogenases which can deliver electrons to the system, and two or three reductive enzymes which can reduce either molecular oxygen (oxidases) or electron acceptors such as nitrate (NO_3^-), nitrous oxide (N_2O) and nitrite (NO_2^-) (reductases) (see Refs. 45–51).

The bacterial oxidases are a group of structurally diverse haemoproteins, and haem *a* and Cu^{2+} are not always required as prosthetic groups as they are in their mitochondrial counterparts. In fact, several bacteria contain enzymes with protohaem IX (haem *b*) as a prosthetic group operating either as cytochrome *c* – or ubiquinol – oxygen oxido-reductases: these are termed *o*-type oxidases. Examples of this kind are the cytochrome *c* oxidases of *P. aeruginosa* [52,53], *Azotobacter vinelandii* [54–56] and *Vitreoscilla* [57,58]. Note that in all these species the enzyme is isolated tightly bound to *c*-type cytochrome subunit (*co*-type oxidase). Conversely, in *Escherichia coli* the *o*-type haem is complexed with another haem of *b*-type [59–61].

Cytochrome *o* is generally identified on the basis of a CO-binding *b*-type haem in CO-difference spectra (see Ref. 62). In this respect, the purple non-sulphur bacterium *Rhodobacter capsulatus* appears to contain a peculiar *co*-type oxidase: the enzyme (molecular mass as a monomer of 54 kDa, 0.4 mol haem/mol) presents a high and pH-dependent mid-point potential, it is sensitive to N_3^- and CN^-, but does not bind to CO [63–65]. This 'anomalous cytochrome *o*' will be hereafter operationally defined as CO-insensitive *b*-type oxidase.

Cytochrome *o* may serve as a sole oxidase or, more commonly, as an oxidase in one limb of a branched system where the other branches may be terminated by *bd*, aa_3, caa_3 or perhaps CO-insensitive *b*-type oxidase (see Refs 45–51). Depending on the growth conditions, one of the oxidases may predominate. For example, in *P. aeruginosa*, the membrane-bound cytochrome *o* terminal oxidase is predominant when the cells are grown with high aeration, and a cytochrome cd_1 soluble complex is predominant when the oxygen tension is low and in the presence of nitrate [66]. Another feature to note is that some bacterial reductases utilize cytochrome *c* as substrate similar to the mitochondrial cytochrome *c*/oxygen oxido-reductase; others, deficient in cytochrome *c*, utilize ubiquinol as substrate and, as such, function as ubiquinol/oxygen oxido-reductases. This is the case of the phytopathogen *P. syringae* [67], whereas the closely related species *P. cichorii* contains oxidases of both types [67]. However, under microaerophilic conditions, the cytochrome *c*-containing pathway of *P. cichorii* is drastically repressed and respiration is by

means of the remaining ubiquinol/oxygen oxido-reductase [68] (see Section IV).

IIB. Composition of the aerobic respiratory chain in P. aeruginosa

The aerobic respiratory chain of the pathogen *P. aeruginosa* consists of primary dehydrogenases including D-glucose, D-gluconate, NADH, malate, and succinate dehydrogenases, coenzyme Q-9, and cytochromes of type *b*, *c* and *o* [69–71] (see Fig. 1). In membrane particles, the cytochrome *c* oxidase, measured as ascorbate-TMPD oxidation, is blocked by 10^{-4} M KCN ($K_i = 0.4$ μM) while both NADH and D-glucose oxidase-dependent activities are inhibited only 23 and 46%, respectively, at the same CN^- concentration [72]. This residual activity is highly resistant to KCN ($K_i = 30$ mM) and it is also insensitive to antimycin A (not more than 10 μM), a specific inhibitor of bacterial and mitochondrial ubiquinol/cytochrome *c* oxido-reductase complexes. These data are clearly indicative of the presence of a branched respiratory chain. The isolation of the respiratory mutant T105, deficient in ascorbate-TMPD dependent activity, supports the view that *P. aeruginosa* possesses two terminal oxidases. Unfortunately, since no appreciable differences were detected in cytochrome patterns between the parent and T105 strain, the molecular nature of the mutation affecting T105 respiration remains obscure [72]. Recently, the effect of nitrite, coupled to low temperature (4°C), on cytochrome *c* oxidase of *P. aeruginosa*, has been analysed [73]. It is in fact well known that this bacterial species is responsible for massive food contamination and sodium nitrite is widely employed by the food industry in food preservation although nitroso compounds, originated by NO_2^- in combination with amines, are carcinogenic in animals [74]. Sodium nitrite was found to inhibit the isolated cytochrome *c* oxidase uncompetitively, this inhibition pattern being therefore

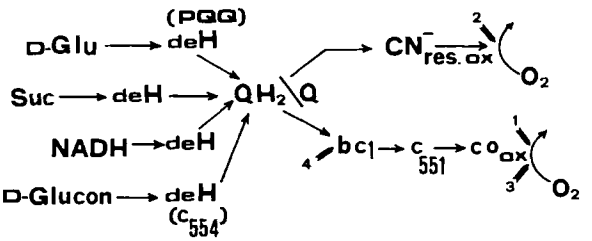

Fig. 1. Schematic representation of the respiratory chain in aerobically grown *P. aeruginosa*. Abbreviations: D-Glu, D-glucose; D-Glucon, D-gluconate; deH, dehydrogenase; Suc, succinate; PQQ, pyrroloquinoline quinone; *c*-554, cytochrome *c*-554; QH_2/Q, ubiquinol/ubiquinone pool; bc_1, bc_1 oxido-reductase complex; *c*-551, soluble cytochrome *c*-551; co_{ox}, cytochrome *c* oxidase of *co*-type; CN^- res. ox, cyanide resistant oxidase. The solid arrows indicate the sites of inhibition by low- and high-concentrations of KCN (1 and 2, respectively), nitrite (3) and antimycin (4).

different from typical competitive inhibition kinetics of NaN_3 and KCN.

The high-resistance of *P. aeruginosa* to cyanide might be considered, at first sight, both puzzling and unexpected. However, it must be underlined that radiolabelling studies with *P. aeruginosa* have shown that cyanide is formed in cultures supplemented with glycine, serine or threonine [75,76]. Indeed, cyanide production by *P. aeruginosa* in patients suffering massive septaecemia from infections of severe burn wounds can be intimately related to the high mortality caused by this bacterium [77]. Cyanide is produced in batch cultures of *P. aeruginosa* at the end of the exponential growth phase and is coincident with the time of minimal oxygen content of the medium [78]. Interestingly, oxygen can be replaced by artificial electron acceptors such as phenazine methosulphate (PMS), methylene blue or 2,6-dichlorophenol indophenol (DCIP) when cyanogenesis is catalyzed by isolated membrane fragments [79–82]. Thus, the cyanide synthase system is likely to be membrane-bound and the natural electron acceptor for the process is not O_2 itself.

The number, type and thermodynamic characteristics of the cytochromes involved in the membrane-bound electron-transport system of aerobically grown *P. aeruginosa* are largely unknown. Spectral studies suggest that membrane particles contain the *b*- and *c*-type elements of a putative b/c_1 complex [72]; however, the electron-transport step between the ubiquinol/cytochrome *c* oxido-reductase and cytochrome *c* oxidase has never been defined. The *c*-type haem/protohaem ratio in *P. aeruginosa* membrane particles is close to 1.3 [72]. Gel'man et al. [83] have reported the existence of cytochromes *c* with α bands at 551 and 554 nm in *P. aeruginosa* grown aerobically while anaerobic cultures are endowed with cytochromes *c* with α bands at 550, 551, 555 and 557 nm (in Ref. 62). In this latter type of growth, cytochrome *c*-550 and cytochrome *c*-555 were estimated as less than 2% of cytochrome *c*-551 level (one of the two electron donors to nitrite reductase, see subsection IID). Conversely, cytochrome *c*-557 seems to be part of a peroxidase enzyme which can only be reduced by dithionite [84–87]. Since cytochrome *c*-554 of aerobically grown cells has been purified by Matsushita et al. [88] as a component of the D-gluconate dehydrogenase, it can be presumed that cytochrome *c*-551 of aerobic particles might be analogous to cytochrome *c*-550 found in very low amounts in anaerobically grown cells. If this is true, cytochrome *c*-551 would be the electron donor to the CN^--sensitive membrane-bound cytochrome oxidase (shown in Fig. 1). However, thermodynamic and kinetic evidence is required to test this latter assumption.

The cytochrome *c* oxidase of aerobically grown *P. aeruginosa* consists of *b*- and *c*-type haems. The *b*-type cytochrome was judged to be a cytochrome *o* from its

CO-binding properties. Similarly to *Azotobacter vinelandii*, which also contains a cytochrome *co* oxidase [55,56], the isolated enzyme shows a high TMPD oxidizing activity [52]. However, unlike *Azotobacter* oxidase, the *c*-type cytochrome contained in the *P. aeruginosa* oxidase is able to react with CO. The purified oxidase consists of four polypeptides (molecular masses of 29, 21, 12 and 10 kDa) with a suggested ratio of two iron atoms per copy of each subunit. If these two iron atoms correspond to haem *b* and haem *c*, the oxidase could operate as a four-electron carrier. Interestingly, the polypeptide composition of the *P. aeruginosa* cytochrome *c* oxidase is remarkably similar to that found in rather diverse bacteria such as *E. coli* and *Vitreoscilla* [89]. The *bo*-type oxidases of *E. coli* and *Vitreoscilla* consist of four subunits with molecular masses, calculated by SDS-PAGE analysis, of 60, 36, 18 and 13 kDa and 55, 35, 19 and 10 kDa, respectively [88]. Both enzymes possess 2 mol of protohaem IX (cytochrome *b*-555 and *b*-562) and 2 mol of copper per mol of enzyme, and bind to CO. It has been speculated that the two haems-two Cu^{2+} structure is reminiscent of the mitochondrial aa_3 type oxidase, this suggestion being supported by both ESR and resonance Raman spectroscopy [90–92]. According to this, the cytochrome *b*-562 of the complex would be analogous to cytochrome *a* and it would be unable to bind to CO or O_2, whereas the cytochrome *b*-555 component would be analogous to the O_2 binding cytochrome a_3 component of the mitochondrial oxidase.

There are at least two reports on the presence of an a_1-type oxidase in aerobically grown *P. aeruginosa* [93,94]. Unfortunately, more recent experiments [69,71] do not confirm previous evidence and this discrepancy might be due to difficulty in the actual definition of this cytochrome species. Indeed, unequivocal evidence for a functional role of this cytochrome is restricted to those organisms that have been examined by photochemical action spectra (see Ref. 45). For other bacteria, such as *P. aeruginosa*, the presence of cytochrome a_1 might strictly depend on the growth mode, since it is generally accepted that cytochrome a_1, in addition to *o* and *d*, is commonly contained in bacteria adaptable to unstable environmental conditions [95]. Although it is by now clear that the prosthetic group of what is generally referred to as cytochrome a_1 is not *a*, but protohaem IX [96,97], for historical reasons we have maintained the original nomenclature (for a review see Ref. 45).

IIC. Composition of the aerobic respiratory chains in P. cichorii and P. aptata (syringae group)

Among the phytopathogenic pseudomonads, *P. cichorii* and *P. aptata* (syringae group, see Table I) are the best characterized species in terms of respiratory chain composition [67,68,98–101] (see Fig. 2). A study

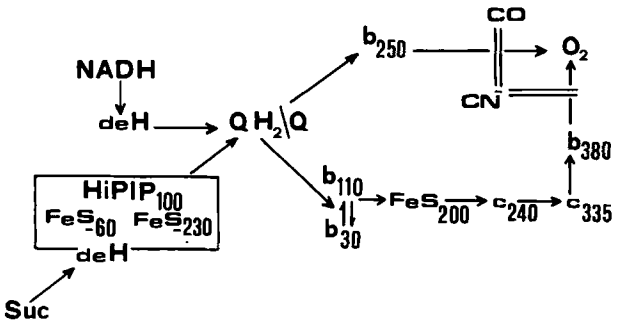

Fig. 2. The respiratory chain of *P. cichorii*. Iron-sulphur centers and haems of *b*- and *c*-type have been indicated with their actual mid-point potentials at pH 7.0. Suc, succinate; deH, dehydrogenase; HiPIP, high-potential iron-sulphur protein; CN^-, cyanide; CO, carbon monoxide. See text for more details. The data are from Refs. 67 and 101.

of their membrane-bound cytochromes has revealed that a linear respiratory chain is functional in *P. aptata*, cytochromes *c* being absent and the terminal oxidase being a ubiquinol/oxygen oxido-reductase of *b*-type (*o*-oxidase) [67]. Conversely, *P. cichorii* retains a similar cytochrome *o*-containing pathway, but has in addition a second pathway, involving cytochromes *c*, to a distinct cytochrome *b*-oxidase [67]. The *o*-type and the *b*-type oxidases have tentatively been associated to haems with mid-point potentials at pH 7.0 of +250 mV and +380 mV, respectively [67]. These two membrane-bound oxidases also differ in their sensitivities to CN^- and CO. The cytochrome *b*-oxidase is in fact blocked by low CN^- concentrations ($K_i = 2$ μM) and insensitive to CO (CO-insensitive *b*-type oxidase), in contrast to the *o*-type oxidase which is fully inhibited by 1.5 mM CO. The *o* type oxidase shows a CN^--inhibition constant of 0.1 mM. The results of kinetic studies have demonstrated that in *P. cichorii*, the pathway less sensitive to cyanide, hereafter named 'alternative oxidase', is inhibited by KCN in an uncompetitive manner. Conversely, CN^- blocks the oxidation of ascorbate-TMPD in a purely non-competitive manner with K_i for CN^- and K_m for TMPD of 6 μM and 100 μM, respectively [67]. As reported by Dawson and Jones [102], non-competitive inhibition is generally associated with aa_3 type oxidases. However, the lack of *a*-type cytochromes in both *P. aptata* and *P. cichorii* makes such a general statement rather uncertain. A more detailed analysis of the different kinetics involving the great variety of bacterial oxidases is therefore needed.

It has been reported that organisms such as many fungi that are phytopathogenic to cyanogenic plants form cyanide hydratase to detoxify cyanide by conversion into formamide [103]. It might therefore be expected that bacteria pathogenic to cyanogenic plants would also form cyanide hydratase or other cyanide-degrading enzyme systems. Rust et al. [104] have tested a range of bacteria for their sensitivity to CN^- by inclu-

sion of cyanide in a glucose plus nutrient broth medium. The bacteria tested included several strains of *P. syringae* (pathogenic to sorghum) as well as a range of bacteria pathogenic to non-cyanogenic plants. In all cases growth was 50% inhibited by 0.06–0.17 mM cyanide. These concentrations are considerably lower than the local concentration of cyanide that would form in the leaves of sorghum if even a small proportion of their cyanogenic glycosides were degraded on infection. Conversely, these results tend to support the above-reported experiments in vitro indicating the presence of an 'alternative oxidase' with a K_i for cyanide of 0.1 mM. Clearly, the extrapolation of results obtained with cultured bacteria to the situation in vivo is rather complex. It is possible that in vivo the bacteria might degrade cyanide released from the plant cyanogenic glycosides; in this case the bacterial degrading system would be activated by the infection process.

Rotenone and thenoyltrifluoroacetone (TTFA), potent inhibitors of NADH and succinate dehydrogenases, respectively [105–107], are ineffective on respiration by membranes from both *P. cichorii* and *P. aptata*. From a physiological point of view, the rotenone-insensitivity is not surprising. Rotenone is an isoflavonoid which derives from formononetin, a well-known constituent of many leguminous plants (see Ref. 108). It might, therefore, be expected that bacteria pathogenic to plants would be insensitive to a secondary plant product. In this respect, a close correspondence is generally observed among cluster N-2 of NADH dehydrogenase, rotenone and piericidin sensitivity, and site I of energy coupling [109,110]. A similar correlation is also verified in membranes from *P. cichorii* and *P. aptata* [67,101].

The TTFA-resistance, unusual in mitochondrial system, has frequently been observed in bacteria [111]. This inhibitor is a metal-chelating agent which appears to perturb the interaction between the high-potential iron-sulphur centre of succinate dehydrogenase (centre S-3 or HiPIP) and two or more ubisemiquinone molecules [112–114]. Thenoyltrifluoroacetone abolishes the EPR signal that arises from the interaction of ubisemiquinones. The molecular nature of TTFA-insensitivity of phytopathogenic pseudomonads is obscure.

Membranes isolated from *P. cichorii* and *P. aptata* contain a large EPR signal, observed at 8 K in the oxidized sample, with a peak at $g = 2.015$. This centre is similar to centres described in succinate dehydrogenases, fumarate reductases and some other enzymes such as aconitase, and is due to a four or three iron sulphide cluster which is paramagnetic in its physiological oxidized form (HiPIP) [115–119]. Quantification of this HiPIP centre indicated that it was present at a concentration similar to that of $g = 2.02/1.93$ centres (0.13 nmol/mg protein), which are also typical of succinic dehydrogenase. Oxidation-reduction titration of the $g = 2.015$ signal gave a mid-point potential (pH 7.0) of +100 mV, whereas the $g = 2.02/1.93$ ferredoxin titrated biphasically, suggesting two components with $E_{m\,7.0}$ at −60 mV and −230 mV [101].

In *P. cichorii* membranes an additional centre characterized by a $g_y = 1.90$ was seen at 22 K [101]. This resonance was similar to those of the Rieske iron-sulphur centres observed in other respiratory and photosynthetic electron-transport chains in terms of its high mid-point potential ($E_{m\,7.0} = +200$ mV), g-values and temperature dependence [120]. The Rieske-type ferredoxin centre is lacking in *P. aptata* [101]. The correlation between the presence of cytochromes *c* in *P. cichorii* and the Rieske centre, compared to their absence in the closely related *P. aptata*, is of particular interest and suggests that in these phytopathogenic pseudomonads cytochromes of *c*-type and the Rieske centre are likely to be genetically coexpressed. Such a correlation has also been suggested in other bacterial species such as *Rb. capsulatus, E. coli* and *P. denitrificans* in which the presence of the Rieske centre is taken as diagnostic of a membrane-bound ubiquinol/cytochrome *c* oxido-reductase complex [121,122].

The results of spectral and kinetic studies on the membrane-bound respiratory components of *P. cichorii* and *P. aptata* have clearly demonstrated that indeed the latter species contains a defective b/c_1 complex [100]. Indeed, although *P. aptata* seem to contain two *b*-type species with analogous thermodynamic properties of the haems which are an integral part of the bc_1 complex, the two haems do not participate in respiratory electron transport [100]. This conclusion is strongly supported by evidence that respiration by membranes from *P. aptata* is completely insensitive to the antibiotics antimycin A and myxothiazol. Myxothiazol, which contains β-methoxyacrylate as structural segment, binds to a cytochrome *b* region close to cytochrome *b*-566, one of the two haems of the ubiquinol/cytochrome *c* oxido-reductase complex [123–125]. According to the protonmotive 'Q-cycle' scheme [126], the binding of myxothiazol to cytochrome *b*-566 induces a block of the electron flow to both cytochrome *b*-566 and Rieske iron-sulphur centre at a redox centre defined as '*o*'. Conversely, antimycin A blocks reduction of ubiquinone by cytochrome *b*-562 (reoxidation of *b*-562) at a redox centre defined as 'i', through binding to cytochrome *b*-562 [127,128]. The binding of both antibiotics can also be demonstrated by a perturbation of cytochrome *b* absorbance signals and the latter evidence is not verified in membranes from *P. aptata* [100,129–131]. In this respect, a key observation which had been reported before the postulation of the 'Q-cycle' scheme was that of 'oxidant-induced reduction' of cytochromes *b*. An elegant explanation for this phenomenon was originally offered by Wikström and Berden [132] in terms of two $n = 1$ redox couples of ubiquinone, one of which

(QH^-/Q) operated at high and the other (QH_2/QH^-) at low potentials. Due to the extreme instability of the semiquinone species in the membrane, it was later realized that this ordering should be reversed [126]. At present, there is general consensus about a mechanism by which the reaction sequence at the '*o*' site would be:

$$QH_2 + FeS \rightarrow Q^- + FeS^- + 2H^+ \quad (1)$$

$$Q^- + b \rightarrow Q + b^- \quad (2)$$

As shown in Figs. 3 and 4, this sequence is in accord with the mid-point potential value calculated for the couples QH_2/Q^- and Q^-/Q if the '*o*' site is fully accessible to water [133]. Although several objections to the mechanism have been raised [134–136], in the light of the counter-arguments reported in the literature [136,137], the mechanism represented in Eqs. 1 and 2 reasonably explains the reaction sequence in a linear-type redox chain. Conversely, the operativity of an orthodox 'Q-cycle' scheme must necessarily be ruled out, when the respiratory chain is branched at the level of the ubiquinone pool. In this connection, a fundamental observation is that the quinone analogue 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) can discriminate electron flow between the two respiratory branches. UHDBT is a hydroquinone compound introduced by Roberts et al. [138] as an inhibitor of mitochondrial respiration, and was characterized as a reactant for the Rieske Fe-S centre in mitochondria

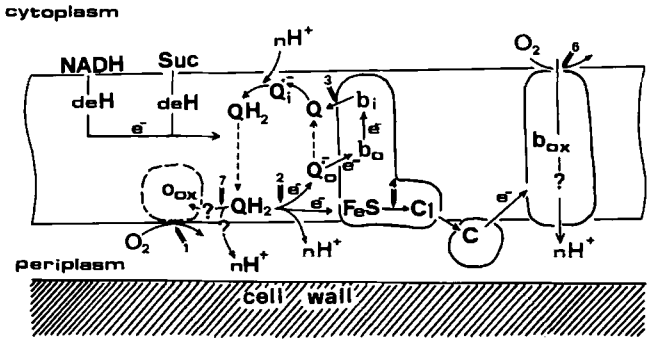

Fig. 3. A model for respiratory electron transport in *P. cichorii*. The numbered solid arrows indicate the postulated sites of inhibition by KCN (1 and 6), CO (1), myxothiazol (2), antimycin A (3), UHDBT (4 and 7) and mefloquine (4 and 7) (see text for more details). The scheme depicts electron transport from NADH and succinate (Suc) dehydrogenases (deH) to a branched chain leading to two oxidases of *b*-type: a cytochrome *c* oxidase (b_{ox}) inhibited by low-KCN concentrations and insensitive to CO plus an *o*-type oxidase (o_{ox}) inhibited by high-KCN concentrations and by CO. Low- and high-potential haems of the bc_1 complex are represented as b_o and b_i, respectively, since they interact with the semiquinone species at the level of centre 'o' (out) and centre 'i' (in). The bc_1 and the b_{ox} are shown with sectors spanning the plasmamembrane. A 'Q-cycle' scheme is shown tentatively. The *o*-type oxidase is shown facing the periplasmic space owing to uncertainty about its role as a proton pump. For simplicity the protons required for both oxidation-reduction of ubiquinone and reduction of oxygen are not shown.

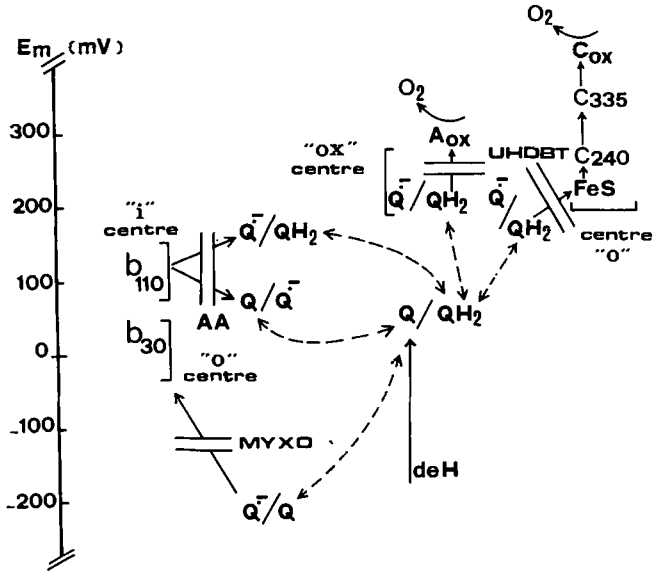

Fig. 4. A thermodynamic representation of a hypothetical 'Q-cycle' model for electron transport by *P. cichorii* showing the postulated redox couples involved and the sites of inhibition by antimycin A, myxothiazol and UHDBT. The scheme depicts electron transport in the forward direction, from a dehydrogenase to either branch, under pre-steady conditions. Cytochromes of type *b* and *c* are indicated with their $E_{m,7.0}$. For the sake of simplicity the pathway of protons is omitted. The interrupted arrows symbolize the mobility of the different redox couples of ubiquinone. Abbreviations: AA, antimycin A; MYXO, myxothiazol; Cox, cytochrome *c* oxidase; Aox, alternative oxidase (ubiquinol/oxygen oxido reductase); UHDBT, 5-*N*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; deH, dehydrogenase. The data are from Refs. 133, 137, 67, 100 and 101. See text for further details.

[139,140], chloroplasts [141,142] and bacteria [143,144]. It is preferentially bound to the reduced form of the centre, shifting the redox potential to higher values and changing the EPR spectrum [143,46]. The results of experiments on membrane fragments from both *P. cichorii* and *P. aptata* have clearly shown that UHDBT acts in two different regions of the chain, namely: (i) at the 'cyanide-resistant' pathway level (high-affinity site, $K_i = 1\ \mu M$); and (ii) in the bc_1 region (low-affinity site, $K_i = 0.1$ mM) [100]. This conclusion was strongly supported by evidence that cytochrome *o* reduction in *P. aptata*, the strain deficient in Rieske- and *c*-type components, was blocked by 15 μM UHDBT. Thus in bacterial membranes, the obligate interaction of semiquinone molecules at the redox centre 'o' does not seem to be so stringent as predicted by the 'Q-cycle' scheme. As schematically drawn in Figs. 3 and 4, in *P. cichorii*, centre 'o' would be that at which quinol oxidation occurs through reduction of both the Rieske iron-sulphur protein (UHDBT high-affinity site) and cytochrome b_{30} (also referred to as b_L). Conversely, the second quinol reaction-site, operationally defined here as centre 'ox', would be that at which quinol oxidation occurs through the alternative oxidase containing pathway (UHDBT high-affinity site).

In addition to antimycin A, myxothiazol and UHDBT, the respiratory chains of fluorescent pathogenic pseudomonads are sensitive to the antimalarial drug mefloquine [145]. Mefloquine (DL-*erythro*-2-piperidyl-2,8-bis[trifluoromethyl]-4-quinolinemethanol-hydrochloride) is a well-known quinolmethanol blood schizontocide which is highly effective against drug-resistant as well as drug-sensitive *Plasmodium vivax* and *P. falciparum* (see Ref. 146). Mefloquine binds to both phospholipids and iron porphyrin components such as plasmodial hemozoin and affects the integrity of *E. coli* inner membrane in which has been proposed to inhibit NADH oxidation through interaction with membrane-bound *b*-type cytochromes [147–150]. This hypothesis has further been supported and extended through evidence that mefloquine induces a strong red-shift of cytochrome *b*-566 reduced absorbance spectrum [145]. In *P. cichorii*, mefloquine, when used in combination with antimycin A, was reported to cause an increase in cytochrome *b* reduction by ubiquinol. Conversely, the combination of mefloquine and myxothiazol drastically blocked cytochrome *b* reduction. Unlike myxothiazol, mefloquine does not affect the formation of ubisemiquinone (Q^-) at the redox centre 'o', since it does not inhibit the oxidant-induced extra reduction of *b*-type cytochromes. Additional experiments revealed that mefloquine mainly affects the quinol/oxygen oxido-reductase, i.e., the alternative pathway, of *P. cichorii* (K_i of 15 μM) with a second inhibitory site in the bc_1 region of the chain ($K_i = 50$ μM).

IID. Nitrate-, nitrite- and nitrous oxide reductases

P. aeruginosa can use nitrate as the sole nitrogen source for growth (nitrate assimilation) and can also grow under anaerobic conditions using nitrate as the terminal electron acceptor providing a pathway for oxidative phosphorylation (nitrate respiration). Nitrate respiration depends on nitrate (NO_3^-) and nitrite (NO_2^-) reductase activities which are produced under anaerobic conditions in the presence of NO_3^-. It is important to note that nitrate cannot, however, support anaerobic growth on compounds for which oxygen is required for the initial metabolic reaction (see subsection IB).

The best characterized respiratory nitrate reductase systems are those of *E. coli* (see Ref. 46) and *Paracoccus denitrificans* [151]. In each case, the enzyme is known to consist of three polypeptides: α (M_r = 150000), β (M_r = 60000) and γ (M_r = 20000), the γ subunit being a *b*-type cytochrome. *P. denitrificans* and *E. coli* nitrate reductases are also known to utilise both nitrate and chlorate (ClO_3^-) as substrates. Studies on the enzyme from *P. aeruginosa* have shown the presence of subunits similar in molecular mass to the α and β polypeptides of the *E. coli* and *P. denitrificans* enzymes but a subunit analogous to γ has not been identified [152]. There is general consensus on the cytoplasmic location of the catalytic site for reduction of nitrate to nitrite (see Ref. 46). This view implies that there exists a mechanism for nitrate entry into the cell. The K_m (NO_3^-) value for intact *P. denitrificans* cells has been determined to be less than 5 μM [153] while for purified preparations for dissimilatory nitrate reductases from *P. denitrificans* [154], *E. coli* [155,156] and *P. aeruginosa* [152], $K_m(NO_3^-)$ lies in the range 0.3–1.5 mM. It has been proposed that the very low $K_m(NO_3^-)$ in intact cells might arise from either a transport process or a nitrate-specific pore that allows access of nitrate directly to the active site of its reductase from the periplasm [153]. Although recent observations on purified nitrate reductase from *P. denitrificans* indicate that the $K_m(NO_3^-)$ with quinol as substrate is considerably lower than the value obtained with viologens as reductants [151], the problem of access of nitrate to the active site of the reductase is still unsolved. As pointed out by Ferguson [157], the existence of a nitrate/nitrite antiporter is an attractive possibility because it would obviate the thermodynamic problem faced by nitrate anions attempting to move by uniport against an opposing membrane potential. It would also explain how nitrate generated at the cytoplasmic surface of nitrite reductase could be delivered to its reductase which is located in the periplasmic space (see below). Although the nitrate reductase from *P. aeruginosa* is far less characterized than its counterpart in *E. coli* and *P. denitrificans*, it can be presumed that electrons flow from the ubiquinol pool on the periplasmic side of the membrane to the NO_3^--reductive site at the cytoplasmic side. This electron transport is insensitive to antimycin A and myxothiazol, suggesting that ubiquinol may operate as a branch-point in the respiratory chain with exclusion of the bc_1 complex [153] (see Fig. 5).

Nitrite reduction is located in the periplasmic space of the cell [81]. In *P. aeruginosa*, this process involved

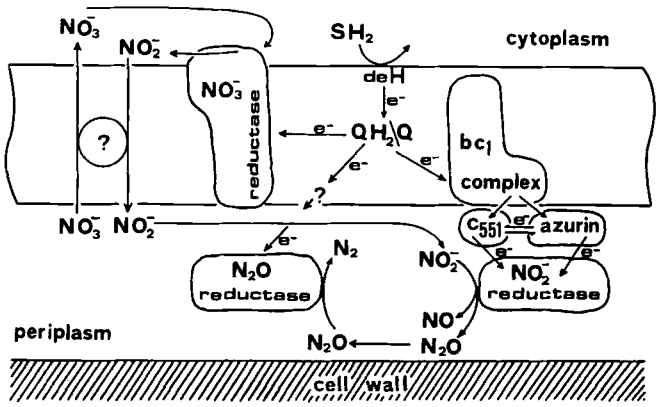

Fig. 5. The postulated organization of enzymes of nitrate, nitrite and nitrous oxide reduction with respect to the anaerobic respiratory chain of *P. aeruginosa*. A nitrate-nitrite antiporter is shown tentatively. See text for further details.

soluble redox carriers (cytochrome *c*-551 and the Cu^{2+}-containing protein, azurin) catalysing electron flow to a nitrite reductase enzyme [158,159]. Enzyme synthesis, induced by nitrate, is repressed in aerated cultures, even in the presence of nitrate (see Ref. 160).

The nitrite reductase is generally referred to as ferrocytochrome *c*-551/oxygen oxidoreductase (EC 1.9.3.2), since it is also able to catalyze a four-electron reduction of O_2 to water [161]. There is still uncertainty about the product(s) of in vivo nitrite reduction. In intact cells of *P. aeruginosa*, the nitrite-reducing activity of the enzyme is thought to catalyze the single-electron reduction of nitrite to nitric oxide (NO) and thence to nitrous oxide (N_2O) [162–165]. On the other hand, there is evidence that in *P. denitrificans* nitrous oxide is formed by a discrete membrane-bound nitric oxide reductase enzyme which presumably generates a $\Delta\tilde{\mu}_{H^+}$ [166–168].

There are two types of nitrite reductases, namely: (i) the Cu^{2+}-containing enzyme of *Alcaligenes faecalis* [169,47]; (ii) the enzyme containing *c* and d_1 haems of *P. aeruginosa* (see below).

The ferrocytochrome *c*-551/oxygen oxidoreductase of *P. aeruginosa* is historically important, since it was the first-described bacterial nitrite reductase and it remains the most intensively studied enzyme of this *Pseudomonas* species (see Ref. 160). The enzyme is purified as a dimer of M_r 120000–130000 with two apparently equivalent subunits of 58000–65000 [162,170–172]. Each monomeric unit contains one *c*-type haem and one haem designated as d_1 [162,172]. Cytochrome d_1 structure has recently been elucidated and it appears to be derived from protohaem IX by a reductive dihydroxylation in pyrrole ring C [173]. As isolated, both *c* and d_1 type haems have been shown to be low-spin ferric species, haem *c* giving rise to EPR signals at $g = 3.01$, 2.30 and 1.4 and haem d_1 to signals at $g = 2.51$, 2.43 and 1.71, which are abolished by cyanide [172–179]. Binding studies have also suggested that both *c*- and d_1-oxidized haems will undergo substitution with a variety of ligands, such as cyanide, imidazole and nitric oxide, but that, conversely, only haem d_1 shows any reactivity with azide and fluoride [162,176–181]. The near-IR MCD spectra of isolated cd_1-complex, which are diagnostic of the state of axial ligation [182,183], have clearly established that haem *c* is ligated by histidine and methionine [184]. Cyanide binds to the *c* haem to displace the methionine ligand and form a cyano-histidine-coordinated haem [179]. The nature of the axial ligands of haem d_1 have been analysed on the basis of the optical and EPR properties of model iron (III)-chlorin and iron(III)-isobacteriochlorin bis-ligated complexes [184]. These studies have demonstrated that the d_1 haem in the oxidized state of nitrite reductase of *P. aeruginosa* is ligated by two histidine residues. A similar conclusion has been drawn for the ligation state of the haem d_1 from *Thiobacillus denitrificans* [185].

The function of the nitrite reductase in *P. aeruginosa* is to catalyze the reduction of nitrite, using reduced cytochrome *c*-551 as electron donor. However, the blue copper protein azurin may also act as electron donor, either to cytochrome *c*-551 or to the nitrite reductase itself. Azurin is by far the most extensively studied prokaryotic blue copper protein to date. Its 3-dimensional structure was reported in 1981 [186] and its redox kinetics have been studied exhaustively [187]. The hypothesis has been formulated that two areas on the protein surface may be involved in electron transfer, namely a hydrophobic patch around copper ligand His-117 and a second region close to more buried His-35 [187,188]. The latter residue has also been implicated in the pH dependence of the spatial conformation on the protein and in the pH dependence of its redox activity, as observed in the electron-transfer reaction with cytochrome *c*-551 [189–191].The electron-transfer kinetics from cytochrome *c*-551 and/or azurin to nitrite reductase suggest competition between the two carriers for the same active site on the enzyme [192]. At low concentrations of substrates (less than 50 μM) the values obtained for K_m and catalytic-centre activities are 15 μM and 77 min, respectively, for azurin and 2 μM and 66 min, respectively, for cytochrome *c*-551 in line with the general concept that cytochrome *c*-551 shows the higher affinity for the enzyme whereas azurin has a slightly higher catalytic rate [159]. This behaviour changes dramatically when cytochrome *c*-551 concentration is extended beyond the K_m value, the initial velocity being almost doubled [192].

Azurin and *c*-551 haem retain different mid-point potentials with the haem *c* more negative (approx. 70 mV) (255 mV for cytochrome *c*-551 and 320 mV for azurin) [193–195]. It is well established that electron transfer between azurin and cytochrome *c*-551 might be very rapid and also involves a complex mechanism characterized by pH-dependent effects to which a regulatory significance has been assigned [196,191]. On the other hand, it is important to notice that the actual physiological significance of having two electron substrates for the nitrite reductase of *P. aeruginosa* is far from clear.

Electrons enter the cd_1 complex at the haem *c* and are then transferred to haem d_1. Similarly to azurin and cytochrome *c*-551, the two haems of the nitrite reductase complex present different redox mid-point potentials with haem d_1 ($E_{m\,7.0} = +220$) more negative than haem *c* ($E_{m\,7.0} = +288$ mV) [197,198,159] (but see also Refs. 199 and 200). The intramolecular electron transfer is complex. Haem–haem interactions have been demonstrated at various levels: (i) between the d_1 haems in the dimeric form of the complex, by the existence of a cooperativity in CO and CN^- binding properties of the reduced species [181,201], and in the electron uptake during redox titrations [200]; (ii) between the *c*-haems,

by the existence of a negative cooperativity in the electron uptake during redox titrations of the dimeric complex [200]; (iii) between *c* and d_1 haems by the existence of cooperativity in redox behaviour of the ligated haems [159,202]. In particular, the binding of CO to ferrohaem d_1 induces a decrease in the redox potential of the *c* haem of at least 80 mV [159]. It is therefore evident that all four of the haem groups in the dimeric form of the enzyme interact. Although sometimes conflicting, all these lines of evidence, taken with the heterogeneity of pre-steady-state kinetics reported in the literature [203,204], and the observed 'half-hearted' behaviour of the enzyme under steady-state conditions [192], give a picture of a dimeric enzyme in which a rather complicated modulation and regulatory phenomenon arises from the assembly of two structurally identical subunits and from their interaction with the substrates.

The nitrous oxide (N_2O) reductase is a water-soluble protein that is located in the periplasm [47,205] (shown in Fig. 5). Until recently there was a general consensus about the presence of nitrous oxide reductase in several N_2-producing denitrifying species such as *Pseudomonas denitrificans, Pseudomonas stutzeri, Pseudomonas perfectomarinus, P. denitrificans, Rhodobacter sphaeroides* and *Rhodobacter capsulatus* [206–208]. *P. aeruginosa* was an exception. Quite recently, Bazylinski et al. [209] have been able to show that *P. aeruginosa*, strains PAO1 and P1, can indeed grow to some extent on exogenous N_2O under strict anaerobic conditions, the growth yield being considerably enhanced by nitrite or nitrate. Unfortunately, the metabolic defect that prevents abundant growth of *P. aeruginosa* on exogenous nitrous oxide remains presently obscure.

The nitrous oxide reductase of *P. denitrificans* accepts electrons from soluble cytochrome *c* via the ubiquinol/cytochrome *c* oxido-reductase. In this bacterial species, the connection of the enzyme is thus the same as for nitrite reductase. Conversely, in both *Rb. sphaeroides* and *P. aeruginosa* there is uncertainty about the respiratory-chain electron donor to the enzyme.

The molecular characterization of nitrous oxide reductase has successfully been achieved only with the enzyme from *Pseudomonas perfectomarinus* [205]. The reductase is a dimer (subunit molecular mass of 74 kDa) with approx. eight Cu^{2+} atoms per molecule. Visible spectra and EPR measurements indicate that many of the copper ions are in an unusual 'type-1' environment not previously found in proteins, with 'type-2' copper being absent [205]. The intimate mechanism involved in the two-electron reduction of N_2O has not yet been clarified.

III. Respiratory-linked energy transduction

It has widely been demonstrated that the exergonic electron-transport reactions catalysed by the bacterial plasmamembrane generates a proton electrochemical gradient across the membrane. The proton electrochemical gradient, also termed protonmotive force when it is quantified in electrical units, comprises both a concentration gradient of protons (ΔpH) and a membrane potential ($\Delta\psi$). Their relative contributions vary amongst bacteria according to the environment of the cell, but, in general, it can be expected that the membrane potential term is predominant [210–214]. The currently accepted value, at an external pH 7.0, lies between 160 and 170 mV (positive outside), with the pH gradient being equivalent to 30 mV (relatively acidic outside) [211,212]. In bacteria the proton electrochemical gradient can drive not only ATP synthesis but also, amongst others, active transport, motion of flagella and reversed (endoergonic) electron flow (see Ref. 215). The last of these is likely to be of minor importance in anaerobic bacteria, since they can generate electrons at a very negative redox potential during fermentation. Conversely, obligate aerobes such as most of the *Pseudomonas* species, utilize reversed electron transport to produce reducing equivalents.

In *P. aeruginosa* glucose metabolism can proceed via phosphorylation to glucose 6-phosphate or by direct oxidation to gluconate, catalyzed by the membrane-bound glucose dehydrogenase (EC 1.1.99.17) [216,217]. This enzyme is inducible by glucose, gluconate, mannitol, and glycerol [218] and it has been shown to belong to the class of quinoproteins which have a pyrroloquinoline quinone (PQQ) as the prosthetic group [219,220]. Glucose dehydrogenase is therefore a membrane-bound aldolase dehydrogenase transferring electrons from the aldose sugars directly to the electron-transport chain. Besides *P. aeruginosa*, the enzyme has also been detected in a wide variety of bacteria, including *Rb. sphaeroides* [221], *Acinetobacter calcoaceticus* [222], *Klebsiella aerogenes* [223] and *Aerobacter aerogenes* [224].

The activity of the glucose dehydrogenase in vitro can be manipulated by addition of limiting amounts of PQQ and the electrons transferred to oxygen through the respiratory chain leads to the generation of a protonmotive force [225] (shown in Fig. 6b). Evidence has been presented that glucose oxidation by right-side out membrane vesicles of *P. aeruginosa* generates a $\Delta\psi$ of -70 mV, provided the presence of saturating concentrations of PQQ.

The proton-translocating segments within the respiratory chain of *P. aeruginosa* have tentatively been determined by Matsushita et al. [71] through comparison of the H^+/O ratios in wild type and cytochrome *c*-oxidase-deficient cells (strain T105). The H^+/O ratios were 5.6 and 4 in wild-type and T105 cells, respectively. Assuming an H^+/P ratio of 2 gram ion H^+ per mol ATP the measured H^+/O would be approximately equal to twice the number of potential energy sites. This has

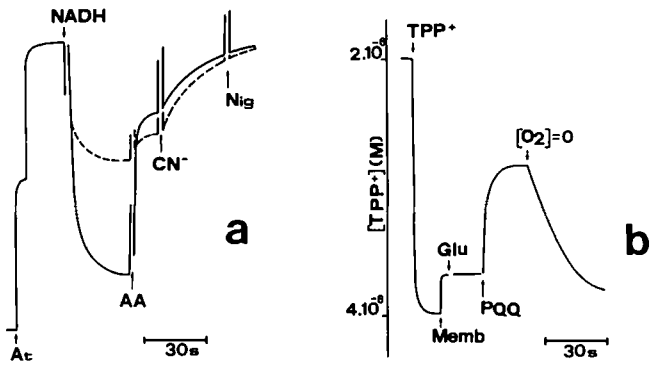

Fig. 6. Membrane energization dependent on glucose and NADH oxidation. (a) Formation of an electrochemical potential of protons coupled to NADH-dependent respiration in membrane fragments from *P. cichorii* (solid line) and *P. aptata* (interrupted line) as monitored with atebrine (At) fluorescence. A downward deflection of the signal indicates the atebrine quenching as a consequence of an active uptake of this diamine into the inner compartment, following acidification of this phase. Additions: atebrine, 1 $\mu g \cdot ml^{-1}$; NADH, 2 mM; cyanide (CN^-), 5 mM; nigericin (Nig), 2 $\mu g \cdot ml^{-1}$; membranes, 2–3 $mg \cdot ml^{-1}$ (adapted from Ref. 99). (b) Uptake of tetraphenylphosphonium ions (TPP^+) by right-side out membrane vesicles from *P. aeruginosa* energized by electron transport via the PQQ-dependent glucose dehydrogenase. Additions: TPP^+, 4 μM; membranes (memb), 0.7 $mg \cdot ml^{-1}$; glucose (Glu), 10 mM; pyrrolo-quinoline quinone (PQQ), 0.3 μM (adapted from Ref. 225).

been and still is much debated, but the current consensus view is that the actual stoichiometry is three per ATP synthesised [226]. Even so, it is apparent that the CN^--resistant oxidase of *P. aeruginosa* can sustain a respiratory electron transport generating more than one energy coupling site.

Electron transport from ubiquinol to nitrate is thought to generate a proton electrochemical gradient [153]. As already mentioned, reduction of nitrate generates nitrite which is reduced by the nitrite reductase located on the periplasmic space. This location has two consequences. First, its connection to soluble cytochrome *c*-551 has an important implication for the energetics of nitrite respiration. This particular point has recently been discussed in great detail [153]; transfer of one electron from ubiquinol to nitrite will be theoretically associated with the net movement of one positive charge out of the cell. This means that the possible ATP yield linked to nitrite respiration will be the same as mentioned earlier for nitrate respiration. The second consequence is, as underlined earlier, that the cytosolic nitrite produced by nitrate reductase must be exported to the periplasm.

The recent observation that *P. aeruginosa* can grow anaerobically on exogenous N_2O provided the maintenance of highly anaerobic conditions is still open to several questions in relation with energy coupling [209]. In *Rb. sphaeroides* f.sp. *denitrificans*, for example, the bc_1 complex, although present, is not involved in nitrous oxide reduction [47]. The latter complex is, however, suggested to operate in electron flow to nitrite and nitric oxide [47]. On the other hand the stoichiometry of charge translocation, and therefore of ATP synthesis, in *P. denitrificans* is believed to be identical for electron transport to either nitrate, nitrite or nitrous oxide [168]. These considerations tend to blur the general concept that energy conservation reflects the redox potential of the electron-acceptor couples involved. The N_2O/N_2 redox couple ($E^{0\prime} = +1.1$ V) is more oxidized than that for NO_3^-/NO_2^- ($E^{0\prime} = +0.42$ V), yet the net proton (charge) translocation stoichiometries for electron flow to either N_2O or NO_3^- are the same. Other factors must therefore be taken into account to explain these unexpected results, namely: (i) the N_2O/N_2 ratio in vivo might lower the operating redox potential of the N_2O/N_2 couple; (ii) N_2O is chemically rather inert and a large drop in free energy may be required for kinetic activation of the enzyme. Point (ii) might explain why *P. aeruginosa* does not seem to grow abundantly on exogenous N_2O although no appreciable dysfunction in terms of the respiratory proton pump and proton permeability of the membrane, are reported [168].

The role in energy transduction of the two pathways containing the CN^--resistant oxidase and the cytochrome *c*-oxidase, respectively, has also been examined in membranes isolated from the phytopathogenic bacteria *P. aptata* and *P. cichorii* [99]. As expected by their insensitivity to rotenone, both species contain a non-energy transducing membrane-bound NADH-dehydrogenase which is, however, capable of catalyzing a very fast NADH/UQ oxido-reductase activity. This finding is rather puzzling, since it suggests that energy transduction in *P. aptata*, the strain deficient in the bc_1 containing pathway, must be linked to the ubiquinol/oxygen oxido-reductase, i.e., CN^--resistant pathway. In this latter species, the generation of a protonic gradient by NADH oxidation, as monitored by the quenching of the diamine atebrine, is insensitive to antimycin A but strongly affected by high concentrations of KCN (shown in Fig. 6a). In this respect, it is important to appreciate that surprising similarities exist between the respiratory chains of several fluorescent *Pseudomonas* species and those found in facultative photosynthetic bacteria [67,48]. Phototrophic bacteria can grow on highly reduced substrates and they must therefore have means for disposing of excess reducing equivalents [48]. According to this general concept, CN^--insensitive oxidative pathways have been regarded as uncoupled 'electron sink mechanisms' operating under some circumstances a redox control at the ubiquinol region of the chain [227,228]. The situation is however more complex, since in membranes isolated from cytochrome bc_1-deficient mutants of *Rb. capsulatus* quinol oxidation catalysed by the alternative pathway is coupled to both ATP synthesis and ΔpH generation [229]. This energy coupling is not observed in *Rb. sphaeroides*

in which a cytochrome *o* similar to those observed in *Rb. capsulatus* and fluorescent pseudomonads is thought to be involved in CN^- insensitive respiration [48,230–232]. A possible explanation for this discrepancy might be based on the fact that electron transport in *Rb. sphaeroides* mainly proceeds via ubiquinol/cytochrome *c* oxido reductase while the alternative pathway catalyses a significant activity only in the presence of inhibitors such as KCN or antimycin A [225]. The opposite situation is observed in *Rb. capsulatus* and *P. cichorii* in which CN^--resistant respiration may be physiologically important, being linked to energy transduction [67,48]. Another interesting example of quinol oxidase coupled to energy transduction is the *b*-562/*b*-555 complex of *E. coli* [46]. The isolated complex can in fact generate a membrane potential of 145 mV when functionally incorporated into liposomes [233]. It is therefore evident that in bacterial species such as *E. coli, Rb. capsulatus* and phytopathogenic pseudomonads, quinol oxidation plays an important role in energy transduction. If this is true, under excess of reducing equivalents there must exist a way for dissipating reducing power. As reported elsewhere in this article (section IV), this result is achieved in *P. cichorii* by means of a strong repression of the cytochrome *c*-containing pathway [68] while in *Rb. capsulatus* both cytochrome *c* inhibition and induction of a non-energy transducing NADH-dehydrogenase is observed [234].

In isolated inside-out membrane vesicles energy transduction at site II (ubiquinol/cytochrome *c* oxido-reductase) and site III (cytochrome *c*/oxygen oxido-reductase or cytochrome *c* oxidase) can successfully be analysed by means of artificial dyes such as DAD or TMPD. These redox mediators are lipid-soluble compounds that deliver reducing equivalents from an external non-permeable donor (ascorbate) to an acceptor located inside the membrane vesicles (cytochrome *c*) and vice versa. Although DAD and TMPD might be used interchangeably in electron-transport reactions, their mechanism relative to energy transducing is quite different when used as substrates [235]. DAD is in fact a well-known electron and proton translocator and the generation of a pH gradient following its oxidation does not therefore reflect the actual presence of an energy-transducing step. Conversely, TMPD does not produce any artificial redox loop being a mere electron donor. In membrane vesicles isolated from *P. cichorii*, rapid ascorbate-TMPD oxidation was shown to induce a significant decrease in the fluorescence of the diamine atebrine, this quenching being reversed by anaerobiosis or by the proton translocator nigericin. The presence of a site for transmembrane ΔpH generation between cytochrome *c* and oxygen is also supported by evidence of CN^--sensitive ATP-synthesis linked to ascorbate-TMPD oxidation ($P/2e^- = 0.1$) [99]. When used as electron acceptor, DAD can sustain a rapid succinate oxidation under anaerobic conditions which is coupled to the onset of an antimycin A-insensitive proton gradient.

IV. Genotypic and phenotypic modifications of electron-transport chains

As reported in the preceding paragraphs there are several unresolved problems concerning the organization, physical nature and functional role of aerobic and anaerobic respiratory chains of *Pseudomonas* species. Few attempts have been made to address these questions by physiology-oriented genetics. Van Hartinsveldt and Stouthamer [236] isolated a mutant (*aer* strain S1276) affected in aerobic growth and which can grow only under anaerobic conditions, or at low oxygen tension, in the presence of nitrate. After anaerobic cultivation no differences in respiration rate, cytochrome spectra, or nitrate and nitrite reduction were observed between *aer* mutant and wild-type bacteria. When shifted from anaerobic to aerobic conditions *aer* mutants did not synthesize haem and released coproporphyrin to the medium [237]. Examples of mutants in which the aerobic conversion of coproporphyrin into protoporphyrin is blocked have been isolated from *E. coli* [238], *Rb. sphaeroides* [239], and *Saccharomyces cerevisiae* [240].

A preliminary analysis of chemically induced mutants from *P. aeruginosa* with defects in the nitrite respiratory system suggested involvement of several gene loci in the *Nir* phenotype [236,241]. It is interesting to note that other mutations in the *NirA* (= *nirR*) locus of *E. coli* affected the regulatory gene *fnr* for anaerobic gene expression but not nitrite respiration per se [242–245]. Quite recently, a series of Nir^- mutants of *P. stutzeri* have been biochemically characterized [245]. It was concluded that in this *Pseudomonas* species cytochrome cd_1 and the nitric oxide reducing system are separate entities. The latter finding is quite important, since also confirms previous evidence that in *Rb. sphaeroides* f.sp. *denitrificans* such an enzyme co-purifies with the cytochrome bc_1 complex [47]. Nitric oxide reductase may therefore be an enzyme that is firmly associated with the cytoplasmic membrane.

In *P. aeruginosa* several genes are involved in the reduction of nitrate to nitrite [232]. Amongst them, four (*nar* A, B, D and E) have pleiotropic effects, as have most of the *chl* (chlorate) mutations of *E. coli* (see Ref. 46). In *nar* B, D and E a molybdenum-containing cofactor, common to dissimilatory and assimilatory nitrate reductase and xanthine dehydrogenase, might be affected. In *nar* D mutants molybdenum might not be incorporated in this factor or converted into the proper redox state while a defect in a part common to both dissimilatory and assimilatory nitrate reductase might account for *nar* A mutation.

The azurin gene from *P. aeruginosa* has recently been cloned and sequenced [246]. It appears that the gene codes for a pre-protein (pre-azurin) with a 19 amino-acid long-signal sequence which possibly assists in the transport of the azurin over the periplasmic membrane. The length of the signal sequence (19 amino acids) concurs with the average length of 20 amino acids observed for prokaryotic signal peptides [247]. Interestingly the hydrophobic region of the signal peptide contains two serines. In fact, it is often assumed that this part of the peptide may adopt an α-helical conformation, at least when it is embedded in a membrane [248]. The presence of two serines seems therefore partly to counteract this tendency.

The ability to denitrify characterizes a heterogeneous group of bacteria of which *P. aeruginosa* PAO1 is the best understood strain genetically [249]. This strain shows the genotypic characteristics of two transposon *Nos*$^-$ mutants of *P. stutzeri* unable to grow anaerobically in nitrous oxide [206,250,251]. Since growth-yield studies demonstrated that nitrous oxide produced in vivo was actively respired, but not nitrous oxide supplied exogenously, the defect has tentatively been associated with electron transport or, more likely, with nitrous oxide uptake [206].

As reported elsewhere in this article, nitrous oxide (N_2O) reductase has a novel type of copper chromophore which is not readily accommodated within the current classification of the principal types of copper proteins [205,252]. Several gene products are required for the assembly of the copper prosthetic group in an as yet unknown way [253–255]. By mapping transposon Tn5-induced mutations, a *nos*-coding region of approx. 8 kb, which comprises functions related to the biosynthesis of N_2O reductase, has been identified [256]. Mutagenesis of a region of approx. 3.5 kb resulted in chromophoreless, apoenzyme-synthesizing strains [254,255]. The same phenotype was observed in a class of frameshift mutants that had lost a 61 kDa protein from the outer membrane [253]. Quite recently, the cloning of the entire *nos* cluster, previously identified by insertional mutagenesis [254] and the determination of the primary structure of the structural gene, *nosZ*, of the N_2O reductase, has been described [256]. The gene comprises 1914 nucleotides, together with 282 nucleotides of 5′-flanking sequences and 238 nucleotides of 3′-flanking sequences. An open reading frame coding for a protein of 638 residues ($M_r = 70\,822$) includes a signal sequence of 35 residues possibly required for protein export. The pre-sequence is in line with the periplasmic location of the enzyme (subsection IID).

In many denitrifying bacteria nitrate reduction is inhibited in the presence of oxygen. Conversely, the cd_1 complex is only induced in the presence of nitrate. The control of nitrate reductase activity is probably the result of an indirect regulatory mechanism on the movement of nitrate across the plasma membrane [257]. A similar hypothesis has recently been advanced by Hernandez and Roewe [258] in *P. aeruginosa*. The molecular basis of this control by oxygen is obscure.

The composition of the membrane-bound electron-transport system of the phytopathogenic species *P. cichorii* is dramatically changed in response to oxygen supply [68]. Growth adaptation to low oxygen concentrations (20–30 μM) is characterized by repression of cytochromes involved in ubiquinol-cytochrome *c* oxido-reductase and cytochrome *c* oxidase activities. By contrast, the cytochrome *c* containing pathway, i.e., alternative oxidase, is unaffected by low oxygen tension. In this respect, *P. cichorii* behaves differently from the soprophyte *P. putida*, in which there is spectral evidence that a *d*-type oxidase is synthesized and that the total cytochrome amount, relative to protein concentration, increases up to severalfold in response to low oxygen tension [259]. The appearance of a new oxidase can suggest a more efficient oxygen utilization under oxygen restriction which in turn would be the cytochrome *d*-triggering induction mechanism by limited supply of energy. The latter type of control mechanism is unlikely for *P. cichorii* since both respiratory branches have shown quite similar K_m values for oxygen (8–10 μM), severalfold lower than the critical oxygen concentration required for repression of the cytochrome *c*-containing pathway. By analogy with the facultative phototroph *Rb. capsulatus* it has therefore been suggested that excess of reducing power under oxygen-limited conditions would be the induction mechanism promoting the restriction of the cytochrome *c* oxidase branch of the chain with a parallel activation of the cyanide less-sensitive one. This control mechanism has two consequences: the first is that both site II and site III of phosphorylation are suppressed; the second is that the alternative pathway must constitutively be linked to energy transduction, since the site I of phosphorylation in *P. cichorii* is uncoupled (Section III).

Conclusions and Outlook

This review analyses some aspects connected with anaerobic and aerobic respiratory chains of three selected pathogenic pseudomonads. This choice was necessary, since the bioenergetics of pathogenic bacteria is a rather unexplored field, medical and metabolic aspects being the most common research topics.

The current upsurge of interest in microbial biotechnology is particularly spectacular in phytopathogenic bacteria because of the economic advantages offered by genetic manipulation of these organisms. It is clear, however, that growth conditions may alter significantly the pathways of electron transport and the components for electron transfer, and the necessity for a more articulated biochemical and genetic approach to *Pseu-*

domonas respiratory components is rather stringent. The case of the nitrite reductase is an example of this kind. While in the seventies, the enzyme was proposed as a suitable model for understanding mammalian cytochrome *c* oxidase, the 'model' has turned out to be nearly as complicated as the other enzyme.

A subject hardly touched on by the present biochemical research is the molecular nature of the so-called 'cyanide-resistant oxidases'. These respiratory pathways, alternative to the classic cytochrome *c* oxidase (when present), are widespread among aerobic bacteria. Indeed, except for few cases, a simplistic linear flow of electron transport to oxygen is not a realistic model and it is certainly more appropriate to regard the bacterial respiratory systems as a complex frame of segments in which a certain interplay of electron flow takes place. Inside of this complicated array, 'cyanide-resistant pathways' might be considered a sort of 'Arabian phoenix', since their actual role and structure remain elusive. The hypothesis that alternative oxidases are primarily a defence mechanism against naturally occurring respiratory inhibitors and therefore plays an important selective advantage, although quite attractive, requires verification.

In conclusion, studies on respiratory chains of *Pseudomonas* species claim a new input of biochemical research, since it is reasonable to presume that the results can be of general relevance to bioenergetics.

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