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Microbial Biocatalytic Processes and Their Development

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I. Introduction

With recent advances in genomic and genetic engineering, interest in the use of biocatalysis for industrial synthetic chemistry is growing rapidly. Likewise, the range of characterized enzymes and the applications of biocatalysts continue to expand (Rich *et al.*, 2002). In particular, biocatalysis is starting to fulfill its promise as a key tool for the production of optically pure small organic molecules (for the pharmaceutical and agrochemical sector), in part due to the high chemo-, regio-, and stereospecificity and selectivity inherent in enzymatic reactions. In addition, the use of microbial (whole-cell) catalysts containing these enzymes for biocatalytic synthesis is gaining considerable industrial interest. Microbial processes offer possibilities for biocatalysis, which cannot always be met by use of isolated enzymes. The cellular environment protects the protein, which may lead to improved catalyst stability. Furthermore, reactions involving expensive cofactors [e.g., NADH

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or NAD(P)H] and multistep conversions can be simplified in a whole-cell format.

However, despite the increased interest from chemists in the application of enzymes and whole-cell catalysts, it remains the case that the majority of biocatalytic processes implemented in recent years use isolated enzymes. In this chapter, the focus is on microbial conversions with particular emphasis on the so-called ''resting cell'' mode of operation (i.e., where the cells are not growing during the conversion phase) in order to distinguish this from directed fermentations.

II. Rationale for the Use of Microbial Biocatalysts

Few decisions in the design and development of a new biocatalytic process for industrial implementation are as important as the choice of whether the reaction is to be catalyzed (at scale) by an isolated enzyme or by a whole (intact) cell catalyst (Lilly and Woodley, 1996). Processes implemented before the 1960s were predominantly based on whole-cell catalysis (usually with growing cells in fermentative mode) since techniques were not available to efficiently isolate proteins. However, through the 1960s and 1970s methods for the effective isolation of proteins were developed resulting in the possibility of isolated enzyme operation. Attaching the isolated enzyme to a solid support (Cao, 2005) allows reuse of the catalyst at the end of each batch, thus reducing the overall cost contribution from the enzyme. The subsequent introduction of recombinant DNA technology to enhance protein expression has resulted in still further reductions in catalyst cost (Lilly *et al.*, 1996) and led to implementation of many new biocatalytic processes (Fig. 1).

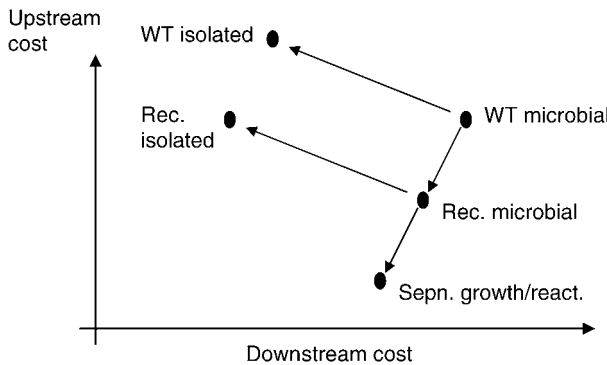

FIG. 1. Scientific developments leading to cost reductions for biocatalysts.

Currently around 150 processes are operating industrially with the majority using isolated enzyme catalysts (Schmid *et al.*, 2001; Straathof *et al.*, 2002; Yazbeck *et al.*, 2004). More recently, the focus has shifted to discovery of new enzymes and their implementation in chemical syntheses in industry. For many bioprocesses, the development route is complex and in recent years we have been developing tools to assist in the rapid evaluation of processes at an early stage (Lye *et al.*, 2002). As part of this activity we have also started to provide guidelines for the evaluation of alternative catalyst forms (isolated enzyme, immobilized enzyme, whole cell or immobilized whole cell). The choice of catalyst form is fundamental to the architecture of the process. In general, isolated enzyme processes require investment upstream of the reactor (Bycroft *et al.*, 2001) and whole-cell processes require investment downstream (May and Groger, 2005). Figure 2 is a generalized biocatalytic process structure indicating the upstream and downstream parts to the process. However, a straightforward upstream–downstream trade-off is often not sufficient to enable a decision to be made. Table I lists some of the key considerations when implementing whole-cell and isolated enzyme catalysts. Clearly not all considerations

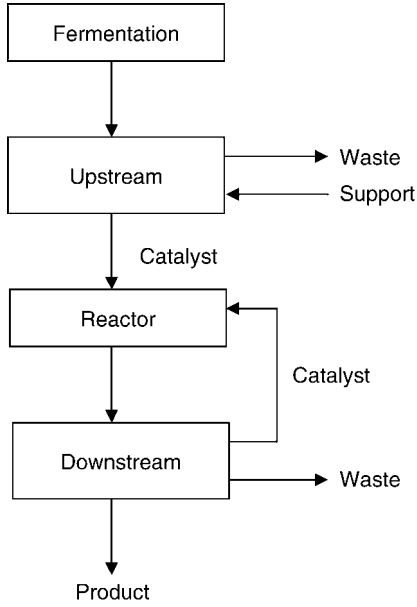

FIG. 2. Generalized process structure for biocatalytic processes.

TABLE I

KEY CONSIDERATIONS WHEN IMPLEMENTING WHOLE-CELL AND ISOLATED ENZYME CATALYSTS

Process step	Whole-cell catalyst	Isolated enzyme catalyst
Upstream		
Preparation	Cell concentration	Isolation Immobilization
Reactor		
Selectivity	By-products	
Media	Fermentation broth	
Organic solvents	Lysis Emulsification Interfacial damage	 Emulsification Interfacial damage
Cofactors		Additional recycle
Kinetics	Substrate access Oxygen supply Product inhibition	 Product inhibition
Reagents	Host specific	Protein specific
Downstream		
Product	By-products	
Catalyst	Cell softening	Protein recovery

are required for every reaction type, and the scientific literature discusses these issues in more detail.

III. Mode of Operation

Isolated enzyme catalysts have the virtue of easy control of kinetics. Addition of a known amount of enzyme catalyst to the reactor gives a known rate of reaction. However, for whole-cell catalysts, the cell concentration is dependent on that which comes from the fermentation. The advent of high cell density fermentation (producing up to 100 g dcw l^{-1}), in particular for *Escherichia coli*, into which many enzymes have been cloned, means that the upstream stages can now be more cost effective. However, some of the benefit of this is lost if the subsequent reaction has to be carried out at the same cell concentration. Hence, it is preferable if the optimization of the fermentation and bioconversion are independent. Separating the two operations means that media as well as concentration can be changed after fermentation reducing both

bioconversion and downstream product recovery costs (Doig *et al.*, 2002). A number of industrial processes (some at very large scale) now operate this strategy. More widely this is referred to as ''resting cell'' biocatalysis since the cells remain metabolically active but are not growing.

IV. Conversions Particularly Suited to Microbial Biocatalysts

A limited number of biocatalytic reactions are impossible to carry out using isolated enzymes, for example, where the enzyme is a multicomponent assembly of proteins or where the protein is membrane bound and inseparable from the cell. In these cases microbial biocatalysts must be used. However, in the majority of situations there is a choice between either whole-cell or isolated enzyme operation as explained previously. Nevertheless, a number of bioconversions are particularly well suited to the use of whole cells and these will be discussed in the following section.

A. Conversions as Part of a ''Cell Factory''

Some reactions are particularly well suited to the use of whole-cell catalysts. Those reactions which use natural substrates, can exploit the cell metabolism, to help provide the substrate from far cheaper starting compounds, further up the metabolic pathway. Multistep conversions [in which two or more enzymatic steps operate in sequence (or potentially in tandem)] also can benefit from whole-cell operation whether existing pathways, modified pathways, or *de novo* pathways are used in a sort of ''cell factory.'' In such cases, the expense of multiple protein separation and isolation operations is avoided.

B. Reactions Involving Cofactor-Requiring Enzymes

Most of the biocatalysts in current industrial use for synthetic purposes are limited to cofactor-independent enzymes such as hydrolases, which perform relatively simple chemistry. In comparison, cofactor-dependent enzymes, such as oxidoreductases and transferases, are capable of performing much more complex chemistry and catalyzing a large number of synthetically useful reactions. Certain cofactors, such as pyridoxal phosphate and biotin, are tightly bound to the enzymes and are therefore in effect self-generating. However, others such as pyridine nucleotides act as functional group transfer agents and are therefore reagents that must be used in stoichiometric amounts. As these

cofactors are too expensive to be used stoichiometrically at process scale, they must be generated *in situ*.

For example, monooxygenases have great potential for symmetric hydroxylations and epoxidations as well as hydroxylations at nonactivated positions for which currently no synthetic organic catalyst exists. Although there are several notable examples of the use of monooxygenases in cell-free systems using enzymatic cofactor regeneration, most uses thus far involve either metabolically active or nongrowing cells of bacterial or yeast origin. Similarly many applications of dehydrogenases use whole cells, which either have intrinsically high activities and stereoselectivities or have been genetically engineered to contain the desired enzymes.

Each of these applications circumvent the expensive protein isolation steps and furthermore take advantage of the cellular machinery for NAD(P)H recycling from cheap cosubstrates such as glucose or glycerol (van der Donk and Zhao, 2003; Zhao and van der Donk, 2003). The natural NAD(P)H regeneration rate is sufficient for oxygenase–dehydrogenase activities of about 100 U g^{-1} dcw, but may become limiting at higher activities. In the case of isolated enzymes, such recycle has to be carried out by an alternative method by addition of a second enzyme or electrochemical method (Eckstein *et al.*, 2004; Hollman and Schmid, 2004; Hummel, 1999). Clearly for such processes this adds to the expense and complexity of the process. It is for this reason, despite many inventive approaches to the isolated enzyme cofactor regeneration problem, that the majority of scaled cofactor-dependent processes use whole-cell catalysts. Finally, cofactor regeneration can also reduce the cost of synthesis by driving the reaction to completion, simplifying product isolation, and preventing the accumulation of inhibitory by-products.

V. Processing Considerations for the Application of Microbial Biocatalysts

While the potential for whole-cell-based biocatalysis is clear, there are a number of special considerations that need to be examined for the design of a scalable bioprocess using this form of biocatalyst. These considerations are outlined in the following section.

A. Industrial Process Requirements

Industrial processes need to fulfill the required metrics of product concentration (g product/liter) and yield of product on catalyst

(g product/g catalyst) to give an economically viable process. However, in addition, in order to be efficient they must use a fast-growing strain (to avoid contamination) and crucially achieve a high cell concentration. Most important is that achieving this concentration cannot be at the expense of protein expression. The amount of expressed enzyme per mass of cell protein is vital and over 10% should always be the aim following cloning. Although we, and others, have reported far higher concentrations (up to 40%) the risk of inclusion body formation is significant at such levels. Furthermore, for industrial operation the use of defined media and controlled growth by carbon-limited feeding is preferable.

B. Reactions Using Organic Solvent-Based Media

In the last two decades, work on the use of biocatalysis in the presence of varying amounts and types of organic solvent has been carried out (Schmid *et al.*, 1998) with the primary goal of enabling higher concentrations of poorly water-soluble substrates to be converted. Many compounds of interest to the chemical and pharmaceutical industries fall into this category. The more polar solvents damage cells (Laane *et al.*, 1987) and may also lead to cell softening (making catalyst recovery difficult) and cell lysis (making product recovery difficult). In the case of nonpolar solvents at concentrations beyond aqueous saturation, the presence of a second phase may give rise to interfacial damage and emulsification [although this has been shown to be partly overcome by immobilization techniques (Schroen and Woodley, 1997; Woodley *et al.*, 1991)]. Careful consideration must be given to the particular cell–solvent combination for process implementation. In recent years, some solvents have been replaced with ionic liquids (with a potentially improved environmental footprint) where possibilities have also been shown to exist for whole-cell operation.

C. Oxygen-Requiring Reactions

Many of the most interesting bioconversions suited to whole-cell catalysis involve the insertion of oxygen into a molecule and therefore require the supply of molecular oxygen (Duetz *et al.*, 2001). For an isolated enzyme the requirement is stoichiometric for the conversion, but the presence of oxygen can be harmful to the protein via oxidative damage or interfacial effects. However, in the case of whole-cell catalysts the situation is rather different. Here, the primary limitation comes from the need to supply oxygen not just for reaction but also

for cell maintenance (or even cell growth). This may preclude operation at high catalyst concentrations with consequent deleterious effects on overall productivity (Duetz *et al.*, 2001). Where volatile reactants and products are used this may be of particular concern.

D. Substrate Access

The cell membrane and the cell wall have to protect the inside of the cell from the external environment, and they create and preserve good conditions for cell metabolism. Crucially, they play an important role in the regulation of the substrate exchange between cell and the surrounding environment. In nature, microorganisms take up nutrients and natural substrates by virtue of an active transport mechanism (Felix, 1982). However, application of biocatalysis for industrial synthetic chemistry is most usually with nonnatural compounds, and mechanisms for uptake may not exist. Hence, the penetration of some molecules can be limited or totally prevented (e.g., phosphorylated compounds cannot enter an intact cell). This leaves the transport into the cell to diffusion and may rapidly become the rate-limiting step. In a number of comparative studies, far higher activities have been found in isolated enzyme compared to cellular systems (Doig *et al.*, 2003). During the last decade a series of methods has been developed to overcome this barrier partially or totally (Ni and Chen, 2004). Cells can be permeabilized without lysis or destruction of the whole inner organization of the cell. In most cases the cell exterior is damaged in such a way that the morphology of the cells remains intact (which is good for subsequent catalyst recovery), yet low-molecular-weight molecules can freely enter and leave the cell (which is good for enzyme activity).

E. Product Recovery

One interesting consequence of whole-cell operation is the presence in the reaction environment of a range of enzymes rather than just the enzyme required for a given conversion. This can have two effects. First, the substrate can be degraded by the other enzymes, reducing the available concentration for conversion and in addition producing unwanted by-products. Second, the product of the desired reaction can also be degraded by other enzymes, either as part of a pathway (overmetabolism) or specific enzyme catalysis to that compound. The consequence of either form of degradation (substrate or product) is reduced concentrations of final product and an increased number of small molecular weight compounds from which it needs to be separated.

Thus, the burden for whole-cell processes is particularly placed on product recovery. This has driven the need for specific product removal methods, overexpression of the desired enzyme and knockout mutants to prevent overmetabolism.

VI. Bioprocess Development

The particular considerations outlined previously will in part drive the development of a given whole-cell process. Alongside this are further requirements for evaluation during bioprocess development. Some of these are enzyme-substrate specific and others are cell-substrate specific. Hence, the possibilities of genetic engineering for cloning into an alternative host are particularly attractive in some cases. The concentration of both substrate and cell determine a considerable amount about the limiting factors in a given conversion. Illustrative of this is a model we have devised for the conversion of cyclic ketones to optically pure lactones using a recombinant strain of *E. coli* containing cyclohexanaone monooxygenase (CHMO). A representative reaction is shown in Fig. 3, where the overexpressed CHMO is used to oxidize bicyclo[3.2.0]hept-2-en-6-one to its two corresponding lactones at very high enantiomeric excess (Alphand *et al.*, 2003; Doig *et al.*, 2001). The reaction is a useful example to study biocatalytic oxidation. The major limitations to a highly productive biocatalytic oxidation in this case were found to be oxygen supply, product inhibition, and biocatalyst stability (Fig. 4).

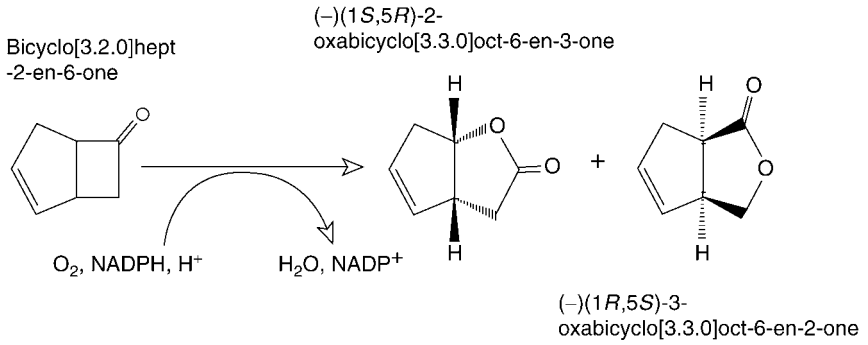

FIG. 3. Biotransformation schematic: *E. coli* TOP10 [pQR239] catalyzed oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones (–)(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (–)(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one.

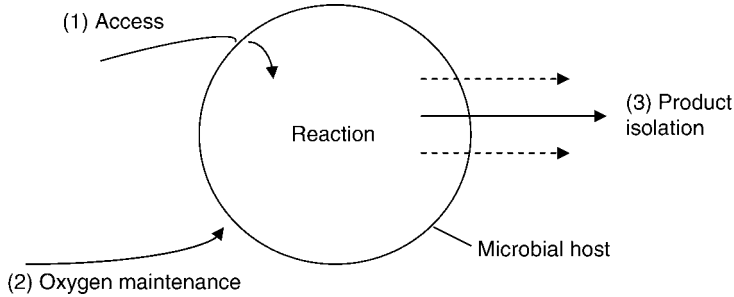

FIG. 4. The process limitations of the whole-cell biocatalytic oxidation of cyclic ketones to lactones. Oxygen supply limits rate of reaction, product inhibition limits final yield and biocatalyst stability limits total reaction time.

Biocatalyst stability has been studied in CHMO containing nongrowing cells. The activity of the cells was linked to intracellular stability of the enzyme which could be prolonged by carrying out the conversion at lower temperatures (Walton and Stewart, 2004). The final limitation, which is common to all oxygenases, is the requirement for molecular oxygen. This requirement is twofold: first as a substrate for the enzymatic conversion and second for oxidation of a carbon source by the bacteria for metabolism (Li *et al.*, 2002). At low cell concentrations (<2 g dcw l^{-1}), the maximum specific rate (0.65 g h^{-1} g dcw^{-1}) is observed. However, at higher cell concentrations (>2 g dcw l^{-1}), the reaction becomes oxygen limited and both the specific rate and absolute rate decrease with further increases in cell concentration.

Providing that substrate inhibition can be overcome by a robust feeding strategy that integrates process monitoring to avoid overfeeding (Bird *et al.*, 2002), we have identified three limitations with our current process (Doig *et al.*, 2003). These limitations are oxygen supply, product inhibition, and biocatalyst stability. The rate at which we can provide oxygen to the reactor will define the maximum rate of reaction assuming that the biocatalyst is not limiting. The product inhibition will define the maximum final concentration and hence yield of the reaction, and finally biocatalyst stability will define the maximum time of the reaction. From these results we proposed a model for the role of oxygen in limiting the rate of reaction. There is a stoichiometric requirement for molecular oxygen as a substrate for the CHMO catalyzed oxidation of ketone and glycerol for NADPH recycle. However, in addition, oxygen, NAD(P)H, and glycerol are all consumed by bacteria during oxidative

phosphorylation in order to produce ATP required for cell maintenance. Our model assumes that oxygen is preferentially used by metabolism of the *E. coli* in order to recycle the NADPH and carry out maintenance of the cell. Once this aspect of the whole-cell reaction has been accounted for, any remaining oxygen can then be utilized in the Baeyer-Villiger monooxygenase (BVMO) reaction (Fig. 5). Previously it has been reported (Nesheim and Lipscomb, 1996) that the turnover number for NAD(P)H-dependent oxygenases is lower than that for NAD(P)H-independent oxygenases due to the electron transfer step of oxygenase reactions being a relatively slow step. This assumption is supported by observations that monooxygenases which do not involve NAD(P)H show a 10- to 1000-fold higher k_{cat} value (Duetz *et al.*, 2001). It is likely that above a certain expression limit of an NAD(P)H-dependent oxygenase in a whole-cell system, the apparent k_{cat} will be limited by the regeneration of the cofactor. The K_m for most oxygenases falls within the range of 10–60 μM (Shaler and Klecka, 1986) whereas the K_m for the electron-transport chain is ∼1 μM. It is therefore likely, as seen in our results, that oxygen-limited conditions would lead to a dramatic decrease in space-time yields because the oxygenase would not be able to compete for the available oxygen within the cell (Duetz *et al.*, 2001).

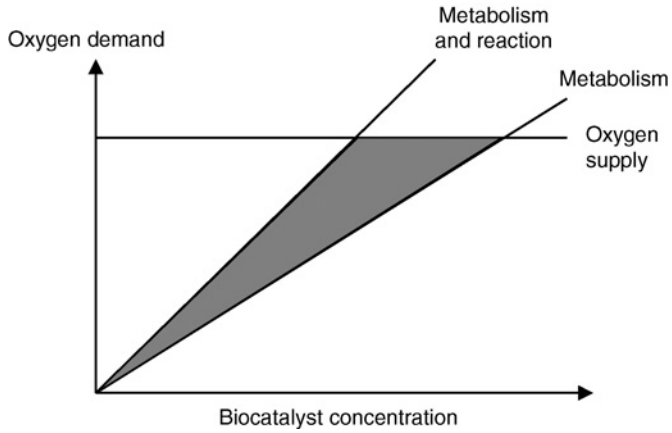

FIG. 5. Schematic representation of oxygen supply and demand in a whole-cell mediated biocatalytic oxidation. The shaded area represents the oxygen available under given conditions for reaction and therefore determines specific activity of the catalyst.

VII. Future Outlook

The future application of whole-cell catalysts (in particular in ''resting cell'' format) seems likely to increase in the coming years. Genetic engineering possibilities are part of the reason for this, but an increasing emphasis on the potential for a bio-based economy is also a strong driver. In the following section, two of the primary motivations for developing further this biocatalyst format for scaled reactions are discussed.

A. Cell Engineering

The ability to alter the properties of a biocatalyst (via cell or protein engineering) is a powerful characteristic of biological catalysts. Targets to date have focused on alteration of enzymes to improve activity or stability under given conditions (Arnold, 1996; Dalby, 2003; Hibbert *et al.*, 2005). Higher activities ultimately mean much reduced fermentation costs. Recently work has been started on cell engineering, for example, altering expression levels of multiple enzyme systems (Hussein and Ward, 2003). More work in this activity is required, in particular to devise more stable cells in industrial conditions. While microbial cells may be stable in a given environment in nature, this may not prove the ideal environment for industrial chemistry. Hence for widespread application, microbial catalysts need to be resistant to industrial environmental features such as high concentrations of substrate and product, high salt concentrations, organic solvents, and extremes of pH. The possibility of altering biocatalysts to have this resistance may come through the use of genetics, screening, or selection [e.g., the use of alternative hosts where Gram-negative organisms have been found to confer greater solvent tolerance than Gram-positive (Woodley *et al.*, 1991)].

B. Toward Bulk Chemical Bioprocessing

A second driver for increased application of whole-cell processes is the move of biocatalysis toward lower value products. We already see the start of this with some processes for bulk chemical manufacture now coming onstream. Bulk chemicals put huge pressure on the cost structure of a process and necessitate processes with high concentrations of product (>250 g l^{-1}) and low catalyst costs. The latter could be achieved by whole-cell operation provided interfering enzymes do not put an undue pressure on product recovery and cheap media can be used.

VIII. Concluding Remarks

While in the past the upstream costs were usually dominant in a biocatalytic process, in recent years the development of rDNA technology has brought these costs down. For pharmaceutical products the molecules have become more complex and frequently reactant conversion becomes the dominating issue. For the future, more emphasis will probably be placed on whole-cell catalysis (Buckland *et al.*, 2000; Ishige *et al.*, 2005; Liese *et al.*, 2000). First, pharmaceutical processes are becoming ever more complex with molecules containing multiple chiral centers. In these cases enzyme cascades are being used increasingly. A recent example uses both whole-cell and isolated enzymes together in subsequent steps of a synthesis (Rustoy *et al.*, 2004) and increasingly metabolic engineering will come to bear. Second, biocatalysis is now expanding beyond the pharmaceutical sector where the balance of costs is different. Use of biocatalysis for the production of bulk chemicals will place a huge burden on costs, and here too processes are only likely to be sustainable via implementation of whole-cell catalysts. It is impossible to generalize what makes the ideal catalyst (and the form of that catalyst) in a given process (Burton *et al.*, 2002; Cheetham, 1998), but while the balance of processes may move to whole-cell catalysis, tools for effective biocatalyst evaluation in a given case will increasingly be required using methods such as software tools, miniaturized experimentation (Lye *et al.*, 2003), and flow cytometry to assess the state in given industrial conditions of particular host cells (Amanullah *et al.*, 2002).

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Occurrence and Biocatalytic Potential of Carbohydrate Oxidases

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I. Introduction

Carbohydrate oxidases are oxidoreductases that use molecular oxygen as electron acceptor and subtract two electrons from a CH–OH (EC 1.1.3.x) or CH–NH group (EC 1.5.3.x) of their carbohydrate substrate. In doing so they oxidize a substrate (S) into a product (P) while dioxygen is reduced to hydrogen peroxide:

$$S + O_2 \rightarrow P + H_2O_2 \quad (1)$$

In order to catalyze such an electron transfer reaction, oxidases contain a redox cofactor. For carbohydrate oxidases this cofactor is often flavin adenine dinucleotide (FAD) which can be covalently or noncovalently bound to the protein. Occasionally, copper in combination with a radical site is employed as cofactor like in the case of galactose oxidase from the fungus *Dactylium dendroides*.

Carbohydrate oxidases appear in all kingdoms of life but are mostly found in fungi (Table I). Their natural role is not always clear. Usage of molecular oxygen as electron acceptor is not a logical choice when the

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TABLE I

List of Characterized Carbohydrate Oxidases

Enzyme	Abbreviation	Year of cloning	Cofactor	Source	Localization	Family
Xylitol oxidase	XYO	2000	FAD	Bacteria	Intracellular	VAO
Sorbitol oxidase	SOX	1998	FAD	Bacteria	Intracelullar	VAO
Fructosyl-amino acid oxidase	FAO	2002	FAD	Bacteria	Intracellular	
		1997	FAD	Yeast, fungi	Peroxisome	
Fructosyl peptide oxidase	FPO	2003	FAD	Fungi		
Glucose oxidase	GOX	1989	FAD	Fungi, plants	Extracellular	GMC
Galactose oxidase	GAO	1992	Cu	Fungi	Extracellular	
Pyranose oxidase	PYO	1996	FAD	Fungi	Periplasma	GMC
Gluconolactone oxidase	GLO	2004	FAD	Fungi	Extracellular	VAO
D-Arabinono-1,4-lactone oxidase	ALO	1998	FAD	Yeast	Mitochondria	VAO
Lactose oxidase	LAO	2001	FAD	Fungi	Extracellular	VAO
Glucooligosaccharide oxidase	GOO	2005	FAD	Fungi	Extracellular	VAO
Hexose oxidase	HOX	1997	FAD	Plant	Extracellular	VAO
Nectarin 5	NEC5	2004	FAD	Plant	Floral nectar	VAO
Carbohydrate oxidase	CHO	2004	FAD	Plant	Extracellular	VAO
L-Gulonolactone oxidase	GUO	1988	FAD	Animals	Endoplasm ret	VAO
Mannitol oxidase	MOX	–	?	Mollusks	Mannosomes	

Abbreviations: FAD, flavin adenine dinucleotide; VAO, vanillyl-alcohol oxidase; GMC, glucose-methanol-choline oxidoreductases.

enzyme is part of a catabolic pathway. By shuttling the electrons directly to dioxygen, the electrons liberated during the oxidation reaction do not enter the respiratory chain prohibiting energy production. In contrast, dehydrogenases typically are dedicated energy-producing enzymes as they deliver electrons by forming reduced coenzymes (e.g., NADH). As a result, they are typically involved in catabolic routes. This is reflected in the observation that the amount of carbohydrate dehydrogenases by far exceeds the amount of known carbohydrate oxidases.

An advantage of producing an oxidase may lay in the inherent property to produce H_2O_2, a signaling molecule that can cause oxidative stress. It has been suggested that the production of H_2O_2 could serve as a mechanism to compete with other microorganisms for growth substrates. Alternatively, it is produced to serve as cosubstrate for peroxidases. For example, the degradation of lignin, a major component of plant material, can only be effectively catalyzed by extracellular fungal peroxidases. These peroxidases rely on the action of oxidases generating the required H_2O_2. For this, lignin degrading fungi typically excrete a variety of oxidases including carbohydrate oxidases.

The first carbohydrate oxidases were purified and characterized more than 40 years ago. These enzymes have extensively been studied for their mechanistic and biocatalytic properties. Nevertheless, it took about 30 years before the first crystal structures of galactose oxidase (Ito *et al.*, 1991) and glucose oxidase (Hecht *et al.*, 1993) were elucidated. An important breakthrough was the notion that many carbohydrate oxidases contain a covalently bound FAD cofactor and that the occurrence of the covalent linkage can be predicted on the basis of the amino acid sequence (Fraaije *et al.*, 1998). Since then, the number of available (recombinant) oxidases has increased significantly (Table I).

Carbohydrate oxidases are valuable enzymes for several applications. They are relatively stable and do not need expensive coenzymes. Carbohydrate oxidases are widely used in diagnostic applications, in the food and drinks industry, and for carbohydrate synthesis. They are also used for bleaching (production of H_2O_2) and as oxygen scavenger.

In this chapter, we first give an overview of the occurrence and properties of carbohydrate oxidases. The physiological role of the different enzymes is discussed in relation to their origin, and the catalytic and structural properties are discussed in relation to their family background. In the second part of this work a summary is given about the biocatalytic applications of carbohydrate oxidases. Issues not discussed here can be found in related reviews (Fraaije and van Berkel, 2006; Giffhorn, 2000; Leskovac *et al.*, 2005; Mattevi, 2006; van Berkel *et al.*, 2006).

II. Physiological Role and Catalytic Properties

A. Microbial Carbohydrate Oxidases

1. *Bacterial Enzymes*

a. *Xylitol Oxidase and Sorbitol Oxidase.* Xylitol oxidase (XYO, xylitol:oxygen 1-oxidoreductase; EC 1.1.3.41) and sorbitol oxidase (SOX, sorbitol:oxygen 1-oxidoreductase; EC 1.1.3.x) are the only carbohydrate oxidases which are exclusively found in bacteria. They catalyze the oxidation of xylitol or sorbitol at the C1 position yielding respectively D-xylose and D-glucose as products. They are unique in their ability to use molecular oxygen as electron acceptor since most bacterial carbohydrate oxidizing enzymes are NAD^+-dependent dehydrogenases. XYO and SOX are found in *Streptomyces* sp. (Hiraga *et al.*, 1997; Yamashita *et al.*, 2000) and the latter enzyme has also been isolated from *Xanthomonas maltophilia* (Hattori *et al.*, 1995; US Patent 5,472,862). The biological functions of XYO and SOX are unknown but might be related to hydrogen peroxide production as a mechanism to compete with other species or as provision of substrate for peroxidases.

XYO and SOX from *Streptomyces* sp. are intracellular monomeric FAD-dependent oxidases with a molecular mass of approximately 45 kDa. No structural data for these enzymes are available and their catalytic mechanisms have not yet been elucidated. XYO and SOX share 51% amino acid sequence identity and belong to the vanillyl-alcohol oxidase (VAO) family of flavoenzymes (Fraaije and Mattevi, 2000; Fraaije *et al.*, 1998). The FAD cofactor of XYO and SOX is suggested to be covalently linked to a histidine residue (Hiraga *et al.*, 1997; Yamashita *et al.*, 2000). The pH optimum for activity of both oxidases is around 7 with an optimum temperature of 50–55 °C. XYO from *Streptomyces* sp. IKD472 and SOX from *X. maltophilia* show a broad pH stability (pH 5.5–10.5 and pH 5–11, respectively) while SOX from *Streptomyces* sp. H-7775 is stable between pH 7.5–10. XYO and SOX are mesophilic enzymes with melting temperatures of 55°C (SOX) and 65 °C (XYO), respectively.

The substrate specificity of XYO and SOX is rather similar. For XYO, the best substrate is xylitol ($K_m = 0.6$ mM; $V_{max} = 29.2$ U mg^{-1}) while D-sorbitol is still oxidized at a reasonable level ($K_m = 3.5$ mM; $V_{max} = 10.6$ U mg^{-1}). For SOX from *Streptomyces* sp. H-7775, the K_m values for D-sorbitol and xylitol are 0.38 and 0.26 mM, respectively, with similar relative activity. SOX from *X. maltophilia* has a twofold higher K_m value for D-sorbitol compared to the *Streptomyces* enzyme and is also active with xylitol (Hattori *et al.*, 1995; US Patent 5,472,862). Besides xylitol and D-sorbitol,

D-mannitol, D-galactitol, and D-arabitol are oxidized by XYO and SOX, while shorter alditols are worse substrates. Oxidation of xylitol by XYO gives xylose as a product, as identified by high performance liquid chromatography (HPLC) (Yamashita *et al.*, 2000). However, since only the D-enantiomer was used as reference compound, the absolute configuration of the product remains unclear. For SOX from *X. maltophilia*, the reaction product of D-sorbitol oxidation was shown to be D-glucose, which has been confirmed by ^{1}H NMR (Hiraga *et al.*, 1997).

b. *Fructosyl Amino Acid Oxidases.* Fructosyl amino acid oxidases (FAOs, fructosyl-α-L-amino acid:oxygen oxidoreductase; EC 1.5.3.x), also called amadoriases, are oxidases which are not active toward carbohydrates as such but act on amadori compounds. These products are formed by a nonenzymatic process called glycation (to distinguish from the enzymatic glycosylation of proteins). During glycation, reducing sugars react with the amino groups of proteins forming Schiff bases which rearrange to ketoamine products (Fig. 1A). FAOs catalyze the oxidative cleavage of these protein–carbohydrate linkages. Although FAOs are mostly found in eukaryotic species, they do occur in bacteria.

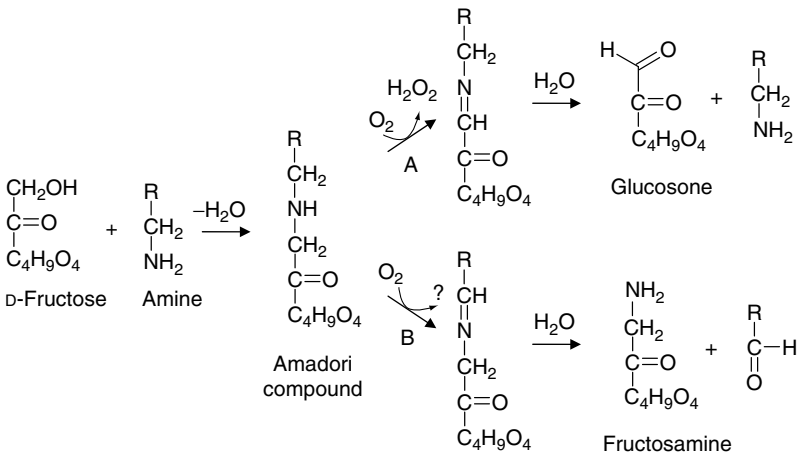

FIG. 1. Reactions catalyzed by FAOs (adapted from Wu and Monnier, 2003). Amadori compounds are formed by a chemical condensation reaction between the carbonyl group of carbohydrates and the free amino group of amino compounds, yielding an unstable Schiff base, which rearranges to the amadori compound. Most FAOs cleave the amadori compound at the ketoamine bond, yielding glucosone (A), however, the *Pseudomonas* enzyme cleaves it at the alkylamine bond resulting in fructosamine (B).

A FAD-containing FAO has been isolated from *Corynebacterium* sp. 2-4-1 (Horiuchi *et al.*, 1989) and a similar enzyme was found in *Agrobacterium tumefaciens* (Hirokawa and Kajiyama, 2002). A membrane-bound deglycation enzyme from a *Pseudomonas* sp. has also been described (Gerhardinger *et al.*, 1995; Saxena *et al.*, 1996). The exact role of FAO in bacteria is unknown but it has been suggested that the enzyme is needed to be able to use fructosyl amino acids as the only carbon and nitrogen source (Hirokawa and Kajiyama, 2002).

FAO from *Corynebacterium* sp. 2-4-1 (*Co*FAO) is the best characterized bacterial FAO. Its gene has been cloned and expressed in *Escherichia coli* (Sakaue *et al.*, 2002). It is a dimeric FAD-containing protein consisting of two identical subunits of 44 kDa each. The flavin cofactor is noncovalently bound which is in contrast with fungal FAOs that often bind FAD covalently (Wu and Monnier, 2003). The amino acid sequence of *Co*FAO does not show any similarity with fungal FAOs, but the GXGXXG motif indicative for an ADP-binding site is present near the N-terminus (Sakaue *et al.*, 2002). It is suggested that, together with the AgaE-like protein, *Co*FAO forms a novel family of prokaryotic FAOs (Hirokawa and Kajiyama, 2002). No three-dimensional (3D) structure has been resolved yet, but a preliminary crystallographic analysis was reported (Sakaue *et al.*, 2005). *Co*FAO has a pH optimum of 8.3 and its optimum temperature lies around 40 °C. The enzyme is stable between pH 7.5 and 9.5 and up to 37 °C. A thermostable mutant has been engineered by directed evolution which is stable up to 45 °C (Sakaue and Kajiyama, 2003). FAO from *A. tumefaciens* (AgaE-like protein) has 54% similarity with *Co*FAO and similar properties (Hirokawa and Kajiyama, 2002).

The substrate specificity of *Co*FAO is mostly restricted to D-fructosyl-L-amino acids yielding glucosone, the corresponding amino acid and H_2O_2 as products (Fig. 1A). D-Fructosyl-D-amino acids are not accepted as substrates, the same holds for D-fructosyl-β-amino acids. *Co*FAO and the AgaE-like protein are inactive against amadori compounds which are glycated at the ε-NH_2-group of lysine like N^{ε}-fructosyl N^{α}-Z (benzyloxycarbonyl)-lysine, while many fungal FAOs and the *Pseudomonas* enzyme do convert this kind of substrates. The substrate specificity is important for the determination of glycated blood proteins in diabetes patients (Hirokawa and Kajiyama, 2002).

The FAO identified from *Pseudomonas* sp. is totally different from *Co*FAO and the AgaE-like protein. This 106-kDa membrane-bound enzyme does not contain FAD. Instead, copper was suggested as cofactor. The enzyme is unique in cleaving the amadori product at the alkylamine bond instead of the ketoamine bond, resulting in fructosamine as a

product (Fig. 1B). Besides that, H_2O_2 was not detected although oxygen consumption was measured for the purified enzyme (Saxena *et al.*, 1996).

2. *Fungal and Yeast Enzymes*

a. *Glucose Oxidase.* Of all carbohydrate oxidases, glucose oxidase (GOX, β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is by far the most well known. It catalyzes the oxidation of D-glucose into D-glucono-1,5-lactone, which spontaneously hydrolyzes to gluconic acid. GOX has been isolated and characterized from *Aspergillus niger* (Hatzinikolaou *et al.*, 1996; Pazur and Kleppe, 1964; Swoboda and Massey, 1965), *Penicillium amagasakiense* (Kusai *et al.*, 1960), *Talaromyces flavus* (Kim *et al.*, 1990), and *Phanerochaete chrysosporium* (Kelley and Reddy, 1986b). The physiological role of GOX in fungi could be related to the production of H_2O_2 in order to be able to compete with bacteria for nutrients. GOX was initially discovered by its antibactericidal properties in the presence of glucose by Fleming (Wilson and Turner, 1992). In *P. chrysosporium*, GOX has been related to the supply of H_2O_2 as a substrate for peroxidase-catalyzed lignin degradation (Kelley and Reddy, 1986a). GOXs from *A. niger* and *P. amagasakiense* have been extensively characterized and will be discussed here in more detail.

GOX from *A. niger* (*An*GOX) is an extracellular homodimeric flavoprotein of approximately 160 kDa and is highly glycosylated. It contains one noncovalently but tightly bound FAD molecule per monomer. Its complete amino acid sequence was first described by Kriechbaum *et al.* (1989) and contains 583 residues. Its optimum pH lies around 5.6 (Pazur and Kleppe, 1964; Swoboda and Massey, 1965).

The 3D structure of *An*GOX is known at 1.9 Å resolution (Hecht *et al.*, 1993; Wohlfahrt *et al.*, 1999). The enzyme belongs to the glucose-methanol-choline (GMC) oxidoreductases, a family of flavoproteins which all contain an ADP-binding $\beta\alpha\beta$-fold (Wierenga *et al.*, 1986) and also includes cholesterol oxidase and methanol oxidase (Cavener, 1992). Each monomer of *An*GOX consists of two domains: a FAD-binding domain and a substrate-binding domain. The FAD is bound near the $\beta\alpha\beta$-fold in a channel that is locked by a lid. When FAD binds to the apoprotein, this lid will close and form the interface of the dimeric protein (Hecht *et al.*, 1993). In this way dissociation of the cofactor is being prevented. The substrate domain consists of a large antiparallel β-sheet supported by helices. This β-sheet region shows similarity with the substrate-binding region of cholesterol oxidase (Vrielink *et al.*, 1991) and forms one side of a deep funnel-shaped cleft where near the bottom the isoalloxazine ring of the flavin cofactor is

located. Besides the interfacial contact between the monomers formed by the lid, another region of contact is formed by a carbohydrate moiety attached to asparagine residue Asn89.

The catalytic cycle of *An*GOX can be divided into two parts. In the reductive half-reaction, D-glucose is oxidized into D-gluconolactone while FAD is reduced to $FADH^-$. In the oxidative half-reaction, $FADH^-$ is reoxidized to FAD by molecular oxygen, yielding H_2O_2 as a second product. *An*GOX obeys ping-pong bi-bi kinetics, which implies that D-gluconolactone is released from the enzyme before dioxygen binds (Weibel and Bright, 1971):

$$E_{ox} + S \rightarrow E_{red} + P \quad (2)$$

$$E_{red} + O_2 \rightarrow E_{ox} + H_2O_2 \quad (3)$$

When glucose binds to the active site a water molecule and a proton are expelled (Leskovac *et al.*, 2005). Two different base-catalyzed mechanisms have been proposed for the next step (Bright and Appleby, 1969; Weibel and Bright, 1971) of which hydride abstraction at the C1 position of glucose, preceded by removal of a proton by His516, is the most likely one (Wohlfahrt *et al.*, 2004). During the oxidative half-reaction, $FADH^-$ is reoxidized to FAD via two single-electron transfer steps (Roth and Klinman, 2003; Su and Klinman, 1999). The rate of formation of the superoxide-flavin semiquinone pair is strongly enhanced by the protonation of His516 (Roth and Klinman, 2003; Roth *et al.*, 2004). The presence of a positive charge close to the reactive locus of the flavin seems to be a recurrent feature in flavoenzymes with enhanced oxygen reactivity (Mattevi, 2006).

The substrate specificity of *An*GOX is rather narrow and mainly restricted to the physiological substrate β-D-glucose. α-D-Glucose, pentoses, other hexoses, and various deoxy analogues of β-D-glucose are poor substrates except for 2-deoxy-D-glucose, which is a reasonable substrate (Leskovac *et al.*, 2005). The specificity for flavin reoxidation is more relaxed. Several quinones can act as an electron acceptor under anaerobic conditions (Wohlfahrt *et al.*, 2004).

GOX from *P. amagasakiense* (*Pa*GOX) shares 65% sequence identity with *An*GOX and has many properties in common with it. *Pa*GOX is also a dimeric, highly glycosylated enzyme with a noncovalently bound FAD. The structure of *Pa*GOX is solved up to 1.8 Å (Wohlfahrt *et al.*, 1999) and is very similar to the one from *An*GOX. However, *Pa*GOX has a sixfold higher K_m and a tenfold higher catalytic rate constant for β-D-glucose and is less stable than *An*GOX. Also, the optimum ranges of temperature and pH for activity are more narrow

compared to that of *An*GOX (Kalisz *et al.*, 1997). The gene encoding *Pa*GOX has been cloned and expressed in *E. coli* which resulted in the formation of deglycosylated inclusion bodies, which could be refolded and purified (Witt *et al.*, 1998).

b. *Galactose Oxidase.* After GOX, galactose oxidase (GAO, D-galactose: oxygen 6-oxidoreductase; EC 1.1.3.9) is probably the most extensively studied carbohydrate oxidase. GAO catalyzes the oxidation of a wide range of carbohydrates (including galactose) and primary alcohols into the corresponding aldehydes with the reduction of O_2 into H_2O_2. The enzyme is highly regioselective and only oxidizes the hydroxyl group at the C6 position of hexoses. GAO was first isolated by Cooper *et al.* (1959) from the fungus *Polyporus circinatus* Fr., which was later renamed to *D. dendroides* (Nobles and Madhosing, 1963) and is identical to *Fusarium* sp. strain NRRL 2903 (Ogel *et al.*, 1994). Besides *D. dendroides*, GAO has been isolated from *Gibberella fujikuroi* (Aisaka and Terada, 1982) and a gene encoding a putative galactose oxidase has been found in the bacterium *Stigmatella aurantiaca* (Silakowski *et al.*, 1998).

The enzyme from *D. dendroides* (*Dd*GAO; Amaral *et al.*, 1963; Avigad *et al.*, 1962) is the best characterized GAO. It is a monomeric extracellular copper-containing oxidase with a molecular mass of 69 kDa. *Dd*GAO belongs to the family of the radical-copper oxidases that also includes glyoxal oxidase from *P. chrysosporium* (Kersten, 1990; Whittaker *et al.*, 1996). The gene encoding *Dd*GAO, *gaoA* has been isolated and encodes an amino acid sequence which includes an N-terminal leader peptide. This leader peptide is cleaved off, resulting in the mature protein of 639 amino acids (McPherson *et al.*, 1992). Intracellular GAO is more heavily glycosylated than the extracellular protein (Mendonça and Zancan, 1987).

The 3D structure of *Dd*GAO at 1.7 Å resolution has been solved by Knowles and coworkers (Ito *et al.*, 1991, 1994). The enzyme contains three domains and is dominated by β-structures. The N-terminal domain consists of a ''β-sandwich'' structure, where two antiparallel β-sheets are facing each other. This domain contains a pocket for a small ligand like a sodium ion and a carbohydrate-binding module (CBM), which belongs to carbohydrate-binding module family 32 and binds to D-galactose and lactose (http://www.cazy.org/CAZY/fam/CBM32.html) and could assist the enzyme in reaching its substrate. The second domain forms a seven-petaled flower, where each petal consists of a four-stranded antiparallel β-sheet. This type of propellor-like structure has also been found in neuraminidase (six-bladed propellor) (Varghese *et al.*, 1983), methylamine dehydrogenase (seven-bladed propellor)

(Vellieux *et al.*, 1989), and in methanol dehydrogenase (eight-bladed propellor) (Xia *et al.*, 1992). At the bottom of this flower structure the copper cofactor is located and three out of four of the copper ligands are provided by this domain. The C-terminal domain lies above the ''top'' of the second domain, on the other side as the copper ion and consists mostly of antiparallel β-sheets. Two of these β-sheets are inserted in the second domain, all the way through the flowerlike structure, providing the fourth equatorial ligand of the copper.

The active site of *Dd*GAO is located at the bottom of the second domain and close to the protein surface. The mononuclear copper is coordinated in a distorted pyramid structure by two tyrosine residues (Tyr272 and Tyr495), two histidine residues (His496 and His581), and one solvent molecule (Ito *et al.*, 1991). The presence of a mononuclear copper which catalyzes a two electron redox reaction can be explained by the presence of a free radical site, which is posttranslationally and autocatalytically formed by a covalent thioether bond between Tyr272 and Cys228 (Ito *et al.*, 1991; Rogers *et al.*, 2000) and is shielded from the solvent by a tryptophan residue (Trp290).

The mechanism of *Dd*GAO has been extensively investigated, both experimentally as well as theoretically (Himo *et al.*, 2000; Whittaker and Whittaker, 1988). The catalytic cycle of GAO is believed to start with the enzyme in the fully oxidized state, Cu(II) and a tyrosine radical present. After binding of the substrate, a proton transfer has been proposed from the alcohol substrate to Tyr495, followed by the C2 hydrogen atom transfer from the substrate to the tyrosine radical. This results in the intermediate redox state of the enzyme [Cu(II), no radical present]. Electron transfer from the tyrosine radical to the copper atom and release of the aldehyde product brings the enzyme in the fully reduced state [Cu(I), no radical present]. The reduced enzyme can then be reoxidized by molecular oxygen. The rate-limiting step during this catalytic cycle is the hydrogen atom transfer to the tyrosine radical.

*Dd*GAO has a very broad substrate specificity. Besides galactose, it is active toward many galactose derivatives (Avigad *et al.*, 1962; Schlegel *et al.*, 1968), polysaccharides (Avigad *et al.*, 1962), dihydroxyacetone (Zancan and Amaral, 1970), and benzyl alcohol derivatives (Whittaker and Whittaker, 2001). Despite its broad specificity, the enzyme shows high regioselectivity, it only oxidizes the hydroxyl group at the C6 position. *Dd*GAO is very stereoselective; L-galactose is not a substrate and oxidation of glycerol only results in the formation of S-glyceraldehyde, while in the case of chiral alcohol substrates only the enantiomer which resembles the D-galactose conformation is being converted (Klibanov *et al.*, 1982).

c. *Pyranose 2-Oxidase.* Pyranose 2-oxidase (PYO) or glucose 2-oxidase (pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) catalyzes the oxidation of D-glucose and other aldopyranoses at the C2 position, yielding the corresponding ketoaldoses and H_2O_2. PYO was first found in *Polyporus obtusus* (Janssen and Ruelius, 1968) and has been isolated and characterized from a number of wood-degrading white-rot basidiomycetes like *P. chrysosporium* (Volc and Eriksson, 1988), *Trametes versicolor* (Machida and Nakanishi, 1984), *Phlebiopsis gigantea* (Schäfer *et al.*, 1996), and *Tricholomona matsutake* (Takakura and Kuwata, 2003). The occurrence and properties of fungal PYOs have recently been reviewed by Giffhorn (2000).

The physiological role of PYOs has been related to lignin degradation. Since their occurrence is restricted to wood-degrading fungi, they exhibit a broad substrate specificity and are active toward wood-derived sugars. Therefore, they are believed to represent the major class of carbohydrate oxidases responsible for the H_2O_2 supply for lignin degrading peroxidases (Daniel *et al.*, 1994). Another natural role for PYO has been described to the synthesis of the antibiotic cortalcerone. This antibiotic, which biosynthesis starts with the conversion of D-glucose into 2-keto-D-glucose, has been found in several white-rot fungi (Baute and Baute, 1984). The reduction of quinones might be another biological role of PYO (Leitner *et al.*, 2001b).

PYO is a relatively large homotetrameric flavoenzyme which is mostly localized in the hyphal periplasm. It has an overall mass of 280 kDa for the tetramer. Each monomer contains one molecule of FAD, covalently linked to a histine residue (Halada *et al.*, 2003). Like GOX, PYO is a member of the GMC oxidoreductase family (Albrecht and Lengauer, 2003).

Recently, the 3D structures of PYO from *T. versicolor* (Hallberg *et al.*, 2004) and from *Peniophora* sp. (Bannwarth *et al.*, 2004) have been elucidated. Each monomer consists of a ''body'' domain, an ''arm,'' and a ''head'' domain. The head domain contains the FAD $\beta\alpha\beta$-motif and a substrate-binding domain, which consists of a six-stranded β-sheet that forms the bottom of the active site. Contact between the monomers happens via an ''oligomerization loop'' and the arm domain. The head domain consists of an uncommon $\alpha\beta\beta\alpha$-motif with no clear function. However, due to the extremely flat structure of the two β-sheets, it could act as a potential interaction surface for a substrate, membrane, or another protein (Hallberg *et al.*, 2004).

The tetrameric composition of PYO can be described as a dimer of dimers and forms a big cavity filled with water in the center of the molecule with entrance to the four active sites. Thus, the substrate

must first enter this cavity which can be reached through channels that connect the cavity with the surface of the tetramer. The existence of this cavity and solvent channels could explain the strict preference of PYO for monomeric carbohydrates (Hallberg *et al.*, 2004).

The active site in PYO is closed off from the internal cavity by an active site loop, which is similar to the lid structure found in other GMC oxidoreductases (Hallberg *et al.*, 2002). In the active site of PYO, an acetate ion, which originates from the crystallization buffer, is bound in close proximity to an asparagine residue (Asn593) and a histidine (His548). Based on this structural information and homology with cellobiose dehydrogenase (Hallberg *et al.*, 2002), the substrate β-D-glucose was modeled in the active site in a way that the C2 and O2 bind in a similar position as the carbon and oxygen atom in the acetate ion. However, positioning of C3 and O3 near Asn593 and His548 was also possible, which agrees with the observation that PYO can also catalyze C3 oxidation (Freimund *et al.*, 1998). The proposed reaction mechanism of PYO proceeds via a general base-catalyzed mechanism, where like in GOX and other GMC members a histidine acts as the general base. After proton abstraction, hydride transfer to the N5 of the isoalloxazine ring yields the $FADH^-$ intermediate, which is then reoxidized by O_2.

The substrate specificity of PYO differs in several ways from that of GOX. First, PYO catalyzes the oxidation at the C2 position and does not act at C1 at all. It does also react at the C3 position when the C2 hydroxyl is absent like in the case of 2-deoxyglucose. Second, PYO does not exclusively react with the β-anomer of D-glucose, but is also active with α-D-glucose. Third, PYO is active with a wide range of aldopyranoses like D-xylose, L-sorbose, D-allose, and L-galactose (Leitner *et al.*, 2001b), while GOX is only active with D-glucose or its derivatives. The substrate specificity of PYO toward electron acceptors is very broad. PYO accepts a wide range of substituted benzoquinones and many of them are in fact a better substrate for PYO than oxygen (Leitner, 2001b). Although PYO exhibits activity with a broad range of sugars, D-glucose and its deoxy analogues are by far the best substrates. The catalytic efficiency k_{cat}/K_m using D-glucose as a substrate is 73 $mM^{-1}s^{-1}$ for PYO from *T. multicolor* and 107 $mM^{-1}s^{-1}$ for PYO from *P. gigantea* (Giffhorn *et al.*, 2000; Leitner *et al.*, 2001). For other sugar substrates these values drop typically 100-fold, an effect which is mainly caused by an increase in K_m.

Pyranose oxidase is a rather thermostable enzyme. The optimal temperature for activity of PYO ranges from 45 °C for the *Peniophora gigantea* enzyme (Bastian *et al.*, 2005) to 65 °C for PYO from *P. gigantea* (Schäfer

et al., 1996). PYO is also active in a wide pH range, its optimal pH differs between pH 5.5 (Leitner *et al.*, 2001b) and pH 8 (Artolozaga *et al.*, 1997).

d. *D-Arabinono-γ-lactone Oxidase and Gluconolactone Oxidase.* Next to carbohydrate oxidases acting on monosaccharides and amadori compounds, a class of enzymes exists which is mainly active with aldonolactones. These enzymes (EC 1.1.3.x) catalyze the oxidation of aldonolactones into the corresponding ketones. In animals (except humans, other primates and guinea pigs), L-gulono-γ-lactone oxidase (EC 1.1.3.8) catalyzes the final step in the biosynthesis of L-ascorbic acid (vitamin C), while in plants this step is catalyzed by L-galactono-γ-lactone dehydrogenase (Ostergaard *et al.*, 1997; Fig. 2). Many lower eukaryotes do not contain vitamin C but its C-5 epimer, D-erythorbic acid (isovitamin C) or D-erythroascorbic acid, a five carbon analogue of vitamin C. In the yeasts *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae*, an enzyme called D-arabinono-γ-lactone oxidase (ALO) is found, which catalyzes the final step in the biosynthesis of D-erythroascorbic acid (Huh *et al.*, 1994, 1998; Fig. 2). This enzyme has been earlier described as an L-galactonolactone oxidase (Nishikimi *et al.*, 1978). In *Penicillium cyaneo-fulvum* ATCC 10431, gluconolactone oxidase (GLO) catalyzes the oxidation of D-glucono-1,5-lactone to D-erythorbic acid (Salusjärvi *et al.*, 2004).

The gene encoding ALO from *S. cerevisiae* has been sequenced and the enzyme has been characterized (Huh *et al.*, 1998). The gene encodes a protein of 526 amino acids and shares sequence identity with L-gulono-γ-lactone oxidase from rat (32%) (Koshizaka *et al.*, 1988) and L-galactono-γ-lactone dehydrogenase from cauliflower (21%) (Ostergaard *et al.*, 1997). ALO is a monomeric mitochondrial membrane protein of 60 kDa, which contains one molecule of covalently linked FAD attached to a histidine residue (Kenney *et al.*, 1979). The enzyme oxidizes L-gulono-1,4-lactone, D-arabinono-1,4-lactone, and L-galactono-1,4-lactone. The enzyme of *C. albicans* ATCC 10231 has been characterized in more detail. Also, L-xylono-1,4-lactone has been reported as a substrate and the kinetic parameters have been determined for D-arabinono-1,4-lactone and L-galactono-1,4-lactone. The former has proven to be the best substrate ($k_{cat} = 21\ s^{-1}$ and $K_m = 44$ mM). The optimum temperature is 40 °C and the enzyme is most active at pH 6.1.

Gluconolactone oxidase (GLO; EC 1.1.3.x) is a fungal lactone oxidase and has been isolated from *P. cyaneo-fulvum* ATCC 10431 (Takahashi *et al.*, 1976). Recently, the GLO-encoding gene has been sequenced, cloned, and expressed in *Pichia pastoris*. The GLO gene encodes a 480-amino acid prepropeptide which contains a 20-amino acid signal

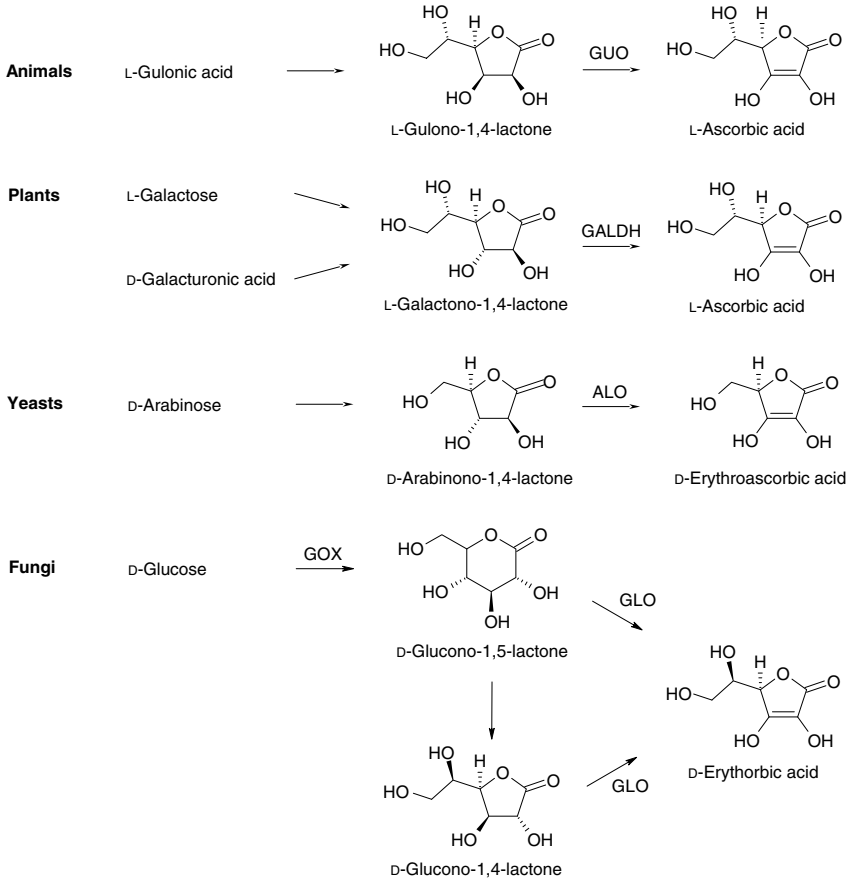

FIG. 2. Biosynthesis of vitamin C and analogues (adapted from Huh *et al.*, 1994; Salusjärvi *et al.*, 2004; Valpuesta and Botella, 2004).

peptide. GLO is an extracellular homodimeric glycoprotein of 160 kDa and does not contain any transmembrane regions like ALO. Like in ALO, the FAD cofactor is covalently attached to a histidine residue. The enzyme does not only oxidize D-glucono-1,4-lactone (glucono-γ-lactone) but also D-glucono-1,5-lactone (glucono-δ-lactone) (Salusjärvi *et al.*, 2004). It has an optimum pH of 6.3 and optimal activity at 42°C. The enzyme is stable under alkaline conditions but is sensitive toward repeated freezing and thawing and loses its activity rapidly at 50°C.

e. *Lactose Oxidase and Glucooligosaccharide Oxidase.* Over the last years, two novel oligosaccharide oxidases have been described: lactose oxidase (LAO; EC 1.1.3.x) from *Microdochium nivale* and glucooligosaccharide oxidase (GOO; EC 1.1.3.x) from *Acremonium strictum* (Lin *et al.*, 1991; Xu *et al.*, 2001). LAO and GOO both contain a covalently linked FAD and are able to oxidize mono-, oligo-, and polysaccharides in contrast to earlier described oxidases, for example, GOX. This expanded substrate specificity for carbohydrates makes these enzymes especially interesting for industrial and diagnostic applications. For instance, the use of LAO in an amperometric glucose biosensor has been studied, yielding some advantages over the use of GOX (Kulys *et al.*, 2001a). Both oxidases catalyze the C1 oxidation of aldoses by using molecular oxygen as electron acceptor, yielding the corresponding acid and H_2O_2. The physiological role is not known, but these oxidases may be involved in biotransformation, utilization, or detoxification of lignocellulosic compounds.

The LAO gene encodes a 495-amino acid (54.7-kDa) protein including a tetrameric propeptide and an 18-amino acid Von Heijne signal peptide. Next to this, two nucleotide-binding domains and three putative *N*-glycosylation sites were detected by sequence analysis. The gene shows 25% sequence identity with 6-hydroxy-D-nicotine oxidase from *Arthrobacter oxidans* and reticuline oxidase (berberine bridge enzyme) from California poppy. LAO has a p*I* of 9, a pH optimum of 5–7, and an optimal temperature at pH 6 of 50 °C. At pH 6 and 30 °C, using cellobiose as a substrate LAO exhibited an apparent k_{cat} of 4 s^{-1} and a K_m of 19 mM. For O_2 under the same conditions, a K_m of $\geq$0.25 mM was determined (Xu *et al.*, 2001). Besides O_2 as electron acceptor, artificial electron acceptors are also accepted by LAO. At pH 6.0 and 25 °C, the reoxidation rate of the reduced enzyme was measured using O_2 and a selection of artificial electron acceptors, for example, 10-propionic acid phenoxazine cation radical ($PAPX^+$). The rate constant of reoxidation was found to be highest with $PAPX^+$ rather than with O_2, respectively, $30 \pm 4 \times 10^6$ and $135 \pm 16 \times 10^3$ $M^{-1}s^{-1}$ (Kulys *et al.*, 2001b).

A 499-residue polypeptide containing a 25-residue N-terminal signal sequence is encoded by the GOO gene. This signal sequence is being cleaved off to form the mature protein, as has been confirmed by N-terminal sequencing. Three possible glycosylation sites have been identified at Asn305, Asn341, and Asn394. Significant sequence homology was found with, for example, reticuline oxidase from *Eschscholzia californica* and carbohydrate oxidase from sunflower (Lee *et al.*, 2005). The mature protein has a molecular mass of 61 kDa, a p*I* of 4.3–4.5, a pH optimum at pH 9–10.5, and an optimal reaction

temperature at 50 °C. GOO oxidizes the reducing end glycosyl residues of oligosaccharides linked by α- or β-1,4 bonds and glucose. Cellotriose is the substrate converted with the highest catalytic efficiency (Fan *et al.*, 2000; Lin *et al.*, 1991). Steady-state parameters were determined with a number of malto- and cellooligosaccharides. For cellotriose ($k_{cat} = 13\ s^{-1}$, $K_m = 0.026$ mM) and cellobiose ($k_{cat} = 5\ s^{-1}$, $K_m = 0.048$ mM), steady-state parameters were determined at pH 8.0 and 37 °C. Also, GOO is capable of using artificial electron acceptors for reoxidizing the reduced FAD during catalysis. For 2,6-dichlorophenol-indophenol, an apparent K_m of 0.85 mM and a k_{cat} of 6.2 min^{-1} were determined using 2 mM cellobiose as substrate (Lee *et al.*, 2005).

Recently, the crystal structure of GOO has been solved in the absence and presence of the substrate analogue 5-amino-5-deoxy-cellobiono-1,5-lactam (Huang *et al.*, 2005). This structure shows the first flavoprotein to contain a double covalently linked FAD via 6-*S*-cysteinyl and 8α-N_1-histidyl linkage (Fig. 3). Furthermore, it becomes clear from this structure that oligosaccharides can be accommodated because of the open carbohydrate-binding groove.

f. *Fructosyl Amino Acid Oxidase and Fructosyl Peptide Oxidase.* Besides their occurrence in bacteria, FAOs and fructosyl peptide oxidases (FPOs) are mostly found in fungi. They catalyze the oxidative cleavage of amadori products into glucosone, the corresponding amino acid or peptide and hydrogen peroxide. Fungal FAOs have been isolated from *Aspergillus* sp. (Jeong *et al.*, 2002; Takahashi *et al.*, 1997a), *Fusarium* sp. (Sakai *et al.*, 1995), *Gibberella* sp., and *Penicillium* sp. (Yoshida *et al.*, 1995). Ferri *et al.* (2004) cloned and expressed an

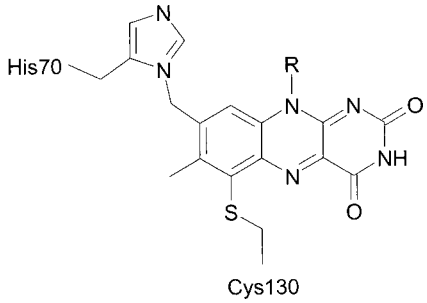

FIG. 3. In glucooligosaccharide oxidase (Huang *et al.*, 2005), FAD is linked via 6-*S*-cysteinyl and 8α-N_1-histidyl linkages. This is the first example of a double covalently bound flavin.

FAO from the marine yeast *Pichia* sp. N1–1. Also two FAO homologues have been cloned from the yeast *Schizosaccharomyces pombe*, but they did not show any activity with fructosyl amino acids (Yoshida *et al.*, 2004).

FPOs differ from FAOs in that they can accept fructosyl peptides like $N^{\alpha\alpha}$-fructosyl valyl histidine (Fru-ValHis). Recently, Hirokawa *et al.* (2003a) screened their collection of fungi for the presence of FPOs. Out of 420 strains, they found 21 strains with FPO activity. They characterized the enzyme from *Achaetomiella virescens* (Hirokawa *et al.*, 2003a) and two FPOs from *Eupenicillium* and *Coniochaete* (Hirokawa *et al.*, 2003b).

In contrast to the bacterial FAOs, the enzymes from fungal origin often contain covalently bound FAD as a cofactor linked to a highly conserved cysteine residue. They contain the GXGXXG ADP-binding motif and most of them are monomeric proteins with a molecular mass of ±50 kDa. For the enzymes of *Aspergillus terreus* and *Penicillium janthinellum*, it has been demonstrated that they are located in the peroxisomes (Sakai *et al.*, 1999). Their pH optimum usually lies around 7.5 and they show highest activity around 30 °C.

FAO and FPO are rather selective in their substrate specificity. For instance, FAO from *Gibberella fujikuroi* (Yoshida *et al.*, 1995) and FPO from *Fusarium oxysporum* S-1F4 (Sakai *et al.*, 1995) act primarily on ε-glycated amadori compounds like N^{ε}-fructosyl-N^{α}-Z-lysine [Z-Lys (Fru)], while other amadoriases prefer mainly α-glycated substrates like *N*-fructosyl-valine (Fru-Val) (Yoshida *et al.*, 1995). FAO from *A. terreus* shows reasonable activity with both types of substrates (Yoshida *et al.*, 1996) and two FAOs from *Aspergillus fumigatus* (amadoriase I and amadoriase II) show also activity with fructosyl propylamines (Takahashi *et al.*, 1997a).

Of all fungal amadoriases, the latter two enzymes have probably been most extensively studied. Their genes have been cloned and expressed in *E. coli* and the corresponding enzymes have been purified and characterized by Monnier and coworkers (Takahashi *et al.*, 1997b; Wu *et al.*, 2000). They share 51% sequence identity on the amino acid level and are both monomeric flavoproteins of ±50 kDa with a *Cys*-covalently attached FAD cofactor. They differ in their substrate specificity in that amadoriase I has a preference for hydrophobic over anionic amadori compounds [$k_{cat}/K_m = 250\ mM^{-1}s^{-1}$ for propylamine amadori product (PAAP) versus $6.6 \times 10^{-2}\ mM^{-1}s^{-1}$ for glycine amadori product (GAP)], while for amadoriase II the opposite is true ($k_{cat}/K_m = 1.1\ mM^{-1}s^{-1}$ for PAAP vs $14\ mM^{-1}s^{-1}$ for GAP) (Wu *et al.*, 2002).

For amadoriase I, the kinetic mechanism has been studied by stopped-flow studies with PAAP as a substrate (Wu *et al.*, 2001). During the reductive half-reaction, three reaction phases were observed. In the first phase, the absorbance drops significantly and this step corresponds to the reduction of the flavin, after binding of PAAP to the enzyme. PAAP was found to be active as a substrate in the β-pyranose form. The other two phases during the reductive half-reaction represent the formation of a reduced enzyme-intermediate complex ($E_{red} \sim I$) and a reduced enzyme-product complex ($E_{red} \sim P$). The spectra of $E_{red} \sim I$ and $E_{red} \sim P$ are unusual and therefore the actual mechanism is not totally clear. A carbanion mechanism, hydride transfer, as well as a nucleophilic mechanism have been proposed. During the oxidative half-reaction O_2 is reduced to H_2O_2. The last step during the reductive half-reaction, the product release, is slower than the overall turnover, which means that the oxidative half-reaction proceeds when the product is still present.

B. Plant Carbohydrate Oxidases

1. *Hexose Oxidase*

Hexose oxidase (HOX, D-hexose:oxygen 1-oxidoreductase, EC 1.1.3.5) is one of the few carbohydrate oxidases with a rather relaxed substrate specificity. The enzyme was first reported in 1956 by Bean and Hassid, while studying the carbohydrate metabolism of the marine red alga *Iridophycus flaccidum*. It was found that cell-free extract of this organism is able to oxidize D-glucose and several other mono- and disaccharides. The enzyme responsible, HOX, was also identified in the marine red algae *Chondrus crispus* and *Euthora cristata*, and was first isolated from *C. crispus* (Irish moss; Sullivan and Ikawa, 1973).

It has been suggested that HOX is an extracellular enzyme since a putative transit peptide cleavage site was found in the cDNA encoding HOX from *C. crispus* (Hansen and Stougaard, 1997), and HOX was found to be a glycoprotein (Groen *et al.*, 1997; Sullivan and Ikawa, 1973). The physiological role of HOX in the red algae was suggested to be the inhibition of growth of other (green) algae (*Chlorella pyrenoidosa*) competing for the same nutrients, due to H_2O_2 production (Ikawa *et al.*, 1969; Sullivan and Ikawa, 1973).

HOX from *C. crispus* (*Cc*HOX) has been best characterized. The cDNA encoding *Cc*HOX encodes a 546-amino acid protein with a predicted mass of 61.898 kDa (Hansen and Stougaard, 1997). *Cc*HOX consists of two subunits (37 and 25 kDa), which are formed on proteolytic activation of the 62 kDa precursor protein (Groen *et al.*, 1997; Hansen and Stougaard, 1997; Savary *et al.*, 2001; Wolff *et al.*, 2001). *Cc*HOX belongs to the VAO

family of flavoproteins (Fraaije *et al.*, 1998) and contains a covalently bound FAD with a hitherto unknown linkage. Inspection of the amino acid sequence of *Cc*HOX suggests that the FAD is covalently linked to a conserved histidine residue in the FAD-binding domain (Fraaije *et al.*, 1998; Wolff *et al.*, 2001). *Cc*HOX also contains a cysteine residue which in GOO is involved in the 6-*S*-cysteinyl-FAD linkage. *Cc*HOX can be produced in recombinant form in *P. pastoris* with properties similar to the wild-type enzyme (Hansen and Stougaard, 1997; Wolff *et al.*, 2001).

Relatively little has been published about the structure, catalytic mechanism, and applications of *Cc*HOX. The enzyme catalyzes the oxidation of a variety of mono- and dialdose sugars at the C1 position including D-glucose, D-galactose, xylose, lactose, maltose, cellobiose, and arabinose, as well as maltotriose and maltotetraose to their corresponding lactones. The lactone products may spontaneously hydrolyze to their aldobionic acids, depending on pH.

HOX shows the highest affinity for the monosaccharides D-glucose and D-galactose and for the disaccharides cellobiose and maltose (Table II). The enzyme shows specificity to the configuration of the C2 hydroxyl group and is nonspecific with regard to the C4 hydroxyl group, which may be involved in the glycosidic linkage (Bean and Hassid, 1956; Poulsen and Bak Høstrup, 1998). *Cc*HOX has a rather broad pH range with two optima, at pH 6 and 10 (Groen *et al.*, 1997; Savary *et al.*, 2001). The optimal pH for catalysis is around pH 6. The optimal temperature for activity is 25 °C (Sullivan and Ikawa, 1973). The enzyme is very stable on storage at 4 °C and under turnover conditions at room temperature (Groen *et al.*, 1997).

Due to its broad substrate specificity, HOX has a great potential for various applications. For example, oxygen scavenging and the removal of sugars from foodstuff, the production of lactones or aldobionic acids, and the production of hydrogen peroxide (see also later in this chapter).

2. *Sunflower Carbohydrate Oxidase*

In search for novel plant antimicrobial proteins, Custers *et al.* (2004) identified a sunflower (*Helianthus annuus*) carbohydrate oxidase (*Ha*CHO) and a lettuce (*Lactuca sativa*) carbohydrate oxidase (*Ls*CHO). Both proteins were identified in the leaves of the plants after treatment with salicylic acid. The sunflower carbohydrate oxidase was investigated in more detail.

The cDNA encoding *Ha*CHO was isolated from sunflower and encodes a 61-kDa protein with a predicted signal sequence. The deduced amino acid sequence of *Ha*CHO shows high homology with several flavoprotein oxidases, including the tobacco NEC5 protein (Carter and Thornburg, 2004b), BBE (Dittrich and Kutchan, 1991), and 27

TABLE II

APPARENT K_m VALUES FOR HEXOSE OXIDASE

Organism	D-Glucose (mM)	D-Galactose (mM)	Cellobiose (mM)	Maltose (mM)	Lactose (mM)	Xylose (mM)	Arabinose (mM)	O_2 (mM)	References
I. flaccidum	2.5	5.0							Bean and Hassid (1956)
C. crispus	4.0	8.0							Sullivan and Ikawa (1973)
C. crispus	2.7	3.8							Hansen and Stougaard (1997)
C. crispus	8.5	1.7	12.5	28.0				0.3	Groen *et al.* (1997)
C. crispus	2.7	3.6	20.2	43.7	90.3	102.0	531.0		Poulsen and Bak Høstrup (1998)
C. crispus	2.5	3.2	27.0	50.0	97.0				Savary *et al.* (2001)

putative oxidases from *Arabidopsis thaliana* with unknown function. Although the presence of a flavin cofactor was not identified, it is clear from its protein sequence that *Ha*CHO belongs to the VAO flavoprotein family, and most likely contains a covalently bound FAD tethered to a conserved histidine residue in the FAD-binding domain (Fraaije *et al.*, 1998). Like *Cc*HOX and NEC5, *Ha*CHO also contains a cysteine residue which in GOO is involved in the 6-*S*-cysteinyl-FAD linkage.

The carbohydrate oxidase activity of *Ha*CHO was first demonstrated with a fungal cell wall suspension from *Rhizoctonia solani* as a substrate; the purified enzyme released H_2O_2 on incubation with the cell wall suspension, which constitutes mainly polymers of glucose and *N*-acetylglucosamine. It appeared that *Ha*CHO could oxidize glucose but not *N*-acetylglucosamine (Custers *et al.*, 2004).

*Ha*CHO has a rather broad substrate specificity and is able to oxidize a variety of mono- and disaccharide substrates, including D-glucose, D-galactose, D-mannose, D-altrose, maltose, lactose, and cellobiose. *Ha*CHO is also able to oxidize the terminal-reducing sugar residue of cello-oligosaccharides up to five residues in length, which makes *Ha*CHO one of the few known carbohydrate oxidases able of oxidizing oligosaccharide substrates. The K_m value for D-glucose was found to be 0.175 mM (Custers *et al.*, 2004), which is considerably lower than the K_m values of GOX (6.3 mM) and HOX (2.7 mM) for D-glucose (Custers *et al.*, 2004; Hansen and Stougaard, 1997). The product of glucose oxidation was identified as glucono-1,5-lactone, indicating that oxidation takes place at the C1 hydroxyl group.

*Ha*CHO is most likely involved in the defense response of sunflower plants. *Ha*CHO is induced after salicylic acid treatment or inoculation with fungal pathogens. The identification of a signal peptide in the *Ha*CHO sequence indicates that the protein is an extracellular protein. A possible mechanism could be that on fungal infection the plant cell wall is degraded by the pathogen, providing substrate for *Ha*CHO which in turn starts producing the antimicrobial agent H_2O_2 (Custers *et al.*, 2004). *Ha*CHO-like proteins appear to be widespread in the plant kingdom, possibly involved in similar defense-related reactions.

3. *Nectarins*

Nectarins are proteins secreted in floral nectar. The nectarins of ornamental tobacco (*Nicotiana langsdorfii* × *Nicotiana sanderae*) have been extensively investigated. The complete tobacco nectar proteome consists of five proteins, Nectarin I (NEC1) through Nectarin V (NEC5). The primary function of these proteins is thought to be the defense of the floral reproductive organs from pathogens due to the generation

of high levels of H_2O_2 (Carter and Thornburg, 2004a). The nectar redox cycle includes three proteins to maintain this high level of H_2O_2; NEC1, a protein with superoxide dismutase activity, NEC3, a protein with carbonic anhydrase and monodehydroascorbate reductase activities, and NEC5, a BBE-like protein with GOX activity. The high levels of H_2O_2 produced by the actions of NEC1 and NEC5 result in the formation of deleterious hydroxyl-free radicals. These radicals can be scavenged by the antioxidant L-ascorbate, which is abundantly present in floral nectar. The L-ascorbate is regenerated by the action of NEC3 and possibly also NEC5 (Carter and Thornburg, 2004a).

The NEC5 cDNA encodes a glycoprotein of 523-amino acid residues, including the predicted N-terminal signal peptide (Carter and Thornburg, 2004b). NEC5 shows strong homology with the BBE, a flavoprotein oxidase involved in the benzophenanthridine alkaloid biosynthesis in plants (Dittrich and Kutchan, 1991). Homologues of NEC5 have been identified in other plants, including the sunflower carbohydrate oxidase mentioned earlier. NEC5 belongs to the VAO family of flavoproteins (Fraaije *et al.*, 1998) and contains a covalently bound flavin cofactor most likely linked to His104 of the FAD-binding domain (Carter and Thornburg, 2004b). The cysteine residue involved in the 6-*S*-cysteinyl-FAD linkage in GOO is conserved in NEC5.

NEC5 catalyzes the oxidation of glucose while reducing O_2 into H_2O_2. NEC5 shows no activity with galactose or mannose (Carter and Thornburg, 2004b). There is evidence that NEC5 also can use dehydroascorbate as terminal electron acceptor, resulting in the regeneration of the antioxidant L-ascorbate in the nectar (Carter and Thornburg, 2004a). Floral nectar lacks alkaloids but is rich in sugars (35% w/v), mainly sucrose, glucose, and fructose. The function of NEC5 is likely to be the regulation of the oxidative state of nectar by generating high levels of H_2O_2 on oxidation of the simple sugars present in the nectar (Carter and Thornburg, 2004a,b).

C. Animal Carbohydrate Oxidases

1. *L-Gulono-γ-lactone Oxidase*

L-Gulono-γ-lactone oxidase (GUO, L-gulono-1,4-lactone:oxygen 3-oxidreductase, EC 1.1.3.8) activity was first demonstrated by Burns *et al.* (1956) in rat liver microsomes. GUO catalyzes the final step in L-ascorbic acid (vitamin C) biosynthesis in animals, the oxidation of L-gulono-γ-lactone into L-xylo-hex-3-ulonolactone, which spontaneously isomerizes to L-ascorbic acid (Fig. 2).

Most vertebrates are capable of synthesizing L-ascorbic acid from glucose with GUO catalyzing the final step, while insects, invertebrates, and most fishes are incapable of doing so. The localization of L-ascorbic acid synthesis, and GUO activity, shifted during evolution. Amphibians, reptiles, and (primitive) birds synthesize L-ascorbic acid in the kidney, while mammals synthesize it in the liver (Chatterjee, 1973). Humans and other primates as well as guinea pigs and flying mammals have lost the ability to synthesize L-ascorbic acid due to large deletions in the *guo* gene (Nishikimi *et al.*, 1992, 1994a). As noted earlier, homologues of GUO exist in yeast (γALO), plants (L-galactono-γ-lactone dehydrogenase), and fungi (GLO).

GUO has been purified and characterized from rat, and goat liver microsomes (Nishikimi *et al.*, 1976) and chicken kidney microsomes (Kiuchi *et al.*, 1982). GUO encoding cDNA has been isolated from rat (Koshizaka *et al.*, 1988) and mouse (Ha *et al.*, 2004). Both cDNAs encode a 440-amino acid residues protein of 51 kDa with more than 94% sequence homology. GUO is an integral membrane protein associated with the endoplasmic reticulum (ER)/microsomal membrane (Eliceiri *et al.*, 1969; Koshizaka *et al.*, 1988; Nakagawa and Asano, 1970). The enzyme solubilized from microsomes exists as aggregates of 400–500 kDa (Eliceiri *et al.*, 1969; Kiuchi *et al.*, 1982; Nishikimi *et al.*, 1976). GUO belongs to the VAO family of flavoproteins (Fraaije *et al.*, 1998). In GUO, the FAD is linked via an 8α-[N_1-histidyl]-flavin linkage (Kenney *et al.*, 1976; Kiuchi *et al.*, 1982) to a histidine residue of the FAD domain. The covalent binding of the cofactor is not a prerequisite for activity. Incubation of recombinant apoGUO produced in riboflavin-deficient insect cells with FAD resulted in holoGUO with noncovalently bound FAD, which showed catalytic activity (Nishikimi *et al.*, 1994b; Yagi and Nishikimi, 1997).

No crystal structure is available for GUO or its homologues and very little is known about the nature of the active site and the catalytic mechanism. Brush and May (1966) suggested a ping-pong bi-bi kinetic mechanism in which the enzyme-bound flavin is reduced by the substrate and L-ascorbic acid is produced and released before flavin reoxidation. Based on chemical modification studies it has been suggested that a sulfhydryl group is important for GUO catalysis (Nakagawa and Asano, 1970; Nishikimi, 1979; Sato *et al.*, 1976).

GUO shows a relatively broad substrate specificity for hexonic acid lactones with the same configuration of the C2 hydroxyl group as in L-gulono-1,4-lactone (Fig. 4). Both rat liver and chicken kidney GUO are able to oxidize L-gulono-1,4-lactone, L-galactono-1, 4-lactone, D-mannono-1,4-lactone, and D-altrono-1,4-lactone, whereas

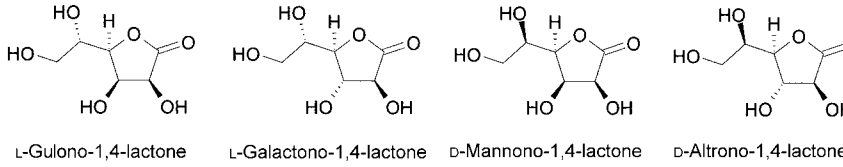

FIG. 4. L-Gulono-γ-lactone substrates (Kanfer *et al.*, 1959; Kiuchi *et al.*, 1982).

the D-enantiomers of L-gulono- and L-galactono-1,4-lactone are no substrates for GUO (Kanfer *et al.*, 1959; Kiuchi *et al.*, 1982). Apparent K_m values for L-gulono-1,4-lactone have been reported for rat, goat, and chicken GUO; 66, 150, and 7 μM, respectively (Kiuchi *et al.*, 1982; Nishikimi, 1979). The optimal pH for activity for chicken GUO is 7.5–8.0 depending on the buffer used (Kiuchi *et al.*, 1982).

GUO shows both oxidase and dehydrogenase activity; phenazine methosulfate and potassium ferricyanide increased the activity in the presence of oxygen and could serve as electron acceptors in the absence of oxygen (Eliceiri *et al.*, 1969). Cytochrome *c*, the physiological electron acceptor of the related plant enzyme L-galactono-γ-lactone dehydrogenase (Ostergaard *et al.*, 1997), is not an electron acceptor for GUO.

2. *D-Mannitol Oxidase*

D-Mannitol oxidase [MOX, mannitol:oxygen oxidoreductase (cyclizing); EC 1.1.3.40] was first identified in digestive gland tissue of the garden snail *Helix aspersa* (Vorhaben *et al.*, 1980). MOXs have been found in the digestive glands of several aquatic and terrestrial snails and slugs, where they are localized in distinct organelles called mannosomes. Both the enzyme and the organelles appear to be unique to mollusks (Large and Connock, 1994; Vorhaben *et al.*, 1986). Mannosomes are tubular membrane systems in which the tubules are assembled in hexagonal bundles within a surrounding membrane (Baumforth *et al.*, 1998). The functions of mannosomes and MOX are as yet not clear, but a role in (heavy metal) stress reactions has been suggested (Knigge *et al.*, 2002).

Mannitol is a common and abundant storage carbohydrate in some plants. Mannitol may be an important source of dietary carbohydrates for herbivorous gastropod mollusks (Vorhaben *et al.*, 1986). The major pathway for mannitol utilization in animals occurs via sorbitol dehydrogenase, an NAD^+-dependent dehydrogenase which converts mannitol into fructose. The presence of MOX in the digestive tract of terrestrial

gastropods suggests a different route for mannitol metabolism, providing nutritional benefit (Vorhaben *et al.*, 1980, 1986).

MOX from *H. aspersa* (*Ha*MOX) has been isolated and characterized. *Ha*MOX is a membrane-bound enzyme of 68 kDa. It is released from the membrane fraction by detergents (Vorhaben *et al.*, 1986). The presence of glycerol in the storage buffer stabilizes the enzyme preparation. Unlike sorbitol dehydrogenase, MOX acts on C1 rather than C2, resulting in the production of D-mannose (Vorhaben *et al.*, 1980). Apparent K_m values for D-mannitol and oxygen are 6 and 40 mM, respectively. Except from D-mannitol, MOX is able to oxidize D-arabinitol, D-sorbitol, galactitol (dulcitol), D-glycero-D-galactoheptitol (perseitol), and D,L-threitol. D-Arabinitol and D-mannitol are the best substrates. The optimal pH for activity is around pH 8–8.5.

No evidence was found for the presence of a flavin or heme cofactor and the enzyme appears insensitive to metal chelators (Vorhaben *et al.*, 1980, 1986). The substrate preference of MOX resembles that of the bacterial xylitol and SOXs. Because the amino acid sequence of MOX is not known, it remains to be seen whether MOX is structurally related to these flavoprotein oxidases.

III. Biocatalytical Applications

A. Biosensors and Diagnostic Applications

Biosensors are devices which incorporate a biological sensing element connected to or integrated within a transducer in order to produce an electronic signal which is proportional to the concentration of a certain chemical compound (Jaffari and Turner, 1995). The biological part of a biosensor often consists of a biocatalyst and so far many carbohydrate oxidases have been applied in biosensors. The detection of specific compounds using oxidase-based biosensors is done either by (1) measuring the consumption of O_2, (2) the use of artificial electron acceptors (mediators), or (3) measuring the formation of H_2O_2.

GOX has been the most widely applied enzyme in biosensors (for a review see Wilson and Turner, 1992). GOX is rather specific for β-D-glucose, but a large number of substrates can replace molecular oxygen in the oxidative half-reaction. Besides from benzoquinones and naphthaquinones, which act as efficient two-electron acceptors, the enzyme is active with many one-electron oxidants including metal-ion complexes. These mediators are widely applied in GOX electrodes.

GOX has been used for self-monitoring of glucose levels in blood for diabetics, for fermentation monitoring, and for measuring glucose in food and drinks (Wilson and Turner, 1992). Besides GOX, also LAO

from *M. nivale* has been used in a biosensor for glucose detection (Kulys *et al.*, 2001a). Immobilized XYO, placed in an enzyme cartridge, in combination with an oxygen detector, has been used in a flow injection method for the determination of xylitol concentrations, which can be used during xylitol-producing fermentation processes (Rhee *et al.*, 2002). Similarly, immobilized GAO in combination with an amperometric detector or peroxidase can be used for the detection of galactose (Kurtz and Crouch, 1991; Szabó *et al.*, 1996; Tkáč *et al.*, 1999). PYO has been applied for the detection of 1,5-anhydro-D-glucitol in serum, which is a marker of glycemic control in diabetic patients (Fukumura *et al.*, 1994; Tanabe *et al.*, 1994) and also for industrial process monitoring (Jürgens *et al.*, 1994).

The substrate specificity of FAOs toward α-glycated or ε-glycated amadori compounds is important for their diagnostic applications of glycated blood proteins in diabetic patients. Enzymes acting on α-glycated compounds can be used to detect glycated hemoglobin, which is primarily glycated at its N-terminal valine residue, while enzymes acting on ε-glycated compounds can be used to detect albumin, which is glycated at the ε-amino group of lysine (Yoshida *et al.*, 1996). FPOs can also be applied for the measurement of glycated blood proteins since they accept protease treated fructosyl hexapeptides, which are released from glycated hemoglobin (HbA_{1c}) by endoproteinase Glu-C (Hirokawa *et al.*, 2003b).

B. Food and Drinks Applications

Food quality can be negatively influenced by the presence of residual oxygen in the package and, therefore, GOX has been tested as an enzymatic O_2-scavenger in order to extend the shelf life of food and drink products (Andersson *et al.*, 2002). Also, for enzymatic preservation of seafood GOX has been used (Dondero *et al.*, 1993). GOX has been commercialized by several companies for dough improvement as an environmental friendly alternative for chemicals like bromate. The exact function of GOX in bread improvement is not completely clear, but is probably related to H_2O_2 production (Vemulapalli *et al.*, 1998).

Due to its broad substrate specificity also HOX has a great potential for various food related applications, for example, oxygen scavenging and the removal of sugars from foodstuff, the production of lactones or aldobionic acids, and the production of H_2O_2. HOX can control the Maillard reaction in foodstuff during baking; HOX oxidizes mono- and disaccharides in food products at the C1 position, which results in sugars that are no longer reducing and thus can not participate in

Maillard reactions (Cook and Thygesen, 2003; Søe and Petersen, 2005, US Patent 6,872,412). In dough, HOX oxidizes mono- and disaccharides with the concomitant production of hydrogen peroxide, which leads to dough strengthening due to disulfide bridge formation in gluten proteins present in the dough (Olsen *et al.*, 2005, US Patent 6,936,289; Poulsen and Bak Høstrup, 1998; Søe *et al.,* 2004, US Patent 6,726,942). *DairyHOX*™ is a commercially available HOX that can be applied in, for example, pizza cheese as oxygen scavenger. This whitens the cheese and extends its shelf life.

C. Synthetic Applications

Carbohydrate oxidases combine the ability to use the cheap, mild, and environmentally clean oxidant O_2 with high regioselectivity toward cheap substrates. Although the production of H_2O_2 can cause enzyme inactivation, this drawback can easily be circumvented by adding catalase to the reaction mixture. Thus, carbohydrate oxidases form an attractive class of biocatalysts for the synthesis of valuable compounds.

GOX has been applied as biocatalyst for the production of gluconic acid (Godjevargova *et al.*, 2004), which is an important antioxidant in food and drinks, and recently also for the synthesis of D-glucosaminic acid, a chiral synthon for the asymmetric synthesis of amino acids (Pezzotti *et al.*, 2005). GAO is an attractive enzyme due to its broad substrate specificity and enantioselectivity toward primary alcohols. Franke *et al.* (2003) applied GAO in combination with dihydroxyacetone phosphatase to synthesize the unnatural nonnutritive sugar L-fructose, starting from glycerol. The LAO-catalyzed oxidation of lactose into lactobionic acid is interesting, since the latter is a valuable antioxidant that can be used for organ preservation during transplantation (Southard and Belzer, 1995).

HOX is presently produced at large scale by Danisco and has shown to be a valuable biocatalyst (Cook and Thygesen, 2003; Wolff *et al.*, 2001). HOX is able to oxidize a variety of carbohydrates, including D-glucose, D-galactose, maltose, cellobiose, and lactose.

PYO probably is the most valuable enzyme to apply for synthetic purposes. Since it oxidizes at the C2 position (and to a lesser extent at the C3 position) and accepts several aldopyranoses, its potential for synthesis of rare sugars and sugar-based compounds has been extensively investigated (Giffhorn, 2000). C2 oxidation of D-glucose by PYO yields 2-keto-D-glucose (glucosone) as a product, which is an important intermediate for the synthesis of rare sugars, fine chemicals, and drugs (Fig. 5). For instance, catalytic dehydrogenation of D-glucosone yields the important sweetener D-fructose as a product. This two-step

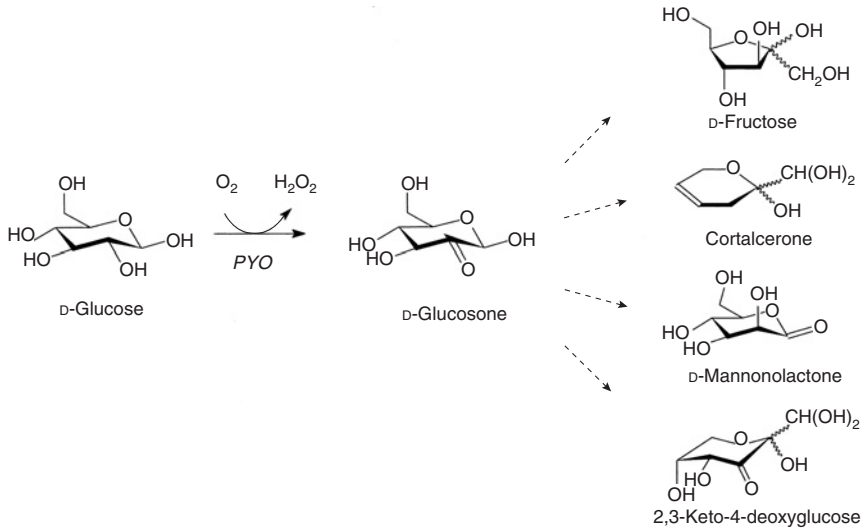

FIG. 5. Possible products of chemoenzymatic reactions catalyzed by pyranose 2-oxidase, starting from D-glucose (Giffhorn, 2000).

chemoenzymatic synthesis is known as the ''Cetus process'' (Geigert *et al.*, 1983). Enzymatic dehydratation of D-glucosone with pyranosone dehydratase results in the formation of the β-pyrone antibiotic cortalcerone (Koths *et al.*, 1992). Due to its broad substrate specificity, PYO can also be used for selective oxidation of other aldopyranoses. In a similar way as D-fructose is synthesized during the Cetus process, D-galactose can be converted into the rare sugar D-tagatose (Freimund *et al.*, 1996). Also, 1,6-disaccharides can be converted into their corresponding ketoses by PYO in combination with an aldose reductase (Leitner *et al.*, 2001a). Besides C2 oxidation, PYO also catalyzes the oxidation of 2-deoxy-D-glucose at the C3 position into 2-deoxy-3-keto-D-glucose which can be easily converted to 1-deoxy-D-xylulose (Freimund *et al.*, 1998), a precursor in vitamin synthesis (Blagg and Poulter, 1999). Glycosyl transfer reactions that might be useful for oligosaccharide synthesis have been observed for PYO as well. However, yields are low and the reactions have not been optimized (Giffhorn *et al.*, 2000).

D. METABOLIC ENGINEERING

The properties of carbohydrate oxidases can also be applied in metabolic engineering. Increased levels of L-ascorbate in plants may have several benefits; the plants may possess an enhanced oxidative stress

tolerance and their nutritional value is increased. Even though plants use different pathways to synthesize L-ascorbate than animals (Fig. 2), it has been shown that the L-ascorbate levels in transgenic plants expressing a rat *guo* gene can be increased up to sevenfold (Jain and Nessler, 2000; Radzio *et al.*, 2003). Furthermore, an adenoviral vector expressing a murine *guo* gene was able to rescue L-ascorbate synthesis deficiency in human cells (Ha *et al.*, 2004).

IV. Conclusions and Future Prospects

Carbohydrate oxidases are valuable enzymes for many applications. By using a flavin or metal cofactor, carbohydrate oxidases catalyze the regioselective oxidation of a wide range of substrates ranging from simple sugars to oligosaccharides. This synthetic property is combined with a unique reactivity toward molecular oxygen, which is used for the reoxidation of the protein-bound cofactor. Cofactor reoxidation results in the formation of hydrogen peroxide, a useful compound in many diagnostic and food applications.

Genome mining suggests that many more carbohydrate oxidases remain to be discovered. It will be a challenge to characterize these enzymes for their structural and functional properties and to find out whether they can be used for novel applications. With the progress in nanosciences, innovative future applications for carbohydrate oxidases can be expected from bionanotechnology.

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Microbial Interactions with Humic Substances[1]

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[1]This article is dedicated to the memory of Robert G. Wetzel for his many contributions to the fields of limnology, microbial ecology, and natural organic matter in the environment.

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I. Introduction

An in-depth understanding of the reaction chemistry of humic substances in soils, sediments, and natural waters and the role played by these compounds in many geochemical cycles has been a major scientific focus for over a century and in that time there has been an accumulation of a vast amount of scientific data. In contrast, however, the interactions between these ubiquitous compounds and microbial populations have only recently gained scientific attention. It is now known that microbial communities in both aerobic and anaerobic environments interact with humic substances through a broad diversity of mechanisms, including catabolic processes, redox cycling, and nutrient bioavailability. HS can be utilized by microorganisms as effective electron acceptors for the oxidative degradation of organic carbon in anaerobic environments. Alternatively, HS in the reduced form can be utilized by microorganisms as effective electron donors for the assimilation of organic carbon coupled to denitrification. These metabolic processes as well as the intrinsic geochemical reactivity of HS are now known to play an important role on the fate and transport of herbicides, pesticides, hydrocarbons, cations, and other important environmental pollutants. This chapter will examine the diverse geochemical and microbial reactivities of humic substances, the role these complex organics play in a diversity of environments, and their potential applicability to bioremediative strategies.

II. Source and Chemistry of Humic Substances

A. HS in the Environment

HS are ubiquitous components in the environment and can be readily isolated from nearly all soils, waters, and sediments. They can account for as much as 10% by weight of the total content of many soils and sediments. In all environments, HS are formed from the decomposition of plant, animal, and microbial cells and tend to be more recalcitrant than the precursor materials. The functional groups of the HS, which determine the physical and chemical characteristics, vary and depend on the origin and age of the material. HS are composed of three basic components,

humins, humic acids (HA), and fulvic acids (FA). These components are traditionally defined according to their solubilities. Humins are the fraction which are insoluble at all pH values, humic acids are insoluble at acidic pH values below pH 2.0, and fulvic acids are soluble at all pH values. HS are thought to consist of a skeleton of alkyl/aromatic units cross-linked mainly by oxygen and nitrogen groups with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Livens, 1991; Schulten *et al.*, 1991). This structure allows HS to bind both hydrophobic and hydrophilic materials and thus they play an important role in the fate and transport of heavy metals and contaminating hydrocarbons in the environment (Buffle, 1984; Hesketh *et al.*, 1996; Moulin and Moulin, 1995; Nelson *et al.*, 1985; Perdue, 1989; Rebhun *et al.*, 1996; Stevenson, 1982b; Zhang *et al.*, 1996).

The overall structure of HS is believed to strongly influence their chemical and physical characteristics (Balnois and Wilkinson, 2002), however, the nature of this structure is still the subject of debate and the focus of several excellent recent reviews (Hayes and Clapp, 2001; Piccolo, 2001; Sutton and Sposito, 2005). Traditionally HS were considered to be macromolecular assemblies of relatively high-molecular-weight compounds while, in contrast, more recent studies suggest that HS are actually aggregations of organic molecules of relatively low molecular weight (Peuravuori, 2005 and references therein). This new view suggests that individual humic molecules may be significantly smaller than previously thought. Overall, it seems likely that individual humic molecules are in the size range 1000–2000 Da (Brown and Rice, 2000; Leenheer *et al.*, 2001; Piccolo and Spiteller, 2003; Stenson *et al.*, 2002).

B. Colloidal Nature of Humic Substances

The range of molecular sizes for the majority of humic and fulvic acid aggregates places them in the colloidal range (0.001–1.0 μm) when in aqueous solution (Gaffney *et al.*, 1996). Chemical and physical reactions are generally enhanced in colloidal systems due to the large surface areas of the colloidal particles. Humic colloidal molecules are thought to consist of coiled, long-chain, or three-dimensional cross-linked macromolecules with electrical charges variously distributed on the particle (Cameron *et al.*, 1972; Chen and Schnitzer, 1976; Ghosh and Schnitzer, 1980; Orlov *et al.*, 1975; Stevenson, 1982a; Visser, 1964). The molecular conformation is characterized by concentration of the HS, pH, ionic strength, and redox state of the environment (Chen and Schnitzer, 1976; Coates *et al.*, 2001; Ghosh and Schnitzer, 1980;

Thieme and Niemeyer, 1997), each of which affects the overall electrical charge distribution on the molecule. Recent studies with transmission electron microscopy have shown that with increasing pH or HS concentrations, an initial finely woven network of elongated fibers coalesce into a sheet-like structure, perforated by voids of varying dimensions which can trap or bind organic and inorganic components in the environment (Schnitzer, 1994). In addition, humic aggregates in solution are thought to be held together by weak intermolecular forces, such as hydrogen-bonding and hydrophobic interactions (Wershaw, 1986), which result in the formation of micelles (Guetzloff and Rice, 1994, 1996 and references therein). In the micellar structure, the hydrophilic ionized groups are located at the interface of the HS with the solution, whereas the hydrophobic portions of the molecule are directed toward the interior of the molecule (Piccolo *et al.*, 1996). Such a structure has been suggested as an explanation for the increased solubility of hydrophobic organic contaminants in the presence of HS (Wershaw *et al.*, 1969).

C. HS Formation

The exact mechanism of humic substance formation is still unknown and is not a major focus of this chapter. However, two main theories have been formulated that describe the genesis of humic substances. Traditionally, humic materials are considered to result from the oxidative modification of organic biopolymers such as lignin (Hatcher and Spiker, 1988; Waksman, 1932). Under this scheme, degradation and transformation of biopolymers first form humin, which is then further oxidized and fragmented to produce humic acid, and eventually fulvic acid (Steinberg, 2003 and references therein). A good early example of this view of HS formation is presented by Waksman's lignin-protein theory in which the genesis of HS is the result of the condensation of modified lignin polymers with protein residues. Several modifications on this theory have subsequently been presented, for example in Waksman, 1932.

Alternatively, more recent studies have proposed that HS form from the condensation of low-molecular-weight organic molecules, such as quinones, into larger structures (Steinberg, 2003; Tan, 2003; and references therein). A number of mechanisms have been proposed to be involved in this type of HS formation. In these theories, low-molecular-weight compounds generally condense to form fulvic acids and further condensations yield humic acids and humins (Stevenson, 1994). One of the prominent models for humic substance formation involves reactions between polyphenols, quinones, and nitrogen-containing

molecules such as amino sugars (Flaig, 1966; Flaig *et al.*, 1975; Kononova, 1966).

D. Metal and Radionuclide Binding and Transport

Humic and fulvic materials are extremely important in the mobilization of toxic metals and radionuclides in the environment. Due to size changes induced by conformational rearrangement and aggregation or dissociation arising from intermolecular hydrogen bonding, humic and fulvic acids form soluble complexes that can migrate long distances or precipitate, carrying bound cations with them. This depends on the metallic ion, the cation charge, the degree of ionization of the organic molecule, the ionic strength of the media, and the amount of metal bound (Choppin and Allard, 1985; Gaffney *et al.*, 1996). For this reason, the transport of these species in the form of humic or fulvic colloids has been emphasized in areas such as metal bioavailability (Buffle, 1984) and safety assessments of nuclear waste disposal facilities (Moulin and Ouzounian, 1992). Trace heavy metals in humate sediments were shown to be enriched by as much as 10,000-fold over concentrations in surface waters (Choppin, 1992). In addition, the concentration of dissolved heavy metal species in surface waters has been demonstrated to directly correlate with humic colloids (Nelson *et al.*, 1985). Due to the complex polyelectrolytic nature of the HS, traditional metal–ligand complexation modeling cannot be used to describe the nature of the metal binding (Rao *et al.*, 1994). Consequently, metal–humate complexation is generally discussed in the context of nonspecific, discrete or continuous multiligand models (Perdue and Lytle, 1983). Although a variety of the HS functional groups (e.g., carboxylic, phenolic, sulfur-containing, and nitrogen-containing groups) are involved in the metal-binding process, the carboxylate groups are considered to be primarily responsible for binding of metals and radionuclides (Buffle, 1984; Choppin and Allard, 1985; and references therein). Conformational changes in the humic structure are thought to have a direct effect on the metal-binding capacity of the HS and on the stability of the metal–humate complex (Coates *et al.*, 2001; Rao *et al.*, 1994; Rate *et al.*, 1993; and references therein). This is because metal binding to HS increases with a higher degree of ionization of the humic material (Coates *et al.*, 2001; Gaffney *et al.*, 1996). The increase in the ionization of the organic polyelectrolytes causes a greater intermolecular repulsion, which results in conformational changes and disaggregation or uncoiling of the polymer chain. The binding of the metal cations neutralizes these repulsive forces and promotes contraction of the polymer (Gaffney *et al.*, 1996).

The presence of high concentrations of metals relative to HS results in a compaction of the humic molecule with a resultant hydrophobic interior (Gaffney *et al.*, 1996 and references therein).

E. Hydrophobic Organic Binding and Transport

The association or binding of nonpolar, hydrophobic compounds to HS has been observed to increase the solubility and thus mobility of various pollutants (Carter and Suffet, 1982; Landrum *et al.*, 1984; Wershaw *et al.*, 1969). The binding of hydrophobic compounds by HS causes a decrease in the sorption of these compounds to sediments and suspended particles, a decrease in their bioavailability to aquatic organisms, a decrease in their volatility rate, a decrease in the rate of alkaline hydrolysis of certain pesticides, and an influence on the photochemistry of various hazardous chemicals in natural waters (Coates *et al.*, 2001; Gaffney *et al.*, 1996; MacCarthy and Suffet, 1989; and references therein). The mechanism by which HS bind hydrophobic compounds is not completely understood (Gaffney *et al.*, 1996). It has been hypothesized that the nonpolar groups in the humic molecule form a micellar or double-layer structure that traps the nonpolar organic in a microscopic hydrophobic environment similar to the behavior of surface-active micelles (Wershaw, 1986). In contrast, evidence exists for hydrogen bonds between HS and hydrophobic compounds (Senesi *et al.*, 1987). It is probable that both types of binding are important depending on the hydrophobic organic and humic materials involved (Gaffney *et al.*, 1996). The extent of this binding depends on the ionization of the humic materials, the ionic strength, and counterion concentration and is most pronounced for the least aqueous-soluble organic compounds (Carter and Suffet, 1982). For these reasons, the binding of nonpolar, hydrophobic compounds by humic materials is also considered to be sensitive to conformational changes within the humic molecule (Coates *et al.*, 2001; Gaffney *et al.*, 1996).

F. Redox Reactions of HS

Humic materials have a relatively high number of free radicals (Gaffney *et al.*, 1996). These radicals are generated by pH changes, chemical reduction, or solar irradiation (Senesi and Schnitzer, 1978). These free radicals play an important role in polymerization and redox reactions of humic materials (Gaffney *et al.*, 1996). HS can reduce metals with estimated reduction potentials of 0.5–0.7 eV (Skogerboe

and Wilson, 1981) and have been demonstrated to transfer electrons from reduced inorganics (sulfide) and organics (ascorbic acid) to various heavy metals, nitroaromatics, and chlorinated solvents (Curtis and Reinhard, 1994; Dunnivant *et al.*, 1992; Kahn *et al.*, 1984; Perdue, 1989; Schindler *et al.*, 1976; Schwarzenbach *et al.*, 1990; Skogerboe and Wilson, 1981; Szilagyi, 1971). This can have a major effect on the migration and toxicity of many of these compounds. It is thought that the redox-reactive components of HS that are involved in these reactions are the quinone moieties (Curtis and Reinhard, 1994; Dunnivant *et al.*, 1992). As the complexity of the HS structure has made definitive redox studies difficult, the potential involvement of the quinone/hydroquinone redox couple in electron transfer has been investigated with model compounds, such as 2,6-anthraquinone disulfonate (AQDS) (Coates *et al.*, 1998, 2001, 2002; Curtis and Reinhard, 1994; Lovley *et al.*, 1996; Tratnyek and Macalady, 1989), which have similar redox properties to the quinone content of HS. However, the effects of other compounding characteristics of HS, such as hydrophobic interactions or cation chelation, will not be apparent with these compounds.

III. Interactions Between Humic Substances and Microorganisms

Microbial interactions with humic materials are dependent on the age and source of the complex organic carbon and the conditions of the environment. Although HS are considered to be recalcitrant in the absence of dissolved oxygen (Stevenson, 1994), several recent studies have demonstrated that these compounds play an important role in the microbial ecology of the natural environment.

A. Degradation of Humic Substances by Microbial Agents

Humic materials are slowly degraded by microbial populations in aerobic environments (Wetzel, 1992; Wetzel *et al.*, 1995; and references therein). White-rot fungi, renowned for their ability to degrade lignin (Hatakka, 2001), have been implicated in the transformation of natural and synthetic humic acids to lower molecular weight compounds and CO_2. Production of extracellular enzymes is likely involved in this process (Blondeau, 1989; Dehorter and Blondeau, 1992; Dehorter *et al.*, 1992; Hofrichter and Fritsche, 1997; Hurst *et al.*, 1963; Willmann and Fakoussa, 1997). In a recent study, the basidiomycete *Collybia dryophila* K209 was shown capable of degrading natural humic acids to lower molecular weight fulvic acids components. This organism also

produced $^{14}CO_2$ when incubated with synthetic ^{14}C-labeled HA in liquid culture media, resulting in almost 50% mineralization of the HA in 42 days when media was supplemented with 200 μM $MnCl_2$ (Steffen *et al.*, 2002). Likewise, prokaryotic microbial populations have been shown capable of degrading humic matter, especially under aerobic conditions (deHaan, 1974; Sederholm *et al.*, 1973; Wetzel, 1992), and decomposition rates of 1–5% per day have been observed for complex dissolved organic matter (Wetzel, 1984). In aerobic liquid nutrient broth, pure cultures of *Pseudomonas fluorescens*, as well as microbial consortia from environmental samples, were capable of degrading/transforming a mixture of HA and FA (Hertkorn *et al.*, 2002). Exposure of HA and FA to UV radiation from normal sunlight significantly enhanced this catabolic process. This is because the absorption of UV rays by organic macromolecules, such as humic and fulvic acids, can result in the production of biologically labile organic compounds via photolytic decomposition (Wetzel, 1992, 1993; Wetzel, *et al.*, 1995). The ability of HS to be degraded under various conditions indicates they may be sources for carbon, nitrogen, or sulfur for microorganisms in many environments.

B. Oxidation–Reduction Reactions Between Bacteria and Humic Substances

HS contain a variety of potentially redox-active moieties including (hydro)quinone, sulfhydryl, carboxylic, and phenolic functional groups. Likewise, HS often contain tightly bound metals, such as iron or manganese, which may participate in oxidation–reduction reactions (Lovley and Blunt-Harris, 1999). Bacteria are known to exploit the redox-active nature of humic substances for microbial respiration. In this vein, HS have been shown to function as electron sinks for anaerobic respiratory bacteria and fermentative bacteria-stimulating mineralization of complex organic carbon compounds in the absence of O_2 (Benz *et al.*, 1998; Bradley *et al.*, 1998; Coates *et al.*, 1998). Electron spin resonance studies have confirmed that the quinone moieties are the redox-active components of the HS for these microbial reductive reactions (Scott *et al.*, 1998), and the HS have been shown to act as soluble electron carriers between microorganisms and insoluble terminal electron acceptors such as Fe(III) oxides (Bradley *et al.*, 1998; Lovley *et al.*, 1996). When the reduced HS interact with the Fe(III) oxides, they are reoxidized and can thus be recycled (Lovley *et al.*, 1996). In contrast, reduced humic substances have also been shown

to function as electron donors for microbial respiration of anaerobic electron acceptors such as NO_3^- (Coates *et al.*, 2002). A basic survey of oxidation–reduction reactions between HS and microorganisms is given in following section. A later section of this chapter will explain how these properties may be exploited for the purposes of bioremediation and environmental modeling.

C. HS as Electron Acceptors and Electron Shuttles

HS were first demonstrated to function as electron acceptors for microbial respiration during investigations of the mechanisms of iron reduction by the model organisms *Geobacter metallireducens* and *Shewanella alga* (Lovley *et al.*, 1996). In this study, HA as well as the quinone-containing compound, AQDS, were shown to stimulate reduction of poorly crystalline synthetic Fe(III). Subsequently, *G. metallireducens* was demonstrated to oxidize ^{14}C-acetate to $^{14}CO_2$ when humic substances were present as the sole electron acceptor in anaerobic, bicarbonate-buffered media. After 2 hours of incubation, the HS were removed from these anaerobic bottles and filtered to remove cells. Addition of the microbially treated HS to Fe(III) citrate led to production of Fe(II), whereas HS forgoing incubation with *G. metallireducens* was unable to produce significant Fe(II). These observations suggested a two-step model for microbial respiration of humic substances. In this model, bacteria first reduce the HS as an electron acceptor. The reduced HS are then capable of diffusing through media and abiotically reducing insoluble Fe(III), thus recycling the humic material to the oxidized state. The quinone-containing compound AQDS was found to mimic the effects of humic substances during microbial iron reduction. In this instance, *G. metallireducens* and *S. alga* reduced AQDS to its hydroquinone counterpart 2,6-anthrahydroquinone disulfonate (AHDS) during the experimental time period (Lovley *et al.*, 1996). These observations gave the first indication that the quinone moieties within HS may perform as the redox-active components during interactions with microorganisms. In subsequent research, other model quinones including lawsone, menadione, and anthraquinone-2-sulfonate were shown to similarly enhance reduction of poorly crystalline amorphous Fe(III) by *G. metallireducens* (Lovley *et al.*, 1998). To various degrees, AQDS and humic acids similarly enhanced both the rate and extent of reduction of various crystalline iron phases including goethite, hematite, and ferruginous smectite by *G. metallireducens* (Lovley *et al.*, 1998).

D. Quinone Moieties Act as Electron Acceptors in HS Reduction

Due to their complex nature, HS contain many functional groups that could potentially function in redox reactions. Studies with quinone analogues suggested that microbial reduction of HS was primarily mediated by the quinone moieties. In fact, previous work on abiotic redox interaction of HS with organic contaminants had also highlighted the importance of (hydro)quinones functional groups (Curtis and Reinhard, 1994; Dunnivant *et al.*, 1992). However, the participation of quinone functional groups in reductive interactions between microorganisms and HS had yet to be experimentally determined. During reduction, quinones undergo a two-electron transfer to the hydroquinone form, generally proceeding through a semiquinone intermediate (Chambers, 1988; Clark, 1960; Meisel and Fessenden, 1976). This chemical property was exploited by Scott and coworkers to demonstrate that the quinone functional groups of HS behaved as microbially available electron acceptors. In this study, electron spin resonance measurements indicated that microbial reduction of HS led to an increase of semiquinone radicals within the HS itself, and also that a positive correlation existed between the ability of an HS to function as an electron acceptor and its overall semiquinone content (Scott *et al.*, 1998), supporting the suggestion that quinone groups were the primary electron-accepting moieties for HS-reducing bacteria.

In addition to quinone functional groups, HS are known to contain complexed iron species, which could also potentially explain the ability of Fe(III)-reducing bacteria to use HS as an electron acceptor. Previous studies demonstrated that humic-bound Fe(III) was preferentially reduced as an electron acceptor by *G. metallireducens* with acetate as the electron donor; however, the electron-accepting capacity of the HS far outstripped the Fe(III) concentrations, and Fe(II) generated from bound Fe(III) did not function as an electron-shuttling agent to added insoluble iron oxides (Lovley and Blunt-Harris, 1999). Likewise, natural HS with undetectable Fe(III) concentrations also served as functional electron acceptors for *G. metallireducens* further indicating that bound Fe(III) plays a minimal role in the electron-accepting capacity of microbially reducible HS (Lovley and Blunt-Harris, 1999).

Importantly, chemical reactions between HS and strong oxidants, such as I_2, indicate that (hydro)quinones are not the sole significant redox-active compound in HS, although such data may not hold true for microbially mediated redox reactions (Struyk and Sposito, 2001). Quinones do not represent the only functional groups within HS that may be capable of microbial reduction or oxidation. In fact, bacteria

have been shown to reduce other nonquinoid organic molecules. Microorganisms, such as *Shewanella oneidensis* and *Escherichia coli*, have been shown to use various phenazines as electron acceptors or shuttles (Hernandez *et al.*, 2004; McKinlay and Zeikus, 2004). These functional groups could theoretically exist as a component or bound entity of any given humic molecule and may participate to some degree in its redox properties. However, it is generally agreed that quinones and their hydroquinone counterparts are the major contributors to redox interaction between HS and microorganisms. This concept is circumstantially supported by the success of model quinone compounds, such as AQDS, in predictably imitating redox behavior of HS and reliably being used to isolate confirmed HS-reducing organisms from the environment (Coates *et al.*, 1998).

E. A Survey of Microorganisms Capable of Reducing Humic Substances

Many organisms have been shown capable of reducing HS or model quinones. Generally, HS are shown to act as terminal electron acceptors or electron shuttles between an organism and a more electropositive insoluble electron acceptor. In many senses, the differences between an HS electron acceptor and an HS electron shuttle are nominal and will largely be considered together in this chapter. However, HS and quinones have also been shown to interact in a redox fashion with fermenting organisms in what must, by definition, be a nonrespiratory process. A brief, nonexhaustive survey of bacteria known to reduce HS or quinones is described later.

Both HS and model quinones have been shown to function as terminal electron acceptors for microorganisms under anaerobic conditions in the absence of any ''shuttling'' activity. For example, *Desulfobulbus propionicus* could grow by oxidizing pyruvate, propionate, lactate, and hydrogen to the reduction of AQDS (Holmes *et al.*, 2004). In one study, a number of Geobacteraceae isolates were enriched from sediment sources by coupling acetate oxidation to AQDS reduction (Coates *et al.*, 1998). These organisms were also able to grow via reduction of purified soil humic acids and Fe(III). In fact, the connection between AQDS, HS, and Fe(III) reduction appears to be relatively intimate. One early study screened pure cultures of various Fe(III)-reducing bacteria and found that all organisms capable of AQDS reduction reduced HS (Lovley *et al.*, 1998). Likewise, organisms which could reduce Fe(III) could reduce AQDS, while organisms incapable of Fe(III) reduction could not. However, many organisms not traditionally associated with

Fe(III) reduction are capable of respiring quinones. For example, a member of the Enterobacteriaceae, *Pantoea agglomerans* SP1, is a facultative anaerobe that can grow by coupling hydrogen or acetate oxidation to AQDS reduction (Francis *et al.*, 2000). In addition, several organisms including halorespiring and sulfate reducing bacteria as well as methanogenic archaea, have been shown capable of HS reduction (Cervantes *et al.*, 2002).

Iron reduction is commonly considered a geochemically important microbial metabolism, and the connection between Fe(III) reduction and quinone-based electron shuttles is well documented (Hernandez and Newman, 2001; Luu *et al.*, 2003). Pure cultures of many different bacterial species are capable of utilizing HS or quinones as an electron shuttle to increase the rate or extent of insoluble Fe(III) mineral respiration. For example, *Shewanella putrefaciens* CN32 could improve both the rate and extent of Fe(III) reduction with AQDS and many other terrestrial and aquatic organic matters (Dong *et al.*, 2003; Kukkadapu *et al.*, 2001). Likewise, both the radiation-resistant *Deinococcus radiodurans* and the chlororespiring *Anaeromyxobacter dehalogenans* could more efficiently reduce Fe(III) oxides in the presence of AQDS (Fredrickson *et al.*, 2000; He and Sanford, 2003). A *Thermus* isolate, whose optimal temperature for growth is approximately 65 °C, was greatly enhanced in growth and Fe(III)-reducing ability in the presence of AQDS (Kieft, 1999).

The ability of AQDS and HS to act as electron shuttles between microorganisms and Fe(III) minerals is not limited to Gram-negative Eubacteria. The Gram-positive *Desulfitobacterium dehalogenans* could similarly reduce both AQDS and HS in washed cell suspensions, which could then theoretically shuttle electrons to Fe(III) species (Lovley *et al.*, 1998). In addition, several members of the domain Archaea are capable of similar metabolisms. *Methanosarcina barkeri* MS and *Methanococcus voltaie* A3 were able to increase their reduction of Fe(III) with small additions of AQDS (Bond and Lovley, 2002). In the same study, additional methanogens tested were shown capable of AQDS reduction. The nonmethanogenic hyperthermophilic archaea, *Pyrodictium islandicum,* also displayed AQDS-stimulated reduction of poorly crystalline Fe(III) oxide, goethite, and hematite; while several other hyperthermophilic and thermophilic archaea could reduce AQDS with hydrogen or lactate as suitable electron donors (Lovley *et al.*, 2000).

It also appears that the ability to reduce AQDS, and potentially HS, is not restricted to respiratory microorganisms. Both *Propionibacterium freudenreichii* and *Lactococcus lactis* shifted their fermentations to

more oxidized endproducts in the presence of HS (Benz *et al.*, 1998). When *P. freudenreichii* ferments lactate, it forms propionate and acetate in a 2:1 ratio. In the presence of Fe(III) alone, this fermentation pattern does not shift, and no Fe(II) is produced. However, in the presence of HS and Fe(III), a lactate fermentation by *P. freudenreichii* produces twice as much acetate as propionate. Further, Fe(II) is produced during the course of the fermentation. This illustrates that at least some fermentative bacteria can exploit extracellular quinones to shed reducing equivalents and shift fermentation balances.

F. Humic Substances as Electron Donors

Humic substances have been shown to chemically reduce various environmentally relevant chemical species, indicating that HS in a reduced valence state can act as reductants. For example, reduced soil fulvic acids were shown capable of reducing Hg(II) to Hg(0), Fe(III) to Fe(II), and V(V) to V(IV) (Skogerboe and Wilson, 1981; Wilson and Weber, 1979). In a similar fashion, HS are also capable of transferring electrons from reduced organic or inorganic species to heavy metals, nitroaromatics, and chlorinated solvents (Alberts *et al.*, 1974; Curtis and Reinhard, 1994; Dunnivant *et al.*, 1992; Kahn *et al.*, 1984; Perdue, 1989; Schindler *et al.*, 1976; Schwarzenbach *et al.*, 1990; Skogerboe and Wilson, 1981; Szilagyi, 1971). The ability of humic substances to act as reducing agents is not limited to abiotic chemical reactions. Microorganisms are also capable of using reduced HS as electron donors for anaerobic respiration and carbon assimilation. This capability has also been illustrated with HS analogues, such as AHDS, the reduced counterpart of the quinone AQDS. An initial study investigating AHDS as a functional electron donor found several organisms, including *G. metallireducens*, *Geobacter sulfurreducens*, *S. alga*, *Geothrix fermentans*, and *Wolinella succinogenes*, could oxidize AHDS coupled to reduction of nitrate or fumarate in cell suspensions (Lovley *et al.*, 1999). While the aforementioned organisms were also known to reduce AQDS and/or HS; *Paracoccus denitrificans*, unable to reduce Fe (III), AQDS, or HS, could also oxidize AHDS or reduced HS coupled to nirate reduction in cell suspensions. *S. alga* was similarly found capable of oxidizing reduced Suwannee river humic acids coupled to reduction of fumarate. Electron spin resonance was utilized in this study to again implicate the (hydro)quinone moieties of HS as agents involved in humic–microbe redox reactions. Although a number of the organisms evaluated in this study did not appear to grow by metabolisms relying on AHDS as an electron donor, both

G. sulfurreducens and *W. succinogenes* could be repeatedly transferred in media containing AHDS and electron acceptor, with amino acids and acetate (respectively) presumably fulfilling the need for cell carbon. *S. alga* cell numbers increased approximately fivefold in the presence of AHDS and fumarate, but were unable to grow in controls lacking either electron donor or acceptor (Lovley *et al.*, 1999).

Perchlorate-reducing microorganisms of the *Dechloromonas* genus have also been shown capable of oxidizing AHDS coupled to nitrate or perchlorate reduction (Bruce *et al.*, 1999; Coates *et al.*, 1999, 2001, 2002). For example, *D. agitata* is capable of growing by this metabolism if 0.1 mM acetate is provided as a carbon source. This organism is not capable of either dissimilatory Fe(III) reduction, or of reduction of oxidized HS. Subsequent most probable number (MPN) enumeration studies found that the ability to grow by oxidizing AHDS coupled to nitrate reduction was environmentally prevalent (Coates *et al.*, 2002). MPN measurements indicated that bacteria capable of AHDS oxidation coupled to nitrate reduction existed in pristine and hydrocarbon-contaminated sites from both freshwater and marine systems. Concentrations of AHDS-oxidizing organism ranged between 1.33×10^1 cells/g for sediment from a hydrocarbon-contaminated aquifer to 4.17×10^6 cells/g for ''pristine'' lake sediment. Enrichments containing AHDS, nitrate, and 0.1 mM acetate were performed in this study and led to the isolation of several bacteria, including representatives from the alpha, beta, gamma, and delta subgroups of the proteobacteria. All of these isolates were able to oxidize reduced natural HS coupled to nitrate reduction. Importantly, these isolates were tested for their ability to degrade the model anthraquinone used to isolate them and were found incapable of this metabolism.

Overall, this study highlights the prevalence, as well as the phylogenetic diversity, of bacteria capable of anaerobically oxidizing hydroquinones within humic substances or model quinoid compounds. A recent study added to the catalog of bacteria known to oxidize hydroquinones through anaerobic respiration. In this investigation, several species of the chlororespiring *Desulfitobacterium* and *Sulfurospirillum* genera were shown capable of oxidizing AHDS with nitrate, tetrachloroethene (PCE), and 3-chloro-4-hydroxyphenylacetate as electron acceptors (Luijten *et al.*, 2004). Although many of these organisms did not grow by these metabolisms, *Desulfitobacterium hafniense* DP7, *Sulfurospirillum deleyianum*, *Sulfurosprillum barnesii* SES3, and *Sulfurosprillum arsenophilum* could be maintained for at least two transfers in media containing AHDS and the relevant electron acceptor.

The phenomenon of microbial oxidation of extracellular hydroquinones is both tantalizing and limited. In such a life strategy, the organisms obtain carbon from readily degradable limited sources, such as acetate, and simply use the reduced HS as an energy source. Such a metabolism gives these organisms a potential competitive advantage over other heterotrophs in the environment that may require a limited organic compound like acetate as both a carbon and energy source, thus requiring significantly greater concentrations for growth (Coates *et al.*, 2001). It appears that the ability to perform this metabolism is environmentally prevalent and that a vast phylogenetic diversity of bacteria can perform the metabolism under experimental conditions. This metabolism may be considered even more environmentally relevant considering recent reports that find HS throughout sediment depths retain some reducing capacity, even in oxic zones. This observation suggests that in many environments, reduced HS present may constitute a previously unrecognized electron-donating capacity supporting a microbial ecology and thus likely influencing nutrient availability and geochemical cycling of any particular system.

G. Adsorption of Bacteria to HS

Previous studies have indicated that microbial cells, such as *Bacillus subtilis*, may be adsorbed by HS (Maurice *et al.*, 2004). The adsorption behavior of *B. subtilis* to HS was shown to be pH dependent with the greatest interaction occurring at a pH where both bacterial cells and HS were expected to exhibit a net negative charge (Fein *et al.*, 1999). This implies that the adsorption behavior is due to hydrophobic rather than electrostatic interactions. The hydrophobic nature of humic–bacterial binding is supported by another study indicating that Cd metal ions, suspected to largely interact electrostatically with carboxyl or phenolic groups of HS, did not impact adsorption of humic acid to *B. subtilis* (Wightman and Fein, 2001). In contrast, this last study did observe an inhibition of Cd adsorption onto the surface of *B. subtilis* in the presence of humic acid. This last point is of particular interest, as humic material in various redox states is known to alter toxicity of metals including Cd and Cr to bacteria such as *E. coli* (Coates *et al.*, 2001). In more general terms, the ability of a microorganism to adsorb to HS bears implications for how processes, such as HS degradation or HS-based respiration, proceed with organisms known to exhibit such characteristics. Likewise, these data suggest that HS may impact the bioavailability of organic and inorganic pollutants to microorganisms by binding the bacteria as well as particular pollutant. Natural organic

matter, such as HS, may also help dictate the transport of bacteria through aquifer systems, likely due to adsorptive reactions between organic matter, minerals, and bacteria (Scholl and Harvey, 1992).

H. Complexation of Enzymes by HS

Humic substances have been shown to form complexes with various extracellular and membrane-bound enzymes, with a variety of consequences for enzyme activity and stability (Boyd and Mortland, 1982; Ladd and Butler, 1975; Ruggiero and Radogna, 1988; Skujins, 1976). HS can cause inactivation of microbial extracellular enzymatic activity by binding of proteins to organic acids, which can result in the aggregation, complexation, and precipitation of the humic–protein complex (Wetzel, 1993 and references therein). Enzymic inactivation is noncompetitive and can be either partial or complete, for example HS released from decaying *Juncus effuses* were observed to reduce the activity of surface-bound β-glucoside, α-glucosidase, and alkaline phosphatase in suspended bacterial cultures from a mixed-species biofilm (Espeland and Wetzel, 2001). In general, the humic–enzyme complex is moderately stable and can be maintained in an inactivated state for extended periods and subsequently reactivated by exposure to mild UV photolysis (Wetzel, 1993).

In contrast, HS may offer photolytic protection to some enzymes. For example, the activity of commercially purified α-glucosidase was tested as a function of time in the presence of photolytic agents UV-A, UV-B, and photosynthetically active radiation (PAR) (Espeland and Wetzel, 2001). In this case, α-glucosidase activity was retained in the presence of humic material, but rapidly dropped off when HS were absent, suggesting that HS protected the enzyme from photolysis. Even in the dark, α-glucosidase activity was better maintained over approximately 100 min in the presence of HS than in the absence of HS, suggesting that HS may either stabilize the enzyme activity or prevent enzymes from interacting with other assay materials that could inactivate them. Another recent study investigated the activity of purified alkaline phosphotase when complexed and immobilized with soil humates. In this study, the humate–enzyme complex retained 57–88% of the activity of the unbound enzyme itself, and K_m values for free and bound enzymes were remarkably similar (Pilar, 2003). However, activity of alkaline phosphatase was altered somewhat, and the effects of pH and temperature were different between humate-bound and unbound enzymes.

The ability of humic matter to impact the stability and activity of both extracellular and membrane-bound enzymes has profound implications for how microorganisms and HS interact in environmental situations. For example, α-glucosidase and β-glucosidase are involved in the environmentally important processes of hydrolyzing the glycosidic bounds of starch and cellulose, while alkaline phosphatases are responsible for hydrolyzing organic phosphorous compounds. If the activity of enzymes with such important ecological roles is significantly impacted by HS, then HS again play a significant de facto role in regulating microbial activity in natural systems. As such, interactions between enzymes and HS are also important factors to consider in any application potentially involving both microorganisms and humic material.

I. AQDS and Microbial Taxis

A recent report indicated that *S. oneidensis* MR-1 was capable of recognizing gradients of AQDS and taxed toward the potential electron acceptor (Bencharit and Ward, 2005). This phenomenon was noted in both ''plug-in-pond'' assays, as well as in swarm plate assays. Unfortunately, the taxis behavior was not shown for humic substances, and it was not illustrated whether *S. oneidensis* moves toward AQDS, or perhaps away from the reduced form of the quinone, AHDS. In addition, it was not noted if the tactic behavior was true chemotaxis or the phenomenon commonly known as ''energy taxis.'' Regardless, these results are interesting and suggest that microorganisms may be well adapted to respond to extracellular electron shuttles in their environment.

IV. The Biochemistry of Microbial HS Reduction and Oxidation

One of the more important interactions between HS and microorganisms is the ability of bacteria to use HS as either an electron donor or electron acceptor in the process of respiration. Unfortunately, the genetic and biochemical basis for these respiratory processes is largely unknown. However, investigation of the microbial respiration of insoluble electron acceptors, such as Fe(III) (hydro)oxides, has yielded some mechanistic data relevant to humic substance respiration. In contrast, very little is known about the oxidation of reduced HS as a source of energy and reducing equivalents for microbial metabolism and carbon assimilation. It is important to note that much of the existing data concerning the biochemistry of HS respiration have been elucidated

when using AQDS as a model for humic material. The suitability of this model in terms of redox characteristics appears reasonable based on previous studies (Coates *et al.*, 2002; Lovley *et al.*, 1999). However, it is possible that AQDS interacts with model organisms differently than HS in other ways. For example, the effects of other compounding characteristics of HS, such as hydrophobic interactions or cation chelation, will not be apparent with these compounds. Furthermore, AQDS is a significantly smaller molecule compared to the average humic molecule. This is especially important, given that results of some studies imply that AQDS enters the periplasmic space of Gram-negative bacteria, a characteristic not expected to be shared by humic substances (Shyu *et al.*, 2002). Likewise, bacteria may or may not bind to HS, while no evidence of AQDS sorption to bacterial surfaces has been presented. AQDS does not encompass the full battery of potentially redox-active components within any given HS molecule. Finally, AQDS is a soluble quinone-containing compound, while portions of any given HS may be soluble or insoluble. With these criticisms in mind, a brief description of the data available of the genetic/biochemical mechanisms of HS respiration is outlined in the following sections. These data are largely assimilated from work concerning either the mechanism of iron reduction, or expression of electron shuttles by bacteria; these processes are reviewed in depth elsewhere.

A. A Speculation: Relationships Between HS Respiration and Endogenous Electron Shuttles

Because of the ubiquitous nature of HS and other redox-active biomolecules (e.g., components of root exudates and certain antibiotics) in the environment, it is conceivable that bacteria evolved specific systems to exploit the redox activity of certain organic molecules without degrading them. In contrast, it is possible that respiration of HS is coincidental. Many microorganisms are known to produce extracellular compounds capable of shuttling electrons to insoluble electron acceptors such as Fe(III). Many of these endogenous shuttling compounds are described as quinones or phenazines and therefore may resemble redox-active functional groups in HS. For example, *S. oneidensis* MR-1 excretes a compound, likely a quinone, which appears to behave as an electron shuttle to inaccessible electron acceptors entrapped in alginate or glass beads (Lies *et al.*, 2005; Newman and Kolter, 2000). The organism *G. fermentans* was similarly found capable of excreting an electron-shuttling compound to access both Fe(III)

entrapped in alginate beads and insoluble electrodes (Bond and Lovley, 2005; Nevin and Lovley, 2002a). *Shewanella algae* strain BRY can access Fe(III) through micropores by producing an electron shuttle, indicated to be a form of melanin (Nevin and Lovley, 2002b; Turick *et al.*, 2002). Recently, the archaeon *Pyrobaculum aerophilum* was reported as capable of reducing poorly crystalline Fe(III) through dialysis tubing in the absence of obvious chelation activity (Feinberg and Holden, 2006). Addition of spent cell-free supernatant of *P. aerophilum* to new incubations allowed for Fe(III) reduction without a lag phase, and the authors proposed the existence of an electron-shuttling compound produced by this organism.

It is possible that the respiration of HS is based on the similarity of humic functional groups to the endogenous electron shuttles produced by the bacteria themselves. Of course, the reverse relationship may also be true. Regardless, the structural similarities between microbially produced electron shuttles and HS may imply a shared biochemistry between use of microbially produced shuttling compound and use of HS in microbial respiration.

A similar parallel may be drawn to microbial respiration of other prevalent organic molecules capable of participating in oxidation–reduction reactions. It was recently observed that phenazines produced by *Pseudomonas chlororaphis* PCL1391 allowed the organism to reduce insoluble Fe(III) in a manner not associated with increased growth (Hernandez *et al.*, 2004). The phenazines produced also allowed for hastened Fe(III) reduction by *S. oneidensis* MR-1, as shown with experiments using spent cell-free extracts from *P. chlororaphis* cultures (Hernandez *et al.*, 2004). No enhanced growth was observed on addition of the phenazine shuttle, likely because *S. oneidensis* is already capable of producing its own quinoid shuttle over time. Additionally, redox-active antibiotics including pyocyanin and bleomycin were also able to accelerate Fe(III) by *S. oneidensis* and a diversity of other gamma proteobacteria (Hernandez *et al.*, 2004).

B. Pathways of Quinone Reduction as a Respiratory Process

As investigators attempt to elucidate the nature of microbial respiration of insoluble electron acceptors, data have slowly accumulated concerning respiration of the model compound AQDS. An attempt to pool together data from a variety of sources yields some agreement on how gene products from a variety of model organisms accomplish AQDS reduction. Much of the work in the biochemistry of AQDS reduction was elucidated in the model organism *S. oneidensis* MR-1.

Various gene products known to be involved either directly or indirectly in electron transport are responsible for transferring electrons to AQDS during AQDS reduction. One of the first components of the electron transport chain shown to be involved in AQDS reduction is menaquinone, which is located in the cytoplasmic membrane of *S. oneidensis*. Menaquinone is required for electron transport to a variety of electron acceptors, including fumarate, nitrate, DMSO, Fe(III), and thiosulfate (Myers and Myers, 2004). Transposon-generated and acridine orange-generated mutants deficient in menaquinone synthesis have been shown incapable of respiring AQDS as well as both soluble and insoluble electron acceptors requiring menaquinone (Myers and Myers, 2004; Newman and Kolter, 2000). In one study, menaquinone mutants were also shown incapable of reducing humic acids (Newman and Kolter, 2000). This was the first explicit example that the pathways for AQDS and HS reduction are at least partially shared in *S. oneidensis* and suggests that AQDS may be a good HS analogue in biochemical/genetic as well as physiological studies.

Similar type studies indicated that *S. oneidensis* strains deficient in outer membrane cytochrome content were also impaired for AQDS reduction activity, suggesting that outer membrane components are also involved in the reductive metabolism of AQDS. For example, mutations in the *omcB* gene (outer membrane cytochrome B) were significantly impaired in both rate and extent of AQDS reduction under growth conditions (Lies *et al.*, 2005). More drastic inhibition of AQDS reduction was seen with MR-1 mutants for *cymA* (a tetraheme cytochrome C) and *mtrB* (required for proper cytochrome localization in inner and outer membranes) (Lies *et al.*, 2005; Myers and Myers, 2000, 2002). A similar pattern of results was observed with the mutant strains respiring insoluble Fe(III) (hydr)oxides. These results suggest that a significant portion, although perhaps not all, of the reduction of AQDS by *S. oneidensis* runs through outer membrane components of the respiratory chain. These results identify specific midpoints along the path of electrons from the initial oxidation of an electron donor to the final reduction of AQDS to AHDS. However, the terminal cell component involved in the final AQDS reduction step has escaped unequivocal identification.

C. Regulation of AQDS Reduction in the Presence of Alternative Electron Acceptors

Little is known about the genetic or posttranscriptional regulation of AQDS reduction activity in cells capable of the metabolism. There

is at least one report which suggests that the presence of nitrate may be sufficient to inhibit reduction of AQDS by *S. putrefaciens* DK-1, which is perhaps unsurprising given the thermodynamic favorability of nitrate reduction compared to AQDS reduction (Lee *et al.*, 2000). However, this observation does demonstrate that only certain cellular components under certain conditions will respire quinones. Other investigations have noted that methanogenic communities rapidly respond to the presence of AQDS and shift away from methanogenesis and toward AQDS-based respiration (Cervantes *et al.*, 2000). However, this metabolic shift was perhaps associated with a change in the methanogenic community structure and may not speak for the regulation of methanogenesis and AQDS reduction for a given microbial species.

D. Hydroquinone Oxidation: A Black Box

Although limited data exist on the biochemical mechanism of quinone reduction, essentially no data exist concerning the microbial oxidation of extracellular hydroquinones coupled to respiratory processes. A recent paper concerning Fe(III) reduction by the archaeon *P. aerophilum* indicates that whole cell extracts of the archaea grown on Fe(III) citrate contain AHDS-oxidizing activity, while extracts of nitrate-grown cells have minimal ability to oxidize AHDS (Feinberg and Holden, 2006). This suggests that certain cellular components capable of oxidizing AHDS must be induced by specific conditions. The vast majority of AHDS-oxidizing activity of Fe(III)-grown cells was located in the membrane, rather than cytoplasmic, fraction of cell extracts. The experimental assay in this study did not include a clear electron acceptor, apart from cellular components. Thus, it seems reasonable to assume that the major electron acceptor for AHDS oxidation in these assays was a component of the membrane fraction in Fe(III)-grown cells. It was further illustrated that AHDS-oxidizing activity in the membrane fraction of *P. aerophilum* cell extracts was sensitive to both temperature and trypsin digest, suggesting the activity may be either directly enzymatic or indirectly associated with the protein complement of *P. aerophilum* membranes. It is possible that the AHDS-oxidizing activity of cell membranes in this study is incidental, having more to do with the cytochrome complement of Fe(III)-grown cells than with a dedication of the cell membrane to oxidize extracellular hydroquinones. This seems likely, given that during growth on Fe(III), one would expect a cell to be primed to reduce a quinone as part of an electron-shuttling process rather than oxidize a hydroquinone, especially in the presence of sufficient electron donor. Unfortunately, further work concerning

the nature of hydroquinone oxidation by both archaea and bacteria is required to elucidate the biochemical nature of the metabolism.

E. Potential Toxicity of AQDS

A number of antibiotics are thought to be toxic by virtue of their ability to generate reactive oxygen species through oxidation/reduction reactions. The compound AQDS shows remarkable structural similarity to many redox-active organic compounds commonly recognized for their antimicrobial activity. In an attempt to address the mechanism of AQDS reduction, previous studies used a transposon system to generate *S. oneidensis* mutants defective in the ability to reduce AQDS (Shyu *et al.*, 2002). One of these mutants, also unable to reduce commercially produced humic acids, was found to have a mutation in a gene encoding a putative *tolC* efflux pump (Shyu *et al.*, 2002). This mutation led to a sensitivity of the organism to 10 mM concentrations of AQDS, indicative of a toxic effect. This study also indicated that 5 mM AQDS concentrations were sufficient to upregulate expression of the *tolC* gene in *S. oneidensis.* It was proposed that the lack of AQDS-reducing activity in the *tolc* mutant was due to AQDS toxicity, although it is interesting to note that this mutant was also defective in the ability to reduce HS, which are presumably too large to enter the cell. It seems possible in light of the new supramolecular view on the humic substance structure (Sutton and Sposito, 2005) that small humic molecules could diffuse through a cell's outer membrane and result in similar toxic effects as those observed with AQDS. However, this interpretation is speculative; it is currently unknown why a *tolc* mutant would be defective in humics-respiring capabilities. It is noteworthy that humic substances are not generally considered toxic to microorganisms.

V. Microbes, Contaminants, and HS: Remediation Promise, Remediation Concerns

Many areas around the world are contaminated with a variety of heavy metals and radionuclides. The behavior of these contaminants in environmental systems can be heavily influenced by both microbial activity and the presence and nature of dissolved organic matter, such as HS. At the outset, understanding possible interactions between microorgansisms, HS, and metal contaminants is important for modeling how these contaminants behave in the environment.

The influence of HS and microorganisms on the environmental behavior of metal contaminants is also of primary importance when searching for appropriate waste disposal sites for particular contaminants. Further, HS and microbial metabolisms have been suggested for use in the remediation of metals in a number of studies. Later, we review how bioremediation strategies for metal contaminant removal may involve both microorganisms and humic substances.

A. Immobilization of Metals

Strategies for the *in situ* remediation of metal contaminants in the environment often involve immobilization of these metals in the environment. Metal immobilization can occur through complexation reactions with a fixed substrate, through chemical transformation of the metal to a more insoluble state, or a combination of these two factors. Once metal contaminants are immobilized, initial spread of contamination is halted and excavation efforts can more efficiently remove the contaminant for proper disposal. Both HS and microorganisms are known to effect the mobility of metals in the environment through either solubilization or precipitation reactions and the nature of the interaction is a function of the pH, ionic strength, redox state, and the ratio of the metal and HS concentrations (Choppin and Allard, 1985; Gaffney *et al.*, 1996). As outlined earlier, HS are able to form complexes with metal cations through interactions that have been extensively reviewed elsewhere (Bradl, 2004; Jones and Bryan, 1998; Livens, 1991; Tipping, 2002). Although there is evidence for multiple binding interactions between HS and cations, carboxylic acid groups in humic molecules are considered particularly important in binding interactions (Stevenson, 1994). Observed binding of metal contaminants by HS has led to speculation that HS could serve as a permeable reactive barrier for the immobilization and eventual removal of metals from groundwater systems (Scherer *et al.*, 2000). In such strategies, HS are produced or extracted in bulk and form a fixed permeable barrier continually loaded with contaminant as the mobile phase passes through the barrier. Along with complexation reactions, reactive barriers made with HS may also subject metal contaminants to oxidation/reduction reactions. Complexation of metals to HS is often associated with reduced bioavailability and/or toxicity, as has been shown for Cu, Ni, Cr, Cd, Pb, Al, and Ni (Gu *et al.*, 2005; Lores *et al.*, 1999; Pandey *et al.*, 2003; Shanmukhappa and Neelakantan, 1990), although these results often vary between studies. Occasionally, contradictions arise. For example, some studies indicate increased toxicity of certain metals in the presence of HS, as

was observed in studies performed with Pb (Tsiridis *et al.*, 2006). It is important to note that although HS are often described as agents promoting the immobilization of metals in the environment, they may also promote the mobility of certain metal contaminants. How HS impact the mobility of a given contaminant will ultimately depend on the chemical characteristics of the contaminant, the chemical characteristics of the humic material, the respective concentrations of these components, and the geochemical characteristics of the surrounding environment. The redox state of the HS present may also be important. As previously outlined, reduced HS have a significantly increased metal-binding capacity relative to oxidized HS (Coates *et al.*, 2001).

Redox reactions can also affect the mobility and toxicity of a variety of metal species. In general, reduction of many metal contaminants appears to lower their mobility and/or toxicity under environmental conditions. For example, reduction of Tc(VII) to Tc(IV) species, such as $TcO_2 \bullet nH_2O$, will likely result in precipitation and immobilization of the radionuclide in sediments (Bondietti and Francis, 1979). Similarly, the reduction of U(VI) to U(IV) is associated with a greatly reduced solubility of the element (Langmuir, 1978). Cr(III) is considered to be less toxic and environmentally mobile than Cr(VI), due to reduced solubility and selective adsorption of Cr(III) cations to sediment components (Moore, 1990; Rai *et al.*, 1989; and references therein). It is also possible that chemical or microbial reduction of oxidized Se and Pb species may be an attractive remediation strategy for these metals (Lovley, 1995). In some cases, metal reduction can lead to enhanced solubility; for instance, plutonium(IV) oxyhydroxides are significantly more insoluble than Pu(III) (Kim and Kanellakopulos, 1989; Morse, 1986). However, for many priority metal contaminants, reductive reactions may play a useful role in preventing the spread of contamination as well as in eventual removal from the contaminated site.

Humic substances alone are capable of mitigating the spread of aqueous metal contaminants through reduction reactions. This fact is important in environmental model of contaminant transport, but has also been suggested as a basis for environmental remediation. For example, fulvic acids have been shown capable of reducing Cr(VI) to Cr(III) (Nakayasu *et al.*, 1999; Wittbrodt and Palmer, 1995), and studies indicate that after reduction, much of the Cr(III) produced remains complexed to humic materials (Fukushima *et al.*, 1997). Humic material has been shown capable of reducing Hg(II), Np(VI), Pu(VI), Np(V), and Pu(IV) (Nash *et al.*, 1981; Skogerboe and Wilson, 1981). In fact, HA and FA are likely to reduce many metals with reduction potentials higher than 0.5 eV. Much as humic substances or humic analogues

facilitate the reduction of Fe(III) by microorganisms, the presence of extracellular electron shuttles may hasten the reduction metal contaminants by microorganisms. As such, synergistic use of humic substances and their analogues may enhance the efficiency of bioremediation strategies centering on the reductive immobilization of toxic metals. HS and their analogues have been shown to increase rates of microbial reduction of toxic metals both directly (by acting as shuttles) and indirectly (via other means). For example, the radiation-resistant *D. radiodurans* R1 can directly reduce Cr(VI) with lactate as an electron donor in cell suspensions, but this activity is greatly enhanced by the presence of 0.1 mM AQDS, which was presumed to act as an electron shuttle to Cr(VI) (Fredrickson *et al.*, 2000). Although this organism was unable to reduce U(VI) and Tc(VII) directly, it could reduce about 95% of 100 μM concentrations of these metals within 21 days when 0.1 mM AQDS was added to cell suspensions. These results indicate that a small concentration of extracellular electron shuttle can greatly enhance the ability of an organism to reduce these particular metal contaminants. Likewise, these results illustrate that with an electron shuttle an organism can indirectly reduce metal contaminants through the abiotic reaction between the reduced shuttle and the oxidized metal. This may broaden the metabolic capabilities of a given organism, making it more useful for bioremediative purposes. Humic substances have been shown to enhance microbial reduction of metal contaminants in other studies as well. One study indicated that *S. putrefaciens* CN32 reduced less than 10% of 200 μM Cr(VI) with H_2 as an electron donor over a 28-hour incubation period (Gu and Chen, 2003). However, the presence of humic acid enhanced Cr(VI) reduction by the organism to about 50% in the same incubation period. This same study illustrated that in cell suspension containing lactate as an electron donor, U(VI) reduction by *S. putrefaciens* CN32 was stimulated in both rate and extent by the presence of natural organic matter, especially humic acids. This result is interesting, given that incubations of reduced humic acids and U(VI) did not lead to substantial U(VI) reduction in the absence of microbial activity, a phenomenon that has been observed in other studies as well (Nash *et al.*, 1981). However, other researchers have found that microbially reduced AQDS is able to abiotically react with and reduce U(VI) (Fredrickson *et al.*, 2000). Still, it is unconfirmed whether the ability of AQDS or HS to enhance U(VI) reduction is due to electron shuttling directly to U(VI).

Although electron shuttling to U(VI) remains unproven, HS have been shown to enhance U(VI) reduction through more indirect effects. For instance, humic acids mitigate the inhibition of U(VI) reduction

by Ca^{2+} and Ni^{2+} with *S. putrefaciens* CN32 in cell suspensions (Gu *et al.*, 2005). The authors hypothesized that beyond perhaps acting as an electron shuttle, humic acids complexed Ca^{2+} and Ni^{2+} and thereby prevented their inhibitory effects. However, this same study found evidence that interactions between HS and uranium formed soluble U(IV) complexes that were sensitive to reoxidation. Another indication that humic material can enhance the reduction of metals through indirect mechanisms was illustrated for the contaminant Tc and the organism *G. sulfurreducens* (Lloyd *et al.*, 2000). In this case, AQDS was not observed to be used by *G. sulfurreducens* as an electron shuttle to Tc(VII). Rather, AQDS enhanced rates of iron reduction in the presence of Fe(III) oxides, which allowed for formation of magnetite. Under these conditions, Tc(VII) reduction to insoluble TcO_2 was greatly enhanced. The authors of this study proposed that the high concentration of Fe(II) on the magnetite surface acted as an abiotic reductant for Tc(VII).

Both HS and microorganisms are able to accomplish the reduction of metal contaminants during a remediation scheme. However, these studies indicate that the reductive transformation of metals by microorganisms may be enhanced by humic substances or humic analogues. Likewise, the ability for humic material to bind metal contaminants and alter their mobility, toxicity, and bioavailabilty offer further benefits to a bioremediation strategy employing the use of HS. Beyond acting as redox shuttles for the reduction of metals, data indicate that the reduction of humic substances may improve the affinity of heavy metal binding by up to 15%. Likewise, the reduction of HS by microorganisms has been reported to decrease the toxicity of metals, such as chromium, to microorganisms (Coates *et al.*, 2001).

B. HS and Bacteria: Roles in Remediation of Organic Pollutants

Organic pollutants (xenobiotics) are a matter of environmental concern in many areas of the world. The presence of humic substances, as well as the activity of microorganisms are major determinants of the fate and transport of xenobiotics in the environment. A variety of mechanisms exist by which microorganisms and humic substances can synergistically interact to remediate organic pollutants (Field *et al.*, 2000).

Humic substances have been shown to participate directly in redox reactions with organic pollutants including chlorinated solvents and nitrobenzenes. Native humic material may contain a sufficiently low redox potential in specific functional groups to directly interact

with oxidized organic pollutants. Alternatively, HS can act as intermediate redox shuttles between strong inorganic reductants (such as sulfide) and the xenobiotic compound of interest. HS can also act as electron shuttles between microorganisms, which reduce HS, and various xenobiotics, which are in turn reduced by the humic shuttles. Reductive transformation of pollutants is important in that such modification may reduce or enhance toxicity of the pollutant. As such, these transformation are important to understand for proper modeling of contaminant behavior in the environment, as well as for possible remediative application.

C. Reduction of Nitrobenzenes

Many studies have implicated NOM as major players in the reduction of nitrobenzenes in environmental systems. In one study, 3-chloronitrobenzene was reduced to 3-chlorophenylhydroxylamine and 3-chloroaniline in the presence of NOM (66 mg C/L), with aqueous hydrogen sulfide as the bulk electron donor (Dunnivant *et al.*, 1992). Little transformation of 3-chloronitrobenzene was observed by hydrogen sulfide alone. Similar activity was illustrated for a variety of substituted nitrobenzenes in this study, and accelerated reduction by the hydrogen sulfide donor was observed in the presence of NOM in natural waters. The authors of this study suggested that quinone moieties in NOM were at least partially responsible for their observations. Later, low concentrations of the model naphthoquinones, juglone, and lawsone, were shown to shuttle electrons from hydrogen sulfide to a series of substituted nitrophenols and nitrobenzenes, supporting the idea that quinone motieties in HS could participate in these reactions (Schwarzenbach *et al.*, 1990). In anaerobic, abiotic systems the compound AQDS also was capable of reducing nitro groups of the pesticide methyl parathion to the corresponding amines (Tratnyek and Macalady, 1989). Although not directly proven, the ability of microorganisms to reduce HS will likely lead to the same stimulation of nitroaromatic reduction described earlier.

D. Reduction of Azo Dyes

Similar to the way both natural and synthetic quinone-containing compounds accelerate reduction of nitrobenzenes, these compounds also facilitate azo dye reduction. Such activity is important because reduction of azo dyes is integral to their degradation. However, these reductive interactions may also produce toxic intermediates before

complete mineralization occurs. Under anaerobic conditions, bacteria are capable of reducing azo dyes, reductively cleaving the azo bond, and producing aromatic amines (Chung and Stevens, 1993; Chung *et al.*, 1992). However, this activity is greatly accelerated in the presence of quinone-containing redox mediators (Brown, 1981). In recent reports, the addition of exogenous 1,2-naphthoquinone or 1,2-dihydroxynaphthalene increased both the rate and extent of reduction of the azo dye amaranth by the organism *Sphinomonas* sp. strain BN6 presumably by acting as electron-shuttling compounds (Keck *et al.*, 1997). Likewise, the rate of amaranth reduction by whole cells of *Sphinomonas* sp. strain BN6 was accelerated by 2-hydroxy-1, 4-naphthoquinone, AQDS, and 2-anthraquinonesulfonate in anaerobic cell suspensions (Kudlich *et al.*, 1997). Similar results were observed in microcosm studies using activated sludge collected from a sewage treatment plant amended with 2-anthraquinonesulfonate, indicating this ability is not limited to pure cultures of strain BN6 (Kudlich *et al.*, 1997). Batch experiments containing an unnoted concentration of granular sludge under growth conditions have also shown a dramatic increase in the rate of the azo dye Reactive Red 2 (RR2) reduction on addition of AQDS (van der Zee *et al.*, 2001). In this case, the rate of RR2 reduction was proportional to the concentration of AQDS, and a corresponding decrease in methanogenic activity by the sludge community was observed. In this study, an upflow anaerobic sludge blanket reactor was constructed to examine the applicability of microbial azo dye reduction for industrial-scale remediation. Under these conditions, 19 μM concentrations of AQDS led to approximately continuous decolorization of 87% of added RR2, indicating that redox shuttles may be an important part of a successful remediation strategy for this dye. It was hypothesized that oxidation of volatile fatty acids in the reactor led to AHDS production, which in turn reduced RR2. Likewise, it was observed that AQDS addition reduced the long-term toxic effects of RR2 to the methanogenic communities studied under these conditions.

E. Reductive Dehalogenation

Electron-shuttling compounds have been shown to abiotically catalyze dehalogenation reactions of various xenobiotics. For example, hexachloroethane (C_2Cl_6) is reduced to perchloroethylene (C_2Cl_4) when incubated with 500 μM AHDS at pH 7.2 and 50 °C (Curtis and Reinhard, 1994). AHDS was also found to reduce CCl_4 to $CHCl_3$ and $CHBr_3$ to an unidentified compound. Humic acids have also been demonstrated to be capable of reductively transforming C_2Cl_6 and CCl_4 in the presence of

electron donors such as HS^- and Fe^{2+} (Kappler and Haderlein, 2003). In these cases, transformation was more rapid in the presence of HA than with the electron donor alone (Curtis and Reinhard, 1994). HS have also been shown to chelate redox-reactive metals, thereby improving their ability to catalyze dechlorination reactions. For example, abiotic reduction of C_2HCl_3 to ethane by nickel with Ti(III) as an electron donor occurred with humic acids, but not to a significant degree in their absence (O'Loughlin *et al.*, 1999).

Under controlled conditions, electron shuttles have also been shown to hasten dechlorination reactions on reduction by microorganisms. An example of this phenomenon was shown recently for organisms maintained in anaerobic granular sludge. In batch experiments with acetate as an electron donor, the addition of AQDS greatly enhanced the rate and extent of CCl_4 reduction than in unamended or heat-killed controls (Cervantes *et al.*, 2004). AQDS also improved CCl_4 reduction with methanol, glucose, and H_2 as the electron donors. A humus-respiring enrichment culture from the anaerobic sludge used in this study was predominated by *Geobacter* species. This enrichment was unable to reduce CCl_4 in the absence of AQDS or HS. These studies highlight the fact that HA acting as electron shuttles may be capable of participating in chemical reactions that specific microorganisms themselves cannot necessarily catalyze alone. As such, electron shuttles may greatly broaden the scope of chemical reactions that a particular organism may be able to influence. In essence, any organism capable of reducing an electron shuttle can indirectly affect the redox state of any oxidized species that the shuttle can interaction with.

F. Humic Substances as Electron Acceptors for the Mineralization of Organic Contaminants

Microorganisms are the primary factors in the mineralization of organic pollutants in the environment (Alexander, 1994). As electron donor sources, the oxidation of organic contaminants in microbial respiration requires the presence of electron acceptors such as O_2, NO_3^-, Fe(III), SO_4^{2-}, or CO_2. Not only do HS and their analogues provide electron acceptors to a variety of microorganisms as previously described, they can also accelerate the use of insoluble electron acceptors such as Fe(III) (Lovley *et al.*, 1996). A variety of pollutants can be mineralized by bacteria utilizing HS or humic analogues as either lectron acceptors or electron shuttles. One of the first examples of stimulating pollutant mineralization with humic material was shown with the compound benzene (Lovley *et al.*, 1996). These studies

demonstrated that addition of humic acid greatly enhanced benzene degradation by an iron-reducing microbial community in aquatic sediments (Lovley *et al.*, 1996). These studies demonstrated that the stimulatory effect was due to the electron-shuttling capacity of HA rather than the ability to chelate insoluble Fe(III) (Lovley *et al.*, 1996). Subsequent studies have similarly demonstrated that anaerobic toluene (Cervantes *et al.*, 2001) or *o*-xylene (Jahn *et al.*, 2005) degradation by microbial communities in sediment microcosms was also stimulated when supplemented with humus or AQDS. HS or AQDS have also been shown to act as electron acceptors for the anaerobic microbial oxidation of phenol, *p*-cresol, vinyl chloride, and dichloroethene (Bradley *et al.*, 1998; Cervantes *et al.*, 2000). In the case of vinyl chloride, the addition of humic acids or AQDS allowed for significant (~91%) mineralization of the compound, converting the contaminant to CO_2 rather than partial degradation to ethane or ethene as is normally observed (Bradley *et al.*, 1998). In microcosms containing liquid media and sediment, minimal dichloroethene mineralization was observed, and vinyl chloride, ethane, and ethene intermediates were observed. However, microcosms amended with HS mineralized dichloroethene without accumulating detectable concentrations of these potential toxic intermediates (Bradley *et al.*, 1998).

Taken together, these studies suggest that the addition of HS or humic analogues may promote mineralization of organic pollutants in the environment, either by acting as electron shuttles or terminal electron acceptors. The possibility of use of HS for these purposes is bolstered by the fact that a small amount of HS can often greatly stimulate microbial metabolism. Likewise, the creation of HS with enhanced electron-accepting capacities through chemical modification has recently been described (Perminova *et al.*, 2005). Such engineered HS could potentially enhance remediation efforts involving humic material as electron acceptors or electron shuttles.

G. HS and Bioavailability of Pollutants for Degradation

Xenobiotics bind to HS through a variety of mechanisms, including van der Waals, ion-exchange, H-bonding, charge transfer, and hydrophobic interactions (Kahn *et al.*, 1984; Pignatello, 1989). HS binding of organic pollutants in soil is thought to significantly impact the fate and transport of these pollutants (Bollag *et al.*, 1992; Hayes and Clapp, 2001). Although the presence of HS has been shown to promote, inhibit, or have no effect on the mineralization of organic contaminants, a few studies give clear indication that HS can promote the

biodegradation of pollutants through sorptive or solubilizing interactions. For example, one study indicated that the presence of humic acid at 100 μg/ml promoted the aerobic biodegradation of phenanthrene by *P. fluorescens* (Ortega-Calvo and Saiz-Jimenez, 1998). Likewise, the presence of humic acid at 10 μg/ml and clay led to approximately 25% mineralization of 1 μg/ml phenanthrene within 150 hours, while controls without clay or humic acid mineralized only 10% of the contaminant. The authors of this study proposed that the soil components tested may sorb both bacteria and phenanthrene thus increasing the localized concentration of the contaminant to the bacteria, enhancing its bioavailability and rate of degradation. However, these conclusions were not explored experimentally. Subsequent reports indicated that specific microorganisms, including members of the *Burkholderia* and *Delftia* families, were capable of degrading humic acid-sorbed phenanthrene with approximately the same efficiency that they could degrade aqueous phenanthrene (Vacca *et al.*, 2005). The degradation rate of phenanthrene by these organisms exceeded the expected desorption rate of phenanthrene from the humic acid. These results indicate that at least some organisms should be capable of degrading organic pollutants sorbed to humic material. The activity of organisms isolated by their ability to degrade soluble phenanthrene was inhibited when the phenanthrene was sorbed onto HS (Vacca *et al.*, 2005). As such, microbial degradation of sorbed xenobiotics appears to be species specific.

HS are known to be capable of solubilizing large amounts of hydrophobic chemicals through formation of micelles (Guetzloff and Rice, 1994; Wershaw *et al.*, 1969; Yonebayashi and Hattori, 1987). Even though humic and soil-sorbed contaminants are considered somewhat bioavailable, the solubilization of soil-sorbed pollutants into the aqueous phase by HS is thought to increase the effectiveness of bioremedation efforts (Rouse *et al.*, 1994; Volkering *et al.*, 1998). Dissolved HS have been shown to increase the solubility of many hydrophobic contaminants in soil, including some polycyclic aromatic hydrocarbons (Conte *et al.*, 2001; Johnson and Amy, 1995) and some polychlorinated biphenyls (Fava and Piccolo, 2002). This solubilization effect may explain the enhancement of contaminant degradation in the presence of humic material under aerobic conditions. For example, the presence of HS has been shown to enhance both the rate and extent of aerobic biodegradation of naphthalene, 2-methyl-naphtalene, and pyrene in solid or slurry-phase bioreactors (Fava *et al.*, 2004). Similarly, introduction of large amounts of HS enhanced polychlorinated biphenyls recovery from the aqueous phase of soil microcosms, and increased their apparent aerobic degradation by the soil's native microbial population (Fava and

Piccolo, 2002). In microcosm studies, humic acids have also been shown to slightly increase the rate and extent of aerobic pyrene mineralization by native organisms in historically PAH-contaminated soil (Haderlein *et al.*, 2001).

Recent evidence suggests that the stimulation or retardation of an individual organic compound by a microorganism may be dependent on the humic substances concentrations (Bogan and Sullivan, 2003). Different pollutants, and different organisms, may have different responses to the addition of HS during a bioremediative strategy. These observations may represent an interplay between the various interactions that can take place between an organism, a contaminant, and humic substances. This effect can be further complicated by the effects of other environmental parameters such as pH, ionic strength, and redox state (Coates *et al.*, 2001).

VI. Conclusions

In summary, although the specifics depend on the situation at hand, it is clear that HS interact with microbial populations through a diversity of mechanisms. These interactions may potentially impact the microbial ecology of a specific environment and significantly affect fate and transport of organic and inorganic environmental pollutants. By considering these interactions, strategies for bioremediation of particular pollutants may be improved. Likewise, through a better appreciation of these potential interactions, the predictive behavior and longevity of pollutants within the environment may be better modeled resulting in improved design strategies for bioremediative processes.

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Significance of Microbial Interactions in the Mycorrhizosphere

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I. Introduction

The living roots of most plant species are colonized by hyphae of nonpathogenic fungi to form structures known as mycorrhizas (from Greek meaning ''fungus-root''; Frank, 1885). Mycorrhizas normally represent mutualistic interactions, the fungus receiving carbohydrate from its host

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plant, allowing it to form a mycelial network in the soil which assimilates nutrients (N, P, K, and some micronutrients) and water, a proportion of which is transferred directly to the host (Smith and Read, 1997).

There are several major types of mycorrhiza, which are classified according to morphology and the types of fungi involved. The dominant type of mycorrhiza in terms of plant species and distribution are the arbuscular mycorrhizas (AM), which form between fungi from the Glomeromycota and angiosperms, gymnosperms, pteridophytes, and bryophytes. Based on taxonomy, approximately 160 species of AM fungus have been described, although this is likely to be a considerable underestimate of actual diversity (Johnson *et al.*, 2005a; Schussler *et al.*, 2001). In AM associations, the fungus penetrates root cortical cells in which it proliferates and forms arbuscules through which materials are exchanged between the symbionts. The fungus spreads into the soil where it forms extraradical mycelial networks of varying size and structural complexity (Hart and Reader, 2002).

The ectomycorrhizal (ECM) association forms largely between basidiomycete and ascomycete fungi and woody perennials, particularly members of the Pinaceae, Betulaceae, Fagaceae, and Dipterocarpaceae (Smith and Read, 1997). Between 5000 and 6000 fungal species may form ectomycorrhizas (Smith and Read, 1997). Hyphae penetrate the root cortex, ramifying between cells to form a ''Hartig net'' through which the symbionts exchange materials. The fungus forms a mantle of hyphae which covers the root, and an extraradical mycelial network of varying complexity (Agerer, 2001). There are a number of other mycorrhizal types which are limited to specific plant families, including the Ericaceae and Orchidaceae, which will not be considered in the current chapter.

Mycorrhizal fungi convey a range of benefits to their host plant in addition to providing nutrients and water, including increased resistance to foliar-feeding insects (Gange and West, 1994) and soilborne pathogens (Whipps, 2004), and tolerance to salinity (Feng *et al.*, 2002) and heavy metals (Diaz *et al.*, 1996). While plant diversity and age can influence the structure and diversity of AM and ECM fungus communities (Johnson *et al.*, 2005a), mycorrhizal fungi themselves may play a key role in determining the structure and diversity of aboveground plant communities (van der Heijden *et al.*, 1998). There is widespread interest in harnessing the benefits of mycorrhizal fungi in agricultural systems to reduce fertilizer and pesticide inputs, and improve the water relations of crop plants (Gosling *et al.*, 2006).

II. The Mycorrhizosphere as a Soil Compartment

Plants allocate between 5% and 30% of photosynthetic assimilate to their mycorrhizal fungus partner (Johnson *et al.*, 2005a), and mycorrhizal hyphae comprise a major proportion of the soil biomass in many soil ecosystems. In boreal forests, ECM hyphae may comprise over 30% of the microbial biomass (Hogberg and Hogberg, 2002), and AM mycelium forms around 20% of the microbial biomass in prairie and pasture grasslands (Miller *et al.*, 1995). Mycorrhizas therefore have a central position in terrestrial nutrient cycling processes.

It has long been recognized that the physical, chemical, and biological interactions which occur in the soil surrounding mycorrhizas may be distinct from those of the nonmycorrhizal rhizosphere and bulk soil (Rambelli, 1973). Mycorrhizal roots and mycelium have the potential to affect, and be affected by, free-living and pathogenic microbes and fauna during the initiation and formation of mycorrhizas, as extraradical mycelium grows through the soil, and following hyphal and root senescence (Johansson *et al.*, 2004). Mycorrhizal fungi can directly affect soil organisms via nutritional interactions, including the production of hyphal exudates and the provision of living and senescent hyphae as a food source, competition for nutrients, changes in pH (via exudation and nutrient mobilization), and the production of inhibitory compounds. Since mycorrhizal fungi can change soil structure and affect the quality and quantity of rhizodeposits, plant growth and root:shoot ratio, they can also have indirect impacts on the soil microbiota. In turn, free-living soil organisms can directly influence mycorrhizal fungi and their host plant by stimulating the formation of mycorrhizas, changing nutrient availability, enhancing plant growth, changing root:shoot ratio, and using living fungal or plant tissues as a food source.

The region of soil inhabited by, surrounding and influenced by mycorrhizal roots and mycelium has been termed the ''mycorrhizosphere'' (Linderman, 1988). This includes the zone where the hyphae, spores, and fruit bodies of mycorrhizal fungi occur, and within the fungal mycelium and mycorrhizal roots themselves. The region of soil inhabited by extraradical mycelium alone is termed the ''hyphosphere.''

III. The Physical and Chemical Environment of the Mycorrhizosphere

A. Physicochemical Properties

Microbial communities inhabiting the rhizosphere and mycorrhizosphere are subject to spatial and temporal gradients and variation

resulting from the uptake of oxygen, nutrients and water, and release of CO_2 by the plant and fungus (Hinsinger *et al.*, 2005). Mycorrhizal fungi also modify soil structure, with implications for aeration and water retention (Gosling *et al.*, 2006). Many studies have shown that the mycorrhizosphere has a lower pH than the rhizosphere and bulk soil, with increased CO_2 concentrations resulting from the presence of mycorrhizal fungi proposed as one of the mechanisms responsible (Knight *et al.*, 1989). However, both nutrient uptake and exudation of organic acids (see Section III.B) can also contribute to mycorrhizosphere acidification. For example, Bago *et al.* (1998) showed that acidification arising from uptake of NH_4^+ was greater in an AM hyphosphere compartment than a nonmycorrhizal rhizosphere. Mycorrhiza-induced changes in soil physicochemical properties may influence the growth of microbial communities, although information on the direct impact of such changes on microbial community structure and functioning in the mycorrhizosphere is lacking.

B. Release of Organic Materials into the Mycorrhizosphere by Roots and Hyphae

Mycorrhizal colonization can induce a range of qualitative and quantitative changes in rhizodeposition (Jones *et al.*, 2004), although these have rarely been characterized. Several studies have shown that amounts of carbohydrates and amino acids exuded are lower in AM roots relative to nonmycorrhizal roots, with reductions in the amount of organic C exuded reported to be up to 78% (Bansal and Mukerji, 1994; Marschner *et al.*, 1997). Furthermore, these differences in exudation were associated with contrasting population densities of bacteria within the mycorrhizosphere. There are also reports of stimulated release of phenolics within the mycorrhizosphere (Mada and Bagyaraj, 1993). However, other studies have found no quantitative or qualitative impact of AM on rhizodeposition (Azaizeh *et al.*, 1995). A variety of factors could explain these contrasting results, including differences in the plant and fungus species involved, the experimental system used, and environmental conditions. ECM fungi can stimulate or inhibit rhizodeposition of carbohydrates and amino acids depending on the combination of plant and fungus (Leyval and Berthelin, 1993).

Living hyphae of ECM fungi have been shown to exude a variety of soluble organic materials, although there is little information available on exudation by the hyphae of AM fungi. Exudates may influence microbial communities in the mycorrhizosphere by direct impacts, such as by changing pH or by providing a substrate for microbial growth, although

to date there is little direct evidence linking mycorrhizal fungus exudates to the structure or functioning of mycorrhizosphere communities (see Section V.A).

Most attention on exudation by ECM fungi has focused on organic acids. When grown *in vitro*, large amounts of oxalic and citric acids, and smaller amounts of tartaric, glycolic, and formic acids can be exuded by a number of ECM fungi (Lapeyrie *et al.*, 1987). ECM roots increase the amount and change the composition of organic acids in soil (Griffiths *et al.*, 1994; van Hees *et al.*, 2003). While oxalic acid appears to be the major organic acid detected in soil supporting mycorrhizal plants, large amounts of propionic, formic, acetic, citric, shikimic, and lactic acid have also been detected (Ahonen-Jonnarth *et al.*, 2000; van Hees *et al.*, 2006). Although the relative contribution of the plant root and fungus mycelium to the elevated organic acid levels around mycorrhizal roots has not usually been determined, the precise types of organic acids exuded by mycorrhizal roots do depend on fungal species.

The role of organic acids exuded by ECM mycelium and roots appears to be to dissolve mineral nutrients, including K, Al, and Mg, increasing their availability for uptake (Jones *et al.*, 2004). The amount of organic acid exuded into soil by ECM mycelium can be large. van Hees *et al.* (2006) estimated that oxalic acid exudation by the ECM fungus *Hebeloma crustuliniforme* in symbiosis with *Pinus ponderosa* represented 2–4% of the total C received by the fungus, which was equivalent to 0.2% of the total C fixed by the plant. Organic acids produced by ECM plants are degraded rapidly in soil, with elevated degradation rates in the mycorrhizosphere compared to nonmycorrhizal rhizosphere soil (van Hees *et al.*, 2003).

The external mycelium of some hydrophobic and hydrophilic ECM fungi can exude drops of liquid at the hyphal tip (Unestam and Sun, 1995). In the case of *Suillus bovinus*, carbohydrates comprised 32% of the exudate mass. Ten different polyols (sugar alcohols) and sugars were identified, with the major components found to be inositol, erythritol, ribose, threitol, and mannose (Sun *et al.*, 1999). The drops also contained significant amounts of peptides, which accounted for up to 14% of the exudate mass. Oxalic and acetic acids were also detected in the hyphal drops, but no amino acids were found. The molecular and environmental factors controlling exudation, and the extent to which ECM hyphae exude carbohydrates while growing in symbiosis and in natural soil, is not known.

A number of hydroxymate siderophores have been detected in pure cultures of ECM fungi (Haselwandter, 1995) and soil colonized by ECM

mycelium (van Hees *et al.*, 2006). These cyclic peptides sequester Fe when it is in short supply, making it more accessible for assimilation. The available data suggest that exudation rates of siderophores by ECM mycelia are small, and around 10,000 times lower than for oxalic acid (van Hees *et al.*, 2006). Although siderophores can be readily catabolized by soil bacteria (Pierwola *et al.*, 2004), it seems that siderophore production by mycorrhizal mycelium does not represent a major C input to soil. However, by changing the availability of Fe, siderophores released by mycorrhizal fungi could influence the growth of free-living microbial communities, although this has not been tested.

A number of other organic materials are exuded by ECM hyphae. Some ECM fungi exude phenolic compounds when grown *in vitro* (Sun *et al.*, 1999). These compounds have antifungal activity against plant pathogens (Yamaji *et al.*, 2005), although the extent to which they suppress the growth of mycorrhizosphere organisms, or represent a substrate for saprophytic organisms, is not known. The ECM fungus *Pisolithus tinctorius* exudes an indole alkaloid, hypaphorine, which counteracts the action of the plant hormone indole-3-acetic acid (IAA), resulting in the inhibition of root hair development (Beguiristain and Lapeyrie, 1997). However, direct and indirect effects of hypaphorine on mycorrhizosphere microbes have not been studied.

Information on exudation from AM hyphae is scarce. Attention has focused on glomalin, a glycoprotein which has been shown to accumulate in concentrations up to 21 mg g^{-1} soil (Wright and Upadhyaya, 1999). Glomalin is highly persistent in soil, with a turnover time of 6–42 years (Rillig *et al.*, 2001). Alongside AM hyphae, glomalin may play a role in promoting soil aggregation. Analysis of glomalin production *in vitro* showed that less than 20% of glomalin produced by *Glomus intraradices* was exuded, with the remainder forming part of the hyphal and spore cell wall (Driver *et al.*, 2005). The significance of glomalin, and its impact on mycorrhizosphere organisms are unclear, and furthermore the processes controlling the fate of glomalin in soil are poorly understood.

IV. Interactions Between Microbes and Symbionts Prior to and During Mycorrhiza Formation

A. Mycorrhization Helper Bacteria

The term ''mycorrhization helper bacteria'' (MHB) was coined by Garbaye (1994) to describe bacteria which can enhance the rate of

mycorrhiza formation. Such MHB may also suppress pathogens in mycorrhizosphere soil (Budi *et al.*, 1999; Schelkle and Peterson, 1996). The helper effect of soil bacteria on mycorrhiza formation was initially investigated in nursery soils (Garbaye, 1983; Ridge and Theodorou, 1972). In these soils, ECM formation was reduced following fumigation with methyl bromide, suggesting that bacteria are important in the formation of mycorrhiza. Further work demonstrated that inoculation of microbial communities into sterilized soils containing *P. radiata* with either *Paxillus involutus, Rhizopogon luteolus*, or *H. crustuliniforme* resulted in enhanced mycorrhiza formation (Garbaye and Bowen, 1987).

In many subsequent studies, pure bacterial strains which promote mycorrhiza formation have been isolated and characterized. Early work on specific MHB interactions focused on the ECM symbiosis *Laccaria bicolor* S238N (formerly *Laccaria laccata*)–*Pseudotsuga menziesii* (Duponnois and Garbaye, 1990, 1991; Duponnois *et al.*, 1993), with bacteria isolated from either the sporocarp or the ECM mantle. Further MHB have been isolated from ECM symbioses involving the fungi *Lactarius rufus, Pisolithus* spp., *Suillus luteus*, and *Amanita muscaria* (Bending *et al.*, 2002; Founoune *et al.*, 2002b; Poole *et al.*, 2001; Schrey *et al.*, 2005). MHB have also been studied to a lesser extent in AM symbioses involving *Glomus* spp. (Duponnois and Plenchette, 2003; Mamatha *et al.*, 2002; Xie *et al.*, 1995).

For both ECM and AM symbioses, MHB strains are predominantly *Bacillus* and *Pseudomonas*, but examples have also been found in the genera *Bradyrhizobium, Burkholderia, Paenibacillus, Rhodococcus*, and *Streptomyces*. Bacteria have been categorized as MHB using a variety of contrasting experimental systems, including laboratory microcosms and glasshouse and nursery systems (Duponnois and Garbaye, 1991; Duponnois and Plenchette, 2003; Poole *et al.*, 2001). Examples of laboratory microcosm and glasshouse systems are shown in Figs. 1 and 2.

B. Characterizing Mycorrhization Helper Bacteria Effects

Numerous studies have been conducted to characterize the helper effect of specific MHB. For example, the bacterial strain BBc6 consistently stimulates mycorrhizal root formation in the *L. bicolor* S238N–*P. menziesii* symbiosis. In bare root forest nurseries, stimulation of mycorrhiza formation in the presence of this MHB is typically in the order of 20–30% over controls receiving no bacterial inoculum

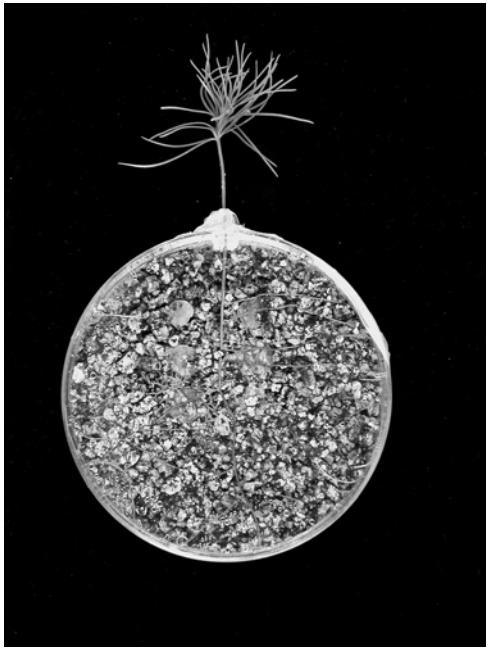

FIG. 1. Photograph of Poole microcosm. A Petri dish–based system containing peat vermiculate and inoculated with *P. sylvestris, L. rufus*, and, as appropriate, bacterial strains.

(Duponnois and Garbaye, 1991). Similarly, for *Acacia auriculiformis–Pisolithus alba*, mycorrhiza formation can be stimulated from 45.8% in controls to 70.3% in the presence of *Pseudomonas fluorescens* HR13 (Duponnois and Plenchette, 2003).

The concentration at which such MHB are applied has important consequences for the degree of stimulation. *P. fluorescens* BBc6R8 increased mycorrhiza formation to a greater extent at lower than high doses (Frey-Klett *et al.*, 1999). Similarly, Aspray *et al.* (2006) found using microcosms and the *P. sylvestris–L. rufus* symbiosis that for *Burkholderia* sp. EJP67 the effect on mycorrhiza formation was dependent on bacterial inoculation concentration, although in the same system, *Paenibacillus* sp. EJP73 stimulated mycorrhiza formation at a greater range of inoculum concentrations. Therefore, in terms of bacterial concentration it seems that a fine equilibrium must exist, as least for certain MHB, in order for the helper effect to be realized.

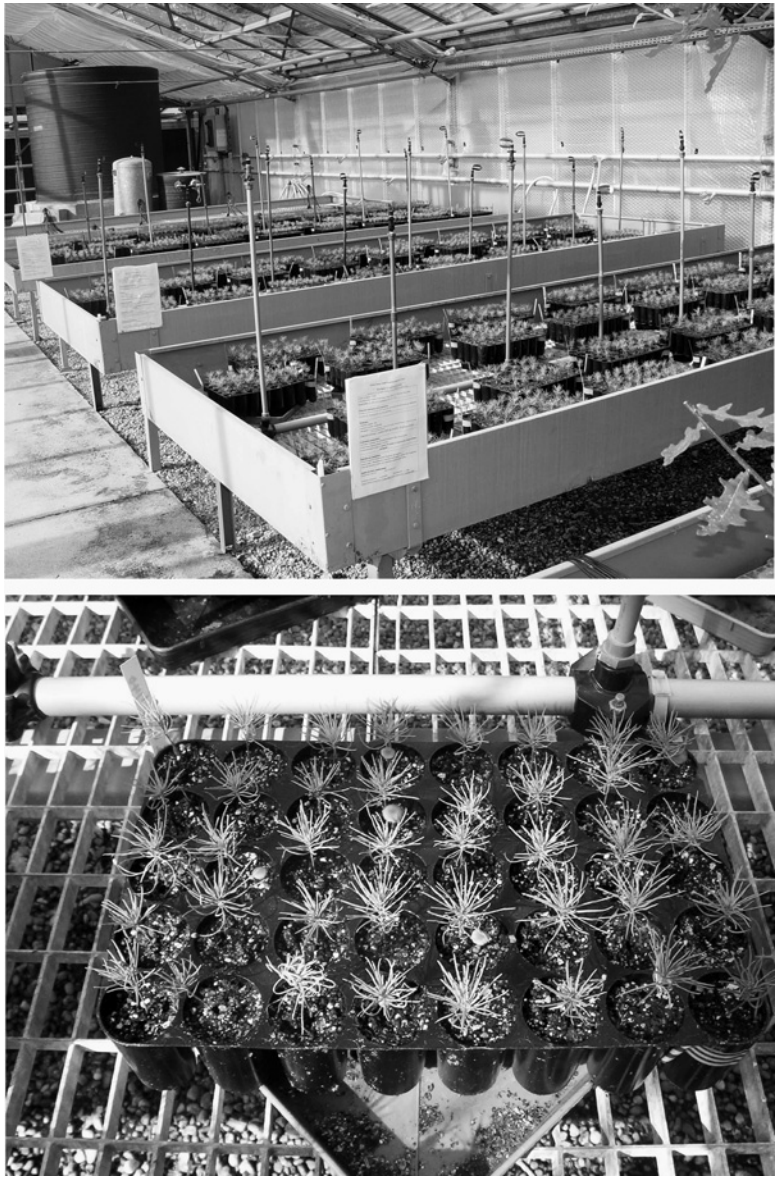

FIG. 2. Photographs of typical glasshouse experiment used to investigate MHB–ectomycorrhiza interactions, as conducted at INRA Nancy, Research Unit ''Tree-Microorganism Interactions, France'' (Courtesy of Béatrice Palin). Three-month-old *P. sylvestris* seedlings inoculated with ectomycorrhizal fungi and MHB.

Although MHB were initially thought to be fungus specific (Dunstan *et al.*, 1998; Duponnois *et al.*, 1993), MHB strains have since been shown to stimulate mycorrhiza formation of several fungal strains. Duponnois and Plenchette (2003) found that MHB *P. fluorescens* HR13 was able to stimulate ectomycorrhiza formation by *P. alba* and *Scleroderma* spp. In fact, HR13 also stimulated mycorrhiza formation by the AM fungus *G. intraradices*. The nonfungal specificity of certain MHB has been confirmed using the strain *Paenibacillus* sp. EJP73, which stimulates *L. bicolor–P. sylvestris* mycorrhiza under glasshouse conditions and *L. rufus–P. sylvestris* mycorrhiza in laboratory microcosms (Poole *et al.*, 2001; Aspray *et al.,* in press).

C. Mycorrhization Helper Bacteria Mechanisms

A key focus for MHB research has been to understand the mechanisms responsible for producing the helper effect. Potential mechanisms were hypothesized by Garbaye (1994) to include: (1) enhancing receptivity of the root to colonization by mycorrhizal fungi, (2) affecting root-fungus recognition processes, (3) stimulating presymbiotic fungal growth, (4) affecting germination of fungal propagules, and (5) modifying soil properties. Garbaye (1994) provided a thorough overview of potential MHB mechanisms. As such, this section of the chapter will cover general concepts and focus on more recent developments in this area.

1. *Enhancing Receptivity of the Root to Colonization by Mycorrhizal Fungi*

One way in which MHB may enhance the colonization of plant roots by fungi is by increasing the actual number of short roots available for colonization (Garbaye, 1994). Bacteria can produce a wide array of molecules which may alter the physical and chemical properties of plant roots, including signaling molecules such as phytohormones, and enzymes. In particular, the phytohormone IAA is important in plants for controlling fundamental cellular processes, including cell division and tissue differentiation (Leveau and Lindow, 2005). Some MHB have been found to produce large amounts of IAA, which can stimulate the initiation of short roots by *P. menziessii* seedlings (Duponnois, 1992; Gamalero *et al.*, 2003).

MHB may also enhance the receptivity of the plant root by softening the cell wall and middle lamella of the root cortex (Duponnois, 1992), and making physical growth of the fungus through the root easier. Soil bacteria can produce a range of enzymes such as endoglucanases,

cellobiose hydrolases, pectate lyases, and xylanases. The MHB *P. fluorescens* 92 and *P. fluorescens* BBc6 have both been shown to produce cellulase (Gamalero *et al.*, 2003), although its role in MHB activity has not been demonstrated. Enzymes such as endoglucanase and pectate lyase are also involved in virulence and pathogenicity of bacterial plant pathogens (Ham *et al.*, 2004; Liao *et al.*, 1992), which could suggest a fine balance between helper and pathogenic effects.

Finally, one area recently receiving attention in relation to both pathogenic and mutualistic interactions between plants and microbes is the involvement of bacterial secretion systems. The type III secretion system (TTSS) in particular has been demonstrated to be involved both in pathogenic and mutualistic interactions with plant roots (Buttner *et al.*, 2006; Molina *et al.*, 2006). TTSS inject proteins into the cytosol of eukaryotic cells, where the translocated proteins interfere with host cell signal transduction and other cellular processes, resulting in changes to host physiology. Further work is needed to clarify the role of such systems in MHB interactions.

2. *Affecting Root–Fungus Recognition Processes*

In mycorrhizal symbioses, fungi and plants produce signal molecules such as phytohormones, enzymes, polysaccharides, phenolic compounds, adhesins, and volatiles during the initiation of mycorrhiza formation (Akiyama *et al.*, 2005; Smith and Read, 1997). Bacteria in the mycorrhizosphere may be able to synthesize many of these chemicals, and thereby affect mycorrhiza formation. In addition, degradation or transformation of signal molecules by bacteria could affect root-fungus recognition.

Production of IAA is a mechanism by which some plant growth-promoting bacteria operate (Patten and Glick, 2002), and production of IAA could also be an MHB mechanism. However, as the ECM fungus *L. bicolor* also produces IAA, it is not clear what contribution bacterially produced IAA has on enhancing this symbiosis. Bacteria, for example *Pseudomonas putida* strain 1290†, may also degrade IAA (Leveau and Lindow, 2005). However, whether such bacteria can enhance or inhibit mycorrhiza formation is unknown.

3. *Stimulating Presymbiotic Fungus Growth*

The third hypothesis, that MHB stimulate presymbiotic growth of the fungus, has been studied widely due to the simplicity of experiments involving cocultures of the fungus and bacterium. One way in which MHB can stimulate fungal growth is through the production of metabolites which can be used as nutrients or anabolic growth factors by the

fungus. A variety of studies have shown that bacterial isolates or their culture filtrates can stimulate growth of ECM fungi on low nutrient agar (Brulé *et al.*, 2001; Duponnois and Garbaye, 1990). MHB may also detoxify metabolites produced by the fungus that inhibit mycelial growth (Duponnois and Garbaye, 1990).

Whether the stimulation of fungal growth on agar or in liquid media is an MHB mechanism remains to be proven conclusively. In the absence of the plant partner, enhancing presymbiotic fungus growth cannot be correlated with enhanced mycorrhiza formation. For example, *P. monteilii* HR13 stimulated radial growth of *Pisolithus* isolates, but not *Scleroderma dictyosporum* or *S. verrucosum*, and yet the bacterium significantly enhanced mycorrhiza formation of all three fungi (Duponnois and Plenchette, 2003).

4. *Affecting Germination of Fungal Propagules*

Bacteria have been shown to stimulate the germination of mycorrhizal fungus spores. For example, surface sterilization of *G. versiforme* spores reduced the rate of spore germination compared to those with naturally associated microbial communities (Mayo and Davis, 1986). Furthermore, the addition of bacteria (including *Pseudomonas* and *Corynebacterium* strains) isolated from nonsurface disinfected spores also increased spore germination compared to disinfected spores. Both volatile and nonvolatile metabolites released by bacteria have been suggested to be responsible for increased spore germination (Azcón, 1987; Carpenter-Boggs *et al.*, 1995; Mayo and Davis, 1986). However, like the previous hypothesis a clear link between enhanced spore germination and increased mycorrhiza formation remains to be demonstrated.

D. Mycorrhization-Inhibiting Bacteria

Some bacteria can have inhibitory effects on *in vitro* fungus growth or on mycorrhiza formation itself and have been termed mycorrhization-inhibiting bacteria (MIB; Bending *et al.*, 2002; Bowen and Theodorou, 1979; Varese *et al.*, 1996). MIB include strains of *Pseudomonas* sp. and *Bacillus* sp. The close phylogenetic similarity between MHB and MIB, and the fact that MHB are able to stimulate mycorrhiza formation of certain fungi and inhibit that of others suggests that bacteria are able to act both as MHB or as MIB, depending on the particular fungal and plant partners involved (Brulé *et al.*, 2001). Furthermore, Brulé *et al.* (2001) found that *P. fluorescens* BBc6R8 could act as an MHB or MIB of

the *L. bicolor–P. menziesii* symbiosis under different environmental conditions. Such findings emphasize the dynamic nature of bacteria in the mycorrhizosphere rather than the presence of discrete functional groups.

V. Interactions Between Mycorrhizas and Free-Living Nonpathogenic Organisms

A. Bacterial Biomass and Community Structure

Most studies which have examined the impact of mycorrhizas on bacterial communities have compared the rhizosphere of mycorrhizal and nonmycorrhizal plants. Since colonization of roots by mycorrhizal fungi can alter root physiology, including patterns of rhizodeposition (see Section III.B), it is not clear to what extent these impacts reflect direct or indirect effects of mycorrhizal fungi. Furthermore, despite evidence that mycorrhizal fungi can alter the chemical and physical environment within the mycorrhizosphere (see Section III), most studies have failed to provide understanding of mechanisms driving community structure in the mycorrhizosphere, or in many instances the functional consequences of altered community structure.

Mycorrhizal fungi have been shown to have negative (Christensen and Jakobsen, 1993), neutral (Olsson *et al.*, 1998), and positive (Andrade *et al.*, 1998) effects on amounts or activity of total bacterial biomass, or specific genotypic groups. Using plating techniques, Meyer and Linderman (1986) showed that *G. fasciculatum* had no effect on the number of bacteria or actinomycetes in the rhizosphere of *Zea mays* or *Trifolium subterraneum*. However, relative to nonmycorrhizal plants, the rhizosphere of mycorrhizal plants supported higher populations of facultative anaerobic bacteria and chitinase-producing actinomycetes, but lower populations of fluorescent Pseudomonads. Numbers of bacteria in the mycorrhizosphere of ECM plants can also be higher than those of bulk soil or nonmycorrhizal rhizosphere, although the extent of differences can vary between mineral and humus horizons (Heinonsalo *et al.*, 2001).

Andrade *et al.* (1997) showed that more bacterial species occurred in soil from the hyphosphere relative to that from AM-colonized rhizosphere and that the precise communities found varied between AM fungus species. A number of specific bacterial genera have been found in greater abundance in the hyphosphere of AM fungi relative to nonmycorrhizal rhizosphere or bulk soil, including *Burkholderia* spp., *Arthrobacter* spp. (Andrade *et al.*, 1997), and *Paenibacillus* spp.

(Artursson *et al.*, 2005). These genera, together with *Bacillus* spp., *Pseudomonas* spp., and *Rhodococcus* spp. can also be abundant within ECM roots (Bending *et al.*, 2002; Izumi *et al.*, 2006; Poole *et al.*, 2001). The mycorrhizosphere can also induce more subtle change to communities. Frey-Klett *et al.* (2005) found that both the genotypic and functional diversity of *P. fluorescens* strains within the mycorrhizosphere was greater than in bulk soil.

In contrast to eubacteria, interactions between mycorrhizas and archaea have received little attention. In a humus soil, Bomberg *et al.* (2003) found greater diversity of archaea in mycorrhizal roots and soil colonized by ECM mycelium relative to uncolonized soil, with no archaea detected in nonmycorrhizal roots. However, the size of these communities and their significance is unclear.

B. Fungal Biomass and Community Structure

A variety of nonsymbiotic microfungi occur within ECM roots or in soil containing extraradical mycelium, although there is evidence that the size of the saprophytic fungus community is reduced in the mycorrhizosphere relative to the bulk soil or nonmycorrhizal roots (Olsson *et al.*, 1998; Summerbell, 2005). However, population sizes of individual fungus species can be inhibited, stimulated, or not affected by the presence of mycorrhizal mycelium (Larsen *et al.*, 1998; Tiunov and Scheu, 2005; Zadworny *et al.*, 2004). Intriguingly, the ECM fungus *L. laccata* has been shown to be a mycoparasite of the microfungus *Mucor hiemalis in vitro* (Werner and Zadworny, 2003), although an antagonistic interaction between these fungi, without mycoparasitism, occurred in the rhizosphere of *P. sylvestris* (Werner *et al.*, 2002). ECM fungi are known to compete with litter-inhabiting saprotrophic macrofungi, which can exploit the same spatial niche as ECM fungi. The outcome of competition between such ECM and saprotrophic macrofungi depends on the species involved and can also depend on C availability to each of the interacting fungi (Lindahl *et al.*, 2001). Much less is known about AM–saprophytic fungus interactions. Some microfungal inhabitants of the mycorrhizosphere are known to be antagonistic to AM fungi (McAllister *et al.*, 1997), and *Trichoderma harzianum* has been reported to be a mycoparasite of AM spores and mycelium (Rousseau *et al.*, 1996).

C. Interactions with Fauna

A variety of micro- and mesofauna inhabit the mycorrhizosphere, and these organisms are likely to affect microbial communities through feeding, inputs of fecal material, and disturbance, although these interactions are poorly understood. Mycorrhizal hyphae and their associated free-living bacterial and fungal communities are grazed by a variety of soil animals. Populations of protozoa have been found to be both lower and higher in ECM roots and soil supporting extraradical mycelium relative to nonmycorrhizal roots (Jentschke *et al.*, 1995; Timonen *et al.*, 2004). Ronn *et al.* (2002) found that numbers of protozoa were reduced in the AM mycorrhizosphere relative to the nonmycorrhizal rhizosphere, with lower bacterial biomass in the presence of the AM fungus given as the reason.

Oribatid mites show varying feeding preferences for the mycelia of ECM fungi (Schneider *et al.*, 2005), while collembola can feed on the mycelium of both AM (Klironomos and Ursic, 1998) and ECM fungi (Hiol *et al.*, 1994) and may also physically sever hyphae as they move through soil (Johnson *et al.*, 2005b). Tiunov and Scheu (2005) showed that three species of collembola fed on saprotrophic rather than AM hyphae in the mycorrhizosphere of the grass *Cynodon dactylon*, altering community structure of the saprotrophic community. Additionally, the feeding preference for saprotrophic microfungi increased the extent to which *G. mosseae* altered the structure of the saprotrophic community. The direct and indirect impacts of collembola on bacterial community structure and functioning within the mycorrhizosphere are not known. Numbers of free-living nematodes can be stimulated in the mycorrhizosphere of ECM plants (Villenave and Duponnois, 2002), and furthermore AM fungi can override the selective influence of host plant on the structure of soil nematode communities (Villenave *et al.*, 2003). The effects of nematodes and other important faunal groups, such as enchytraeid worms (Didden, 1993), on the structure and functioning of microbial communities within the mycorrhizosphere have not been considered.

D. Decomposition Processes in the Mycorrhizosphere

Mycorrhizal fungi coexist with saprotrophic organisms, and interaction between these groups of organism can have consequences for the degradation of organic matter and xenobiotics (Cairney and Meharg, 2002). In forest soil, the presence of ECM roots can inhibit litter decomposition (Gadgil and Gadgil, 1971, 1975), an observation which has

been termed the ''Gadgil effect.'' A number of mechanisms have been proposed for the apparent inhibition of saprotroph communities which underlies the Gadgil effect (Bending, 2003). The simplest explanation is that ECM fungi directly inhibit saprotrophic bacteria, fungi, and fauna (see Sections V.A–C). The capacity of ECM fungi to degrade litter components is low relative to saprotrophic fungi, and the colonization and exploitation of litter by ECM fungi in place of saprotrophic fungi would result in reduced rates of decomposition. Other explanations include the selective exploitation and translocation of available forms of N and P by ECM fungi, which lower the quality of the substrate remaining to saprotrophs (Abuzinadah and Read, 1989; Bending and Read, 1995), and the uptake of water by ECM roots, which could result in availability of water limiting the activities of saprotrophic organisms (Koide and Wu, 2003). However, the Gadgil effect does not occur universally, and several studies have shown that the presence of mycorrhizal roots can enhance decomposition rates (Zhu and Ehrenfeld, 1996). Similarly, the presence of AM mycelium can stimulate decomposition rates of organic matter in soil (Hodge *et al.*, 2001).

The degradation of xenobiotics in the mycorrhizosphere has received little attention. The mycorrhizosphere is a compartment in which xenobiotic catabolizing communities can be enriched, and in which survival of inoculated catabolic communities can be enhanced (Sarand *et al.*, 1998). Joner and Leyval (2001) showed that the presence of *G. mosseae* increased the degradation of several polycyclic aromatic hydrocarbons (PAH) by up to 25% after 16 weeks, although using the same fungus species, Binet *et al.* (2000) found no difference in the catabolism of a mix of eight PAH between nonmycorrhizal rhizosphere and mycorrhizosphere soil. Genney *et al.* (2004) demonstrated that catabolism of the PAH fluorine was inhibited in the presence of ECM mycelium, extending the significance of the Gadgil effect. However, ECM roots may have no effect on degradation of other PAH (Genney *et al.*, 2004; Koivula *et al.*, 2004). Since mycorrhizal fungi are considered to have little or no capacity to degrade complex organic materials, direct stimulation of the activities of saprotrophic organisms by mycorrhizal fungi has been proposed as the mechanism responsible for enhanced organic matter or xenobiotic degradation in the mycorrhizosphere, although the communities involved, and the mechanisms underlying the interactions are unclear.

E. Interactions with Microbes Contributing to N and P Cycling

Availability of N and P is commonly the factor most limiting to plant growth, and enhancing availability of these nutrients to the host is generally believed to be the key function of mycorrhizal fungi (Smith and Read, 1997). Many studies have indicated that organisms which improve N and P availability to mycorrhizal fungi are specifically enriched in the mycorrhizosphere. However, it is not clear whether these effects reflect direct effects of the mycorrhizal fungus, or indirect effects arising from mycorrhizal fungus-induced changes to soil moisture content or impacts on nutrient status.

Li *et al.* (1992) found that N_2 could be fixed by *Bacillus* spp. located within tuberculate ECM of *P. menziessii*. However, actual numbers of N_2 fixing bacteria in the mycorrhizosphere may be no different to those in nonmycorrhizal roots (Rozycki *et al.*, 1999). Amounts of N_2 fixed within the mycorrhizosphere are likely to contribute a relatively small proportion of total atmospheric N inputs to soil (Barkmann and Schwintzer, 1998). However, since the fixed N_2 directly enters the root zone, it could nonetheless be important for tree nutrition. The number of free-living N_2 fixing bacteria has been shown to be elevated in the mycorrhizosphere of several AM fungi growing individually with the grass *Panicum maximum* (Secilia and Bagyaraj, 1987), although N_2 fixation rates were not determined.

Many studies have shown that the AM symbiosis improves both nodulation and symbiotic N_2 fixation, with the extent to which this occurs depending on the specific strains of AM fungus and N_2 fixing bacteria involved (Requena *et al.*, 1997). Improved P and/or N nutrition of the host by the AM fungus is thought to be responsible for determining these interactions (Barea *et al.*, 2002). However, the relative competitiveness of nodule N_2 fixing bacteria can be altered within the mycorrhizosphere relative to the rhizosphere, with implications for patterns of nodulation and N_2 fixation (André *et al.*, 2003). AM colonization can also protect nodules and N_2 fixation from drought, although AM species vary in their effectiveness (Ruiz-Lozano *et al.*, 2001). Enhanced water uptake by AM fungi, in addition to reduction of oxidative damage, may be responsible for these effects.

The population sizes of other organisms involved in N cycling can be altered within the mycorrhizosphere. Numbers of autotrophic NH_4^+-oxidizing bacteria were higher in the mycorrhizosphere of *G. mosseae* and *G. fasciculatum* growing with *Z. mays* relative to nonmycorrhizal rhizosphere soil, with the reverse situation for numbers of denitrifying and NH_4^+-producing organisms (Amora-Lazcano *et al.*,

1998). However, actual rates of N transformation processes were not determined in this study, so the significance is unclear.

The main functional role of AM is thought to be to enhance P uptake to the host plant by obtaining P from beyond the depletion zone surrounding plant roots (Smith and Read, 1997). A variety of free-living soil bacteria and fungi are extremely effective at mobilizing P from insoluble minerals through the production of organic acids (Richardson, 2001), and there has been much interest in the interaction of native and coinoculated P-solubilizing bacteria with AM fungi. In *Medicago sativa*, populations of native P-solubilizing bacteria can be enhanced in the mycorhizosphere of *G. mosseae* relative to the nonmycorrhizal rhizosphere (Toro *et al.*, 1998), while Frey-Klett *et al.* (2005) showed that the ectomycorrhizosphere selected P-mobilizing strains of *P. fluorescens*. Furthermore, several studies have found that the effectiveness of inoculated P-solubilizing bacteria (Villegas and Fortin, 2002) and fungi (Osorio and Habte, 2001; Tarafdar and Marschner, 1995) is stimulated within the mycorrhizosphere, with the inoculants acting synergistically with AM fungi to enhance P uptake by the host plant. However, in other studies, no interactive relationships between AM fungi and inoculant P-solubilizing bacterial strains have been found (Toro *et al.*, 1998).

F. Localization of Microbes Within the Mycorrhizosphere

There is considerable spatial variability in the localization of bacteria within the mycorrhizosphere. Nurmiaho-Lassila *et al.* (1997) showed that within the *S. bovinus–P. sylvestris* mycorrhizosphere, bacteria occurred inter- and intracellularly within the mantle and Hartig net of the root, and while fungal rhizomorphs supported few bacteria, the fungal front, which was composed of dense mycelium, supported an extensive biofilm of bacteria. The localization of bacteria on *P. involutus–P. sylvestris* mycorrhizas was shown to be different, with bacteria mostly absent from mycorrhizal roots. Furthermore, the precise structure of bacterial communities can vary between root and hyphosphere locations within the mycorrhizosphere (Timonen *et al.*, 1998).

The spores and hyphae of some AM fungi, including *Gigaspora margarita*, contain obligate endocellular bacteria, which have been identified as a new taxon, *Candidatus Glomeribacter gigasporarum* (Jargeat *et al.*, 2004). Similarly, living hyphae of the ECM fungus *L. bicolor* can harbor diverse endobacteria, mainly belonging to the α-proteobacteria (Bertaux *et al.*, 2005). However, the functional significance of endocellular bacteria within mycorrhizal hyphae has yet to be elucidated.

Much less is known of the localization of fungi within the mycorrhizosphere. Bending and Read (1995) showed that conidiophores of *Penicillium* sp. were associated with senescent areas of mycelium behind the fungal front and were absent from active mycelium.

G. Nutritional Aspects of Mycorrhizosphere Interactions

Frey *et al.* (1997) demonstrated that the structure of fluorescent Pseudomonad communities associated with *P. menziesii–L. bicolor* mycorrhizas and mycorrhizosphere soil was different to that of the bulk soil, and that those from mycorrhizal compartments preferentially utilized the fungus sugar trehalose. Similarly, Izumi *et al.* (2006) found that diverse endophytic bacteria isolated from a range of *P. sylvestris* mycorrhizas had a preference for trehalose relative to plant sugars. These studies indicate that exudation and specific nutrient availability could be a key driver determining the community structure of organisms inhabiting the mycorrhizosphere surrounding active hyphae, although such links remain to be proven.

The major routes by which C is released from mycorrhizal roots and hyphae into the soil are likely to be through senescence and following ingestion by fauna. The impact of these processes in determining the structure and functioning of mycorrhizosphere bacterial and fungal communities is poorly understood. In the case of AM, hyphae have been shown to have a life span of just 5–6 days, following which they senesce (Staddon *et al.*, 2003). Many ECM fungus species form dense mycelia at the foraging front, connected to plant roots by rhizomorphs (Agerer, 2001). For *S. bovinus*, the time from initial colonization of substrate by the fungal front to senescence was less than 40 days (Bending and Read, 1995). Chitinolytic bacteria are frequently encountered within the mycorrhizosphere of ECM (Bending *et al.*, 2002) and AM fungi (Meyer and Linderman, 1986), and these organisms could potentially utilize chitin from living or senescent mycorrhizal hyphae. Some microfungi which show antagonism to AM fungi have been shown to penetrate living spores and hyphae, and proliferate inside, although chitinolytic activity only occurred at the infection point (Rousseau *et al.*, 1996). Endophytic bacteria associated with ECM and AM fungi must clearly derive all their nutrition from their host fungus, although it is not clear how this is achieved. Furthermore, it remains to be seen whether endophytic bacteria have any positive or negative impact on the mycorrhizal fungus itself. Although *Candidatus Glomeribacter gigasporarum* has been shown to possess N_2 fixation genes (Minerdi *et al.*, 2001), their significance is not known.

VI. Pathogen Interactions with Mycorrhizas

A. Control of Pathogens by Mycorrhizas

The majority of studies concerning pathogen–mycorrhiza interactions have been focused on developing mycorrhizal fungi for biological disease control and this topic has been comprehensively reviewed in recent years (Harrier and Watson, 2004; Whipps, 2004). Consequently, in the main, only general concepts and principles associated with this topic are presented with key or more recent references cited where necessary.

Both AM and ECM fungi have been reported to provide control of numerous plant pathogens and some examples are given in Table I. There is considerable diversity in mycorrhizal fungi capable of reducing diseases caused by a number of different plant pathogens, but *Glomus* spp., especially *G. intraradices* and *G. mosseae* are the most studied AM fungi and *L. bicolor, L. laccata*, and *P. involutus* are the most widely studied ECM fungi. Fungal pathogens, such as *Fusarium* spp., *Rhizoctonia solani, Cylindrocarpon destructans*, and *Phytophthora* spp., have been examined numerous times along with nematodes including *Meloidogyne* spp. and *Pratylenchus* spp., reflecting their widespread nature and economic importance. Control of bacterial diseases has been little studied, although there are a few examples (Garcia-Garrido and Ocampo, 1989; Zhu and Yao, 2004).

The level of control achieved by any AM or ECM fungus can depend on the cultivar of plant (Duchesne, 1994; Mark and Cassells, 1996), the aggressiveness of the pathogen (Strobel and Sinclair, 1991), the isolate of mycorrhizal fungus, and the substrate and environment used for plant cultivation, but control is never complete. Combinations of mycorrhizal fungi may also give improved control of pathogens in comparison with those used individually (Requena *et al.*, 2001). However, it should be noted that there are reports that some soilborne diseases are increased by mycorrhizal infection (Davis and Menge, 1980; Garmendia *et al.*, 2004; Ross, 1972) suggesting that, in some instances, a healthy mycorrhizal plant may be more susceptible than a poorly developed nonmycorrhizal one (Dehne, 1982).

B. Mechanisms and Microbial Interactions Associated with Disease Control

Research on mycorrhiza–pathogen interactions has focused on understanding the mechanisms by which mycorrhizal roots resist attack by a plant pathogen. But more recently, it has been realized that

TABLE I

EXAMPLES OF MYCORRHIZAS EXHIBITING CONTROL OF PLANT PATHOGENS

Mycorrhizal fungus	Pathogen	Host plant	References
Arbuscular mycorrhizal fungi			
Gigaspora margarita	*Fusarium oxysporum* f. sp. *asparagi*	*Asparagus officinalis* (Asparagus)	Matsubara *et al.* (2001)
	Meloidogyne incognita	*Gossypium hirsutum* (Cotton)	Roncardori and Hussey (1977)
Glomus spp.	*Meloidogyne incognita*	*Lycopersicon esculentum* (Tomato)	Talavera *et al.* (2001)
	Pratylenchus penetrans	*Daucus carota* (Carrot)	Talavera *et al.* (2001)
Glomus aggregatum	*Cylindrocarpon destructans*	*Prunus persicaria* (Peach)	Traquair (1995)
Glomus clarum	*Rhizoctonia solani*	*Vigna unguiculata* (Cowpea)	Abdel-Fattah and Shabana (2002)
Glomus coronatum	*Rhizoctonia solani*	*Vigna radiata* (Mung bean)	Kasiamdari *et al.* (2002)
Glomus etunicatum	*Phytophthora fragariae* var. *fragariae*	*Fragaria x ananassa* (Strawberry)	Norman *et al.* (1996)
Glomus fasciculatum	*Fusarium oxysporum* f. sp. *asparagi*	*Asparagus officinalis* (Asparagus)	Matsubara *et al.* (2001)
	Radopholus similis	*Musa acuminata* (Banana)	Umesh *et al.* (1988)
Glomus fistulosum	*Phytophthora fragariae var. fragariae*	*Fragaria vesca* (Wild strawberry)	Mark and Cassells (1996)
Glomus intraradices	*Fusarium solani* f. sp. *phaseoli*	*Phaseolus vulgaris* (Bean)	Filion *et al.* (2003)
	Meloidogyne javanica	*Musa* sp. (Banana)	Pinochet *et al.* (1997)
Glomus mosseae	*Aphanomyces euteiches*	*Pisum sativum* (Pea)	Larsen and Bødker (2001)
	Pratylenchus vulnus	*Prunus domestica* (Plum)	Camprubí *et al.* (1995)
Glomus proliferum	*Cylindrocladium spathyphylli*	*Musa acuminata* (Banana)	Declerk *et al.* (2002)

(*continued*)

TABLE I (*Continued*)

Mycorrhizal fungus	Pathogen	Host plant	References
Glomus versiforme	*Ralstonia solanacearum*	*Lycopersicon esculentum* (Tomato)	Zhu and Yao (2004)
	Verticillium dahliae	*Gossypium hirsutum* (Cotton)	Liu (1995)
Ectomycorrhizal fungi			
Clitocybe claviceps	*Fusarium moniliforme*	*Picea glauca* (White spruce)	Chakravarty *et al.* (1999)
Hebeloma crustiliniforme	*Phytophthora cambivora*	*Castanea sativa* (Chestnut)	Brazanti *et al.* (1999)
Hebeloma sinapizans	*Phytophthora cambivora*	*Castanea sativa* (Chestnut)	Brazanti *et al.* (1999)
Laccaria bicolor	*Fusarium moniliforme*	*Picea glauca* (White spruce)	Chakravarty *et al.* (1999)
Laccaria laccata	*Phytophthora cinnamomi*	*Castanea sativa* (Chestnut)	Brazanti *et al.* (1999)
	Rhizoctonia solani	*Pinus sylvestris* (Scots pine)	Chakravarty and Unestam (1987)
Paxillus involutus	*Cylindrocladium floridanum*	*Picea mariana* (Clack spruce)	Morin *et al.* (1999)
	Phytophthora cinnamomi	*Castanea sativa* (Chestnut)	Brazanti *et al.* (1999)
Pisolithus spp.	Natural plant parasitic nematodes	*Acacia* spp.	Founoune *et al.* (2002a)
	Meloidogyne javanica	*Acacia* spp.	Duponnois *et al.* (2000)
Pisolithus tinctorius	*Rhizoctonia solani*	*Pinus sylvestris* (Scots pine)	Chakravarty and Unestam (1987)
Scleroderma spp.	*Tylenchorenchus gladiolatus*	*Afzelia africana* (African hardwood)	Villenave and Duponnois (2002)

mplex interactions of mycorrhizal fungi with other microorganisms in the mycorrhizosphere can also influence the ability of a pathogen to infect a plant, particularly where bacterial and fungal biocontrol agents are combined with mycorrhizal fungi as inocula. These areas are considered below.

Four major groups of modes of action have been identified (Whipps, 2004): (1) direct competition or inhibition; (2) enhanced or altered plant growth, nutrition, and morphology; (3) biological changes associated with plant defense mechanisms and induced resistance; and (4) development of an antagonistic microbiota. Thus, in (1) the mycorrhizal fungus acts directly on the pathogen, in (2) and (3) it acts on the plant, and in (4) it acts on the microbiota around the root.

Direct competition or inhibition may involve competition for photosynthate in or on the root, or for exudates and rhizodeposits external to the roots. There may also be competition for infection sites or space on the roots, and for ECM fungi, mechanical sheathing of the root forming a defensive barrier. The quantity and quality of exudates from the roots or mycorrhizal fungus could inhibit the pathogen including production of low levels of antibiotics or defense compounds, and there may be direct competition in the soil.

Enhanced or altered plant growth, nutrition, and morphology can involve increased nutrient uptake (particularly P), increased uptake of trace elements, drought tolerance, and decreased toxicity to salt and heavy metals, all providing alleviation of abiotic stress. The age of the plant when a pathogen attacks the root can also influence the biocontrol level seen (Idoia *et al.*, 2004). Similarly, there may be changes in plant hormone levels and damage compensation. All these effects would provide a healthier plant potentially more tolerant to pathogen attack.

Biochemical changes associated with plant defense mechanisms and induced resistance have been a major focus in recent years. Production of phenolics, terpenes, phytoalexins, specific amino acids, internal structural barriers, defense-related proteins, and increased DNA methylation and respiration have all been reported as involved in pathogen control. Combinations of these responses may also give rise to systemic-induced resistance throughout the plant. Potentially, colonization by mycorrhizal fungi could enable the plant to respond more rapidly to subsequent pathogen challenge by resistance mechanisms being preactivated.

Molecular approaches are now being applied to dissect the changes in signaling and defense mechanisms related to disease resistance induced in response to mycorrhizal colonization (Colditz *et al.*, 2005; Pozo *et al.*, 2002; Requena *et al.*, 1999). In a proteomics study, the

proteins expressed in *G. intraradices*-colonized *Medicago truncatula* roots in response to infection by *Aphanomyces euteiches* showed similar changes to those induced by *A. euteiches* alone except for a proteasome subunit alpha type 4 which was increased in abundance (Colditz *et al.*, 2005). This protein is involved in protein degradation by the ATP/ubiquitin-mediated proteolysis pathway, which has been shown to play a key role in regulation of plant disease resistance responses in other systems, with ubiquitin-associated proteins acting as signaling components in defense response signal cascades.

Development of a microbiota antagonistic to pathogens in soil around roots in response to mycorrhiza formation is a relatively recent concept (Andrade *et al.*, 1998) but as evidence has accumulated that the microbiota around the root can be changed in the presence of mycorrhizal fungi (see Section V), the need for further work in this area has been highlighted. One result of this has been the concept of utilizing combinations of biological disease control agents with mycorrhizal fungi to enhance disease control. A huge number of potential candidates that control soilborne plant pathogens when applied to seeds, roots, or soil are known (Whipps, 2001), but relatively few have so far been tested with mycorrhizal fungi. Dual inoculations of *Glomus* spp. with a variety of bacteria, including *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp., and *Rhizobium* spp., and fungi, such as *Gliocladium* and *Trichoderma* spp., have resulted in either improved plant growth or decreased severity of several pathogens (Berta *et al.*, 2005; Whipps, 2004). With ECM fungi, a number of bacteria have been utilized as ECM helper bacteria (see Section IV) resulting in enhanced plant growth but there have been no studies involving pathogen control. Combinations of ECM fungi with other fungi are restricted to a single *in vitro* synthesis experiment involving *P. sylvestris, L. laccata*, and *Trichoderma virens* (Werner *et al.*, 2002) but there were no significant effects of the introduction of *T. virens*.

An important feature of these studies is to ensure that the biocontrol agents do not affect the activity of the mycorrhizal fungi and vice versa. Numerous studies have investigated these interactions and there are examples of bacteria and fungi stimulating, having no effect or inhibiting growth of mycorrhizal fungi; bacteria and fungi enhancing mycorrhiza formation and development; and cases where bacteria and fungi inhibit mycorrhizal formation (Barea *et al.*, 2005; Whipps, 2004). Similarly, mycorrhizal fungi can also stimulate or inhibit specific bacteria and saprotrophic fungi (see Section V.A and B), illustrating the diversity of interactions that are possible between mycorrhizal fungi and

the soil microbiota including plant pathogens. These deserve further study.

VII. Conclusions

There is clearly considerable spatial and temporal variability of microbial community structure and functioning within the mycorrhizosphere with the region representing a mosaic of spatial habitats, resulting from rhizoexudation and hyphal exudation, hyphal and root senescence, and the feeding habits of grazers. The mycorrhizosphere microbial community may play a role in supporting plant growth, by mobilizing nutrients and suppressing plant pathogens. The importance of these processes clearly depends on the characteristics of the mycorrhizal fungus species itself, but also on the host plant and soil and environmental variables.

The biological and chemical interactions which take place within the mycorrhizosphere are still largely unexplored, and furthermore the relative importance of the host and mycorrhizal fungus mycelium for directing interactions largely remains to be resolved. One of the key research challenges is to elucidate the mechanisms driving microbial community structure and functioning within the mycorrhizosphere, including the role of exudates and signal molecules. A variety of techniques have recently become available which will prove valuable to address these issues. These include stable isotope probing (Radajewski *et al.*, 2000) and the use of bromodeoxyuridine immunocapture and mRNA (Artursson and Jansson, 2003) to identify metabolically active organisms, and microarrays (Wu *et al.*, 2001) and metagenomic techniques (Tringe *et al.*, 2005) to profile microbial community structure and functioning. Furthermore, transcriptional profiling of mycorrhizosphere interactions (Duplessis *et al.*, 2005; Morel *et al.*, 2005; Schrey *et al.*, 2005), and the genome sequencing of mycorrhizal fungi and their host plants (Town, 2006; Tuskan *et al.*, 2004) will generate understanding of the mechanisms involved in mycorrhiza formation, including the role of free-living mycorrhizosphere organisms. Ultimately, this information should provide new possibilities to exploit biological interactions within the mycorrhizosphere for agricultural and environmental management.

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Escherich and *Escherichia*

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> Scarcely two hundred years back can Fame recollect articulately at all; and there she but maunders and mumbles.
>
> —(Thomas Carlyle, 1843)

> It is a truth universally acknowledged that there are only two kinds of bacteria. One is *Escherichia coli*, and the other is not.
>
> —(J. Allen Downie and J. Peter W. Young, 2001)

I. Introduction

The purpose of this essay is threefold: to give an outline of the life and the various achievements of Theodor Escherich, to provide a background to his discovery of what he called *Bacterium coli commune* (now *Escherichia coli*), and to indicate the enormous impact of studies with this organism, long before it became the cornerstone of research in bacteriology and in molecular biology.

II. The Naming of Bacteria and the ''Mystery'' of ''*E.*''

The names of bacteria provide a double challenge, for, following the footsteps of Carolus Linnaeus for botanical and later for zoological names, one has the convention, convenient and often obscurantist, of giving two names to bacteria. It has been remarked at times that bacterial names are cumbersome. One can say the same of the binomial names of plants and animals, but microbiologists are perhaps more outspoken in their disapprobation:

> Everyone who has worked with bacteria is more or less familiar with such foreign-sounding names as *Bacillus subtilis, Pseudomonas fluorescens, Phytomonas hyacinthi, Thiobacillus thiooxidans, Streptococcus lactis,*

Mycobacterium tuberculosis, Escherichia coli, Proteus vulgaris, Clostridium botulinum, Actinomyces lavendulae, etc.

—(van Niel, 1946, p. 285)

One can add names such as *Shewanella oneidensis, Sulfolobus solfataricus, Moraxella lwoffii, Capnocytophaga ochracea*, and so on. ''Based on the whims of discoverer and committees, bacterial names, both general and species, are largely a curious hodge-podge of derivations of the names of scientists, such as *Escherichia coli*—which honors Theodor Escherich—and purely morphological and physiological descriptions, as in *Thiobacillus denitrificans*'' (Bibel, 2001, p. 163). One of the two names of bacteria is usually more obviously informative than the other, when it refers to disease (*Vibrio cholerae, Mycobacterium tuberculosis*), source (*Leuconostoc mesenteroides*) or an aspect of chemistry (*Methanobacterium thermoautotrophicum, Propionibacterium freu denreichii*). Frequently, one of the two names refers to people, either as the first name, in capitals, such in *Neisseria, Pasteurella, Klebsiella, Ehrlichia, Escherichia, Rickettsia, Yersinia*, or the last name, not in capitals, as in *Pseudomonas stutzeri, Clostridium kluyveri, Bacillus schlegelii, Methanobrevibacter thaueri*.

Such names within names frequently succeed to cloak well-meaning intent in celebratory obscurity. Remembrance fades, and a name remains, glorying in splendid isolation. Matters are particularly nonrevealing when, following convention, the first name is shrunk to the first letter. Thus, while ''*E.*'' may stand for a person such as Ehrlich or Escherich as in *E. equi* or *E. coli*, it may also stand for *Enterobacter* as in *E. aerogenes*, or *Epulopiscium* as in *E. fishelsoni*, or *Eubacterium* as in *E. limosum*. Clarity is retained by the convention of writing out the full name when first encountered in a chapter or an article, but this convention is not always followed. For instance, in newspapers and in Watson's celebrated textbook ''The Molecular Biology of the Gene'' (e.g., Watson *et al.*, 1987, pp. 96 and I-7), one encounters ''*E. coli*'' like a hieroglyphic, without elaboration or explanation. The present essay is an attempt to rescue the ''*E.*'' in *E. coli* from neglect and obscurity, not as much out of a sense of pedantry, but as a piece of historical remembrance that encapsulates bacteriological insight and bacteriological development.

III. A Paradox: The Golden Age of Bacteriology, and Persistence of Noncontagious Notions of Transmissible Diseases

The name Escherich, if remembered at all, is nowadays connected almost exclusively with the name of *Escherichia coli*. It might seem, therefore, that the activities of a well-neigh forgotten physician provide

nothing more than a sentimental journey into remote regions of little present-day interest. It will become abundantly clear, however, that the discovery of *Escherichia coli* is the direct result of Theodor Escherich's combination of superb clinician with the recognition that the practice of bacteriology is an intrinsic and necessary part both of clinical medicine and of basic science. Escherich's discovery in 1885 of the organism eventually named after him falls into the remarkably short and remarkably productive period, 1870s and 1880s, aptly called the golden age of bacteriology (Waller, 2002; Berg, 2004), which saw the discovery (present-day names) of *Mycobacterium leprae* (1873), *Bacillus anthracis* (1877), *Neisseria gonorrhoeae* (1879), *Salmonella typhi* (1880), *Mycobacterium tuberculosis* (1882), *Corynebacterium diphtheriae* and *Vibrio cholerae*[1] (1883), *Clostridium tetani* (1884), *Streptococcus pneumoniae* (1886), *Neisseria meningitidis* (1887), *Salmonella enteriditis* (1888) (cf. Madigan *et al.*, 2003, p. 883). Escherich started his work on digestive diseases in 1884. He was intrigued by the circumstance that *Vibrio cholerae* was at the time the only one of the various pathogenic bacteria known to be directly related to a digestive disease (cf. Coleman, 1987). ''It is widely known that the comma bacilli found by Koch in cholera asiatica are the first well-characterized microorganisms cultured from intestinal contents and in turn causing pathogenic activities in the intestinal tract; it can be assumed that all remember their discovery and history'' (Escherich, 1886a, p. 3). He was interested in determining the cause of childhood diarrhea, a devastating disease that caused the deaths of a large percentage of children. Escherich's aim, however, went far beyond an attempt to correlate a given disease with a given microorganism. At the time of his work, it was not generally accepted that diarrhea in children was brought about by bacteria (cf. Levinson, 1936, pp. 80–85). Thus Hermann Widerhofer (1832–1901), the highly respected pediatrician whose chair at the University of Vienna Escherich was to occupy upon Widerhofer's death, maintained a pathological and not a bacterial origin of the disease, and stated this in a section of Gerhardt's Handbook of Pediatrics (Widerhofer, 1880).[2] These views were in accord with those of Max von Pettenkofer who, as we shall see, at over 70, more or less to cap his influential career, drank a culture of cholera bacteria to prove, once

[1] Actually it was Filippo Pacini (1812–1883), professor of anatomy at the University of Florence, who already in 1854 discovered the causative agent of cholera, and firmly believed that the disease was contagious (Bentivoglio and Pacini, 1995; Bibel, 2001, pp. 289–293; Vinten-Johansen *et al.*, 2003, pp. 228, 316).

[2] Widerhofer, incidentally, was the personal physician of the old emperor Franz Joseph I (Schick, 1957, p. 116), an intriguing combination of pediatrics and geriatrics, perhaps in accord with the insight that age is second childishness.

and for all, that the comma bacillus did not cause this disease. The view that diarrhea was mainly chemical and only in part bacteriological was advanced among others by the US physician Charles Delucena Meigs (1792–1869), author of ''Observations on Certain of the Diseases of Young Children'' (Meigs, 1850) and his son John Forsyth Meigs (1818–1882) (with W. Pepper) in ''A Practical Treatise on the Diseases of Children'' (Meigs and Pepper, 1877). Such ideas, including the notion that foods, especially fats, were the fundamental factors in diarrhea (cf. Czerny, 1939), were subsequently broadened (cf. Gronowicz, 1954, p. 52). We do not know for certain whether these notions, held in contrast to advancing proofs of bacterial infections, were limited to intestinal diseases, or whether they were thought by some to have wider applications. Even Escherich, early in his career, had his uncertainties. As pointed out by Clemens von Pirquet, his eminent pupil, Escherich, who became known as the father of the school of thought that maintained bacteria to be the cause of diarrhea (cf. Gronowicz, 1954, pp. 51–52; Schick, 1957, p. 114) could not in his observations on the 1884 Naples cholera epidemic distance himself from the doubts of his Munich teacher, Pettenkofer, concerning the contagiousness of cholera injections (cf. von Pirquet, 1911) (see Fig. 1).

IV. The Invention of the Word ''Bacteriology'' and the Rapid Rise of Bacteriology as a Distinct Discipline

Many of Escherich's late appraisals stressed that he was unusual in being both a microbiologist and a physician. The distinction or division between these disciplines as well as among many others did not, however, exist in his day. Distinctions both divide and enrich. In retrospect, a unified concept and approach is often seen to have achieved more than the specializations imposed on it by the flowering of knowledge. In the words of the eminent historian of science, Frederick L. Holmes (1986, p. 74), ''Scientific problem areas are more natural than, and often more stable than, the socially constructed disciplines which lay claim to them.'' I found just one review which recognized that it was not unusual in Escherich's time for physicians to become proficient bacteriologists (Bettelheim, 1986).

The beginning of the word ''bacteriology'' appears to have been as explosive as the discovery of its objects of attention. The new term pops up independently in 1884 in three locations, the United States, the Continent, and Britain: in March of that year the word is used in a review of an American translation of a French book (Anonymous, 1884a), in August it perceptively emphasizes that a new discipline has arisen,

resulting from Koch's discovery of the tuberculosis and cholera bacteria (Anonymous, 1884b), and in November the word refers to a new body of knowledge that has become both obvious and imperative (Anonymous, 1884c).[3]

The word ''bacteriology'' rapidly elicited an impressively strong appeal, for within a few years it was found in the titles of textbooks in various countries: in France, 1889, with the first of six editions of Eugène Macé's ''Traité Pratique de Bactèriologie''; in Germany, 1890, with the first edition of Carl Günther's ''Einführung in das Studium der Bakteriologie'' (six editions to 1906!) in the United States, 1891, with the first edition of Alexander Crever Abbott's ''The Principles of Bacteriology'' (cf. Bulloch, 1938, pp. 349, 370).

Since *the profession of bacteriologist* did not exist, its initiators had to come from other disciplines, and here the field of medicine predominates by far. The discoverers of the prominent pathogens, such as Hansen, Koch, Albert Neisser, Klebs, Löffler, Babes, Yersin, were all physicians, and so, of course, was Escherich. Daniel Elmer Salmon was a veterinarian. Eminent early bacteriologists who were not physicians, like Pasteur, trained as a chemist, and Ferdinand Cohn, who started his career as a botanist, did not discover human pathogens.

V. Theodor Escherich's Life

A. From Medical Studies to Assistant in Würzburg

Theodor Escherich was not primarily a microbiologist; rather he was one of the most eminent pediatricians of his time. His life is easily outlined. He was born in 1857 in the Bavarian town of Ansbach (also the birthplace of Georg Ernst Stahl of phlogiston fame), son of Ferdinand Escherich (1810–1880), a highly respected physician whose many clinical interests included the problem of the high mortality of newborns and ways to improve the care of the poor. These topics, among many others, eventually occupied his son. It has been claimed (cf. Oberbauer, 1992, p. 303) that the first of Ferdinand Escherich's four

[3] Not everyone respected Gerhardt. In 1885, Gerhardt, an out-and-out clinician, became director of the second internal clinic of the famous Charité Hospital in Berlin. Paul Ehrlich worked in this clinic as a clinician and as a researcher on staining methods. Gerhardt insisted that Ehrlich devote himself to full-time clinical work. This enraged Ehrlich, who developed tuberculosis and left the Charité. He stated later: ''When I felt so miserable and forsaken during the time with Gerhardt, I often stood before the cupboard in which my collection of dyes was stored and said to myself: 'These here are my friends, which will not desert me' '' (Marquardt, 1951b, p. 28).

wives had been a piano student of Beethoven, but this could not be independently verified. The third wife, Theodor's mother, Maria Sophie Frederike Stromer von Reichenbach, daughter of Johann Sigmund Ludwig Karl Freiherr Stromer von Reichenbach, died when he was 5 years old. Five years later his father moved to Würzburg. At age 12, Theodor, a somewhat rough boy, was sent to the Jesuit seminary Stella Matutina in Feldkirch, northwest Austria. He finished his high school back in Würzburg. His medical studies, 1876–1881, as was usual at the time, were spent in several cities, Strasbourg, Kiel, Berlin, Würzburg. In December 1881, he passed his final medical exam in first class. His career progressed rapidly. From 1882 to 1884, he was an assistant in the medical section of the Julius Hospital in Würzburg. Here he obtained his skills in physical techniques and in diagnostics. The director of this section was Karl Gerhardt (1833–1902) an eminent internist and one of the founders of pediatrics, author of the pioneering ''Textbook of Children's Diseases'' (1861), and editor of the first German handbook of pediatrics (''Handbuch der Kinderkrankheiten'' six volumes and addenda, 1877–1893). Escherich's doctoral dissertation and first publication ''Die marantische Sinusthrombose bei Cholera infantum'' (1882) was written under Gerhardt's sponsorship. In 1883, Escherich published six papers on various clinical subjects, none yet on pediatric topics. Gerhardt was the decisive influence on Theodor Escherich's choice to become a pediatrician. At the time, pediatrics was regarded in Germany as a stepchild of German clinical faculties (cf. Pfaundler, 1911). Escherich revered Gerhardt throughout his career. Thus, he dedicated to Gerhardt his monographs ''Etiology and Pathogenesis of Epidemic Diphtheria'' (1894) and in his memory ''The Tetany of Children'' (1909). Among the other assistants of Gerhardt at the same time as Escherich was Friedrich von Müller who became one of the most eminent internists of his time, known among German physicians as Frederick the Great (Friedrich der Grosse).[4] Escherich was promoted to a higher assistantship a year after the start of his employment in Würzburg. A year later, he left to pursue his specialization in pediatrics.

[4] As far as could be determined, Frobenius' name is a footnote to bacteriology, since his only mention in the bacteriological world is as Escherich's bacteriology instructor. Apparently, Frobenius published nothing with Koch and nothing later. In 1888, he changed course and became a medical missionary in the East Indies and in the East Asian German possessions (Frobenius, 1888).

B. Studies in Vienna and Paris: Makings of a Pediatrician

Escherich spent a semester in Vienna to study at the venerable St. Anna Hospital, the oldest German-speaking children's hospital. Here he heard lectures by two of the most eminent pediatricians of the time, Hermann von Widerhofer (1832–1901) and his pupil Alois Monti (1839–1909). He greatly benefited from Widerhofer's ingenious discussions of pediatric cases in clinics (Escherich, 1902). Little did he know that he would, in 1902, become Widerhofer's successor. It was in Vienna that Escherich presumably finally decided to remain in the field of pediatrics. Moreover, rather than devoting himself to clinical studies, he began to occupy himself with bacteriological topics. At the Vienna pathological institute, he was apparently the first anywhere to perform bacteriological analyses of mother's milk. He demonstrated that while the milk taken from the first day after birth until 8 months later was sterile, the milk from febrile mothers contained yellow and white staphylococci. As we will see, the broadening of his perspective from pure clinical work was typical for Escherich. This particular pursuit developed into Escherich's later classical studies on the feces of infants. After Vienna, he spent a short time in Paris, where, fluent in French from his time in Strasbourg, he listened to lectures in the Salpêtrière by the world-famous Jean Charcot (1825–1893) who among others had also attracted Sigmund Freud. Escherich was also interested in the potential therapeutic application of hypnosis, but never used this approach in his own clinics.

C. Work in Munich: Makings of a Bacteriologist

Following his stay in Paris, Escherich moved in August 1884 to Munich. One reason for his choice was that it was possible to obtain a Habilitation in pediatrics in Munich, although at the time no separate department of pediatrics existed there. He exhibited impressively broad activities: he had access to the hygienic institute of Max von Pettenkofer (1818–1901), the bacteriological laboratory of Otto von Bollinger (1843–1909), the physiological institute of Karl von Voit (1831–1908), and the dairy industry facilities of Franz von Soxhlet (1848–1926). His main interest appears to have been to extend his bacteriological work. He increasingly became convinced that bacteriology could solve or illuminate many pediatric problems. He would sit at the microscope for days and nights on end (Kundratitz, 1961). As luck would have it, he encountered an assistant at the

pathological institute, a certain Wilhelm Frobenius,[5] a physician who had learned his bacteriology from the master himself, Robert Koch, during three short visits to Berlin. Escherich came to know Robert Koch's techniques for the cultivation and characterization of bacteria from Frobenius who in addition gave lectures for physicians interested in these topics. So one could say that Escherich learned his applied bacteriology ''straight from the horse's mouth''. Escherich's experience with bacteriological techniques led to his work with fecal matters.

D. A Study of Cholera in Naples

The year 1884, a year after Koch's discovery of what he called the comma bacillus, later known as *Vibrio cholerae*, was a busy one for Escherich. On the urging of Gerhardt he spent two decisive weeks, October to November, in Naples during an epidemic of cholera in order to study clinical and bacteriological aspects of this intestinal disease (cf. Emmerich, 1885, p. 291). It was here that Escherich did his first work on fecal bacteria, published, incredibly, that same year (Escherich, 1884). Escherich accompanied the somewhat more senior Rudolf Emmerich from the University of Munich. Robert Koch, in a letter, December 18, 1884, to his friend Carl Flügge,[6] referring to Escherich's publication

[5] Carl Flügge (1847–1923) established Germany's first Institute for Hygiene at Goettingen and served as its first director. He became professor of hygiene at the University of Goettingen in 1885, at Breslau in 1887, and eventually at Berlin. In 1886, together with Robert Koch, he began publishing the Zeitschrift fuer Hygiene, under their joint editorship. The second edition of Flügge's book, ''Die Mikroorganismen'' published in the same year, already refers to Escherich's 1886 book as a notable beginning of research in this field (ein bedeutsamer Anfang zur Erforschung dieses Gebietes) (Flügge, 1886, p. 591, footnote). One is impressed by the thorough reading of contemporary literature, made possible by impressively rapid publications, achieved when typesetting was very much slower than the much more rapid methods used today.

[6] Emmerich was a student of the influential Max von Pettenkofer (cf. Locher, 2001), Professor of Hygiene in Munich, a man who doubted the bacterial origin of a disease such as cholera, so much so that, at the age of 74, he drank a suspension of cholera bacilli he had asked for from Robert Koch. He developed just a slight bit of dysentery (Köhler and Mochmann, 1968, p. 29; cf. Brock, 1988, p. 183). Koch, guessing Pettenkofer's intent, is held deliberately to have sent him a rather weak suspension of the bacteria. Clearly, therefore, considerations for the life of a fellow scientist outweighed Koch's recognition of acting against the acceptance of his own views on the pathogenicity and infectivity of these and other bacteria. In those days a dispute between the ''contagionists'' and the traditional ''miasmatists'', with Koch on the side of the former and Pettenkofer on the side of the latter, had pretty much been settled in favor of the former, and Koch almost certainly recognized that Pettenkofer's views and impending experiments would have only minimal impact. Emmerich and various others, including Elie Metchnikoff, repeated this ''experiment'' (Hume, 1925). According to Möllers (1950, p. 626) and

(1884), of his work with cholera in Naples, remarked that Escherich had no difficulties in finding these organisms, while Emmerich[7] was a miserable failure (cf. Möllers, 1950, p. 159). A fascinating account of the Naples 1884 cholera epidemic is found in Snowden (1995).

E. To Graz, Austria, as Associate Professor of Pediatrics, and Innovations Therein: Spread of Escherich's Fame

Escherich stayed in Munich until 1890 when, at the age of only 33, he was appointed associate (auβerordentlicher) professor of pediatrics and director of the St. Anna Children's hospital at the University of Graz, Austria. He received these appointments not because of his abilities in bacteriology, but because of his achievements as a pediatrician. He rapidly expanded the renown of this hospital to international attention, and more than tripled the number of patients in his hospital. By 1896, the mortality of the neonates in his clinic had been decreased to about 39%, while in the famous Charitè hospital in Berlin it was still as high as 72%. In 1899, a section of the hospital designed for the care of neonates who needed special attention was opened. By 1900, a novel kind of children's walk-in incubator was constructed with his father-in-law, the physicist Leopold von Pfaundler. While in Graz, he studied as many as 300 cases of tetany. He was the first anywhere to use so-called galvanic current as a diagnostic device (cf. Sperk, 1905). In 1895, influenced by von Behring's discovery of diphtheria serum, he wrote his book on diphtheria, croup and serum therapy (Escherich, 1895), and demonstrated that bladder infection was caused by *Bacterium coli commune*. This was not all. A mere 2 years

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Schlegel (1999, p. 162), the culture of *C. vibrio* that Pettenkofer and Emmerich drank was sent not from Berlin by Koch but from Hamburg by Koch's assistant, Georg Gaffky, who had stayed in Hamburg after the 1892 cholera epidemic. Pettenkofer, according to Schlegel, had approached Gaffky to send him a culture of cholera bacteria for research purposes. Research indeed! It is not clear whether Gaffky, or according to some writers, Koch, sent a fully virulent or a weakened culture. While Pettenkofer developed only minor symptoms of cholera, Emmerich almost died after becoming seriously ill. Koch himself, according to this book, regarded such heroic human experiments to be unnecessary, since nature's experiments with cholera epidemics were proof enough.

[7] Ernst Weber (1901–1996), born in Vienna, was an eminent engineer with a long career in the United States, recipient of six honorary degrees, member of the National Academy of Sciences, recipient in 1987 of the US National Medal of Science. He was married to Escherich's daughter, Dr. Charlotte Weber (Sonya), born 1895, who had been on the faculty of the School of Medicine at Columbia University. As mentioned in the text, Escherich's only other child, Leo, died of appendicitis at age 9.

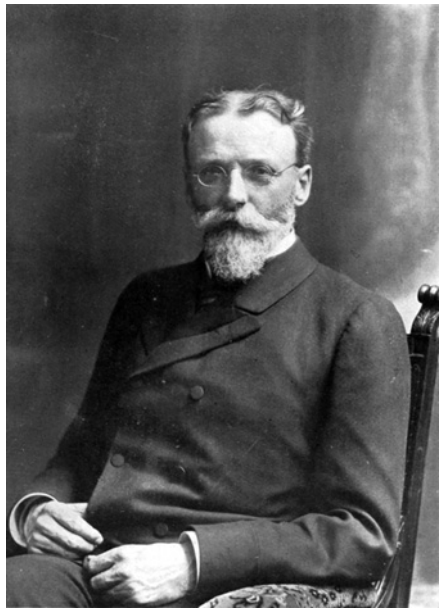


FIG. 1. ''Escherich was young, distinguished-looking, and wore an impressive beard. This was not unusual at that time'': Béla Schick (1957, p. 114) in a moving evocation of his teacher.

after Roentgen's 1895 discovery of x-rays, Escherich was able to obtain funds for the purchase of an x-ray apparatus that he used to follow bone growth in children after feeding them cod liver oil. With two other physicians he was instrumental in changing the requirements for medical studies, including by 1899 obligatory examinations in pediatrics, dermatology, and psychiatry, reforms that were introduced in Germany only 19 years later (Peiper, 1951). Escherich was responsible for expanding research and lecture facilities. He had built a new lecture hall and founded a small library. Four years after his appointment, he was promoted from associate professor (Extraordinarius) to full professor (Ordinarius). His fame spread. He became the pediatrician of nobility and royalty, including the Sultan of Turkey, the son of the King of Bulgaria, and the children of the King of Montenegro. During a journey to Russia, he was called to attend on the hemophiliac son of the czar. Escherich was known, however, not to distinguish between rich and poor patients. As an example, he carried a child suffering from diphtheria from its home to the nearby St. Anna hospital and in so doing saved the child's life (Reichspost Wien, 1911; cf. Oberbauer, 1992, p. 332).

F. Escherich Attracts Brilliant Students

He attracted a coterie of future eminent pediatricians including students from the United States. His most famous students were probably Béla Schick and Clemens von Pirquet. Schick is remembered for his intracutaneous test for diphtheria, using the serum that Escherich and Klemensiewicz had developed as early as 1893 (Escherich and Klemensiewicz, 1893). von Pirquet coined the word ''allergy'', introduced a diagnostic test for tuberculosis by the cutaneous reaction to tuberculin (cf. von Pirquet, 1909; Breathnach, 1985), and worked on serum sickness. In 1909, at age 35, von Pirquet spent about a year as the first chairman of the department of pediatrics at Johns Hopkins University (cf. Silverstein, 2000). After a short stay at the University of Breslau, he became Escherich's successor, 1911, in Vienna.

G. To Vienna as Professor of Pediatrics and Director of a Famous Children's Hospital

In 1902, upon the death of Hermann Widerhofer the eminent pediatrician at the University of Vienna, Escherich was appointed by unanimous decision to succeed him as professor of pediatrics and director of the Vienna St. Anna Children's Hospital. This appointment raised Escherich to one of the most prestigious chairs in pediatrics. [The Vienna St. Anna Hospital was started in 1837. There were only two older pediatric hospitals in Europe, Paris (1802) and St. Petersburg (1834) (cf. de Rudder, 1957, p. 1621).]

H. Various Achievements in Vienna, Medical and Social

Escherich's work in Vienna in the 9 years until his death from a stroke at age 53 was marked by an impressive variety of achievements in many fields of medicine and in the initiation of social organizations devoted to the welfare and health of children. His organizational abilities, already used highly effectively in Graz, again consumed much of his energy in Vienna. He immediately equipped a bacteriology and a chemistry laboratory, and he was the first in Vienna to use x-rays as a diagnostic tool in children. His detailed plan for a completely new children's hospital in Vienna was done by 1906, but bickering with the authorities delayed its completion until after his death. ''Because of delay in the building of the new hospital Escherich renovated and expanded the old one ... and created as a first undertaking in Europe, a children's open air terrace on the roof of the new clinic'' (Weber,

1990, p. 23).[8] He was devoted to social concerns for the welfare of children and was determined to reduce the capital's high infant mortality. In 1903, just a year after his arrival in Vienna, he appealed for support in a pamphlet to the women of Austria. The response was so strong that in following year, with imperial patronage and civic approval he founded the Infants' Care Association (Säuglingsschutz). Previously, newborn babies were not admitted to hospitals because of their high mortality: 20% of all babies died before age 1. His charm and his powers of persuasion induced many high society ladies, including the archduchess, to become members of his organization. Princess Rosa Croy-Sternberg assumed the presidency and each year organized a ball for Vienna's high society that brought in large sums of money for the work of Escherich's baby care society (Hamburger, 1911, p. 266). A dispensary was started as well as a training school for nurses who soon became known all over Austria as ''Escherich Nurses'' (Weber, 1990, p. 23). Public awareness of high infant mortality was very much furthered.

I. Escherich as Vice President and Only European Pediatrician at 1904 Congress of Arts and Sciences, Held as Part of St. Louis World's Fair

A year later, Escherich was invited as the sole European pediatrician to address the International Congress of Arts and Sciences held from September 19 to September 25 at the St. Louis World's Fair. This Congress brought together some of the greatest minds of the time, such as William Osler in Medicine, Jacques Loeb in Biology, Theobald Smith in Pathology and Bacteriology, Adolf Furtwängler (the father of the future conductor Wilhelm Furtwängler) in Archaeology and Classical Greek Art, Ludwig Boltzmann in physics, and a number of past and

[8] (i) A review (Anonymous, 1884a, p. 362), March 21, 1884 of George M. Sternberg's translation of Antoine Magnin's ''Les Bactéries'' (cf. Sternberg, 1884) contains the following: ''Dr. Sternberg is at the head of the American school of working bacteriologists, if, indeed, he is not its only member.'' (Sternberg, author of numerous medical treatises, was the principal physician to two US presidents.) (ii) The August 30, 1884 report of the eighth International Medical Congress at Copenhagen (attended by about 700, including Pasteur and Virchow) has: ''[...] the discovery of the tubercle bacillus by Professor Koch and his later investigations on the spread of cholera have given such an impulse to this branch of knowledge, that in Germany it has become a separate study under the name of bacteriology'' (Anonymous, 1884b, p. 281). (iii) The first sentence of the article ''Bacteriology'' in the November 20, 1884 issue of ''Nature'' states: ''Among the most striking of the recent rapid advances of science is the development of what we may term bacteriology'' (Anonymous, 1884c, p. 49).

soon-to-come Nobel laureates in chemistry: Jacobus Hendricus van't Hoff (1901), Svante Arrhenius (1903), William Ramsay (1904), Henri Moissan (1906), and Wilhelm Ostwald (1909). Escherich, representing Austria as one of the seven honorary vice presidents of the Congress, was one of the speakers at the opening of the Congress. ''On the stage were seated the officials of the Congress, the honorary vice presidents from foreign nations, and the officials of the Exposition'' (cf. Rogers, 1905, pp. 25, 29–30). Two days later, he gave one of the two addresses in the session on pediatrics (cf. Rogers, 1905, p. 69). This important, long, address ''The Foundations and Aims of Modern Pediatrics'' was published five times: in English (Escherich, 1905a), in German as an excerpt (Escherich, 1904b), in the complete form (Escherich, 1905b), in English in the publications of the Congress (Escherich, 1908), where he is impressively ennobled as Theodore von Escherich, and some 75 years later again in English (Hellbrügge, 1979). A detailed paper (Escherich, 1905c,d) reported his American impressions.

J. Escherich Becomes Famous and Initiates Construction of the Imperial Institute of Maternal and Child Care

Escherich's fame soon spread. In March 1906, he was named Hofrat (court counselor) by the Emperor Franz Joseph. He and his wife were invited several times to dinner at court. The Escherich home, with its soirées and formal dinners for over 40, had become a meeting point of society that included the likes of the composer Gustav Mahler (1860–1911, Escherich's almost exact contemporary) and the opera tenor Leo Slezak (Weber, 1990, p. 27; Oberbauer, 1992, p. 362). In 1908, Escherich became president of the Austrian Society for Children's Research. On the occasion of the Emperor Franz Joseph's sixtieth jubilee that year, he again drew attention to the inexcusably high national rate of infant mortality, and his efforts eventually led to the construction of the Imperial Institute for Maternal and Child Care.

K. *Bacterium coli commune*, a Propellant to Fame

It has to be pointed out that the initial fillip to his ascent, the work that spread his name, was his impressively thorough and intensive study of the intestinal bacteria in neonates and small children. *Bacterium coli commune* provides an important background to his career. ''More than a quarter of his publications relate to bacteriology'' (Dolman, 1971). It has been stated, fittingly, that Escherich transferred Robert Koch's bacteriological methods into pediatrics (Lesky, 1981, p. 194). ''Although his claims that *B. coli* could cause cystitis and other localized infections were

undisputed, his contention that some virulent strains provoked infantile diarrhea and gastroenteritis was verified only after sixty years'' (Dolman, 1971, p. 404). ''*E. coli* is the most common cause of bacterial diarrhea in humans worldwide'' (Pickering and Cleary, 2004, p. 617; cf. Pickering *et al.*, 1978, 2006; Sections VI. G and VII). ''The idea of the pathogenicity of various strains of coli bacteria that as we know still plays a special role in the intestinal diseases of babies, undoubtedly originated with Escherich'' (Kundratitz, 1961, p. 723). Escherich's ''observation on intestinal bacteria in young children at once became a classic and fundamental work'' (Gronowicz, 1954, p. 52). ''He certainly deserves credit for realizing how important a role bacteria play in gastrointestinal disorders in infancy. He demonstrated that this normal inhabitant of the intestinal tract [*Bacterium coli*] could become pathogenic and virulent …. The bacterial era [was] inaugurated in pediatrics by Escherich'' (Schick, 1957, pp. 114–115). ''As long as Escherich lived, the bacterial flora of the gastrointestinal tract was his favorite topic of study. Frequently, when a foreign student came to the clinic eager to study a problem, Escherich would suggest his pet subject'' (Schick, 1957, p. 115). Although Escherich's interest and researches subsequently branched out in many different directions, he did not lose his enthusiasm for his first chosen field. He recognized many strains of *Bacterium coli commune*, differing in morphology and biological behavior, and initiated the notion of their pathogenicity. Far in the future lay the insights into enteropathogenic, enterotoxigenic, enteroinvasive, enterohemorrhagic, and enteroadherent *E. coli* (EPEC, ETEC, EIEC, EHEC, and EAEC) (cf. Cohen and Gianella, 1991, p. 413; Donnenberg, 2002; Dobrindt *et al.*, 2003; Krauss *et al.,* 2003; Smith, Willshaw, Cheasty, 2004; Alouf and Popoff (eds.), 2006, pp. 45–46, 120–134, 491–503; Baylis *et al.*, 2006; Lan and Reeves, 2006) especially EHEC 0157:H7, the main culprit in what has been described as Hamburger Disease or Barbecue Syndrome. ''What became of the pathogenic strains that were discovered by Escherich? Today fifty million children still die world-wide of diarrheas caused by them'' (Müller-Hill, 1986, p. 708). The versatility of *E. coli* has gone much further: It ''has been incriminated in infections of almost every human organ system'' (cf. Koneman *et al.*, 1997; Joneja, 2004, p. 144). (cf. Shulman *et al.* (in press) for a review on *E. coli* with a pediatric emphasis.)

L. Escherich's Vienna Hospital: A Mecca For Pediatricians

Escherich's intense interest in research and his fine and pleasant personality (Hamburger, 1911, p. 266) made the Vienna St. Anna Children's Hospital a ''Mecca for pediatricians'' (Schick, 1957, p. 114). It has been

stated that Escherich's clinic exceeded any other scientific training institute in the number of university professors and hospital directors that it produced, both in Germany and in Austria (Finkelstein, 1911). As pointed out by his brother-in-law, Meinhard von Pfaundler, in his obituary: ''Escherich did not recognize his limits, only the urge to more intensive living, to stronger fight, to more work. He could not think that even his amazing energy could give out, that anything could interfere that was stronger or more powerful than his will to create'' (Pfaundler, 1911).

M. Escherich's Books and a Review

In addition to his first book at age 29, on intestinal bacteria (Escherich, 1886a) which included classic descriptions of the two bacteria that he named *Bacterium coli commune* and *Bacterium lactis aerogenes* (later called *Bacterium aerogenes*, often confused with *Enterobacter aerogenes*, and now called *Klebsiella pneumoniae*, cf. Holmes and Aucken, 1998, p. 1007), he published three books or monographs, on epidemic diphtheria (1894), diphtheria, croup and serum therapy (1895), and on tetany in children (1909), and with his brother-in-law, Meinhard von Pfaundler, a lengthy review of the impressive knowledge of *Bacterium coli commune* that had built up in the 18 years since its discovery (Escherich and Pfaundler, 1903). The review ends with about 600 references to authors, most by now forgotten, but an indication of the extent of work and interest in this field (see Fig. 2).

N. Escherich's Clinical Versatility

In Graz and then in Vienna, Escherich built schools of pediatrics. Many of his associates moved with him from Graz to Vienna, among them were Béla Schick and Clemens von Pirquet. It has been stated that there was not a topic in pediatrics in which Escherich was not interested (Neuburger, 1935) and to which he did not contribute. He coined the term idiopathic tetany and established its parathyroid source (cf. Kundratitz, 1961, p. 724) long before the subject of endocrine secretions was known.

He was one of the first, with Klemensiewicz, to show the presence of antitoxins in the serum of children that had spontaneously recovered from diphtheria (Escherich and Klemensiewicz, 1893). He instituted antitoxin therapy in his clinic patients. [He] vigorously sponsored Paul Moser's antistreptococcus serum in scarlet fever treatment. Although he failed to show a direct relation between *Bacterium coli commune*

and diarrhea, he was the first to show that it brought about bladder infection (cf. Schönbauer, 1947, p. 357). Some have regarded this to be among his most important discoveries (cf. Pfaundler, 1911, p. 522). ''The discovery of colicystitis and *Streptococcus enteritis* of infants is exclusively the result of Escherich's researches'' (Rosenthal, 1943, p. 750). ''He was intensely interested in the diagnosis, pathogenesis, and control of tuberculosis. He pioneered in X-ray detection of the disease in children'' (Dolman, 1971, pp. 404–405). In 1889, he confirmed the causal role of the Klebs-Löffler bacillus in a diphtheria epidemic. He made important contributions to the study of infant feeding. His name is associated not only with *Escherichia coli* but also with Escherich's reflex (manifested by a muscular contraction of the lips) (Escherich, 1888; cf. Schachter, 1972). Escherich's name appears in the most unexpected places, a further indication of his versatility. Thus, a compilation of classical papers in clinical dermatology (Shelley and Crissey, 2003, pp. 331–332) included his 1904 paper on erythema infectiosum (Escherich, 1904a). ''He narrowly missed discovering dysentery bacilli, of which he isolated several cultures, only to discard them because they failed to produce gas in carbohydrate-containing media'' (Dolman, 1971, p. 404).

O. Escherich as Teacher, Pediatric Scholar, and Master Educator

In addition to his direct clinical contributions, he ''had no equal in his time, either as a teacher and scholar, or as an organizer'' (Gronowicz, 1954, p. 83). He gave stimulating lectures (Dolman, 1971, p. 404), was an enthusiastic and conscientious teacher, and excelled in describing the pathology of the various diseases of children (Hamburger, 1911, p. 266). As an educator, Escherich was member of an 1892–1893 committee, including the later Nobel laureate Julius Wagner-Jauregg (1857–1940), that separated the study of medicine into a preclinical and a clinical section and that required a preliminary examination in general biology rather than in individual sciences such as mineralogy, botany, and zoology. It took many years, in fact until 1899, for this suggestion to be adopted (Lesky, 1976, p. 268). The fame of the Graz children's hospital expanded well beyond the borders of Austria. The Vienna children's hospital was one of the oldest and most prestigious in Europe. The hospital constructed according to his plans, but which he did not live to see completed, was considered after World War I to be the most beautiful and the largest in the world (Lesky, 1981, p. 198).

Escherich in the course of his career published about 190 papers in addition to his books, and he supervised 271 papers of his associates, whose names were always cited as first authors or by themselves.

He served as head of the German pediatric society. As Pfaundler stated in his Escherich obituary (Pfaundler, 1911), it was hard to imagine German pediatrics without Escherich. His work in pediatrics and in bacteriology was so wide-ranging that it is hard to do justice to all of it. In fact, on comparing various obituaries and later biographical sketches, it is clear that many do not mention some of his diversified activities. A summary is indicated to give a notion of the range of his interests and contributions apart from his discovery and work with *Escherichia*. An excellent brief survey is given by Fischer (1962, p. 375).

P. Escherich's Sudden Death

Escherich died at the comparatively young age of 54. The death of his son Leo from appendicitis some 5 years earlier had strongly affected him with increasing signs of arteriosclerosis (cf. Sackmann, 1985). ''The first symptom of the impending disaster was that Escherich started to talk French at the rounds and complained of headache. He died suddenly several days afterward, apparently from a cerebral hemorrhage'' (Schick, 1957, p. 122). A more sedate version is given in the obituary in *The Lancet* (Anonymous, 1911a): ''Suddenly he began to speak in different languages ... and he had to be conveyed to his home, where he died the next day.'' According to Wagner (1968, p. 87), however, Escherich died during a lecture to his students.

Q. Summary: The Life of a Socio-Pediatrician

Escherich's name should be remembered not only as the discoverer of the bacterium named after him, but as one of the preeminent pediatricians of his time, head of one of the most respected pediatric clinics whose renown he furthered, tireless contributor to a variety of medical and social fields, prolific author of books, monographs, and papers, and outstanding medical educator (cf. Fischer, 1962; Enersen, 2003). A pioneer pediatrician who devoted his efforts to improving child care, particularly infant hygiene and nutrition (Enersen, 2003), ''Escherich believed that pediatrics consisted not only of research and the cure of diseases, but also of prophylactic work'' (Rosenthal, 1943, p. 750). He has aptly been called a socio-pediatrician (Spitzy and Georgopoulos, 1986). ''Less versatility and longer life might have won him greater celebrity and more durable renown'' (Dolman, 1971, p. 405).

R. Obituaries

Escherich's work extended over the whole area of pediatrics, both in terms of clinical work and in terms of strong social interests in the welfare of children and their mothers. In accord with this diversity, contemporary appraisals, evident most clearly from his 1911 obituaries, select one or other of his accomplishments, but most decidedly do not stress his work on children's digestive disorders. Escherich's eminence and unexpected death at an early age stimulated an immediate outpouring of obituaries in leading international medical journals (Anonymous, 1911a,b,c; Finkelstein, 1911; Hamburger, 1911; Moll, 1911; Neurath, 1911; Pfaundler, 1911; von Pirquet, 1911; Zappert, 1911). The one from the United States stated ''His professional reputation was international'' (Anonymous, 1911b). The various writers agreed that he contributed to all regions of pediatrics. They selected different aspects of his medical achievements for special praise such as his studies on tetany, diphtheria, tuberculosis, cystitis, nutrition. Recurring emphases, apart from his prolific medical contributions, were his ''tremendous energy for work'' (Anonymous, 1911b), ''a force of nature with superhuman capacity for work'' (Hamburger, 1911, p. 266), his renown as a teacher, his great organizing skill, manifested particularly in his detailed planning of the splendid new children's hospital in Vienna and in his perseverance in establishing an organization, probably far ahead of its time, whose purpose was to help infants in need of proper nutritional help (Säuglingsschutz). Many of the obituaries, written under the direct spell of his strong personality, emphasized that his unusual ability to apply bacteriology to pediatrics made him the father of bacteriological pediatrics. Some of these evaluations mention but do not especially emphasize what later insight has selected as his most important contribution, the discovery of *Bacterium coli commune*.

A comparison of the recurring tenor of contemporary Escherich obituaries with the numerous appraisals of his work published on the centenary of his birth is highly instructive. Past fame and present acceptance do not always run in parallel. Much that was in the forefront of awareness by his contemporaries moved into the background some 40 years after his death. Thus in a long, highly emotional obituary by Julius Zappert, written on the day of Escherich's death and published in the prestigious *Wiener Medizinische Wochenschrift* a mere 3 days later, one is impressed by the description of Theodor Escherich's strong personality, his organizing ability, and his wonderful way with children. His founding of the organization ''Säuglingsschutz''

(Protection of Newborns) for the distribution of mother's milk to needy mothers, his initiation, through sheer hard work, of the Vienna pediatric society, and his tireless work toward the building of a new children's hospital, whose completion he did not live to see, are emphasized. His principal work was stated to have consisted in the founding of a state-wide organization for the care of mothers and newborns. Praise is given to the fact that some of his many pupils were in charge of three of the largest pediatric clinics in Germany (in Munich, Breslau, and Heidelberg). As a tribute to his impressive energy and versatility, there is a list of his major clinical accomplishments shown by publications on diphtheria, tetany, streptococcal enteritis, pediatric tuberculosis, the demonstration that bladder infection is caused by *Bacterium coli*, and numerous others. The 1886 book, ''The Intestinal Bacteria of Children'' (Die Darmbakterien des Kindes) ''which reported facts completely new at the time and still accepted'' was mentioned to have allowed him to move toward the forefront of pediatricians. However, this work was not discussed any further. Similarly, the three-page obituary by Meinhard von Pfaundler discusses Escherich's work on intestinal bacteria, but his other clinical achievements and his medically related social enterprises are emphasized (Pfaundler, 1911). Clemens von Pirquet, his student and successor in Vienna following Escherich's death, describes him as one of the founders of scientific pediatrics and one of its most important representatives. His detailed obituary ends with the statement ''how proud we are of Escherich's scientific discoveries and the social-hygienic organizations that he initiated and that will ensure his name to be conveyed to posterity''. As stated in connection with the death of another scientist, ''Eulogies and obituaries were a common genre of memorial literature, and many notices, essays and printed speeches appeared in the wake of [one's] death'' (Rupke, 2005, p. 15).

S. Late Appraisals of Escherich's Accomplishments

Later writings about Theodor Escherich, in so far as they understandably stressed his bacteriological work, especially his discovery of *E. coli*, give an incomplete and distorted impression of his many and varied accomplishments. Appraisals were published more or less on the occasion of Escherich's centenary (Neuburger, 1935; Schönbauer, 1947; Gronowicz, 1954; de Rudder, 1957; Schick, 1957; Kundratitz, 1958, 1961; Katner, 1959; Fischer, 1962; Dolman, 1971; James, 1973; Lesky, 1976, 1981) and, later, in celebration of the centenary of his discovery of *Bacterium coli commune* (Breathnach, 1985;

Sussman, 1985; Bettelheim, 1986; Hobom, 1986; Lorenz, 1986; Müller-Hill, 1986; Spitzy and Georgopoulos, 1986; Seeliger, 1987; Irrgang and Sonneborn, 1988). These articles are impressive in their number and in their marked change in emphasis, for now *Escherichia coli* has moved inexorably to the fore, and Escherich's social and varied clinical achievements are subjects, if at all, of dutiful but rather transitory recitation.

There is an Escherich street in Vienna (in the 19th Borough), an Escherich Pavilion existed in the pediatric clinic of the University of Vienna (destroyed in World War II), and the Austrian Pediatric Society issued a plaque ''for merit'' first awarded in the centenary, in 1986, of Escherich's publication of his book on the intestinal bacteria of the newborn (Oberbauer, 1992, p. XI).

T. Escherich's Uniqueness

Although publications that praise and evaluate great men often ignore comparisons with others, it is useful to do so now. He combined, even at an early age, clinical and rigorously scientific attitudes and pursuits; he manifested a clear recognition of objective knowledge separate from direct clinical observations as a prerequisite to eventual clinical insight. His quest for the bacterial cause of devastating childhood dysentery was made difficult by the well-nigh overwhelming variety of intestinal microorganisms. It demanded a thoroughly analytical approach as a prerequisite for an answer to his motivating question. Although his own work did not lead to an answer, his discoveries led to basic insights into fecal bacteria as related to location within the intestine, and the host's age and nutrition. A less-schooled investigator might have missed or ignored these complex interactions. Escherich was impressive in the thoroughness and patience of his work, in his persistence of seeking the bacterial etiology of childhood dysentery, and in his clear recognition, clearly expressed, that an inability to show a direct connection between the presence of his favorite organism *Bacterium coli commune* and dysentery did in no way render his and many others' studies of this organism irrelevant to the clinical problem at hand. We see here, therefore, an admirable scientific attitude that in addition fertilized his numerous other clinical interests and accomplishments. The masterful descriptions in his 1886 book, a work written in his twenties, stimulated early inquiry and discovery on the part of a host of now forgotten investigators. Escherich was lucky to discover an organism that for sheer ease and rapidity of growth in the test tube ultimately led to fundamental discoveries in

bacteriology, biochemistry, and molecular biology of which he had no inklings. In addition to its ready cultivability, Escherich's bacterium lacked the kind of pathogenicity manifested by organisms discovered by his contemporaries, thus stimulating wide-ranging interest into its biology, more and more divorced from its role as an intestinal organism. Luck is not a quality of accomplishment that denigrates a person's renown. As Paul Ehrlich said in an oft-quoted passage, scientific success needs patience, skill, money, and luck (Geduld, Geschick, Geld, und Glück) (cf. Marquardt, 1951a, p. 24). Escherich's versatility and scientific focus deserve one's attention and one's admiration. He made substantial contributions to a variety of medical fields, upheld the social imperatives of his discipline, and possessed an impressive organizational ability. His pleasant and yet inspiring personality, so highly appreciated during his life, helped Escherich's achievements beyond his early clinical and bacteriological observations (see Fig. 3).

VI. First Studies with *Bacterium coli commune*: Search for the Bacterial Cause of Infantile Diarrhea

A. Introduction

Escherich's Naples experiences on cholera stimulated his investigations of children's feces and infantile diarrhea immediately on his return from Naples. In every one of the known, recently discovered pathogens, and many still to come, there was a direct correlation between a given disease and a given pathogen. It was unusual that Escherich did not isolate the causative organism. His motivation was the same, but it developed quite differently. Dysentery was an ancient disease. ''[. . .] the Latin word *pestis*, which was widely used until the seventeenth century, was used to indicate any of the great epidemic diseases, such as plague, typhus, smallpox, or dysentery.'' ''The first book of Samuel in the Old Testament provides the earliest detailed description . . .. Although all retrospective diagnoses are speculative, the disaster was probably an outbreak of dysentery'' (Bourdelais, 2006, 11. 6–7). An epidemic in 580 was described by Gregory, Bishop of Tours, and in 1670 Thomas Sydenham described it as causing ''great torment of the bowels'' (Beck, 2000, p. 28). Escherich hoped that a bacteriological approach would lead to a discovery of the cause of this decimating affliction. He faced a daunting challenge: in contrast to the clear-cut cause and effect relationship presented by other pathogenic bacteria, the unraveling of the cause of dysentery presented a different picture. As he said in an 1885 lecture:

> At a time when Koch's experimental methods reap such rich laurels in the newly revealed fields of the etiology and pathology of infectious diseases it might appear to be a useless and almost thankless task to attempt to disentangle the apparently completely unregulated mass of intestinal bacteria, dependent on a thousand contingencies, of intestinal bacteria in normal stools. When, however, I have by now devoted a year almost exclusively to this special study, I did this in the conviction that the exact knowledge of these conditions is essential not only for the physiology of digestion where intestinal putrefaction remains an unknown and incalculable X, but also for the pathology and therapy of the bacterial intestinal diseases.
>
> —(Escherich, 1885b, quoted by Kundratitz, 1961)

The approach of the author, from a study of normal conditions, to a study of pathology and from microscopy to bacterial cultures, has a thoroughly modern flavor. We find a balance between the attitudes of the pure scientist and the clinician, and an impressive mastery of both.

Escherich's publications leading to *Bacterium coli commune* began with a talk in Munich to the Society for Morphology and Physiology, December 17, 1884 (Escherich, 1885a). He reported preliminary studies indicating that infants in the first days of life had about 20 different types of intestinal and fecal bacteria. The bacteria, grouped according to their appearance in the microscope, included colored sarcinae, 5 types of micrococci, and 10 types of bacilli. No names were given. The short report ends with the statement that bacteria, probably introduced from the food, could be demonstrated in the feces within 12 to 24 hours after birth.[9] This was the opening salvo, greatly

[9] This summary of a talk given by Theodor Escherich at a session, December 17, 1884 of the Gesellschaft (Society) für Morphologie und Physiology at Munich is so short that a complete translation is given here: Escherich (1885a) Ueber die Bakterien des Milchkotes [Concerning the bacteria of milk feces [feces of children fed mother's milk]. Aerztliches [Medical] Intelligenz-Blatt [Publication], Münchner Medicinische Wochenschrift [Weekly] **32**: 243.

''The speaker, following a Referat by professor Tappeiner concerning the work of Bienstock, reports that in the course of his investigations of the feces and the intestinal contents of infants in the first days of life he has for the time being isolated about 20 different types of bacteria: 2 tooth types, several colored sarcinae forms, 5 micrococci and 10 bacillary types. Of the latter, 5 types are constantly found in large amounts as inhabitants of the duodenum and the small intestine, while they appear to form spores in the less suitable conditions encountered in the colon and in the rectum. The occurrence of the micrococci is limited to the large intestine, while that of the sarcinae is restricted to the lowermost part of the rectum. For the latter the entry per anum into the intestinal canal has been established, while the bacilli and cocci are probably introduced with the food and can be detected in the feces as early as 12–24 hours after birth.''

developed in four subsequent publications that inexorably paid more and more attention to *Bacterium coli commune*: a long July 14, 1885 lecture (Escherich, 1885b) before the same society; a year later his celebrated book on the intestinal bacteria of children (Escherich, 1886a); a historical survey in 1887 on intestinal bacteria and diseases (Escherich, 1887); and in 1903, with Pfaundler (cf. Wiskott, 1966, p. 97), a highly detailed 141-page review including 21 pages of densely printed references. The latter was the very first article dealing exclusively with *Bacterium coli commune*. This review, just short of 20 years after the discovery of the organism, was part of the Handbook of Pathogenic Microorganisms, with articles by Victor Babes, Paul Ehrlich, Armauer Hansen, Élie Metchnikoff, and Albert Neisser. Escherich added new observations in each of his various publications. The cumulative effect was one of concentrated hard work, elegance, and critical familiarity with the latest contributions to an exploding field of medicine and bacteriology (see Fig. 4).

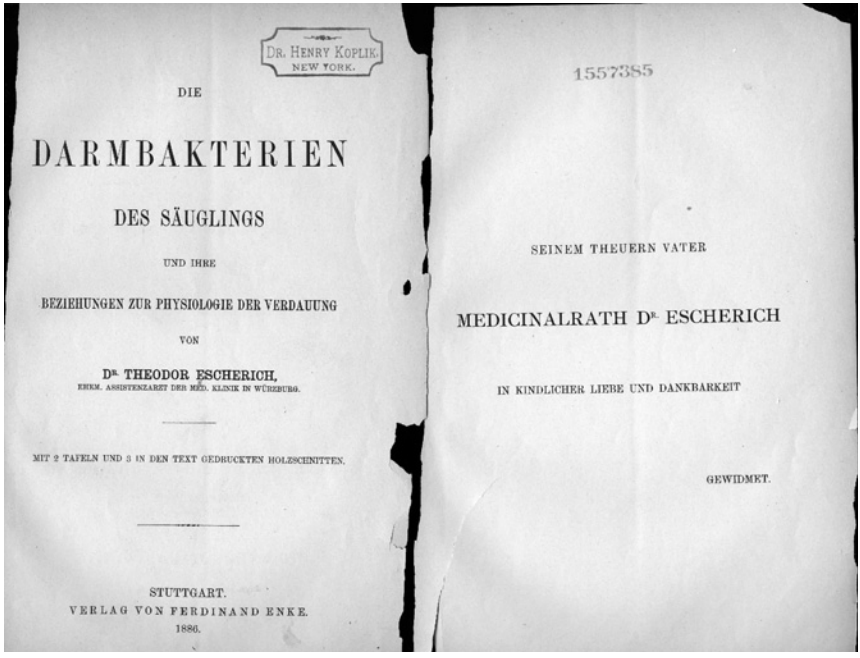
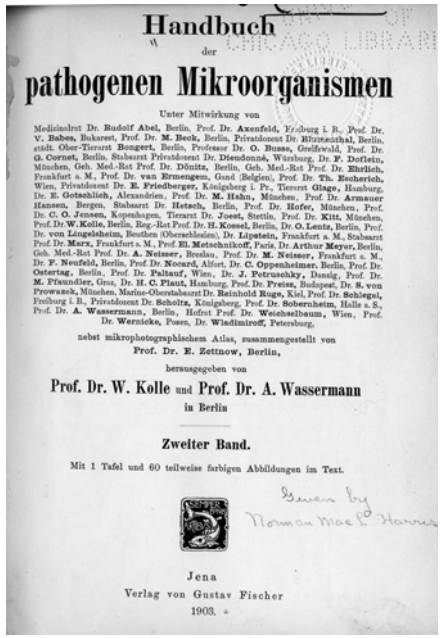


FIG. 2. Title and second page of Theodor Escherich's classic book, 1886, ''The Infant's Intestinal Bacteria and their Relationships to the Physiology of Digestion'', to ''His dear father, Senior Medical Officer (Medizinalrath) Dr. Escherich, dedicated with a child's love and gratitude''.



Handbuch

der

pathogenen Mikroorganismen

Unter Mitwirkung von

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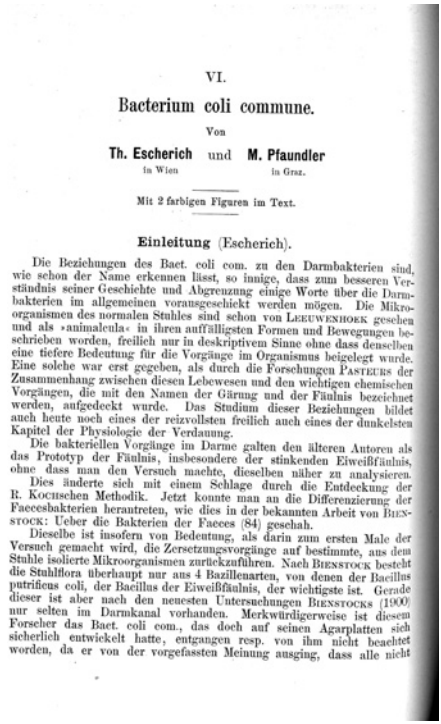
FIG. 3. Title page, volume two of the four-volume comprehensive ''Handbook of Pathogenic Microorganisms'', 1903, edited by W. Kolle and A. Wassermann.

B. THE 1885 LECTURE

In the very first sentence of the 1885 lecture, translated 100 years later into English (Escherich, 1988, 1989), Escherich stated that it may appear pointless and thankless to disentangle the myriad bacteria that occur in the stools and in the intestinal canal, but that he was not going to give up (Escherich, 1885b). The year spent on this topic, to the exclusion of almost everything else, was motivated by the expectation that studies of these bacteria could lead to a better understanding (1) of the physiology of digestion and (2) of the pathology and therapy of microbial intestinal diseases. He emphasized the importance of the latter topic since the mortality from intestinal diseases continued to decimate the world of infants (unsere Säuglingswelt) with unchanged ferocity. His studies did answer some questions concerning microbial processes in the digestive tract, but did not identify the bacterial etiology of intestinal diseases. Nevertheless, his work paid off in other ways. With impressive thoroughness he embarked on a study of the bacterial population found in the intestinal excreta, right



VI.

Bacterium coli commune.

Von

Th. Escherich und M. Pfaundler
in Wien in Graz.

Mit 2 farbigen Figuren im Text.

Einleitung (Escherich).

Die Beziehungen des Bact. coli com. zu den Darmbakterien sind, wie schon der Name erkennen lässt, so innige, dass zum besseren Verständnis seiner Geschichte und Abgrenzung einige Worte über die Darmbakterien im allgemeinen vorausgeschickt werden mögen. Die Mikroorganismen des normalen Stuhles sind schon von LEEUWENHOEK gesehen und als »animalcula« in ihren auffälligsten Formen und Bewegungen beschrieben worden, freilich nur in deskriptivem Sinne ohne dass denselben eine tiefere Bedeutung für die Vorgänge im Organismus beigelegt wurde. Eine solche war erst gegeben, als durch die Forschungen PASTEURS der Zusammenhang zwischen diesen Lebewesen und den wichtigen chemischen Vorgängen, die mit den Namen der Gärung und der Fäulnis bezeichnet werden, aufgedeckt wurde. Das Studium dieser Beziehungen bildet auch heute noch eines der reizvollsten freilich auch eines der dunkelsten Kapitel der Physiologie der Verdauung.

Die bakteriellen Vorgänge im Darme galten den älteren Autoren als das Prototyp der Fäulnis, insbesondere der stinkenden Eiweißfäulnis, ohne dass man den Versuch machte, dieselben näher zu analysieren.

Dies änderte sich mit einem Schlage durch die Entdeckung der R. KOCHschen Methodik. Jetzt konnte man an die Differenzierung der Faecesbakterien herantreten, wie dies in der bekannten Arbeit von BIENSTOCK: Ueber die Bakterien der Faeces (84) geschah.

Dieselbe ist insofern von Bedeutung, als darin zum ersten Male der Versuch gemacht wird, die Zersetzungsvorgänge auf bestimmte, aus dem Stuhle isolierte Mikroorganismen zurückzuführen. Nach BIENSTOCK besteht die Stuhlflora überhaupt nur aus 4 Bazillenarten, von denen der Bacillus putrificus coli, der Bacillus der Eiweißfäulnis, der wichtigste ist. Gerade dieser ist aber nach den neuesten Untersuchungen BIENSTOCKS (1900) nur selten im Darmkanal vorhanden. Merkwürdigerweise ist diesem Forscher das Bact. coli com., das doch auf seinen Agarplatten sich sicherlich entwickelt hatte, entgangen resp. von ihm nicht beachtet worden, da er von der vorgefassten Meinung ausging, dass alle nicht

FIG. 4. First page of Escherich and Pfaundler's 141-page review on *Bacterium coli commune* in the 1903 Handbook. Escherich's introduction mentions Leeuwenhoek, Pasteur, and Koch.

from birth. He discovered that within the first 3 days of life there were three distinct bacterial profiles. The first of these profiles was shown by the meconium, the third by the subsequent feces. At the very start of life, the meconium was sterile, but, in the first profile, a variety of bacteria and yeasts sometimes was present as soon as 4–7 hours after birth.

C. THREE BACTERIAL PROFILES IN INTESTINES OF THE DEVELOPING INFANT: PREVALENCE OF *BACTERIUM COLI COMMUNE* IN EARLY LIFE

Escherich described a number of these bacteria, including some spore-formers, and others that occurred in smaller numbers and that could be grown in culture, including *Bacillus subtilis* and the rods that he called *Bacterium coli commune*. A dense microbial population

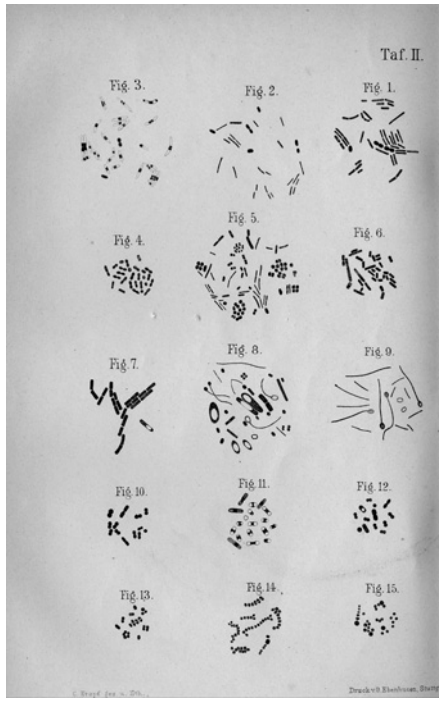


FIG. 5. Photographs and some of the accompanying legends at the end of Escherich's 1886 book. *Fig. 4. Bacterium coli commune* from a 6-day-old potato culture; predominantly short, constricted (eingeschnürte) shapes. *Fig. 6. Bacterium coli commune* from an 8-day-old gelatin plate colony (Gelatineplattencolonie); readily apparent rod-type. *Fig. 7. Bacillus subtilis*; one bacillus at the stage of spore formation. *Fig. 10. Bacterium lactis aërogenes* from an 8-day-old gelatin test tube. All photographs, 970-fold magnified, were taken by Charles Workman, M.D. from Belfast, a visitor in the Bacteriological Laboratory of the Munich Pathological Institute, using isochromatic plates, and copied photographically (durch Lichtdruck vervielfältigt).

developed after about 24 hours. He inferred that this population entered through the mouth with the very first breaths of air and also with air via the anus, since the population varied with the dust content of the air, the temperature, and the time of year. In milk-fed infants he found a sudden change in the bacterial profile, an observation of primary importance in the study of *Bacterium coli commune.*

In the second profile, the earlier diverse bacterial population had been replaced by a single bacterial type, consisting of slender, at times slightly bent, short rods that appeared to be a pure culture. He called it *Bacterium coli commune*. He gave a detailed description of these bacteria, noted over

a 100 years later to be up-to-date (Bettelheim, 1986, p. 256). He emphasized their polymorphism, also observed in cultures, although this disappeared after a number of subcultures on a gelatin medium. He described the appearance of colonies grown on media with agar, blood serum, potatoes, milk, and cane sugar. This thoroughness was accorded to only one of the other bacteria, described almost as an afterthought, which he called *Bacterium lactis aerogenes*. (His concepts were not too clear, since he talked of a ''pure *Bacterium coli commune* culture'' and then mentioned *B. lactis aerogenes*, admittedly present in smaller amounts.)

In a 1900 study, Ernst Moro, one of Escherich's students, confirmed Escherich's observations on the distinctions between the bacteria of meconium feces and mother's milk feces, and extended these distinctions to cow's milk feces. He made two interesting points: he used the adept phrase ''the vegetation from meconium feces to mother's milk feces changes with one blow'' (ändert sich mit einem Schlage), and he indicated that an examination of the baby's feces permitted one to determine even an occasional administration of cow's milk.

The third profile, with its variety of microorganisms, tended to resemble that of the adult.

D. Escherich's Classical 1886 Book on Infants' Intestinal Bacteria: Masterpiece of a 29-Year-Old

In his classical 1886 book on the intestinal bacteria of infants, Escherich addressed three related questions: the normal bacterial composition of the intestinal tract of infants and the changes that these undergo right from birth; the role in nutrition of the decomposition of foods by intestinal bacteria; and the relation of these studies to pathological conditions. There had been but one study on microscopic examinations of feces of normally fed infants, by Uffelmann in 1881, and there had been very few attempts at cultivation of intestinal bacteria from adults by the new methods of Koch. Escherich emphasized that microscopy would furnish only the framework for studies that used bacterial cultures. In contrast to Uffelmann, who detected but two different bacteria in infant's stools with microscopic observation, Escherich succeeded in isolating at least 19 different bacteria from the intestinal contents of infants and carefully described their morphology and cultural requirements (cf. Irrgang and Sonnenborn, 1988, p. 8). He paid special attention to the regularly occurring and numerically predominant *Bacterium coli commune*. He demonstrated that these bacteria could grow in the absence of oxygen, that is, under conditions resembling those found in the intestine (cf. Oberbauer, 1992, p. 313).

He stated: ''They were thus-far found only in the intestinal canal, especially in its lower parts, and therefore they were called 'colon bacteria''' (Escherich, 1886a).

A brief summary of this work provides a rich insight into the background motivation, results, and outlook of Escherich's investigations of children's feces. He spent 15 months in this new enterprise, away from his exclusively clinical work, spending days and nights sitting over his microscope. He started his book by pointing out that the first to show the occurrence of the ''smallest living beings'' in the feces was Leeuwenhoek, in a 1719 letter to Robert Hooke: ''de vivis animalculis existentibus in excrementis'' (Hooke, 1719). In a footnote, Escherich gave a long passage from Leeuwenhoek in Latin, without translation, indicative of his own learning and of his assumption that his readers would not need a translation.[10]

Escherich was very much aware of the distinction to be made between the bacterial picture revealed by direct examination with the microscope and subsequent attempts at bacterial cultures, for not all bacteria will grow in culture. He described in detail his invention of a painless and effective method to remove fecal material from babies, using aspiration with a syringe under sterile conditions. He stressed the difference between the miniscule bacterial content of feces from babies obtained up to 14 hours of birth (meconium feces) and that obtained on milk feeding (milk feces). The meconium feces were sterile or sparsely populated (he described three bacteria, *Proteus vulgaris, Streptococcus coli gracilis*, and *Bacillus subtilis*), while the milk feces had an abundance and variety of bacteria, different from those in the meconium feces. Escherich divided these bacteria into two different kinds, *obligate* and *facultative*. The obligate bacteria, always present in large amounts, consisted of just two novel species which he named *Bacterium lactis aërogenes* (former spelling) and, present in far greater abundance, *Bacterium coli commune* (cf. Escherich, 1885b). As to the facultative fecal bacteria,

[10] In a learned article Dobell indicates, without reference to or apparent knowledge of the 1719 letter, that Leeuwenhoek, ''the first to observe the intestinal protozoa of man'' recorded his discovery in 1681 from his own stools (Dobell, 1920, p. 1), and that it can be concluded from his detailed description that he observed *Giardia* (=*Lamblia*) *intestinalis* (Dobell, 1920, p. 15). Dobell makes the point that all of Leeuwenhoek's letters were written in Dutch, ''the only language which he could read or write'' (Dobell, 1920, p. 3), and that he discovered free-living protozoa earlier than 1675 (Dobell, 1920, p. 1). Dobell (1932, p. 198) quotes the eminent Dutch microbiologist Beijerinck to the effect that Leeuwenhoek's ''animalcules'' ''were undoubtedly bacteria—not protozoa—and that among them were probably (as he found in his own experiments) *Bacillus coli, Azotobacter,* and *Amylobacter saccharobutyricum*'' (Beijerinck, 1913).

Escherich described 14 different species. These occurred in much smaller numbers and were not always present. Of these, only one, *Micrococcus ovalis*, had a distinctive name. As a sign of bacterial classification at the time, four yeast species (*Torula* and *Monilia candida*, as well as ''red yeast'' and ''capsula yeast'') were included. Escherich discussed at great length the relation between facultative intestinal bacteria and anaerobiosis. He was very much aware of facultative anaerobiosis as a possible explanation for the rather limited number of species found in the intestine. He described a device that he has designed to grow bacteria anaerobically. *Bacterium lactis aerogenes* and *Bacterium coli commune*, especially the latter, got by far the most attention. This organism was found from 14 hours after birth, but not before. He demonstrated that these two organisms had different pathogenic activities in different animals. On injections in various ways, he found that *Bacterium coli commune* was highly pathogenic in guinea pigs, pathogenic in cats, less so in rabbits, and nonpathogenic in mice and dogs. He performed detailed autopsies on the succumbed animals. He observed that *Bacterium lactis aerogenes* manifested a similar distribution of pathogenicity. He emphasized the polymorphous character of *Bacterium coli commune* and regarded it as almost certainly identical to Brieger's bacillus, isolated from feces (Brieger, 1883–1884, 1885a,b). He made it clear that *Bacterium coli commune* occurred in the lower part of the small intestine and *Bacterium lactis aerogenes* in the upper part. He emphasized (Escherich, 1886a, p. 39) that not all the fecal bacteria observed microscopically will grow after inoculation on growth medium with gelatin or with agar-agar.

Escherich stressed that the occurrence of bacteria in feces was a perfectly normal phenomenon. In addition, he recognized, without providing data, that the bacterial composition of adults' feces was very different from that of children. He asked whether the facultative organisms, often obtained from children who showed slight digestive symptoms, might be disease related, but he admitted that more clinical and animal experiments were needed to answer this question. The book is impressive in its thoroughness, range, and in its bacteriological and pathological acumen. Moreover, the book is largely a piece of scientific research, with only a few statements as to its possible or hoped for medical promises. Thus, on pp. 53–54 one reads:

> ''Among the endless number of facultative intestinal bacteria I have described in some detail only a few of the more frequently occurring types and groups. Among these we will . . . encounter a predominant number of cocci. The relevant types of rods and spout types (Sprosspilzarten) were obtained from children who suffered or who had suffered from light

digestive disturbances. Did these forms have an etiological relation to the disease? There are various reasons that made it appear very likely that some intestinal diseases of infants are caused by certain microorganisms, and that among the facultative intestinal bacteria some will be found that, starting from the intestine, will bring about disease. However, it is obvious that only the systematic investigation of suitable clinical cases and animal experiments will answer these questions.''

The book ends with the following evocation (p. 177):

''The first and most essential basis of further progress ... consists in the study of the physiological processes of fermentation and of the bacteria occurring in the intestine under normal conditions, as has been the aim of the present work. May the perspectives gained here not remain without use and without practical applications on behalf of the therapy of the most murderous pest of the first year of life, the *mycotic intestinal diseases*.''

Rietschel and Hummel (1927, pp. 1006–1007) pointed out that Escherich's studies on the intestinal flora should be remembered not only for the discovery of *B. coli commune* and *B. lactis aerogenes*, but in addition for stressing the importance of processes of fermentation and putrefaction in infants, for being the first to indicate the relation between nutrition and the bacteria flora, and for initiating studies on the impressive differences in the bacterial flora of different parts of the intestinal canal.

In the part of the book that discussed the properties of *Bacterium lactis aerogenes* and *Bacterium coli commune* in detail (microscopic behavior, growth on a variety of media, macroscopic appearance of colonies, fate of injection into various animals such as guinea pigs, rabbits, cats, dogs, and mice), the former organism was treated in 6½ pages, but the latter received 11 pages. Few, if any, animal experiments were carried out with the remaining bacterial types that could be cultured.

The second part of the book dealt with the oxygen consumption of intestinal bacteria. At least 55 experiments were carried out on facultative anaerobiosis of various microorganisms on various media, intended to throw some light on the possible presence of oxygen in the intestinal canal and on the manner by which the intestinal contents were colonized. The third part of the book discussed the physiology of intestinal fermentation in infants, recognized the limited breakdown of proteins by the intestinal bacteria, investigated the acidity of infants' feces, reported experiments on the origin and types of intestinal gases, and discussed the relation between the activities of intestinal bacteria and nutrition. The shortest and last section, ''Clinical-Therapeutic Considerations'', a mere 4½ pages, reflected the profound lack of

knowledge in this area. The book's last sentence stated: ''May the views that have been obtained here not remain without use and practical application for the therapy of the most murderous plague of the first year of life: the bacterial infectious diseases.''

Escherich's work, undoubtedly facilitated by the application of Koch's bacteriological methods, and buttressed by persistence and impressively hard work, brought the knowledge of intestinal bacteria to a new level. The book impresses one by the application of pure science in the search for a practical solution to a serious clinical problem.

From the welter of intestinal forms that he was the first to describe, Escherich extracted both descriptive and, to some extent, interpretative order. This achievement was in no small measure due not only to the patience and thoroughness that he brought to his 1886 book but also to his decision to ignore those organisms that could not be cultured by measures such as the ones newly introduced by Robert Koch. In addition to *Bacterium coli commune* and *Bacterium lactis aerogenes*, he tested his organisms for growth not only on agar media, but on gelatin media, on potatoes (remarking that certain types of potatoes were more suited than others), as well as on liquid media. He much preferred gelatin media over agar media. In addition, he instituted clinical tests with *Bacterium coli commune* and *Bacterium lactis aerogenes*, and observed that both of the above were on occasion pathogenic in humans and, not always reproducibly, in animals such as guinea pigs, rabbits, cats, and dogs. Escherich made it his task to attempt making some order out of the abundance of bacteria present in infant feces after a few days of life. His persistence and sheer hard work are obvious. He focused on those bacteria that could be cultivated after initial microscopic observation. He distinguished between ''the types that were constant inhabitants of the intestinal tract and the ones that were rarer and present in smaller amounts'' (p. 53). Escherich's initial aim, to discover a bacterial cause for children's diarrhea, was not satisfied, but in its place a matter of at least equal importance came to light, an emphasis on nutritional factors related to children's well being and disease. Escherich initiated the study of nutritional disturbances as related to health (Ballabriga, 1991, p. 14).

E. Early Fascination and Denigration of *Bacterium coli*: Lessons of Methodological Limitations

The 1885 discovery of *Bacterium coli commune* initiated the field of intestinal pathology. There is a paradox in this story. The limitations of methods of bacterial cultivation at the time pushed this readily grown

and undemanding bacterium to the forefront of interest (cf. Barth, 1984, p. 111). Friedrich von Müller denigrated the importance of *Bacterium coli*. He emphasized that this organism always pops up when the usual methods of bacterial culture were used, although it represented only a small part, and not necessarily the most important one, of the bacterial population. He implied, therefore, that *B. coli* was rather a nuisance in the search of possible true intestinal pathogens (Müller, 1898, quoted by Barth, 1984, p. 111 and by Knoke, 1984, pp. 111–112). A researcher from London at almost exactly the same time (Gordon, 1897) praised the hardiness of the organism as a reason for its apparent intestinal prevalence: ''[. . .] it finds thence a large area of distribution, and as a facultative anerobe of great hardiness and fertility, it can thrive in circumstances where many a more delicate microbe would perish.'' This view was in direct contrast to the prevailing opinion that, for a long time, regarded *Bacterium coli* as the most important intestinal bacterium (cf. Strasburger, 1902). We have here a wonderful object lesson in the limitations that available technology places on the pursuit of knowledge. In the intervening century, the source and possible biological function of this organism has become all but irrelevant, and the very ease of cultivation that catapulted it to initial interest has ensured its continuing usefulness. It often has been remarked that the large number of subcultures of various strains of *Escherichia coli* would render it unsuitable for intestinal growth (cf. Hobom, 1986). Most present-day interest in this organism as a research tool for molecular biology completely ignores its long-distant intestinal provenance. Reminders are provided by its many pathological strains and by its use as an indicator of biological contamination (initiated by von Freudenreich, 1895, 1896). We will see below how recognition of *Escherichia coli*'s relatively minor contribution to the bacterial population of the colon constitutes an object lesson in advances in bacteriological techniques.

F. Luck and the Discovery of *Bacterium coli commune*

In terms of the discovery of *Bacillus coli commune*, Escherich was lucky to have studied the stools of infants, for in adult stools the abundance of this organism is rather less. Reports vary, but nowhere else does one find an incidence even approximating that discovered by Escherich in the stools of just a few day old infants. Another criterion that Escherich used, as stressed at the start of his book, was capacity to grow in culture the bacteria observed under the microscope. *Bacillus coli commune* turned out to be an exceedingly easy organism to grow.

It was the ready cultivability, along with a short generation time, rather than a recondite interest in human excreta, that right from the start contributed materially to the preponderance of studies with this organism. The term ''coli'' was intimation of its early history, of direct interest only to the clinicians and pathologists who were saddled with intestinal aberrations, but far indeed from the minds of the myriad researchers who studied bacterial mutants, genes, clones, and used the organism to unravel the genetic code, bacterial conjugation, DNA and RNA synthesis, topoisomerases, bacteriophages, and the mysteries of gene expression. Strains such as *E. coli* ML or K-12 are progeny distant from even a cursory acquaintance with a colon, far removed from their ancestral progenitors. They would, in fact, not be able to exist in human intestines (cf. Hobom, 1986, p. 65). ''Most work on *E. coli* centers around one particular strain, known as Nissle 1917. It was isolated in World War I from a soldier who survived a particularly severe outbreak of diarrhoea. Nissle proposed the use of *Bact. coli* as early as 1916 and showed in the 1930s that administration of this strain improved symptoms in patients with non-infectious bowel disorders'' (Rastall and Gibson, 2004).

G. The Incidence of Intestinal *E. coli*: A Tale of Diminution

Since Escherich's days there has been a flowering, if one is permitted to use this term, of interest and research in intestinal flora. The estimated number of species in the human microflora has been increasing from ''some 300 different types of organisms'' (Finegold *et al.*, 1974; Finegold and Sutter, 1978), ''up to 400 different phenotypes'' (Onderdonk, 1999, p. 165), to at least 400 to 500 different bacterial species (cf. Moore and Holdeman, 1974; Poulsen *et al.*, 1995), and in addition some eukaryotic species, and at least one methanogenic archaeal species (cf. Harmsen and Welling, 2002). For a listing of the main bacterial genera in human feces, see Tannock (2001, p. 410S). On the other hand, the reported percentage of *E. coli* in the human gut has been steadily decreasing. Gordon (1897) calls it ''An extensive occupant of the intestine of man and the higher animals''. Jacob (1909) indicates that it is the predominant inhabitant (der häufigste Bewohner) of the gut. Clifton (1950, p. 12; 1958, p. 13) regards *Escherichia coli* as ''the predominant organism in the intestinal tract of man''. Finegold *et al.* (1974) reported *E. coli* to be among the 25 most prevalent species in the fecal flora from humans fed a Japanese or a Western diet. ''Human faeces usually contain 10^3–10^8 *Escherichia coli*/g, comprising approximately 1% of the total cultivable intestinal flora'' (Hartley *et al.*, 1977). *Escherichia coli* is now accepted as but a minor ingredient of the intestinal flora in the adult

human; numbers range between 0.2% and 1.5% of total cells (Poulsen *et al.*, 1995). It has been regarded by Moore and Moore (1995) as the 22nd most common fecal bacterial species, at 1.21%. They recognize 371 taxa, of which 261 are ''new species yet to be named''. Gerhardt and Iglewski (1976) report one case of an individual who completely lacked or had very low numbers of coliforms in his stools. The problem of the disparity between the number of microscopically observable species and the number that could be grown in culture, already a concern of Escherich, has been partially overcome by the application of new methods for studying population dynamics in the intestinal tract such as quantitative polynucleotide chain reaction (Q-PCR) (Higgins *et al.*, 2001) and fluorescence *in situ* hybridization (FISH) (cf. Harmsen and Welling, 2002). These techniques have changed perspectives on the prevalence of bacteria in many ecosystems related to the human colon. Thus ''In 1995 . . . the results of bacteriological culture . . . [indicated] that obligate anaerobes outnumbered facultative anaerobes 1000:1. Since then, nucleic acid-based techniques have provided a new and more accurate perspective of this bacterial community. Nevertheless, a value of around 0.1% for *E. coli* holds true'' (Tannock, 2003a). ''Facultative species commonly described as 'enterics', such as *Enterococcus faecalis* and *Escherichia coli*, are present at less than 0.1% of the total population'' (Onderdonk, 1999, p. 165). ''As commensal organisms in our gut flora [*E. coli* is] a relatively minor component'' (Thomas, 2004). Numerous references can be found in a review by Paul (2002). For some more publications on the extensive literature on this topic cf. Adam (1922); Rotimi and Duerden (1981); Kuhn *et al.* (1986); Simon and Gorbach (1986); Hall *et al.* (1990); Yoshiota *et al.* (1991); Roberfroid *et al.* (1995); Adlerberth *et al.* (1998); Adlerberth (1999); Kirjavainen and Gibson (1999); Tannock (1999); Schwiertz *et al.* (2003); Tannock (2005); Tuohy and McCartney (2006). Escherich's own comments on the numerical incidence of *Bacterium coli commune* in the intestine are somewhat inconsistent. In one exhaustive review (Escherich and Pfaundler, 1903) the organism was said to constitute ''only a vanishingly small fraction of the bacteria in the feces and in the intestinal contents'' (p. 336), while later, in the same review, one reads about the ''constant and rich occurrence of *Bact. coli com.* especially in the lower parts, in the empty intestinal canal, in the meconium and in diarrheal feces'' (p. 417).

Four further points must be mentioned: (1) The influence of diet on the intestinal microflora, very much a concern of Escherich who stressed the importance of mother's milk in early nutrition, remains a subject of close attention (Finegold *et al.*, 1974; cf. Finegold and Sutter, 1978; Hanson and Yolken, 1999; Tannock, 2003b). (2) It has become

known that ''The composition and functioning of [the intestinal] microflora plays an important role in the protection of the host against several pathogenic conditions such as colonic cancer, gastroenteritis, immunological disorders and developmental atopy'' (cf. Hentges, 1983; Tannock, 1999; Harmsen and Welling, 2002, p. 42, and references therein; for an early reference, cf. Müller, 1898). (3) In spite of the recognized low numbers of *E. coli* in the gut, the mutagenicity of this organism and its occurrence or invasion of other tissues make it a prevalent and important pathogen (cf. Gibson and Macfarlane, 1995).[11] (4) Escherich's pioneering studies on the prevalence of *Bacterium coli commune* in infants have been extended. For example, a paper by Bettelheim and Lennox-King, almost 100 years later (1976), discusses the influence of the newborn's environment, such as the type of birth and, as main vector, the contaminated hands and uniforms of nursing staff [conclusions that forcefully remind one of the famous Semmelweis observations on the transmission of puerperal fever by physicians attending at childbirth (cf. Risse, 1975, pp. 294–297)].

VII. *Escherichia*: Vagaries of a Name

The name *Escherichia* is now so strongly associated with the intestinal bacterium first denoted by Theodor Escherich as *Bacterium coli commune*, that the rather turbulent history of this name is often forgotten. The name, *Escherichia coli*, along with many other bacterial names, apparently was proposed in 1918 by Aldo Castellani and Albert J. Chalmers. Castellani, an eminent Italian physician, was at the time Lecturer at the London School of Tropical Medicine[12] and Chalmers

[11] In a paper from Graz, one of Escherich's associates, a Dr. Robert Eberle, reports on the counting of infants' intestinal bacteria. He finds that only between 4.5% and 10.6% of the stainable bacteria will grow on his nutrient media. He discusses the possibility that this low ratio may be due not only to inadequate nutrients, but to the possibility that many of the fecal bacterial types may be dead or weakened (Eberle, 1896).

[12] Aldo Castellani (1878–1971) had an extraordinary career (cf. Bibel 2001, pp. 261–263; Shelley and Crissey, 2003, pp. 410–411). He obtained his M.D. degree in Florence in 1899, trained in bacteriology in Bonn, in tropical medicine in London, went to Uganda and was the first, near end of 1902, to show *Trypanosoma brucei* in the cerebrospinal fluid of a patient suffering from sleeping sickness, became Director of the Bacteriological Institute and Professor of Tropical Medicine at the Medical College of Colombo in Ceylon (Sri Lanka), discovered *Shigella sonnei* (he had called it *Bacillus ceylanensis*), wrote 200 mycological articles, discovered *Candida tropicalis*, *Candida pseudotropicalis*, *Candida guiliermondii*, and *Trichophyta rubrum*, was simultaneously Professor of Tropical Medicine at Tulane University (later at Louisiana State University), at the London School of Hygiene and Tropical Medicine and at the University of Rome. He also practiced medicine at Harley Street in London (eventually became Sir Aldo Castellani),

was Director, Welcome Tropical Research Laboratories, Khartoum, Sudan. The name and the 1918 date are found in a 1919 and a 1920 Castellani and Chalmers publications: (1) the third edition (1919) of their impressive ''Manual of Tropical Medicine'', p. 941 and (2) Annales de l'Institut Pasteur (1920) near the end of a 21 page paper ''Sur la Classification de Certains Groupes de Bacilles Aérobies de l'Intestin Humain''. The early ''Escherichia'' appellations are highly confusing. In the index to the ''Manual'' there are indeed a few references to *Escherichia coli* and extensive references to *Bacillus coli commune*, but none of these overlap. Similarly, in the ''Annales'' there is one mention of *Escherichia coli* Escherich 1886 (p. 619), and the organism is twice referred to as *B. coli* (pp. 610, 620). A look at the first edition (1923) of ''Bergey's Manual of Determinative Microbiology'' shows that the name, ''*Escherichia*'', had caught on like wildfire, for no less than 22 apparently different types of bacteria were designated as *Escherichia* (Bergey *et al.*, 1923, pp. 194–205). The second edition of Bergey (1925) already indicated the rather uncertain or tentative nature of at least some of these assignments, for by then, although there were again 22 different species of bacteria classified as *Escherichia*, some of the earlier ones had been eliminated and others had taken their place (Bergey *et al.*, 1925, pp. 216–227). The third edition (1930) listed 29 species of *Escherichia* (Bergey *et al.*, 1930, pp. 316–331), the fifth edition of Bergey (1939) had extensive revisions and has *Eschericheae* under Enterobacteriaceae as Tribe I, Genus I (Bergey *et al.*, 1939, pp. 389–396). At this point there were but two species under this genus, *Escherichia coli* and *Escherichia freundii* and three variants of *E. coli* (*acidilactici, neapolitana*, and *communior*). Topley and Wilson (1937, pp. 521–526) had the same three variants of *E. coli*. The appendix to the 1939 Bergey had 10 additional species according to Hauduroy *et al.* (1937, pp. 226–232). Almost two closely printed pages in the 1939 Bergey listed bacteria either closely related to *Escherichia*

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and spent his final years in Lisbon as Professor at the Tropical Disease Institute and personal physician to the exiled Queen of Italy. This is not all. His and Albert Chalmer's Manual of Tropical Medicine is an immense and highly impressive book (the first edition, 1910 has 1212 pages, the third edition, 1919, has 2436 pages) (Castellani and Chalmers, 1910, 1919). In 1959, with Frederick Reiss, he founded the Internationl Society of Dermatology (initially the International Society of Tropical Dermatology) and was its first President. In England his patients included the Maharaja of Mysore, Rudolf Valentino, and Giuglielmo Marconi. To cap all of this, perhaps, he was also at one time the personal physician of Benito Mussolini. Much of this is described in Castellani's fascinating autobiography (1960). Not too many people know that we owe the term *Escherichia coli* to Mussolini's personal physician.

coli or identical to it. In the sixth edition of Bergey (1948, p. 10) one reads: ''One of the most unsatisfactory portions of recent classifications . . . is the treatment given to organisms of the coliform-dysentery-typhoid group.'' The applicability of the term *Bacterium* to this group of bacteria was described (Breed *et al.*, 1948, pp. 444–453). The seventh edition of Bergey (1957) had four species (*coli, aurescens, freundii, intermedia*) and four variants (the ones from the fifth edition plus *Escherichia coli* var. *communis*) (Yale and Breed, 1957, pp. 334–341). Almost a whole page was given to serology. ''Within recent years there has been a great increase in interest in the serology of *E. coli* due to the association of certain serotypes with severe outbreaks of infantile diarrhea . . .. While it is not yet clear how many different strains of *E. coli* may be involved in the etiology of infantile diarrhea, the following have been found repeatedly in association with the disease: 026:B6; 055:B5; 0111:B4; 0127:B8; and O128:B12'' (p. 337). By the time of the eighth edition, almost 20 years later (1974), the situation had changed again, this time drastically (Cowan, 1974, pp. 290–293; Ørskov, 1974, pp. 293–296). ''*Escherichia* is now regarded as a genus with only one species in which there are several hundred different antigenic specificities; together these specificities produce by different combinations of the O, K and H antigens several thousand serotypes. Two species that were in the genus *Escherichia* in the seventh edition of The Manual will now be found in *Citrobacter*, while a fourth species, *E. aurescens*, is thought to be a pigmented form of *E. coli* and is denied specific status'' (p. 292). By 2005, in the second edition of ''Bergey's Manual of Systematic Bacteriology'', 18 pages are devoted to the genus *Escherichia*. A few important facets may be mentioned: (1) Comparative studies with rDNA. These show a close phylogenetic relatedness between *E. coli, Salmonella* spp., and *Citrobacter freundii*. In addition, relationships to *Shigella* are pointed out. ''With the exception of *S. boydii* serotype 13, the DNAs of *E. coli* and the four *Shigella* species show such a high degree of relatedness . . . that these species should be considered as a single species (Brenner *et al.*, 1973). The distinction between these bacteria prevails, however, for reasons of historical/medical precedent and to avoid confusion in the literature and with existing surveillance systems'' (Scheutz and Strockbine, 2005, p. 607). (2) O, K, H, and to some extent F antigens: ''Subdivision of *E. coli* can be carried out in many ways, but serotyping remains one of the most useful ways to subdivide the species on a global basis'' (Scheutz and Strockbine, 2005, p. 613). (3) Pathogenicity in relation to virulence factors: ''Most *E. coli* strains are nonpathogenic and reside harmlessly in the colon; however, certain serotypes or clones play an important

role in both intestinal and extraintestinal diseases .... In hosts with compromised defenses, *E. coli* can also be an excellent opportunistic pathogen'' (Scheutz and Strockbine, 2005, p. 613). The longest part of the review is devoted to the topic of *E. coli* in human intestinal diseases. The complexity of this field is brought home by the discussion of as many as eight ''currently recognized categories of diarrheagenic *E. coli*'' (cf. Hyma *et al.*, 2005; Scheutz and Strockbine, 2005, p. 613) such as for example enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). In addition, it has been recognized that ''*Shigella*, which still stands as a genus with four species ... in reality belongs to the extremely diverse species *Escherichia coli*'' (Lan and Reeves, 2002). The magisterial ''Topley & Wilson's Microbiology and Microbial Infections'' has extensive discussions of the serology of *E. coli* (cf. Altweg and Bockemühl, 1998; Holmes, 1998). One cannot help recognizing, in relation to Theodor Escherich, how far one has had to travel in order to confirm his motivation for studying the bacterial basis of at least one of the causes of childhood dysentery. One also bears in mind the role of *E. coli* in extraintestinal infections (cf. Scheutz and Strockbine, 2005, p. 620), for, as we saw, Escherich was the first to demonstrate the role of the bacterium in the pathogenesis of bladder infections. The 2005 review ends with brief discussions of ''the five species'' of *Escherichia* (*coli, blattae, fergusonii, hermannii, vulneris*). So an initial exuberance in assigning the name *Escherichia* to a variety of microorganisms was eventually replaced by the recognition of *E. coli* as the serologically most prolific microorganism. Escherich was celebrated quite a while before Castellani's appellation: not only does one find quite a few references to *Bacterium coli* (Escherich), a common practice in denoting the discoverer of a microorganism in parentheses after the name of the organism, but already in 1889 one sees ''B. Escherichii Trev. (*Bacterium coli commune* Escherich)'' on p. 15 of a 36-page classification book ''I Generi e le Specie delle Batteriacee'', by Count Vittore Trevisan di Saint-Léon. The death in 1911 of Theodor Escherich, well known and celebrated, very likely stimulated the wide acceptance of the term *Escherichia*. Furthermore, the proposal by Castellani and Chalmers to reclassify a host of bacterial species strongly contributed to the acceptance of this term. No reference to a 1918 publication by these two authors could be found, not even in the exhaustive bibliography found in the 2005 Bergey's Manual (cf. Brenner *et al.*, 2005, p. 940). An Italian reference (Castellani, 1918) which I have not seen in the original, might possibly refer to *Escherichia*.

Monod, in his 1942 doctoral thesis, always uses the term *B. coli* (I counted 61 times in this fascinating work) without initially indicating

the meaning of ''*B.*'' and he never uses *Escherichia coli or E. coli.*[13] As mentioned near the beginning of this essay, Watson in the various editions of his influential textbook, ''The Molecular Biology of the Gene'', uses ''*E. coli*'' throughout, and also does not follow bacteriological practice in initially using the whole designation. There is an amusing disparity in nomenclature in Escherich and Pfaundler's review of Escherich's bacterium: in the clinical part, written by Escherich, one consistently sees *Bacterium coli commune*, while in the physiological part of this highly impressive review, written by Pfaundler, one just as consistently sees *Bacterium coli*, and never *Bacterium coli commune*. Escherich, in a footnote to this review, indicated that the designation ''commune'' in *Bacterium coli commune* refers to the common occurrence of this organism in infants as well as in adults (Escherich and Pfaundler, 1903, p. 335). He eschewed the binomial system for naming his bacteria. He used *commune* to emphasize that it was present in all the specimens that he had tested, and that it was an obligate intestinal bacterium. Although he could find no correlation between the appearance of the bacteria and the symptoms and occurrence of childhood diarrhea, he proceeded, in true scientific and modern-sounding fashion, to continue his work. He proceeded to describe the appearance, the cultural characteristics, and the possible pathogenicity of his bacteria (see Fig. 5).

VIII. Escherich's Neglect of His Discovery of *Campylobacter jejuni* and of Some Other Bacteria

Escherich is celebrated as the undoubted discoverer of what is now known as *Escherichia coli*. He also, and at the same time, discovered what he called *Bacterium lactis aerogenes*. In addition, he is almost certainly the discoverer of yet another organism, *Campylobacter jejuni*, responsible for more cases of diarrhea than those brought about by pathogenic strains of *E. coli*. The story is intriguing and again shows Escherich as a superb bacteriologist and clinician. As we saw, he spent just 2 weeks in Naples during the 1884 cholera epidemic. Scarcely a month later, at the December 3, 1884 session of the Munich Medical

[13] An amusing episode, told by Lwoff about Monod's introduction to E. coli, deserves mention: ''[I] advised him to use a bacterium able to grow in a synthetic medium, for example *Escherichia coli.* ''Is it pathogenic?'' asked Jacques. The answer being satisfactory, Monod began, in 1937, to play with E. coli and this was the origin of everything . . .'' (Lwoff, 1979; cf. Lwoff, 1991, 2003). See Cohen (2002) for an admirable account of the contributions of work at the Paris Pasteur Institute to the development of molecular biology.

Society, he presented a long report on his experiences. In the course of his discussion on observations of Koch's ''commabacteria'' in cholera feces, he mentioned that he frequently found ''tooth spirochetes'' as well as several other bent forms in these feces. Almost as an aside he stated that, several weeks earlier, i.e., presumably before he went to Naples, he found a bent bacterium in the intestine of a 4-day-old baby, and that this organism was decidedly different from ''real'' commabacteria (Escherich, 1884). One can say that his interest in this bacterium was stimulated by a comparison with the cholera bacterium. He observed this organism, therefore, before his discovery of *Bacterium coli commune*. A much more detailed report on the bacteriology of a ''bent'' organism appeared in two reports from 1886, one on what he called *Vibrio felinus* obtained from the feces and intestine of a diarrheic cat (Escherich, 1886b), the other, with illustrations, from the intestinal canal and the feces of infants (Escherich, 1886c). The latter organisms differed from the dental spirochaetes. To study the possible relation between the vibrios and various intestinal disturbances, he observed samples from 72 sick infants under the microscope and found the vibrios in only 41 cases. As a careful clinician, he shied away from concluding that these vibrios were a cause of infant diarrhea, but he indicated that their presence could not contradict a serious prognosis. He was far more conservative a year later, near the end of a historical review on intestinal bacteria and the etiology of intestinal diseases. He stated that there was no relation between the vibrios and the etiology of intestinal diseases (Escherich, 1887). This review is of interest to readers in the United States, for with his customary thoroughness Escherich stated that American physicians were the first to observe the hot summer months to be associated with the enormous, almost epidemic, increase in mortality of infants up to the age of 1 year. It was called ''summer complaint'' or ''summer cholera'', and was regarded until the middle 1800s as ''an entirely American disease''. Escherich indicated that the first detailed descriptions were given by Benjamin Rush in his 1789 ''Medical Inquiries and Observations''. Kist (1986) has suggested that Escherich did not pursue the study of his vibrios since he was not able to grow them in culture. Kist (1986), exactly a 100 years after Escherich's detailed description, resuscitated Escherich's essentially forgotten discovery of *Campylobacter jejuni* (not mentioned in any of his obituaries). The end of Kist's paper is worth quoting: ''Besides the discovery of *Escherichia coli* it has now become highly probable to add the first description of *Campylobacter* to Escherich's scientific accomplishments, although he never fully recognized its actual significance.'' Only in the 1970 was *Campylobacter jejuni* recognized as an emerging

human pathogen, leading cause of enteritis and enterocolitis (Kist and ereswill, 2001; cf. Bereswill and Kist, 2002; Skirrow and Blaser, 2002; Ketley and Konkel, 2005).

There is another matter of a near miss, that of the dysentery bacillus. This is brought out most clearly in Béla Schick's admirable reminiscences of Escherich, written some 46 years after his teacher's death: ''Escherich's preoccupation with the colon bacillus stood in his way of making another important discovery. When he cultured colon bacilli from the gastrointestinal tract, he focused his attention on the gram-negative strains which produced gas in the agar-culture medium and discarded those strains which did not produce gas. This discarded bacillus was the dysentery bacillus later described by Flexner'' (Schick, 1957, p. 115).

IX. Extensive Studies with *E. coli*, Long Before the Advent of Molecular Biology

A. Early Interests

Escherichia coli is often regarded to have been an obscure organism lifted from neglect by the rise of molecular biology. As Lord Byron would have said, it was held to have awoken one morning and to have found itself famous. Thus, for example, one reads in Greame Hunter's book, ''Vital Forces, the Discovery of the Molecular Basis of Life'' (2000, p. 314), ''Arguably, the main achievement of the Phage Group was to introduce the bacterium *Escherichia coli* into biochemistry.'' Similarly, in Franklin Harold's book ''The Way of the Cell'' p. 65, one reads ''Project *E. coli* grew out of the researches of André Lwoff, François Jacob and Jacques Monod, initiated in Paris just before the Second World War'' (Harold, 2001). Hobom (1986) in an article on the occasion of the centenary of the discovery of *E. coli* calls this inconspicuous intestinal inhabitant the ''laboratory mouse of the molecular biologists'', and states that ''the history of *E. coli* is mainly also the history of molecular biology''. This is decidedly not so. *E. coli* was famous right from the beginning. It almost immediately attracted the attention of bacteriologists, physicians, biochemists. As early as 1894, that is, just about 9 years after Escherich's description of the organism, a textbook published in the United States stated: ''The fact that it is always with us in most intimate association with certain of our life processes, together with the fact that it is known to appear in organs other than that in which it is normally located, and that its occurrence in diseased conditions is not rare, justifies the opinion that it is one of the most important of the micro-organisms with which we have to

deal'' (Abbott, 1894, pp. 305–306). Familiarity with *Bacterium coli commune* rapidly spread. Thus some 3 years later one reads: ''few organisms are more frequently met in the every day work of a bacteriologist than *Bacterium coli commune*'' (Gordon, 1897, p. 438). An impressive testimony to the early and extensive interest in the organism is provided by the 141-page review, simply called *Bacterium coli commune*, published in 1903 by Escherich and Pfaundler, that is, just under 20 years after the discovery of the organism, in volume 2 of the highly regarded ''Handbook of Pathogenic Microorganisms''. The cumulative effect of this article is one of concentrated, hard work, elegance, and critical familiarity with the latest contributions to an exploding field of medicine and bacteriology. The inclusion of an article on *Bacterium coli commune* in a ''Handbook of Pathogenic Microorganisms'' is a recognition of the pathogenicity of this organism. The review has 20 pages of references, with about 30 citations per page, many, but by no means all, dealing with *Bacterium coli commune*. The article is a useful milestone for then current bacteriological techniques and approaches, as well as a critical evaluation of the clinical and pathological ramifications occasioned by this organism. The first part of the review, some 88 pages written by Pfaundler, describes the ''Morphology and Biology'' of the organism; the remaining two parts, written by Escherich, deal in a short section with ''*Bacterium coli commune* under physiological and pathological conditions'' and, in a long section, with ''The Bacteria of the Coli-Group as Causative Agents of Disease''. The details do not concern us here, except for the recognition that this organism elicited diversified interest, bacteriological and pathological, right from the start. We read about the morphology of the organism under different conditions of growth, on its fermentative behavior, its formation of lactic and other acids, of indole, skatole, methyl mercaptan, ammonia, hydrogen sulfide, on ways of distinguishing it from *Bacterium typhi*, on its growth under anaerobic versus aerobic conditions, antagonism to growth of other bacteria as a possible role in controlling the variety of intestinal bacteria, on the effects of desiccation, of pressure, and so on. Chemical criteria for distinguishing between different bacteria were probably first used to distinguish between *Bacterium coli* and what Escherich and Pfaundler (1903, p. 383) call ''Typhuskultur''. Thus, while *B. coli* converts glucose to D-lactic acid, *Bacterium typhi* converts it to L-lactic acid (Capaldi and Proskauer, 1896). A 5% lactose solution is rapidly fermented by *B. coli*, but not by *B. typhi*. A long section is devoted to its pathogenicity in animal experiments and to its occurrence outside the intestinal tract. No firm evidence is presented for the increase in incidence of *Bacillus*

coli commune in dysentery and a possible role of this organism as a causative agent of this disease. W. Kruse is cited as the discoverer (1900) of the dysentery bacillus. Escherich is extremely critical about accepting various studies indicating changes of *Bacterium colon commune* populations as associated with pathogenicity in humans. On the other hand, he accepts reports on the occurrence of a fatal intestinal colisepsis in a number of babies. The possibility that this organism may cause peritonitis and diseases of the gall bladder is also discussed. Far more credence is given in a lengthy section (7 pages) to the observations that this organism is the most common cause of diseases of the urinary tract. The possible role of the organism in a large number of other diseases is discussed. The article ends with the statement: ''One cannot doubt the existence of independent coli effects, although the clinical picture and the type of these diseases have to be established by further research.'' Although Escherich was the first to demonstrate and to stress the marked differences in the pathogenicity of his organism to animals as contrasted to humans, the various pathogenic strains that later were discovered were not known in his time. However, Ludwig Brieger had claimed as early as 1895 that there were resemblances between poisons due to *Bacterium coli* and to the organism causing typhus (Brieger, 1895).

B. The Development of Classical Biochemistry Depended Heavily on Studies with *Bacterium coli commmune*

What is of greater interest to contemporary readers is the recognition that work with *Bacterium coli*, later called *Escherichia coli*, led to an impressive variety of fundamental biochemical discoveries, long before the biological insights that initiated and advanced molecular biology. A bedrock of biochemical insights was gained by work with this organism. In the words of Joshua Lederberg: ''During the first half of the twentieth century, *E. coli* was probably the single most studied bacterial species for basic physiological and metabolic investigation, but it was rarely mentioned in general biology texts'' (Lederberg, 1998). Textbooks of biochemistry by and large tend to minimize or to ignore the sources of their substances or extracts of interest, but in textbooks of bacteriology one finds clear correlations between observations and the bacteria or the bacterial material with which they were made. Two of the standard textbooks of bacteriology, one current in the early 1930s (Buchanan and Fulmer, 1929, 1930), the other current in the 1930s and 1940s (Stephenson, 1930, 1939, 1949) were consulted. The ''Index to Microörganisms'' in Buchanan and Fulmer's scholarly work has far

more references to *Bacterium coli* than to any other microorganism. This organism again predominates in the Index of all three editions of Marjory Stephenson's influential treatise. Thus, *B. coli* in the Index to the first edition occupies an entire page (p. 312), with more entries than any other microorganism or indeed any other topic. In the Index to the 1939 edition (p. 380) *Bacterium coli* again has more entries than any other subject; here for the first time one sees *Escherichia coli*, with the cursory remark ''see *Bacterium coli*''. The organism again predominates in the Index for the 1949 edition with all entries (p. 389) under ''*Escherichia coli* (*B. coli*)'' and none under *Bacterium coli*.

We start this survey of pre-molecular biology *E. coli* discoveries with a quotation from Juda Quastel, a major 1920s contributor.[14] He started his work with *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*), but soon abandoned this organism since it tended to form sticky, mucilaginous growth. ''I . . . eventually concentrated on *Bacillus coli* (or *Escherichia coli*) as a very suitable organism, easily grown and washed, and capable of forming saline suspensions that could be handled with great accuracy. . . . Our systematic work with *E. coli* launched this organism into the field of biochemical research. Today it is one of the most popular organisms of investigation in the field of molecular biology'' (Quastel, 1974, pp. 72–73). Experiments with *E. coli* suspensions, performed at the Biochemical Laboratory of Cambridge University, resulted in the recognition of ''at least 56'' specific dehydrogenases (Quastel, 1974, p. 73), leading directly to the fundamentally important idea of ''active centers'' (Quastel, 1926; Quastel and Wooldridge, 1927, 1928; cf. Sung, 1979; Quastel, 1980) and grew into a series of highly important insights into the nature of enzymatic action.[15]

Further, Quastel's interest in the dehydrogenation of succinate to fumarate led directly to his discovery of the phenomenon of competitive inhibition, exemplified by the effect of malonate on succinic dehydrogenase (Quastel and Wooldridge, 1929; Cook, 1930; Quastel and

[14] The high esteem in which J. H. Quastel was held, not only for his pioneering work with *E. coli*, was shown by the fact that he and C. S. Hanes, an eminent enzyme kineticist, served as an honorary president of the XIth International Congress of Biochemistry, Toronto, 1979. The author remembers an outdoor reception at that Congress, with Hanes and a smiling Juda Quastel, resplendent in a spotless off-white suit, shaking hands with a line of attendees.

[15] The idea of what is now called the enzyme active site was advanced by Quastel, following work with *Bact. coli*, in the very year that Sumner with his studies on urease demonstrated the protein nature of enzymes. Quastel's proposal of an active center was, however, independent of the actual chemical nature of enzymes (cf. Friedmann, 1981, p. 394).

Wheatley, 1931). This action of malonate led workers in Szent-Györgyi's laboratory to demonstrate the catalytic role of fumarate in cellular respiration (Annau *et al.*, 1935, p. 33). Furthermore, the phenomena of malonate inhibition and of the role of fumarate played a central role in Krebs' discovery of the citric acid cycle (Krebs and Johnson, 1937). It is not often realized that it was work with *E. coli* that led to these fundamentally important biochemical insights (cf. Quastel, 1983, p. 154). Work by others in Cambridge with *Bacterium coli communis* led to further basic discoveries. Thus, Barnet Woolf's classical work with *Bacterium coli communis* (1929, 1931) first advanced what he modestly called ''a limited hypothesis on enzyme action'' (Woolf, 1929, p. 482) later known as the ternary-complex mechanism, a bedrock of understanding of the mechanism of a host of enzymatic reactions (cf. Bloomfield *et al.*, 1962). J. B. S. Haldane in his classic 1930 book ''Enzymes'' has extensive references to ''*Bacillus coli*'', far more in fact than to any other bacterium, and he devotes a number of pages to what the calls ''Quastel's Theory of Dehydrogenases'', based entirely on work with this organism (Haldane, 1930, pp. 183–185).

A few more discoveries made with *Bacterium coli communis* bring home its importance in the development of biochemistry, long before the advent of molecular biology. Work with *B. coli* led to the term colicin, coined by André Gratia in 1925. *B. coli* was one of the bacteria in which the enzyme hydrogenase was first demonstrated (Stephenson and Stickland, 1931).[16]

A mere 2 years after Eduard Buchner's revolutionary discovery in 1897 of cell-free alcoholic fermentation by yeast extract (cf. Friedmann, 1997), Arthur Harden began his classical studies on the nature of alcoholic fermentation not, as may be assumed, with yeast preparations but with *B. coli* (Harden, 1899, 1901). His interest in fermentation, stimulated by work with *B. coli*, led to his classical studies on alcoholic fermentation by yeast for which he received an early Chemistry Nobel Prize. The first recognition of chemical relationships between fermentation products and fermented substrates were credited to the ''theoretical speculations'' that Arthur Harden (1901) brought to bear on his studies with *B. coli* (cf. Emmering, 1902). Although he soon switched to yeast (Harden, 1903; Harden and Young, 1906), his interest in *B. coli* as a model system remained for many years (Harden and Penfold, 1912).

[16] Leonard Hubert Stickland, in his very first paper (Stickland, 1934) on what became known as the Stickland reaction referred to similarities between his observations with *Clostridium sporogenes* and ''certain anaerobic energy-yielding reactions of *Bact. coli* (Quastel and Whetham, 1924)''.

Kluyver in his 1931 book ''The Chemical Activities of Microorganisms'' gives extensive data on the fermentation of glucose by *B. coli* (Kluyver, 1931, pp. 56–57). *B. coli* was also one of the predominant organisms used in varied and extensive studies on amino acid metabolism. Umbarger states in one of his impressive reviews of amino acid biosynthesis and its regulation:''The material is heavily weighted toward the regulation of amino acid biosynthesis in *Escherichia coli* and *Salmonella typhimurium*. This bias could not be avoided, since these organisms have attracted most of the workers in the field'' (Umbarger, 1978, p. 534). One of the highlights was the isolation of shikimic acid from *Escherichia coli* mutants (Davis, 1951).

C. Bacterial Genetics Originates with *Bacterium coli mutabile*

B. coli communis played a basic role in the development of bacterial genetics. In the earliest years, there was a somewhat perfunctory interest in the possibility that different strains of *B. coli commune* existed. Thus in 1886, just a year after the discovery of the organism, Escherich states in his notable book: ''In an earlier section I indicated that I was more interested in the description of closely related types occurring in feces than in their detailed differentiation. I referred particularly to the large group of colon bacteria that in my opinion share enough properties to justify being summarized under one designation. Individual observations point to the possibility that more precise investigations and perfected methods will lead to the differentiation of further species.'' Some 13 years later, when Escherich was in Graz, Dr. Henry Lee Smith, a visitor from Baltimore, was asked to look into the possibility of different *Bact. coli* strains. He compared morphological and biological characteristics of the organism obtained from the feces of different children and concluded that there were distinct differences in *B. coli* obtained from the feces of different children, particularly in the lack of agglutinating cross reaction by the serum of a guinea pig that had been immunized against one of the strains. He concluded that the differences were not dependent on nutrition, since, at least in one case, there was no change in serological properties in the course of the child's transition from mother's milk to artificial food (Smith, 1899). A year earlier A. Péré (1898) had shown that reported differences in fermentative properties of *B. coli commune* could be traced to different sources of the organism. The 1903 review by Escherich and Pfaundler has many pages of references and discussions of variations in *B. coli*. There was no consensus as to the meaning of such variations. On the one hand, some changes were reversed by simple further cultivations, and on the other hand,

uninfluenced (unbeeinflusste) changes in some strains were reflected by stable morphological, fermentative, and nutritional properties. These latter strains were designated as different types (Spielarten) or variations (Escherich and Pfaundler, 1903, p. 405). There was no discussion of the permanence or the inheritance of these traits.

Max Neisser in a report on the dissertation of his student Rudolf Massini used the term *Bacillus coli mutabilis*, for ''a case of mutation according to de Vries in bacteria'' (Neisser, 1906). A year later Massini's work was published in a 40-page article on what he called *Bacterium coli mutabile* (Massini, 1907), with a clearer statement on his work's genetic significance: ''This work constitutes a contribution from bacteriology to the theory of mutation, as has been carried out by Hugo de Vries in the field of botany . . . the mutation is inherited as soon as it has occurred . . . it seems permissible to use de Vries's theory in bacteriology, especially in our case'' (Massini, 1907, pp. 289–290). This work was done in the Institute of Experimental Therapy in Frankfurt whose director was none less than Paul Ehrlich.[17] Burri and Düggeli (1909, p. 174) also spoke of mutations in coli stems. The idea of bacterial mutations faced criticisms. In a later paper, Burri (1910) strongly attacked Massini's conclusions and claims that one ''did not deal with a mutation in the sense of DeVries but with a phenomenon of adaptation''. ''Although later workers concluded that Burri's culture was apparently not *E. coli mutabile*, Massini's work was largely discounted because of Burri's experiments'' (Beck, 2000, p. 145). The lasting inheritance of new traits in typhus, paratyphus, and related bacteria was vigorously defended by Reiner Müller (1908, 1911) and especially by F. H. Stewart (1926) for the ''paracolon mutabile colon group''. The latter paper began with the statement: ''Mendel's principles of variation, formed from the study of the genetics of higher plants and animals, can be applied also to bacteria, and that acquired virulence in bacteria is the result of Mendelian variation.'' He continued, quoting Neisser and Massini, by stating ''This thesis is not entirely new''.

Some detail on Massini's work may be of interest, exactly a 100 years later, for it was impressively ''modern''. Massini's *Bacterium coli mutabile*, in contrast to his initial *B. coli* strains, was able to ferment lactose. The lactose-fermenting ability of this strain was observed by use of so-called Endo agar (Endo, 1904), which contains lactose, and fuchsin as an indicator of acid formation. After a few days a bacterium

[17] The bacteriologist Max Neisser, head of the bacteriology section of Ehrlich's institute (cf. Neisser, 1914) is not to be confused with the physician Albert Neisser of *Neisseria* fame.

able to ferment this sugar produced dark red papillae on this agar, whereas the bacterium incapable of such a fermentation produced a white colony. The papillae, upon replating, grew as red-colored bacteria. Massini concluded that, during the growth of the colony of initially lactose-negative bacteria, a sudden change to lactose-positive had occurred, without intermediate forms. The bacterial subclones retained the capacity for lactose fermentation, that is, one had obtained lactose utilizing *mutations*. Massini advanced numerous experimental criteria to ascertain that he had always started with single colonies of ''white'' bacteria, that is, that no ''red'' colonies were present from the start. In Massini's day, the genetic nature of these mutations could not be proven conclusively. It is not clear from a reading of Massini's and later papers whether the term *B. coli mutabile* referred to the strain before or after mutation. Thus Arnold Burk, just a year later, confirmed Massini's results, but argued that the mutation resulted in an organism indistinguishable from *B. coli* (Burk, 1908). He held that the original strain was related to *B. coli*, but should not be regarded as this organism. In fact Escherich and Pfaundler's 1903 review (cf. Section IX.A) made the point that *B. coli*, in contrast to *B. typhi*, was a lactose fermenter. Here one had a criterion for the important distinction between *B. coli* and *B. typhi*. Clearly the earlier work on *B. coli* may have dealt with a strain different from Massini's or with an unrecognized mutant, but this in no way invalidates Massini's conclusions. I. M. Lewis, almost 30 years later stated that ''The subject of bacterial variation and heredity has reached an almost hopeless state of confusion'' (Lewis, 1934), but he obtained the valuable measurement that the frequency of the change from *E. coli* to *E. coli mutabile*, that is, from lactose-negative → lactose-positive, was in the order of 1 per 100,000 viable cells. He took care to use the word *variation*, and not *mutation*. After a further ten years, a summary of Massini's paper in the authoritative Topley and Wilson's textbook, third edition, also avoided the terms *mutant* or *mutation*: here *Bacterium* (not yet *Escherichia*!) *coli mutabile* was called ''an interesting type of variability'' and a ''curious organism'' (Wilson and Miles, 1946, pp. 297, p. 677). At the time no less a person than André Lwoff (1946, p. 146) still referred to *Escherichia coli mutabile*. By then the assumption, implicit in many of the early papers in the field, that it was the nutritional environment that brought about bacterial mutations, began to be replaced by the recognition that a given nutritional environment, rather than initiating mutations, simply allowed preexisiting mutants to grow. As noted by Schlegel (1999, p. 136), neither Massini nor Lewis recognized that their data showed the spontaneous nature of their mutations. As an illustration of the recalcitrance of

habit over the elusiveness of epochal conclusions, Lewis for instance left his area of investigation and devoted himself to bacterial cytology (cf. Schlegel, 1999, p. 136). It bears remarking, in an article on *Escherichia coli*, that it was work with *E. coli*, infected with bacteriophage, that initiated the replacement of a Lamarckian by a Darwinian process of selection in bacterial selection (Luria and Delbrück, 1943). Massini and Arnold Burk and Burri and Lewis, and others not cited here, have lapsed into near obscurity, while Luria and Delbrück received a Nobel Prize. From that point, one can say, bacterial genetics came into its own.

X. Summary

Theodor Escherich's success in discovering *Bacterium coli commune* stemmed from a mixture of luck and of the limitations in bacteriological techniques available at the time. Luck came in three forms: the preponderance of this organism in the stools of infants in contrast to adults, the obligate, rather than what he called facultative, presence of this organism in all the stools that he examined, and above all the remarkable ease with which it could be cultivated. More sophisticated techniques for bacterial cultivation, made available long after Escherich's time, would have demonstrated that the stools are populated by as many as 400–500 different kinds of microorganisms, a recognition that might well have relegated *Bacterium coli commune* to a subsidiary position in the pantheon of fecal exuberance. One should probably add technical limitations, when they help to contribute to a discovery, to the confluence of lucky circumstances, since later technical advances or changes cannot possibly be used to deprecate earlier standards of achievement. This recognition applies particularly to the spectacular achievements in bacteriological discovery, due especially to the new techniques of bacterial cultivation introduced mainly by Robert Koch. The very inability to detect more discriminatory organisms, not cultured with the media known at the time, strongly helped in the discovery of *Bacterium coli commune*.

The discovery of *Escherichia coli* in 1885 occurred at a time that saw the confluence of a number of factors. This discovery occurred during the so-called golden age of bacteriology when it became abundantly clear that many diseases were caused by newly discovered pathogenic bacteria. As examples, tuberculosis was shown to be due to an infectious bacterium, and cholera was shown to be due to the so-called comma bacillus. These various discoveries were brought about by a refinement in bacteriological techniques and by the promulgation of Robert Koch's postulates of infection (actually first advanced by Koch's teacher Jacob

Henle, cf. Modlin and Sachs, 2004, p. 449). The isolation of pathogenic bacteria, and the elaboration of criteria to establish their causal relationship to a number of devastating diseases, at long last demolished still prevalent ideas of disease etiology that pitted the traditional miasmatists against the upstart contagionists. (See Bourdelais, 2006, for a discussion of these two views, their relationships, and their applications in the service of preventive medicine.) Theodor Escherich, a highly versatile pediatrician skilled in bacteriological techniques, was very much aware of the devastating toll that childhood dysentery took upon early human lives. More and more convinced that dysentery, in analogy to the cholera epidemic he had studied in Naples, was also of bacterial origin, he was determined to establish this postulate by finding the causative organism. He failed in this effort, as he himself acknowledged. The welter of microorganisms in the human intestine made it very difficult to determine which of these, if any, might be the culprit, since they all occurred in normal as well as in diseased intestines.

Escherich's work was helped by three factors. First, he had learned Robert Koch's brand-new bacteriological techniques from Wilhelm Frobenius, who had learned them in turn from Robert Koch himself. Second, Escherich's careful bacteriological researches on the feces of very young children showed that one of these bacteria, which he called *Bacterium coli commune*, predominated at some early stages of life. Third, this organism claimed attention by its incredible ease in culturing. Its apparent prevalence and its ready growth prompted Escherich and many of his contemporaries to select this organism for further study. An additional advantage, apparent early, but expanded by later work was that this readily cultured organism manifested a highly impressive variety of strains. It became the first bacterium, in the form of the so-called *Bacterium coli mutabile*, to which genetic theories were applied. Thus, studies by Rudolf Massini in 1906 initiated the field of bacterial genetics. In addition, long before the advent of molecular biology with *E. coli* as its flagship organism, *Bacterium coli commune* was a favorite model organism in many basic biochemical studies. Investigations with this organism suggested the notion of the active site of enzymes; work with a large number of dehydrogenases from this organism led to the discovery of competitive enzyme inhibition. Studies with enzymes from this organism suggested the so-called ternary complex mechanism, a standard enzyme mechanism, led to the discovery of hydrogenase, initiated studies on alcoholic fermentation. It was, along with *Salmonella typhimurium*, a favorite organism for the study of amino acid metabolism. Clearly *E. coli* had a venerable history before its adaptation as the organism of choice in the

development of molecular biology, a choice that continues to this day and that shows no signs of abating. By now the ''coli'' in *E. coli* is but a distant echo of its source, as irrelevant to most as is ''*E.*'', intended originally in celebration, but by now, to adopt Thomas Carlyle's quotation at the top of this essay, but maundering and mumbling.

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