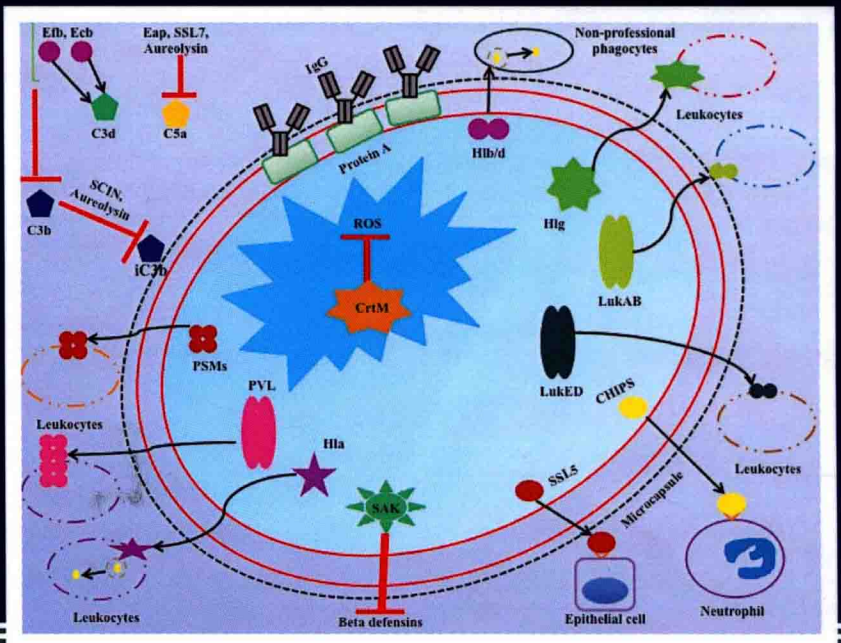
Advances in MICROBIAL PHYSIOLOGY

ADVANCES IN BACTERIAL PATHOGEN BIOLOGY

EDITED BY ROBERT K. POOLE



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VOLUME SIXTY FIVE

ADVANCES IN MICROBIAL PHYSIOLOGY

Advances in Bacterial Pathogen Biology

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525 B Street, Suite 1800, San Diego, CA 92101-4495, USA
225 Wyman Street, Waltham, MA 02451, USA
32 Jamestown Road, London NW1 7BY, UK
The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK

First edition 2014

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ISBN: 978-0-12-800142-4
ISSN: 0065-2911

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CONTENTS

CHAPTER ONE

Energetics of Pathogenic Bacteria and Opportunities for Drug Development

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Contents

Abstract

The emergence and spread of drug-resistant pathogens and our inability to develop new antimicrobials to overcome resistance has inspired scientists to consider new targets for drug development. Cellular bioenergetics is an area showing promise for the development of new antimicrobials, particularly in the discovery of new anti-tuberculosis drugs where several new compounds have entered clinical trials. In this review, we have examined the bioenergetics of various bacterial pathogens, highlighting the versatility of electron donor and acceptor utilisation and the modularity of electron transport chain components in bacteria. In addition to re-examining classical concepts, we explore new literature that reveals the intricacies of pathogen energetics, for example, how *Salmonella enterica* and *Campylobacter jejuni* exploit host and microbiota to derive powerful electron donors and sinks; the strategies *Mycobacterium tuberculosis* and *Pseudomonas*

Advances in Microbial Physiology
ISSN 0065-2911
http://dx.doi.org/10.1016/bs.ampbs.2014.08.001

aeruginosa use to persist in lung tissues; and the importance of sodium energetics and electron bifurcation in the chemiosmotic anaerobe *Fusobacterium nucleatum*. A combination of physiological, biochemical, and pharmacological data suggests that, in addition to the clinically-approved target F_1F_o-ATP synthase, NADH dehydrogenase type II, succinate dehydrogenase, hydrogenase, cytochrome *bd* oxidase, and menaquinone biosynthesis pathways are particularly promising next-generation drug targets. The realisation of cellular energetics as a rich target space for the development of new antimicrobials will be dependent upon gaining increased understanding of the energetic processes utilised by pathogens in host environments and the ability to design bacterial-specific inhibitors of these processes.

1. INTRODUCTION

The majority of current antimicrobials were developed during the golden era of antimicrobial discovery. These compounds target a number of essential processes for the growth of microbial cells, including peptidoglycan biosynthesis, RNA and protein synthesis, DNA replication, and folic acid metabolism. During this period, antimicrobial use became widespread, not only in hospitals but also in agricultural environments. As quickly as new antimicrobials were developed, however, resistance followed increasing the demand for new derivatives through optimisation of existing molecular scaffolds. The burden of antimicrobial resistance was further compounded by the lack of new drugs with unique targets to overcome resistance and by the increasing cost of antimicrobial discovery and development. The number of new antibiotic approvals by the FDA continues to decline contributing to the withdrawal of pharmaceutical companies in this area (Boucher et al., 2013).

To address the emergence and spread of drug-resistant bacterial pathogens, new drug targets and drugs with a novel mode of action are urgently required to expand our antimicrobial armoury. The development of narrow spectrum agents to prevent widespread resistance developing remains a priority. A key to the development of the next generation of antimicrobials will be increased understanding of how new targets function in the physiological context of the pathogen. Deciphering the essential and non-essential roles of these targets in response to the host environment will be an important question to address.

2. BACTERIAL ENERGETICS AS A TARGET SPACE FOR DRUG DEVELOPMENT

A major structural component of bacterial cells is the cytoplasmic membrane made up of a lipid bilayer that forms a continuous barrier around

the cell. The cytoplasmic membrane imparts structure to the cell and allows for the selective (filter) passage of nutrients and wastes into and out of the cell. The membrane also plays an essential role in cellular homeostasis and energy transduction. Several new antimicrobials have been developed that target the bacterial membrane (e.g. daptomycin and lipoglycopeptides), leading to disruption of bacterial membrane integrity, but this target space has remained largely unexplored (Hurdle, O'Neill, Chopra, & Lee, 2011).

2.1. Generation of the proton motive force: An essential property of all bacterial cells

All bacteria require a proton motive force (*pmf*) to grow and remain viable under replicating and non-replicating conditions. During respiration, energy is conserved by the generation of a *pmf* across a proton-impermeable membrane. The electron transport chain components are membrane-bound and asymmetrically arranged across the membrane to achieve net consumption of protons from the cytoplasm and net release of protons on the outside the cell. The *pmf* (electrochemical potential) consists of two gradients: an electrical potential ($\Delta\psi$), due to the charge separation across the membrane ($positive_{outside}/negative_{inside}$) and a chemical transmembrane gradient of protons (ΔpH, $acidic_{outside}/alkaline_{inside}$). At neutral pH, the *pmf* is predominantly in the form of a $\Delta\psi$, but as the external pH drops, the ΔpH increases, and the $\Delta\psi$ decreases to maintain a constant *pmf*. Dissipation of the *pmf* leads to a rapid loss of cell viability and cell death.

A variety of mechanisms are used to generate the *pmf* in bacteria (Fig. 1). In obligately aerobic bacteria, the generation of a *pmf* is mediated primarily by the proton-pumping components of the electron transport chain (Fig. 1, mechanism 4). In facultative anaerobes, when alternative electron acceptors are available (e.g. nitrate and fumarate), proton release is coupled to a terminal reductase (e.g. nitrate reductase) via a *pmf* redox-loop mechanism (Jormakka, Byrne, & Iwata, 2003b; Fig. 1, mechanism 2). Under strictly fermentative conditions, the F_1F_o-ATP synthase can operate as a reversible ATP-driven proton pump to generate the *pmf* (Dimroth & Cook, 2004; Fig. 1, mechanism 3). Furthermore, in some bacteria, end-products (e.g. lactate) efflux can generate a *pmf* (Otto, Sonnenberg, Veldkamp, & Konings, 1980; Fig. 1, mechanism 1). The flexibility of respiration in bacteria under anaerobic conditions is further highlighted by the discovery that endogenous phenazine production by *Pseudomonas aeruginosa* enhances anaerobic

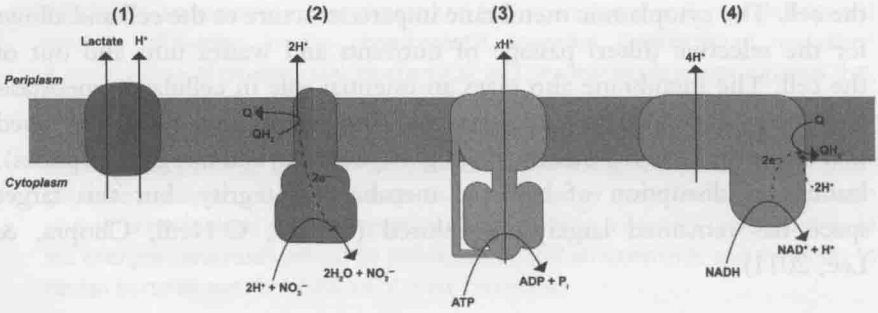

Figure 1 *Mechanisms (1–4) by which a proton motive force can be generated in bacteria.* (1) Co-transport of protons driven by solute (lactate) symport into the periplasm. (2) Redox-loop separation of charge; quinol oxidation results in proton release into the periplasm by virtue of quinol site proximity to the periplasm, while electrons are transferred to reduce a terminal electron acceptor in the cytoplasm that results in neutralisation of charge. (3) Proton export driven by ATP hydrolysis, i.e., ATP synthase working in the reverse direction. (4) Proton translocation mediated by primary proton-pumping complexes. (See the color plate.)

survival through maintenance of the *pmf* (and ATP production) via a redox homeostasis mechanism (Glasser, Kern, & Newman, 2014).

There are a wide range of compounds that target the *pmf* in bacteria (Fig. 2), including agents that inhibit the major proton pumps (e.g. rotenone) (Fig. 2, mechanism 3) and those that facilitate proton transport through the cytoplasmic membrane (protonophores, e.g. carbonyl cyanide *m*-chlorophenyl hydrazine—CCCP) (Fig. 2, mechanism 5). The majority of protonophores are non-specific and functional in both prokaryotic and eukaryotic cell membranes. Individual components of the *pmf* can be collapsed using specific inhibitors. For example, the $\Delta\psi$ can be collapsed by compounds that catalyse electrogenic cation transport across the cell membrane (e.g. valinomycin) Valinomycin is a dodecadepsipeptide that forms a macrocyclic molecule allowing for rapid K^+ movement down its electrochemical gradient (Fig. 2, mechanism 1). The chemical transmembrane gradient of protons (ΔpH) can be collapsed by nigericin through its K^+/H^+ antiporter (electroneutral) activity (Fig. 2, mechanism 2). Nigericin has similar properties to monensin, a Na^+/H^+ exchanger widely used in livestock as a feed additive. Gramicidin is a channel-forming ionophore, making the membrane more permeable to ions (Fig. 2, mechanism 3).

Some bacterial pathogens generate a considerable ΔpH in response to acidification of host tissues (e.g. *Helicobacter pylori*, *Salmonella enterica*, and *Streptococcus pneumoniae*), and collapsing the pH gradient would be an

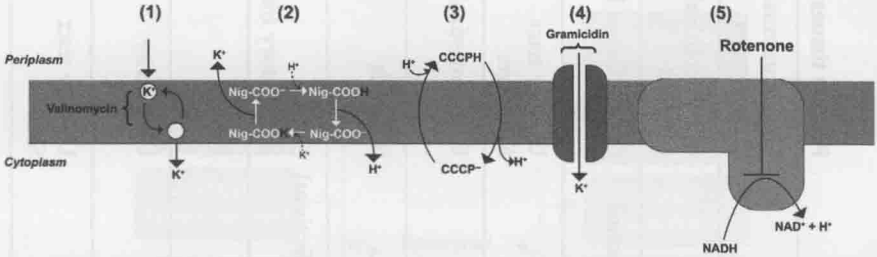

Figure 2 *Traditional inhibitors of proton motive force generation.* (1) Valinomycin is an ionophore, selective for potassium ions, which equilibrates the potassium gradient—dissipating the $\Delta\psi$ (electrogenic). (2) Nigericin is a hydrophobic weak carboxylic acid, which can traverse the membrane as its either protonated acid or neutral salt. It dissipates chemical gradients (i.e. ΔpH) but maintains the charge (one positive charge exchanged for one positive charge—electroneutral). (3) Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) is an electrogenic protonophore. $CCCP^-$ is driven to the periplasm by the $\Delta\psi$, while CCCPH is driven to the cytoplasm by the ΔpH. It can equilibrate both $\Delta\psi$ and ΔpH. (4) Gramicidin is a channel-forming ionophore, making the membrane more permeable to ions. (5) Rotenone inhibits primary proton pumping—preventing the initial generation of a proton motive force. (See the color plate.)

effective strategy in acidic tissues to eradicate these bacteria (Hall, Karem, & Foster, 1995; Matin, Zychlinsky, Keyhan, & Sachs, 1996). The *pmf* has recently been screened as a target for methicillin-resistant *Staphylococcus aureus* using high-throughput screening to identify compounds that dissipate individual components of the *pmf*, i.e., the $\Delta\psi$ or ΔpH and synergistic combinations thereof (Farha, Verschoor, Bowdish, & Brown, 2013).

2.2. Diversity and flexibility of electron transport chains in bacteria

The main pathogens discussed in this review are summarised in Table 1. The electron transport chains both within and between these bacteria show a remarkable diversity with regard to both electron donor and electron acceptor utilisation, enabling growth and persistence in a wide variety of environmental niches (Fig. 3). Bacteria are able to use a range of primary dehydrogenases to deliver electrons from central metabolism into the respiratory chain to generate energy. These electrons pass through various redox carriers to the quinone/quinol pool. In bacteria, the electron transport chain is often branched with multiple routes to terminal respiratory oxidases or reductases (Fig. 3). For example, *Escherichia coli* uses a low-affinity (μM for oxygen) proton-pumping cytochrome bo_3 (haem–copper) oxidase

Table 1 Classification and characteristics of the pathogens discussed in this review

Organism	Classification	Metabolism	Major diseases	Primary tissues
Escherichia coli (pathogenic strains)	γ-Proteobacteria Enterobacteriales	Heterotroph Facultative anaerobe	Gastroenteritis Urinary tract infections	Gastrointestinal tract Urinary tract
Salmonella enterica	γ-Proteobacteria Enterobacteriales	Heterotroph Facultative anaerobe	Gastroenteritis Typhoid fever	Gastrointestinal tract
Pseudomonas aeruginosa	γ-Proteobacteria Pseudomonadales	Heterotroph Facultative anaerobe	Opportunistic infections (e.g. pneumonia)	Cystic fibrosis lungs
Neisseria gonorrhoeae *Neisseria meningtidis*	β-Proteobacteria Neisserales	Heterotroph Facultative aerobe	Gonorrhoea Meningitis	Urinary tract Meninges
Campylobacter jejuni	ε-Proteobacteria Campylobacterales	Heterotroph Microaerobe	Gastroenteritis	Gastrointestinal tract
Helicobacter pylori	ε-Proteobacteria Campylobacterales	Heterotroph Microaerobe	Stomach ulcers Stomach cancer	Stomach
Staphylococcus aureus	Firmicutes Bacilliales	Heterotroph Facultative anaerobe	Opportunistic infections (e.g. skin infections)	Skin Respiratory tract
Mycobacterium tuberculosis	Actinobacteria Actinomycetales	Heterotroph Obligate aerobe	Tuberculosis	Lungs
Fusobacterium nucleatum	Fusobacteria Fusobacteriales	Heterotroph Obligate anaerobe	Periodontitis Lemierre's syndrome	Oral cavity
Treponema pallidum	Spirochaetes Spirochaetales	Heterotroph Obligate anaerobe	Syphilis Yaws	Urinary tract Skin

It only lists those pathogens where a relatively complete overview of their energetics is provided.

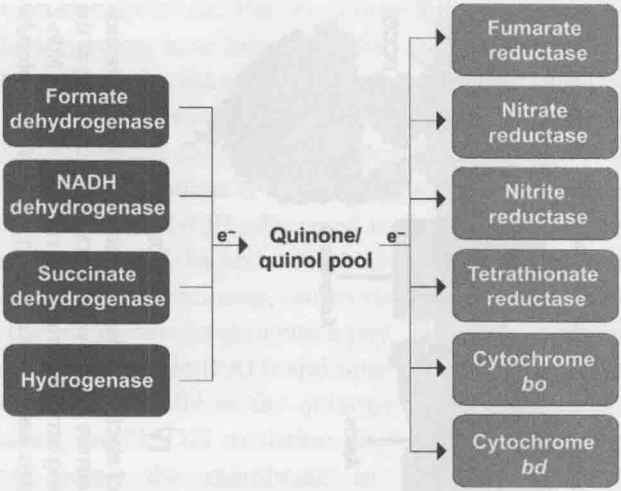

Figure 3 *Generalised schematic overview of relevant electron transfer components.* Complexes indicated in blue oxidise various substrates to reduce quinones. The resulting quinol molecules can be oxidised to result in reduction of various terminal electron acceptors, mediated by the complexes indicated in green. For some electron transfer pathways intermediate complexes and molecules exist, for example, complex III will generally reduce cytochrome *c*, which will serve as the electron donor for complex IV. The complexes used, types of quinones, and intermediates thereof are highly variable between genera. Only complexes relevant to this review are indicated. (See the color plate.)

growing at high oxygen tensions, but switches to a high-affinity (nM for oxygen) non-proton-translocating cytochrome *bd* oxidase when growing at low oxygen tensions (Cotter, Chepuri, Gennis, & Gunsalus, 1990; D'Mello, Hill, & Poole, 1995, 1996; Fig. 3). In *S. enterica*, the electron transport chain shows considerable diversity in response to oxygen tension and will be highlighted throughout this review.

A number of compounds have been shown to inhibit the major components of mitochondrial and bacterial electron transport chains (Fig. 4). However, few if any studies have assessed how specific these compounds are across different bacterial genera.

2.3. Primary respiratory dehydrogenases

2.3.1 NADH dehydrogenases: The roles of bacterial NDH-1 and NDH-2

In many bacterial pathogens, the major entry point to the electron transport chain is the transfer of electrons from reduced nicotinamide dinucleotide (NADH) (reduced by the oxidation of organic carbon) to quinones (e.g.

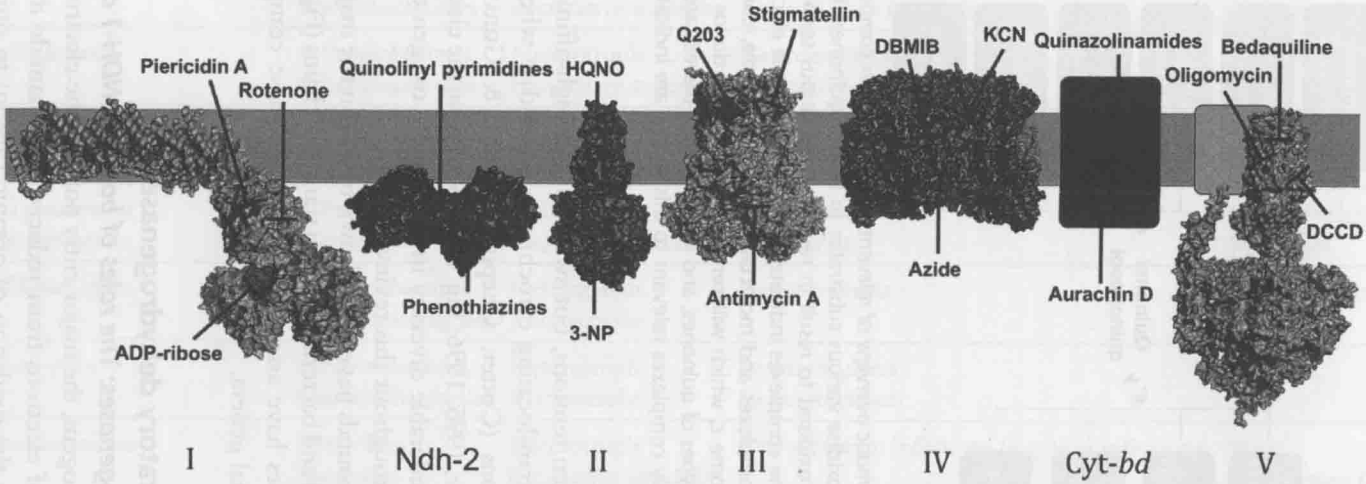

Figure 4 *Diversity of electron transport chain inhibitors.* Structural surface representations of electron transport chain components are indicated where possible. Selected inhibitors of these complexes are indicated with flathead arrows and do not reflect the binding site of the inhibitors. Crystal structures were obtained from RCSB protein data bank from the following accession numbers: complex I, 3M9S; Ndh-2, 4NWZ; complex II, 2WDV; complex III, 3H1J; complex IV, 3ASN; and complex V, 4B2Q. Models were generated using the PyMOL molecular graphics system. (See the color plate.)

ubiquinone or menaquinone; Fig. 3). Three different types of respiratory NADH dehydrogenases have been identified and characterised on the basis of reaction mechanism, subunit composition, and protein architecture (Kerscher, Drose, Zickermann, & Brandt, 2008): the proton-pumping type I NADH dehydrogenase (NDH-1, complex I), the non-proton-pumping type II NADH dehydrogenase (NDH-2; Fig. 4), and the sodium-pumping NADH dehydrogenase (NQR, discussed in Section 2.5). Homologous to mitochondrial complex I, bacterial NDH-1 is encoded by the *nuo* operon and transfers electrons to quinone, conserving energy by translocating protons across the membrane to generate a *pmf*. This multimeric enzyme uses flavin adenine dinucleotide (FAD) and nine iron–sulphur clusters to transport electrons from NADH to the quinone pool. The release of the two electrons during the NADH oxidation produces enough energy to pump four protons across the membrane to generate a *pmf* (Baradaran, Berrisford, Minhas, & Sazanov, 2013).

NDH-2 is more relevant to drug discovery. This small cytoplasmically oriented monotopic membrane protein (40–60 kDa; Fig. 4) catalyses electron transfer from NADH via the flavin cofactor to quinone (Heikal et al., 2014). NDH-2 enzymes are widespread in bacteria and, while also encoded in some eukaryotes (Melo, Bandeiras, & Teixeira, 2004), have not been reported in mammalian mitochondria. This has resulted in the proposal that they may represent a potential drug target for the treatment of pathogenic bacteria (Rao, Alonso, Rand, Dick, & Pethe, 2008; Teh, Yano, & Rubin, 2007; Warman et al., 2013; Weinstein et al., 2005; Yano, Li, Weinstein, Teh, & Rubin, 2006), as well as protozoa (Biagini, Viriyavejakul, O'Neill, Bray, & Ward, 2006; Warman et al., 2013).

In many pathogens, there are copies of both types NDH-1 and NDH-2 in the genome (Melo et al., 2004). In the enteric pathogens *E. coli* and *S. enterica*, these enzymes are differentially expressed, with NDH-2 primarily being synthesised aerobically and NDH-1 being active during anaerobic respiration (Calhoun, Oden, Gennis, de Mattos, & Neijssel, 1993; Unden & Bongaerts, 1997). One potential explanation for the dominant role of NDH-2, even in the presence of NDH-1, is that lack of proton translocation may be desirable during some conditions. NADH oxidation by NDH-2 would not be impeded by a high *pmf*, as would be the case with NDH-1, which could ultimately slow metabolic flux due to back-pressure on the system; NDH-2-mediated NADH oxidation would therefore allow for a higher metabolic flux and increased carbon flow into biosynthetic pathways and ultimately higher rates of ATP synthesis, at the expense of low energetic